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Journal of Chromatography A, 1034 (2004) 139–148
JOURNAL OF CHROMATOGRAPHY A
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Rapid analysis of nucleotide-activated sugars by high-performance liquid chromatography coupled with diode-array detection, electrospray ionization mass spectrometry and nuclear magnetic resonance

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Received 17 November 2003; received in revised form 4 February 2004; accepted 6 February 2004

Abstract

A generally applicable method for HPLC analysis of sugar nucleotides was established. Separation was achieved using ion-pair chromatography on a reversed-phase column. Ion-pair reagents were selected and various parameters optimized with respect to separation of 11 of the most important sugar nucleotides and compatibility with on-line detection by electrospray ionization MS and NMR. The method was applied to the on-line analysis of the GDP-D-mannose-4,6-dehydratase (Gmd) and GDP-4-keto-6-deoxy-D-mannose reductase (Rmd) catalyzed conversion of GDP-D-mannose to GDP-D-rhamnose. By LC–NMR, the intermediate product of the reaction was shown to be a mixture of GDP-4-keto-6-deoxy-D-mannose and GDP-3-keto-6-deoxy-D-mannose. Nucleotide co-factors of enzymatic reactions such as ATP and NADH did not interfere with the analysis of nucleotide-activated sugars.

Keywords: Nuclear magnetic resonance spectrometry; Nucleotide sugars; Carbohydrates; Guanosine diphosphate–mannose; Guanosine diphosphate–rhamnose

1. Introduction

Nucleotide-activated sugars are ubiquitous metabolites for the biosynthesis of carbohydrate containing molecules. Interconversions of monosaccharides occur in their activated forms. Nucleotide sugars serve as building blocks for oligo and polysaccharides; they are involved in glycosidation of proteins and lipids, and in phase 2 metabolization (conjugation) of xenobiotics. Carbohydrates form highly complex structures at the outer surface of microbial, plant, and animal cells, where they serve for molecular recognition in fundamental cellular processes including signal transduction, intercellular communication, protein targeting and recognition of pathogens [1–3]. The bacterial cell wall is particularly rich in complex carbohydrates containing rare sugars which are not found in other organisms such as mammals. Polysaccharides of the outer membrane, such as O-antigens in Gram-negative and S-layer in Gram-positive bacteria, are often essential virulence factors of pathogens. Targetting the biosynthesis of specific carbohydrates is, therefore, of considerable interest for the development of therapeutic agents [4–6].

Investigation of the metabolism of nucleotide-activated sugars requires a generally applicable, rapid and robust analytical assay which should enable the separation, unambiguous structural characterization and quantitation of substrates, intermediates and end products. From the late 1970s until today, several HPLC methods for analysis of nucleotides have been developed. Ion exchange chromatography enables primarily the separation of nucleotides that differ in their degree of phosphorylation. Ion exchange chromatography is, therefore, only of limited use for analysis of sugar nucleotides, and few applications have been reported [7–9]. Long separation times and poor long-term stability of columns are major drawbacks. Reversed-phase liquid chromatography with acetonitrile–phosphate buffer gradients leads to significantly

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doi:10.1016/j.chroma.2004.02.023

faster separations and enables the successful analysis of numerous nucleotide-containing molecules. However, separation of nucleoside phosphates and nucleotide-activated sugars is not satisfactory [10–12]. Finally, ion-pair chromatography was established as an alternative. Addition of tertiary or quaternary ammonium derivatives to the mobile phase provides satisfactory retention of the highly polar analytes on reversed-phase materials. Using acetonitritrile gradients, numerous nucleotide-containing compounds and sugar nucleotides can be separated [13,14]. Ion-pair chromatography has been successfully applied to the analysis of nucleotide sugars from genetically engineered yeast and bacteria [15].

The applications mentioned were carried out with UV detection. All nucleotides exhibit an absorption maximum around 260 nm and are, therefore, easily detected. However, sugar nucleotides cannot be differentiated according to the nature of their nucleotide diphosphate moiety (ADP, CDP, GDP, dTDP, UDP) attached to C-1 of the sugar residue. Thus, HPLC peak identity can only be established by co-chromatography with reference compounds, if available, and no further structural information can be derived. Given the subtle structural conversions in monosaccharide biosynthesis, biochemical analyses of carbohydrate metabolism are severely hampered by the lack of structural information.

In the context of our ongoing investigation of the biosynthesis of lipopolysaccharides (LPSs), rare bacterial sugars and activated 6-deoxyhexoses [16–18], we needed an analytical method providing on-line spectroscopic information for the unambiguous identification of closely related nucleotide sugars, and for structure elucidation of potentially unknown intermediates. At the same time, we wanted to avoid tedious sample preparation which possibly would result in loss of analytes and decomposition of labile compounds. We here describe HPLC conditions suitable for the separation of the most important activated sugars and compatible with on-line electrospray ionization (ESI) MS and NMR analysis. Biosynthesis of GDP-D-rhamnose is taken as an example to demonstrate the detection and structural characterization of intermediate products obtained by enzymatic conversion of GDP-D-mannose.

2. Experimental

2.1. Chemicals

Nucleotide sugars were purchased from various suppliers. ADP-D-glucose (**1**), CDP-D-glucose (**2**) and ADP-D-ribose (**8**) were from ICN Biomedicals (Eschwege, Germany), GDP-D-glucose (**3**), UDP-D-glucose (**5**), UDP-D-galactose (**7**), UDP-*N*-acetyl-D-glucosamine (**10**) and UDP-D-glucuronic acid (**11**) were from Roche (Basel, Switzerland), dTDP-D-glucose (**4**) was from Fluka (Buchs, Switzerland), GDP-D-mannose (**6**) from Calbiochem (Bad Soden, Germany), and GDP-L-fucose (**9**) was from Sigma (Deisenhofen, Germany). Stock solutions (10 mg ml^{-1}) of each compound were prepared in HPLC-grade water and were stored at −26 °C for not more than 1 month. Trietylamine (TEA), tripropylamine (TPA), tributylamine (TBA) and acetic acid were analytical grade (Fluka). HPLC-grade acetonitrile was from Merck (Darmstadt, Germany). HPLC-grade water was prepared with a Milli-RO 3Plus system coupled to a Milli-Q RG module (Millipore, Bedford, MA, USA), followed by filtration through a 0.22 μm cellulose acetate filter (Schleicher & Schuell, Dassel, Germany). Deuterated water (99%) for LC–NMR analysis was purchased from Dr. Glaser (Basel, Switzerland). LC–NMR-grade acetonitrile was from SDS (Peypin, France).

2.2. Instrumentation

HPLC separations were carried out with a HP series 1100 system (Agilent, Waldbronn, Germany) driven by ChemStation software. This system was composed of a solvent degasser module, a binary high pressure mixing pump, an autosampler, a column thermostat, and a diode-array detection (DAD) system. For LC–MS analysis, the HPLC system was connected to a PE Sciex API 165 quadrupole mass spectrometer with a turbo ionspray interface (Applied Biosystems, Foster City, USA). PE Sciex Analyst 1.1. software was used for processing of MS data.

LC–NMR analysis was performed on a Varian Unity Inova 500 MHz spectrometer equipped with a LC–NMR flow-probe cell (60 μl, 3 mm i.d.) (Varian, Palo Alto, CA, USA). The HPLC system consisted of a Varian 9012 pump and a ProStar 320 Varian UV-Vis detector. A Valco stop-flow valve (Valco, Houston, TX, USA) was used for the stop-flow experiments. This valve was controlled by the LC–NMR Varian software. It enables a precise parking of the LC peak of interest in the NMR flow-probe based on a calibrated delay time between UV and NMR detectors. In the stop-flow position, the valve releases into the waste the flow caused by the slow pressure drop over the column after the LC-pump stop.

2.3. Chromatographic conditions

All separations were carried out on Hypersil ODS RP-18 columns (5 μm, 250 mm × 4.6 mm i.d.) (Supelco, Taufkirchen, Germany). The column was thermostatted at 22 °C, or at temperatures indicated in text and legends. Buffers were prepared by adding the desired volume of TEA, TPA or TBA to 1 l of HPLC-grade water, followed by adjustment of pH to 6.0 with acetic acid. Due to volatility of reagents, buffers were prepared immediately prior to use. For binary gradients with low acetonitrile proportions, buffer (mobile phase A) and a buffer–acetonitrile mixture (90:10 (v/v)) (mobile phase B) were used to generate the

desired gradient profile. Eluents were prepared daily and degassed prior to use. Flow rate was 1 ml min^{-1}. The injection volume was 10 μl, and the wavelength of UV detection was 254 nm.

2.4. ESI-MS conditions

Operating conditions in the negative ion mode were as follows: source temperature 350 °C, ion spray voltage –3.100 V, focusing potential −310 V, declustering potential −70 V, entrance potential −6.5 V. The settings in positive ion mode were: source temperature 350 °C, ion spray voltage 3.100 V, focusing potential 200 V, declustering potential 20 V, entrance potential 10 V. Mass spectra were recorded in the *m/z* range 150–800 (0.4 s per scan).

2.5. LC–NMR conditions

For LC–NMR analysis the amount injected on-column was increased for sensitivity reasons. The method was tested by injecting a mixture of GDP-mannose, GDP-fucose and GDP-glucose (200 μg each). The HPLC conditions used for LC–NMR were similar to those described in Section 2.3 using TEA as buffer except that H_2O was replaced by ^2H_2O in the buffer solution and that the isocratic mode was chosen (buffer (mobile phase A) + 0.5% MeCN).

2.5.1. Suppression of signals from ion-pair reagent

Multiple solvent suppression was achieved by calculating automatically a selective pulse which enabled the suppression of all the signal from the solvent and buffer using the WET sequence [19] [MeCN (s, δ 2.0), HO^2H (s, δ 4.9), triethylammonium acetate (TEA) (s, δ 1.8; *t*, δ 1.2; dd δ 3.15)] (see Fig. 7).

2.5.2. Stop-flow LC–NMR two-dimensional correlation experiments

Good quality spectra (NT = 128) were obtained in the stop-flow mode for the standards of GDP-mannose, GDP-fructose and GDP-glucose and D-rhamnose (200 μg each). One milligram of the enzymatic reaction mixture was injected on-column. Time slice experiments were performed on the broad assymmetric LC peak corresponding to GDP-keto-deoxymannoses **13a** and **13b** every 1 min. A WET gCOSY (NT 96 × NI 512) was performed on the mixture of interconverting GDP-keto deoxymannoses **13a** and **13b**. A similar experiment on the standard GDP-mannose and D-rhamnose for reasons of comparison was performed. Specific correlations were evidenced by one-dimensional total correlation spectroscopy (1D-TOCSY) experiments. In this case, no solvent suppression was applied and the crude mixture was directly injected in the flow probe in ^2H_2O without separation. The specific signal irradiations were performed based on the LC–NMR results obtained previously.

2.6. Enzymatic reactions with GDP-D-mannose

GDP-D-mannose-4,6-dehydratase (Gmd, EC 4.2.1.47) and GDP-4-keto-6-deoxy-D-mannose-reductase (Rmd, EC 1.1.1.187) from *Pseudomonas aeruginosa* were cloned, overexpressed in *Escherichia coli* and characterized as described elsewhere [18]. The standard reaction mixture for LC–MS experiments contained 4 mM GDP-D-mannose, 20 μg Gmd and, for synthesis of GDP-D-rhamnose, 1.5 μg Rmd in 80 μl of a 25 mM bicine–ammonium hydroxide buffer (pH 8.0). The reaction mixture was incubated at 37 °C for 20 min. The reaction was stopped by heating for 1 min in a boiling water bath. The denatured protein was removed by centrifugation, and the protein-free supernatant was directly submitted to HPLC analysis.

For LC–NMR studies, the reaction mixture consisted of 15 mmol GDP-D-mannose, 1.5 mg Gmd in 1.5 ml of a 10 mM potassium phosphate buffer (pH 8.5). The mixture was incubated at 37 °C for 4 h. After this time, the reaction was complete, as monitored by HPLC. The enzyme was removed by ultrafiltration with a disposable Microcon YM-10 centrifugal filter Unit (Millipore). For two-dimensional NMR experiments, the solution was desalted over a Sephadex G-10 column (200 cm × 1.6 cm i.d.) eluted with water at a flow rate of 1.1 ml min^{-1}. The effluent was monitored at 254 nm, and fractions containing GDP-sugars were pooled, lyophilized. Samples were stored at −80 °C and redissolved in D_2O immediately prior to LC–NMR analysis.

3. Results and discussion

Ion-pair chromatography is a well established method for the HPLC analysis of highly polar nucleotides [14] and has also been successfully applied to the separation of the significantly more polar sugar nucleotides [15,20,21]. The buffer systems and ion-pair reagents used, typically tetrabutylammonium sulfate in phosphate buffers, are suitable for UV detection but not for hyphenation with ESI-MS and NMR. Another drawback of these ion-pair systems is that free nucleotides and co-factors required for enzymatic reactions, such as NADH, ATP or FAD, show similar retention behavior than the sugar nucleotides and unnecessarily complicate the chromatogram [14]. As a starting point for development of a suitable eluent, we chose the volatile triethylammonium acetate buffer system described by Oba et al. [22].

Preliminary HPLC separations with 11 commercially available sugar nucleotides (see Section 2 and Fig. 1) with 40 mM triethylammonium acetate (TEA; pH 6) at 22 °C led to a good separation of analytes. Retention times for compounds **1**, **4**, **8** and **9**, however, were unacceptably long, as a full chromatographic run lasted 50 min (Fig. 1a). Furthermore, the high buffer concentration led to weak ESI-MS signals of analytes due to ion suppression (see below). Reduction of buffer concentration to 10 mM led to shorter

retention times without undue loss of separation. The 11 sugar nucleotides were eluted within 37 min. However, separation of **1** and **8** could neither be achieved with these conditions nor in later experiments. The epimeric pairs **3** and **6**, and **5** and **7**, on the other hand, were well separated (Fig. 1b). Significant peak broadening at high retention times was typical of isocratic elution. In the isocratic mode, further reduction of TEA concentration, however, lead to unsatisfactory separation.

Further reduction of the concentration of the ion-pairing reagent and improvement of peak shapes were achieved with tripropylammonium (TPA) acetate in conjunction with gradient elution. Fig. 1c shows the chromatogram obtained with 1 mM TPA and a gradient from 0.5 to 2% acetonitrile. We also tested tributylammonium (TBA) acetate buffer systems as ion-pairing reagents. The increased lipophilicity of ion pairs necessitated higher proportions of acetonitrile (2–10%) for elution, leading to a significant loss in separation (data not shown).

Column temperature is known to affect chromatographic separation. We studied the effect of temperature on retention times and peak resolution at 20, 30 and 40 °C (Fig. 2). Increasing temperatures significantly reduced capacity factors of nucleotide sugars and separation of critical analytes. Compounds **6** and **11** co-eluted at 40 °C, and the epimers **5** and **7** could not be reliably separated. A temperature of 20 °C was therefore selected for subsequent analyses.

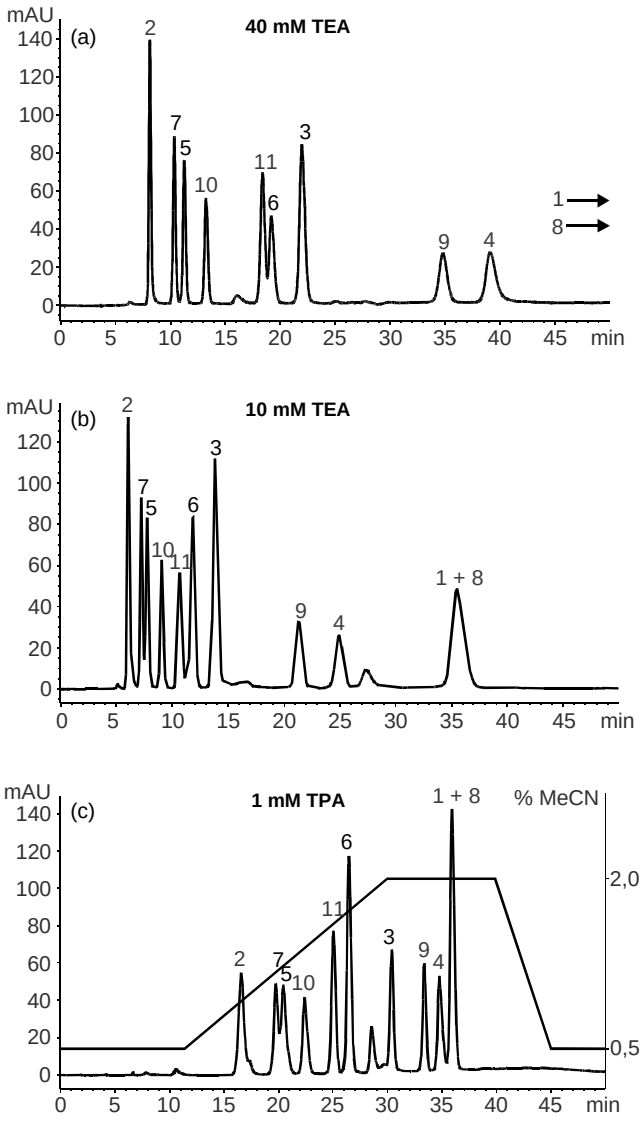

Fig. 1. Ion-pair chromatographic separation of 11 standard nucleotide sugars on a Hypersil ODS column. (a) Isocratic separation with 40 mM TEA acetate buffer; (b) isocratic separation with 10 mM TEA buffer and (c) gradient elution with 1 mM TPA acetate buffer and 0.5–2% acetonitrile. Gradient profile is shown. For details see chromatographic conditions. Peak identification: ADP-D-glucose (**1**), CDP-D-glucose (**2**), GDP-D-glucose (**3**), dTDP-D-glucose (**4**), UDP-D-glucose (**5**), GDP-D-mannose (**6**), UDP-D-galactose (**7**), ADP-D-ribose (**8**), GDP-L-fucose (**9**), UDP-*N*-acetyl-D-glucosamine (**10**) and UDP-D-glucuronic acid (**11**).

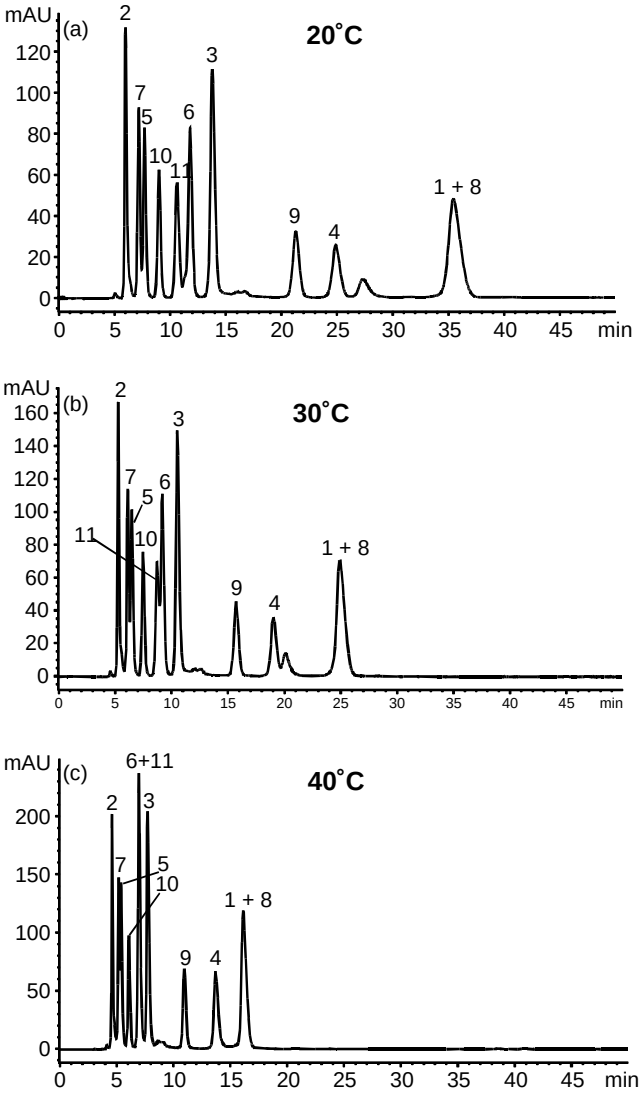

Fig. 2. Effect of increasing column temperature on retention time and peak resolution of nucleotide sugars. (a) 20 °C; (b) 30 °C and (c) 40 °C. Mobile phase: 10 mM TEA actetate buffer. Peak numbering as in Fig. 1.

The elution order of sugar nucleotides corresponds largely with that reported for separations using other ion-pairing reagents [13,14]. Chiefly, retention times increase with increasing hydrophobicity of the nucleotide bases (CDP < UDP < GDP < ADP). dTDP-glucose (**4**) is an exception, as the contribution of the comparably polar thymidine is compensated by the less polar deoxyribosyl moiety of this nucleotide. The nature of the sugar also affects retention behavior. 6-Deoxyhexoses are less hydrophilic than hexoses. Retention time of **9**, for example, is significantly higher than for the corresponding hexosyl nucleotide **3**. The stronger retention of UDP-D-glucuronic acid (**11**) compared to UDP-D-glucose (**5**) may be explained by additional ion-pairing of the carboxylate.

ESI-MS detection enables a mass-selective analysis of co-eluting non-isobaric substances and a sensitive detection of minor components in complex reaction mixture which might go unnoticed by UV detection alone. We investigated the ionization behavior of sugar nucleotides in ESI-MS in positive and negative ion mode and varying concentrations of ion-pairing reagents. In the negative ion mode, all reference compounds were detected with high sensitivity. As expected, the signal intensity was strongly dependant on the concentration and nature of the ion-pairing reagent [23,24]. A plot of ion intensities $[M - H]^-$ measured for GDP-D-mannose (**6**) and UDP-D-galactose (**7**) with increasing concentrations of TEA buffer shows that ionization efficiency was highest in pure water (Fig. 3a). With 1 mM TEA buffer, the ion intensities for the two analytes dropped below 40% of that in water. The concentration of buffer used had thus to be a compromise between optimal separation and ionization conditions which, for TEA, was at 10 mM. Higher MS signal intensities were achieved with TPA (Fig. 3c). For both compounds tested, the signal intensity in 1 mM TPA was more than 2.5-fold higher than in 10 mM TEA. Due to the diphosphate moiety, sugar nucleotides occur in aqueous solutions as preformed anions, and $[M - H]^-$ ions are abundant in the negative ion mode. Increasing concentrations of non-analyte anions, e.g. acetate ions of the buffer system, lead to ion suppression.

The ionization behavior of nucleotide sugars **6** and **7** in the positive ion mode was rather different. At buffer concentrations lower than 1 mM TEA or TPA, no $[M + H]^+$ ions were detected. Maximum signal intensity was reached with 10 mM TEA and remained constant at concentrations up to 40 mM (Fig. 3b). With 4 mM TPA buffer, the protonated molecular ion was detected (Fig. 3d). Given that higher concentrations of TPA led to worsened chromatographic sep-

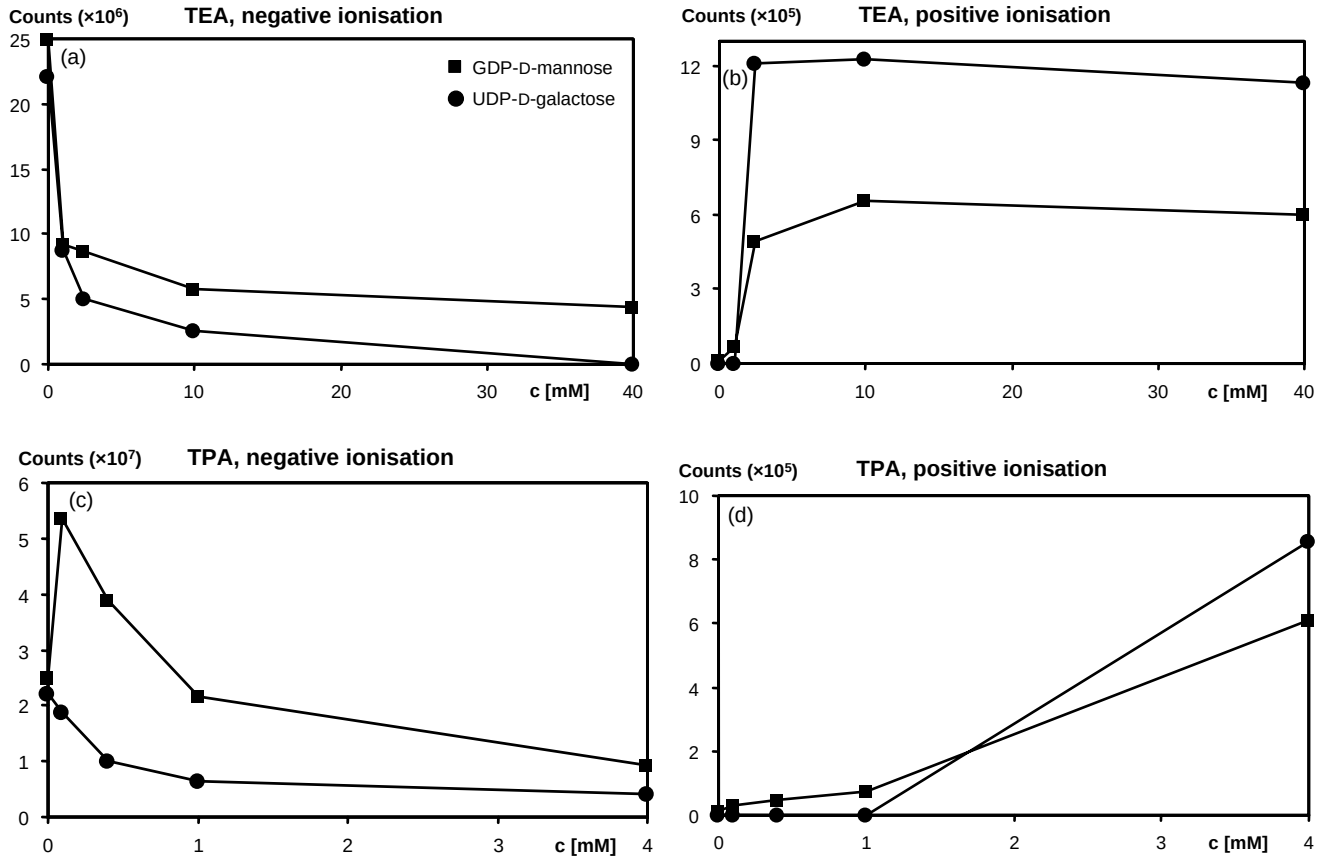

Fig. 3. Effect of ion-pair reagent concentration on signal intensity of $[M - H]^-$ ions (negative ion mode) and $[M + H]^+$ ions (positive ion mode) in ESI-MS of GDP-D-mannose and UDP-D-galactose. (a) TEA acetate buffer, negative ion mode; (b) TEA acetate buffer, positive ion mode; (c) TPA acetate buffer, negative ion mode and (d) TPA acetate buffer, positive ion mode.

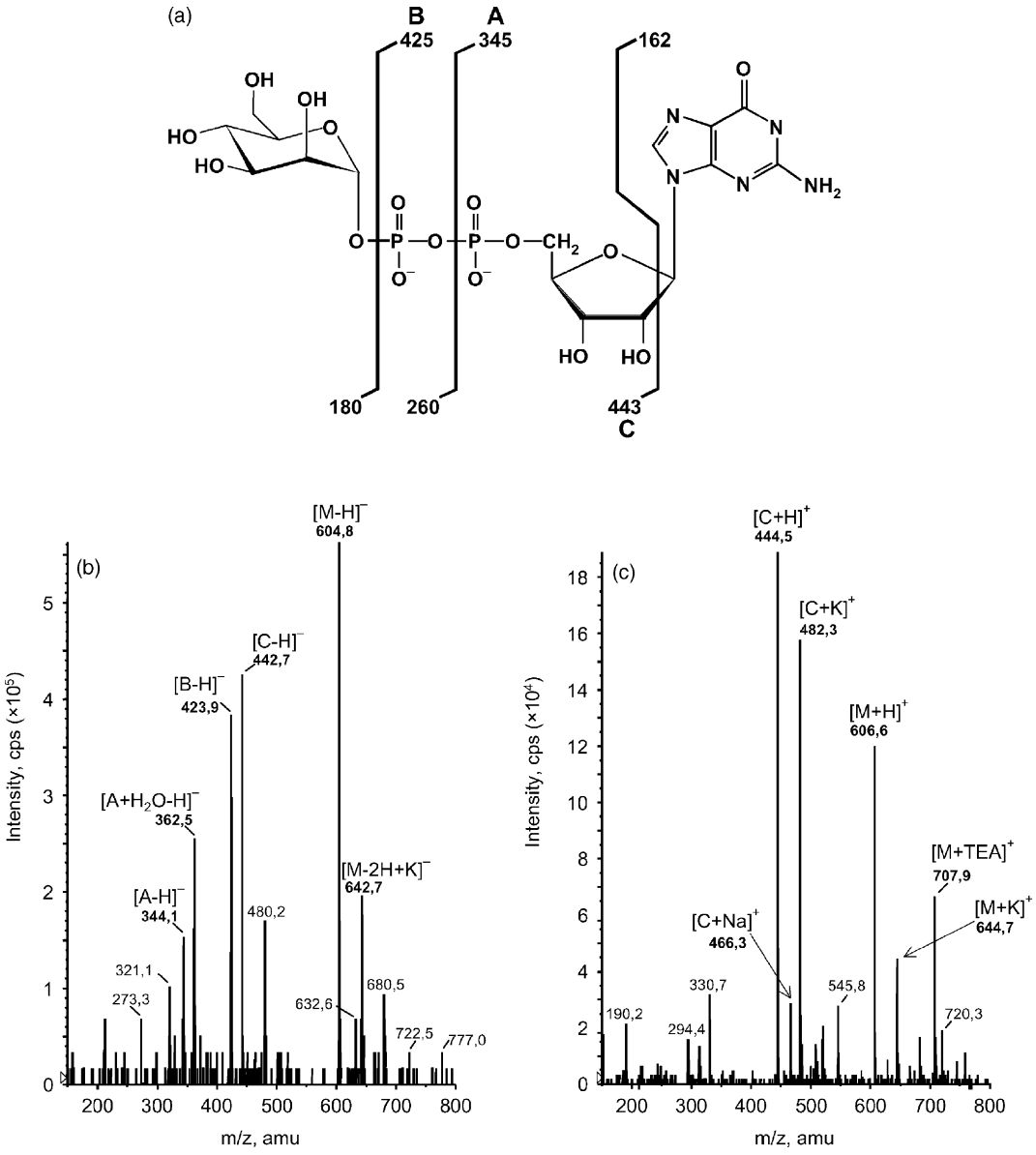

Fig. 4. ESI-MS spectra and proposed fragmentation of GDP-D-mannose (a) in 10 mM TEA acetate buffer; (b) negative ion mode and (c) positive ion mode.

aration (see above), concentrations above 4 mM were not tested for their ionization behavior. Altogether, ESI-MS in the positive ion mode was significantly less favorable. Signal intensities of the $[M + H]^+$ ion for UDP-D-galactose (**7**) in 10 mM TEA and 4 mM TPA were 47 and 21% of that obtained in the negative mode for the $[M - H]^-$ ion. For **6**, only 11 and 6.5% were reached. The poor ionization efficiency was not surprising, given that sugar nucleotides are preformed anions. Formation of $[M + H]^+$ ions necessitates protonation of anionic sites of the diphosphate residue, which is facilitated at higher buffer concentration.

The ESI-MS spectra of sugar nucleotides were analyzed. Representative findings are shown here for GDP-D-mannose (**6**) (Fig. 4). In the negative ion mode (Fig. 4b), the $[M - H]^-$ ion was the dominant species, accompanied by potassium or sodium adducts. Some diagnostic ions resulting from fragmentation at the diphosphate moiety (fragments A and B), or from elimination of the purine residue (fragment C) were observed. In the positive ion mode (Fig. 4c), the protonated molecular ion was accompanied by more abundant fragment ions obtained by elimination of the purine moiety. Potassium and TEA adducts were also observed. Given the more than 10-fold higher signal intensity and the occurrence of prominent molecular ions, the negative ion mode is to be preferred for quantitative analysis and for the analysis of unknown compounds.

The combined use of LC–MS and LC–NMR in the analysis of nucleotide sugar metabolism was explored with the biosynthesis of GDP-D-rhamnose. D-Rhamnose is a rare deoxyhexose which has been found so far only in bacteria [25]. It is the building block for A-band lipopolysaccharide (LPS), the major virulence factor

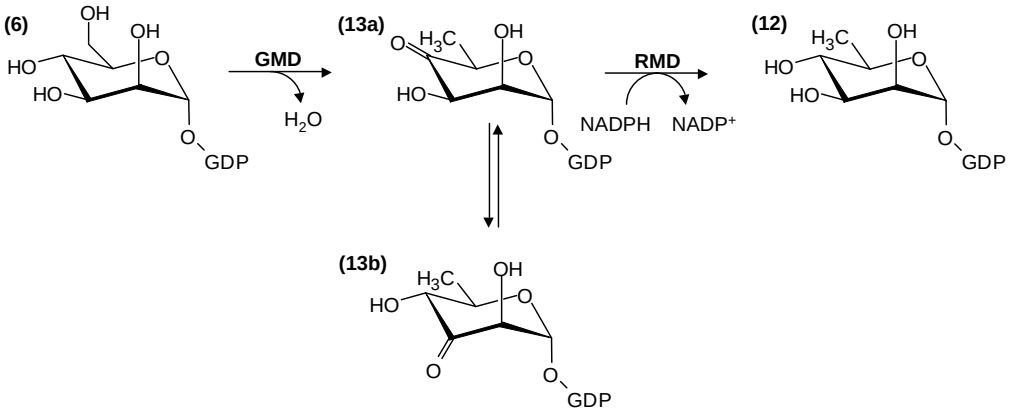

Fig. 5. Proposed pathway for biosynthesis of GDP-D-rhamnose (**12**) from GDP-D-mannose (**6**), via GDP-4-keto-6-deoxy-D-mannose (**13a**) which is in equilibrium with GDP-3-keto-6-deoxy-D-mannose (**13b**).

of *P. aeruginosa* involved in chronic lung infections of cystic fibrosis patients [20,26,27]. Key enzymes of D-rhamnose biosynthesis are promising targets for the development of novel anti-infective agents [28]. Biosynthesis of GDP-D-rhamnose starts from GDP-D-mannose which is dehydrated by GDP-D-mannose dehydratase (Gmd) to GDP-4-keto-6-deoxy-D-mannose. This intermediate is subsequently reduced by GDP-4-keto-6-deoxy-D-mannose reductase (Rmd) to GDP-D-rhamnose (Fig. 5) [29]. We, recently, cloned and characterized Gmd and Rmd from *P. aeruginosa* [18]. The HPLC method described was used for the structural characterization of intermediates obtained in the Gmd catalyzed reaction of GDP-D-mannose. Enzymatic conversion was monitored by direct injection of the deproteinized supernatants. UV detection at 254 nm revealed appearance of a peak (t_R: 19.8 min) which eluted

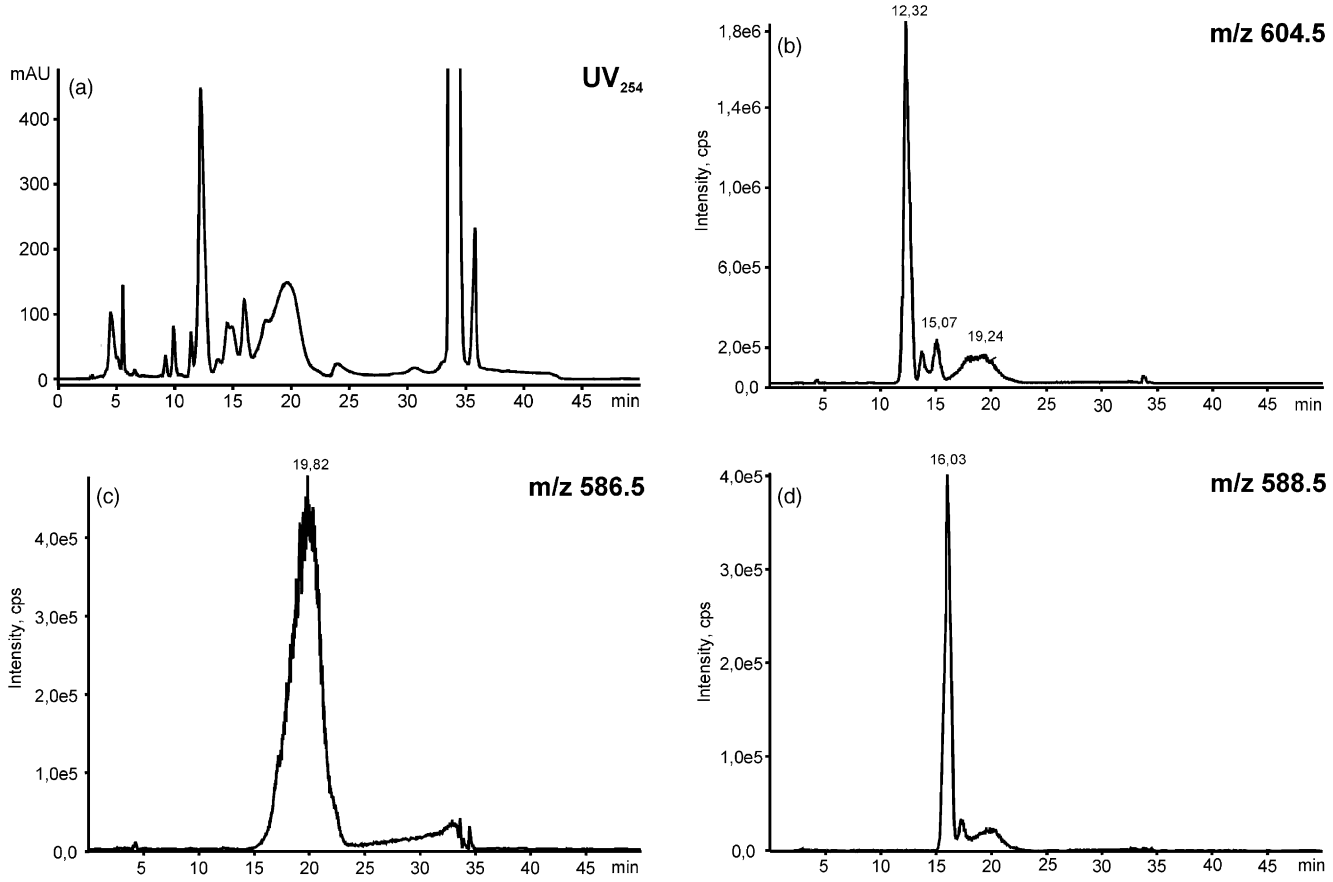

Fig. 6. HPLC analysis of reaction catalyzed by Gmd and Rmd. (a) Chromatogram recorded at 254 nm (standard reaction conditions, eluent 10 mM TEA acetate buffer, 20 °C); (b) ESI-MS trace (negative ion mode) at *m/z* 604.5; (c) at *m/z* 586.5 and (d) at *m/z* 588.5.

after GDP-D-mannose (t_R: 12.3 min) (Fig. 6a and b). The negative ion ESI-MS of the peak showed a $[M - H]^-$ ion at m/z 586.5 (Fig. 6c), as compared to m/z 604.5 for GDP-D-mannose (Fig. 6b), and was thus in accord with a GDP-keto-deoxyhexose. Mass spectra recorded over the entire peak width suggested peak homogeneity despite its unusually broad and asymmetric shape. This fact and earlier investigations on the biosynthesis of keto deoxysugars [30,31] suggested a possible co-occurrence of tautomers, which was clarified with the aid of LC–NMR experiments.

Successful use of LC–NMR relies strongly on a maximal suppression of the strong solvent signals which otherwise obscure the spectra of analytes [32,33]. An additional difficulty in the LC–NMR analysis of nucleotide sugars are the multiple resonances of the ion-pairing reagent which also have to be suppressed. To overcome this problem, modern solvent suppression sequence such as WET [19] are available. Advanced NMR software allows the automatic detection of LC-peak positions in the NMR spectra, using scout scan experiments. A scout scan experiment consists in a single NMR acquisition performed without solvent suppression in between sets of scans acquired with solvent suppression using the WET sequence. The frequency of the signals that have to be suppressed can be precisely measured by this means, taking into consideration shifts of the solvent frequencies related to changes in the eluent composition. Based on this information subsequent solvent and ion-pairing suppression is performed without user intervention and a selective peak shape for solvent suppression is calculated on the fly. If necessary, up to nine solvent signals can efficiently be suppressed by this means. This approach was used for the LC–NMR analysis performed in this study (Fig. 7).

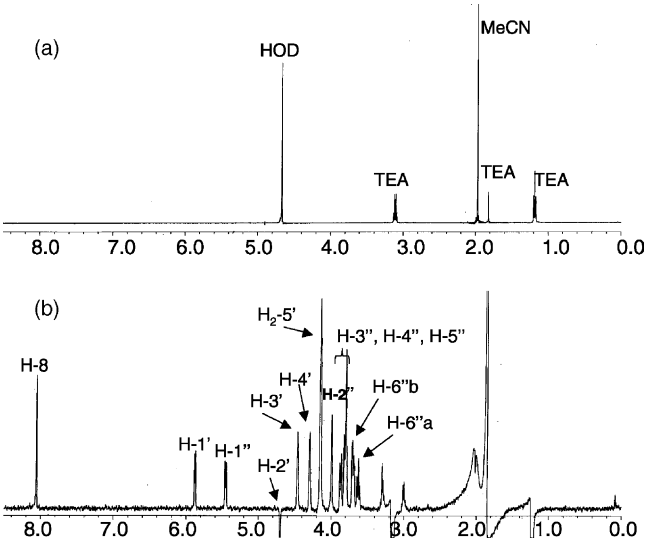

Fig. 7. Suppression of solvent and ion-pair reagent signals in LC–NMR spectrum. (a) Stop-flow spectra recorded without solvent suppression, solvent signals and ion-pair reagents hinder the detection of the signals of the analyte GDP-mannose and (b) stop-flow spectrum of GDP-mannose (200 μg, NT = 128) with suppression of all signals due to the LC eluent.

The Gmd catalyzed conversion product of GDP-D-mannose was deproteinized and desalted (see Section 2) and subjected to LC–NMR analysis in the stop-flow mode. Two resonances at 5.45 and 5.32 ppm were readily attributable to anomeric protons of keto deoxysugars, whereas the signal at 5.78 ppm was due to H-1′ of the ribosyl moiety (Figs. 8 and 9). NMR spectra acquired in the time slice mode over the entire peak width showed a constant ratio for the two anomeric protons (data not shown) and strongly suggested an equilibrium between the two tautomers **13a** and **13b** (Fig. 5). The presence of GDP-4-keto-6-deoxy-D-mannose and its 3-keto tautomer was corroborated by comparison with the GDP-mannose standard in the same conditions, a stop-flow COSY spectrum as well as a series of selective 1D-TOCSY experiments establishing the characteristic connectivity pattern of the two sugars (Fig. 9). As LC–NMR is known to be rather insensitive compared to other LC-hyphenated meth-

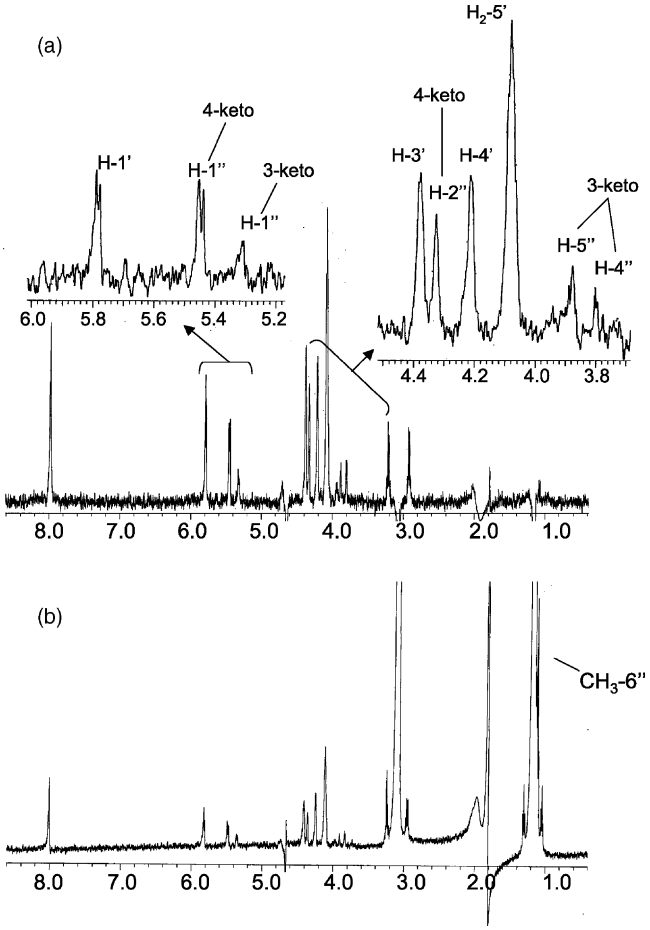

Fig. 8. Stop-flow LC–NMR spectra (NT = 128) of the peak at t_R 19.8 min, corresponding to GDP-keto deoxymannoses **13a** and **13b**. The spectrum was obtained after injection of 1 mg of the deproteinized enzymatic reaction mixture on-column. An expansion of the region of sugar protons is shown. (a) Stop-flow spectrum obtained with suppression of all signals due to the LC eluent and (b) stop-flow spectrum obtained with suppression of the signals due to HO^2H and MeCN only. In this latter spectrum the signal of CH_3-6″ was visible while it was suppressed together with the methyl signal of TEA in (a).

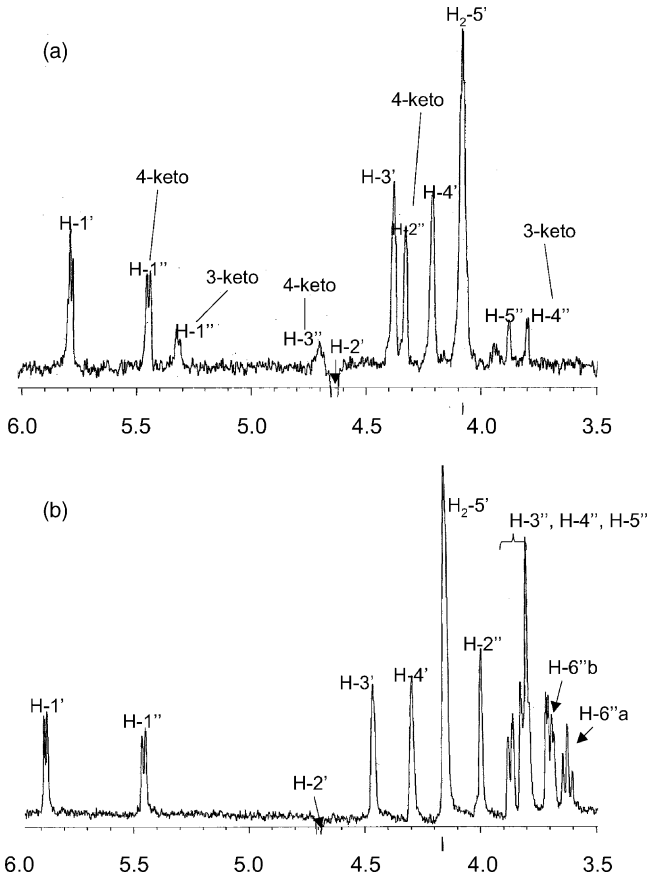

Fig. 9. Comparison of the stop-flow spectra of (a) GDP-keto deoxymannoses **13a** and **13b** (1 mg crude mixture, NT 128) with (b) GDP-mannose (200 μg pure standard, NT = 128).

ods, sensitivity issues are important to be assessed in this type of study. With the LC method described it has been possible to load up to 200 μg of each GDP standards on-column. This did not cause too much loss in LC resolution and LC–NMR spectra could be recorded in the stop-flow mode with a satisfactory S/N ratio with 128 transients. For these standards the corresponding LC peaks were eluting in about 0.5 ml of eluent. As the detection volume of the flow cell is 60 μl, it has been roughly estimated that the spectra were measured on about 40–80 μg of standards in the flow-cell. In the case of GDP-keto deoxymannoses **13a** and **13b** the LC peak was considerably tailing and an injection of 1 mg of the desalted and deproteinised Gmd-catalyzed-conversion products was necessary for LC–NMR. The corresponding LC peak was eluting in 3 ml of eluent and thus about 20–40 μg of the mixture of **13a/13b** were detected in the different time-slice experiments recorded throughout this broad LC peak.

4. Conclusions

A generally applicable method analysis of sugar nucleotides by HPLC coupled to DAD, ESI-MS or NMR detection on-line was established. Conditions for ion-pair chromatography were selected and optimized for separation and compatibility with the various detectors. A mixture of 11 of the most important sugar nucleotides was used for optimization. In the case of nucleotide sugar analysis, the nature and concentration of the ion-pairing reagents were a compromise of chromatographic separation, ionization efficiency, and satisfactory signal suppression. In the negative ion mode, ESI-MS spectra provided information on molecular mass and several diagnostic fragment ions. The example of GDP-rhamnose biosynthesis demonstrated the usefulness of ESI-MS for the monitoring of enzymatic conversions. Reaction mixtures could be analyzed without need for laborious workup, and the mass-selective analysis enabled discrimination of potentially co-eluting co-factors [13] which are required for enzymatic reactions.

LC–NMR has emerged as powerful tool for structural studies of unknown analytes in mixtures [34] and has been successfully applied in combinatorial synthesis, drug metabolism [35], chemical profiling of plant extracts [36], in the analysis of interconverting labile natural products [37], and in some scarce biosynthetic applications [38,39]. The study of the GDP-D-rhamnose pathway as described here is original. It demonstrates the potential of LC–NMR for natural products biosynthetic studies, in particular for proving the presence of an equilibrium between two tautomeric keto sugars forms. Whereas most LC–NMR applications have been in $^{2}H_2O$–acetonitrile eluents and with "spectroscopically silent" inorganic buffers or trifluoroacetic acid [35], the present example showed that multiple resonances from organic ion-pair reagents such as TEA acetate buffer can be successfully suppressed.

The method will be applied to other important steps in the biosynthesis of nucleotide sugars and intermediates, and to the study of transferase reactions leading to oligo and polysaccharide moieties.

Acknowledgements

Financial support was provided by DFG grant RA 596/2-1 (to M.R.), and by the Fonds der Chemischen Industrie (to M.H.).

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