Nuclear Magnetic Resonance Spectroscopy and Reversed-Phase High-Performance Liquid Chromatography of Peracetylated Digalactosyldiacylglycerols

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ABSTRACT: A simple method for the peracetylation of digalactosyldiacylglycerols is described. The peracetylated compounds were isolated by thin-layer chromatography and subjected to nuclear magnetic resonance spectroscopy for structure elucidation. The proton signals at acetylated sites are shifted downfield compared to the nonderivatized compounds, resulting in improved resolution. The peracetylated compounds were also subjected to molecular species analysis by reversed-phase high-performance liquid chromatogrphy. The chromatography behavior of the peracetylated compounds was similar to that of triacylglycerols, displaying narrow and symmetrical peaks yielding a highly resolved molecular species profile.

Paper no. J9445 in *JAOCS 77,* 757–761 (July 2000).

KEY WORDS: Digalactosyldiacylglycerols, molecular species, nuclear magnetic resonance spectroscopy, peracetylation, reversed-phase high-performance liquid chromatography.

Digalactosyldiacylglycerol (DGalDAG, 1,2-diacyl-3-*O*-[α-D-galactopyranosyl-(1->6)-*O*-β-D-galactopyranosyl]-*sn*-glycerol) is a polar lipid found in plants. The amphiphilic nature of DGalDAG makes it a common membrane component, especially in photosynthetic tissues. It is also present in several seeds such as oats, rye, and wheat (1–3). The amphiphilic nature enables the preparation of artificial spherical structures called liposomes. It has been demonstrated that lipid mixtures containing high amounts of DGalDAG are suitable for pharmaceutical formulation work (4). The full potential of liposomes based on DGalDAG has not yet been fully investigated.

The physical properties of DGalDAG depend partly on the molecular species composition, which therefore is desirable to monitor (5). Reversed-phase high-performance liquid chromatography (RP-HPLC) is a common method for separating lipid molecular species (6–8). The hydrophobic character of the stationary phase is well suited for lipid acyl moieties in a liquid-liquid partition process. The polar headgroup of DGalDAG is not likely to interact with the hydrophobic stationary matrix. However, the hydroxyl groups on the polar head may interact with nonderivatized silanol groups on the stationary phase, thereby being retained on the column in an adsorption process. This retention behavior increases band-broadening, causing poor resolution of molecular species. Fewer interactions between DGalDAG and the silanol groups would occur if the polarity of the polar headgroup could be decreased, thus improving the resolution between molecular species.

^{1}H nuclear magnetic resonance (NMR) spectroscopic data of DGalDAG have been reported in a number of studies (9–12). However, the signals from many of the protons in the glycerol and galactosyl moieties appear in a narrow range of the spectrum, resulting in overlapping signals.

The addition of acetyl groups to the hydroxyls of the polar headgroup of DGalDAG should reduce polar interactions that occur in RP-HPLC. Furthermore, additional structural information can be obtained by NMR spectroscopy.

The peracetylated compound gains the characteristics of a triacylglycerol, a feature that previously has been used in conjunction with pancreas lipase in order to release the fatty acids in the *sn*-1 position of DGalDAG (13). Peracetylation of glycoglycerolipids has also been done prior to molecular species separation on silver ion impregnated thin-layer plates (13). Acetylation of deacylated analogs of glycerogalactolipids has been done to determine the structure of the carbohydrate moiety by NMR (14). Peracetylation has also been used for structural determination of the polar headgroup of other glycolipids (15,16).

This paper describes a simple procedure for the peracetylation of DGalDAG, the structural elucidation of the products with NMR spectroscopy, and the molecular species separation of the derivatives by RP-HPLC.

EXPERIMENTAL PROCEDURES

Materials. A fraction from oat containing *ca*. 60% DGalDAG was obtained from Scotia LipidTeknik AB (Stockholm, Sweden). Part of this material was further purified (>99%) by a method previously described (17), and used as a standard. Acetic anhydride, acetic acid, and pyridine were purchased

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from Merck (Darmstadt, Germany). Hexane, chloroform, methanol, acetonitrile, and isooctane were of high-performance liquid chromatography (HPLC) grade from Merck. Ethanol was from Kemetyl (Stockholm, Sweden). Water was ultrapurified on Nanopure equipment (Barnstead, Dubuque, IA). Water-d_2, methanol-d_4, chloroform-d_1, and DMSO-d_6 were from Aldrich (Milwaukee, WI).

Derivatization procedures. To determine conditions for the derivatization, the DGalDAG standard (5 mg) was dissolved in pyridine (0.5 mL) in a test tube. Acetic anhydride (5 μL) was added to the solution. The mixture was shaken for 1 min and held at 60°C for 24 h. The reaction was followed by thin-layer chromatography (TLC). Aliquots (50 μL) were drawn from the reaction mixture at 1, 20, 60, 300 (5 h), and 1440 (24 h) min, respectively. Isooctane (50 μL) was added to each aliquot, followed by aqueous acetic acid (100 μL, pH 4). The mixture was thoroughly shaken and allowed to separate into two layers. The upper layer was isolated for TLC analysis.

For semipreparative purposes, the DGalDAG-containing fraction (100 mg) was dissolved in pyridine (2 mL) and acetic anhydride (100 μL) was added. The mixture was thoroughly shaken for 1 min and held at 60°C for 24 h. Isooctane (1 mL) and aqueous acetic acid (2 mL, pH 4) were added, and the mixture was shaken and separated by centrifugation. The upper layer, containing the peracetylated crude lipid material, was transferred to a test tube for further purification.

TLC. The peracetylation reaction progress was monitored by TLC. Aliquots collected as described above were applied on a 10 × 10-cm silica gel plate (Merck). Development of the plate was completed using a mobile phase of hexane/chloroform/methanol/water/acetic acid (60:31:8:0.5:0.5, by vol), and detection was done with iodine vapor.

Peracetylated DGalDAG was isolated from the acetylated crude lipid material by means of preparative TLC. A solution (100 μL) containing *ca.* 80 mg/mL of acetylated material was applied as a band to a silica gel plate (20 × 20 cm with a 5-cm concentration zone; Merck). The plate was developed using hexane/chloroform/methanol/water/acetic acid (60:31:8:0.5:0.5, by vol). The peracetylated DGalDAG in the mixture was tentatively identified by using peracetylated DGalDAG standard, obtained as described above. The band representing the peracetylated product was scraped off the plate and extracted twice with 10 mL chloroform/methanol (1:1, vol/vol). The procedure was repeated seven times, and the extracts were pooled for further analysis.

NMR spectroscopy. The peracetylated DGalDAG isolated from the preparative TLC step and nonderivatized DGalDAG were subjected to NMR analysis.

DGalDAG standard (17 mg) was dissolved in chloroform-d_1 (400 μL) and methanol-d_4 (400 μL). Peracetylated DGalDAG (10 mg) was dissolved in chloroform-d_1 (500 μL) and methanol-d_4 (500 μL). The sample solutions were transferred to 5-mm (o.d.) NMR tubes.

One-dimensional ^{1}H- and ^{13}C-spectra and two-dimensional (2D) ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) spectra were recorded at 25°C on a 500 MHz spectrometer from Bruker (DMX-500; Karlsruhe, Germany). In the HMQC experiments, the delay for creation of antiphase magnetization was set to 3.45 ms, corresponding to a $^1J_{CH}$ of 145 Hz, the delay for inversion recovery in the BIRD sequence to 0.6 s and the relaxation delay to 4 s. The data were processed with cosine-squared functions in both dimensions, and expressed in magnitude mode.

Molecular species determination. Analysis of the molecular species of peracetylated DGalDAG was performed by utilizing RP-HPLC. The HPLC equipment consisted of a Shimadzu SCL-6A system controller and two Shimadzu LC-6A HPLC pumps (Kyoto, Japan). Detection was done with a light-scattering detector from SEDERE (Sedex Model 45; Cergy, St. Christophe, France) with the nebulizer pressure set to 1.5 atm and nebulizer temperature set to 60°C. Gradient elution was done over 80 min on an octadecylsilane column (Kromasil, 250 × 4.6 mm, 5 μ, 100 Å) from Eka Nobel (Bohus, Sweden). The column was held at constant temperature by a water jacket at 30°C.

The A-eluant was composed of acetonitrile/methanol/ethanol/water/ isooctane (78:7:7:7:1, by vol), and the B-eluant consisted of acetonitrile/ethanol/isooctane (40:35:25, by vol). The solvent system was optimized by using factorial design and partial least squares (PLS) models. Data from previous molecular species elution systems (6,18) generated a single "scouting run" which was used as the base in a factorial design that generated nine experiments in which solvent composition and column temperature were varied on two levels. The experiments were evaluated by their molecular species resolutions, total analysis times, and peak widths. The best experiment was chosen as the basis for a full-factorial fine-tuning design, again varied at two levels, which generated nine additional experiments.

Chromatographic data from all experiments (9 + 9) were used to build a prediction model for the optimal eluant composition according to the PLS strategy previously described (19).

RESULTS AND DISCUSSION

TLC screening of the derivatization progress showed that 24 h at 60°C was satisfactory to obtain complete peracetylation of DGalDAG, and the chromatographically purified reaction product (24 mg) was used for NMR and HPLC analysis.

In the 2D spectra obtained from the HMQC experiments, each one-bond ^{1}H-^{13}C coupling is represented by a cross peak. Thus, for the DGalDAG spectrum (Fig. 1), the signal at ^{13}C-shift 70.8 ppm and ^{1}H-shift 5.11 ppm corresponds to the *sn*-2 position in the glycerol.

Unfortunately, many of the proton signals from the galactose and the glycerol moieties in DGalDAG appear in a rather narrow shift range between 3.5 and 3.7 ppm. For structural elucidation with NMR, this makes signal assignments difficult since most correlation experiments use proton signals in at least one dimension. However, in the peracetylated material protons at acetylated sites in the galactosyl, moieties are

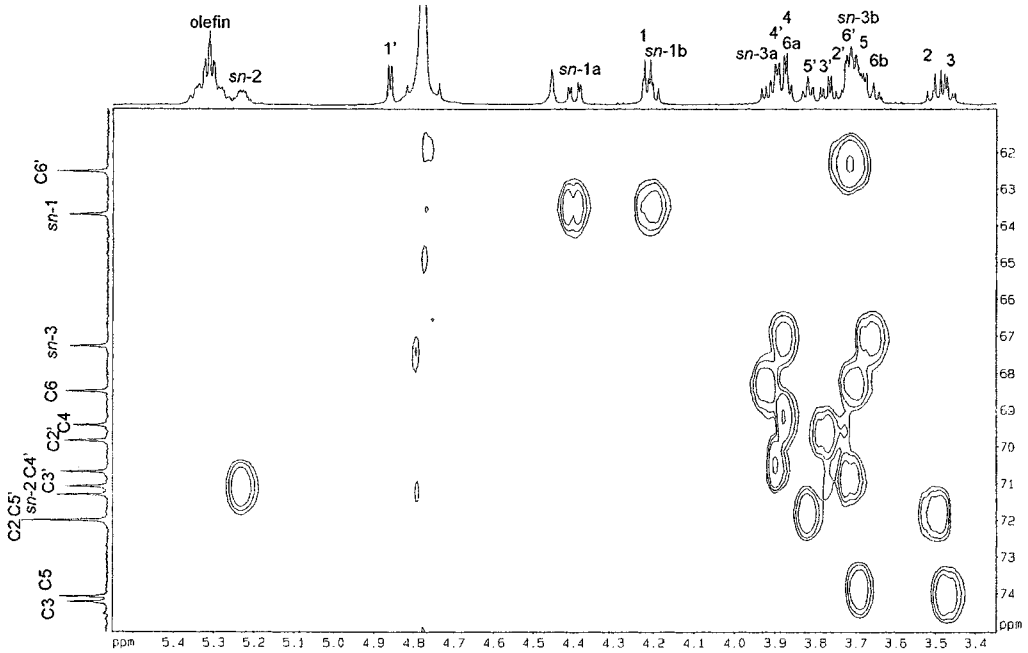

FIG. 1. Detail from the heteronuclear multiple quantum coherence (HMQC) spectrum of digalactosyldiacylglycerol (DGalDAG), showing signals from the galactosyl and glycerol moieties. ^{13}C signals from the anomeric carbons (C1, C1′) are outside the displayed range (δ 101.9 and δ 97.0 ppm, respectively).

shifted downfield, leading to improved resolution between the proton signals (15).

The results from the HMQC experiment on peracetylated DGalDAG (Fig. 2), together with interpretation of ^{1}H-coupling patterns, and comparisons with data on DGalDAG in earlier investigations (9), make it possible to obtain complete assignments for the signals from the glycerol and galactosyl moieties (Table 1).

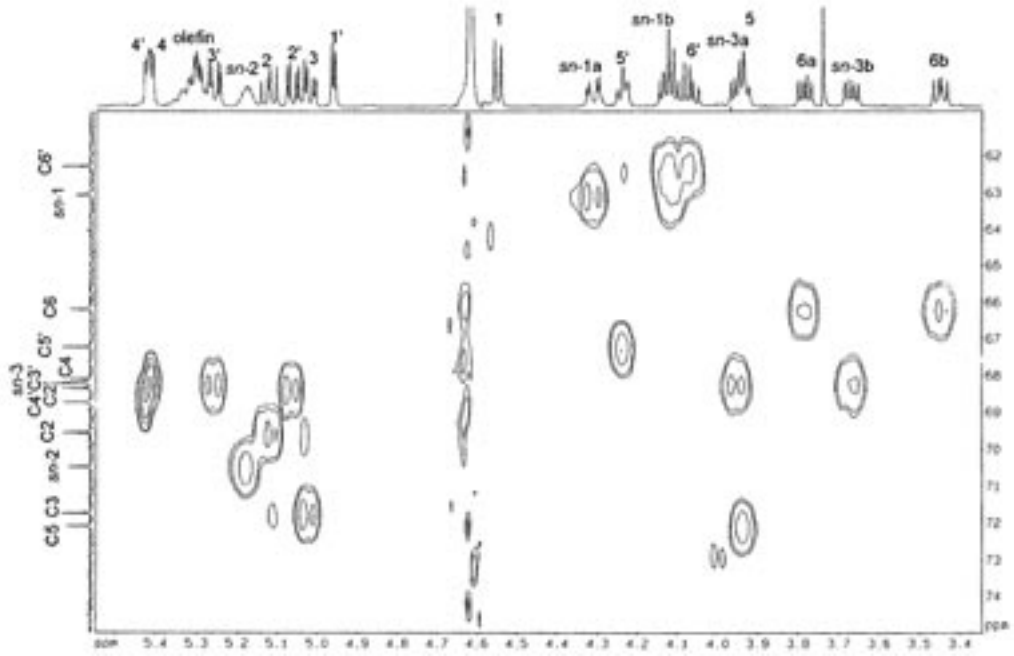

FIG. 2. Detail from the HMQC spectrum of peracetylated DGalDAG, showing signals from the galactosyl and glycerol moieties. ^{13}C signals from the anomeric carbons (C1, C1′) are outside the displayed range (δ 102.1 and δ 97.2 ppm, respectively). See Figure 1 for abbreviations.

TABLE 1
Assignment of Signals and Differences in Chemical Shifts of Glycerol and Galactosyl Moieties in ^{1}H and ^{13}C NMR Spectra of DGalDAG and Peracetylated DGalDAG

	^{1}H chemical shift δ (ppm)[a]			^{13}C chemical shift δ (ppm)[b]		
Signal	DGalDAG	Peracetylated DGalDAG	Δδ	DGalDAG	Peracetylated DGalDAG	Δδ
Glycerol moiety						
sn-1a	4.40	4.31	–0.09	63.7	63.1	–0.6
sn-1b	4.20	4.12	–0.08			
sn-2	5.23	5.17	–0.06	71.3	70.5	–0.8
sn-3a	3.92	3.95	0.03	68.5	68.2	–0.3
sn-3b	3.70	3.67	–0.03			
Galactosyl moiety (inner)						
CH (1)	4.21	4.54	0.33	104.9	102.1	–2.8
CH (2)	3.50	5.12	1.62	72.0	69.6	–2.5
CH (3)	3.48	5.02	1.54	74.2	71.7	–2.5
CH (4)	3.88	5.41	1.53	69.4	68.1	–1.3
CH (5)	3.69	3.94	0.25	74.1	72.1	–2.0
CH (6a)	3.88	3.78	–0.10	67.3	66.2	–1.1
CH (6b)	3.66	3.45	–0.21			
Galactosyl moiety (outer)						
CH (1′)	4.86	4.95	0.09	100.3	97.2	–3.0
CH (2′)	3.73	5.06	1.33	71.1	68.4	–2.7
CH (3′)	3.77	5.25	1.48	69.8	68.2	–1.6
CH (4′)	3.90	5.42	1.52	70.7	68.7	–2.0
CH (5′)	3.82	4.24	0.42	72.0	67.2	–4.8
CH (6′a)	3.72	4.11	0.39	62.5	62.3	–0.2
CH (6′b)	3.72	4.08	0.36			

[a]The signal at δ 3.30 ppm from residual partially deuterated methanol (CD_2HOD) was used as shift reference.
[b]The signal at δ 49.0 ppm from methanol-d_4 was used as shift reference. NMR, nuclear magnetic resonance; DGalDAG, digalactosyldiacylglycerol.

Integrating the signal from the methyl in the acyl groups (δ_H = 0.86 ppm) and the signal from one of the protons in the *sn*-1 position (δ_H = 4.32 ppm) and calculating the ratio (6.6) show that two acyl groups are present. The proton spectrum shows seven signals from methyl protons at acetyls with a total integral ratio to the signal at 4.32 ppm of 21.0, thereby proving that all hydroxyl sites in the digalactosyl moiety have been acetylated.

The coupling constants of the anomeric doublets ($^{3}J_{1,2}$ = 8 and 4 Hz, respectively), their ^{1}H-shifts (4.54 and 4.97 ppm), and ^{13}C-shifts (101.9 and 97.0 ppm) confirm that the inner galactose unit is a β-anomer and the outer is an α-anomer. The coupling constants for the protons at C4 and C4′ ($^{3}J_{3,4}$ = 3 Hz and $^{3}J_{4,5}$ = 1 Hz) suggest equatorial configuration at this position, whereas $^{3}J_{HH}$ coupling constants for other ^{1}H-^{1}H pairs in the sugar moieties are in the range 6–11 Hz, thus suggesting axial configurations and confirming the assumed digalactose structure.

Peracetylation reduces the polarity of the DGalDAG and eliminates the risk of unwanted adsorption interactions with the stationary phase in RP-HPLC, leaving the partition process unperturbed. Thus, the chromatographic conditions for molecular species separation of the acetylated compounds resemble those of the triacylglycerols.

The elution of acylglycerol molecular species in reversed-phase systems is roughly in the order of increasing partition number (PN). The PN can easily be calculated by the formula PN = CN – 2*n*, where CN is the total number of carbons in the acyl moieties and *n* is the total number of double bonds (20). The elution order within the PN groups depends largely on the mobile-phase composition (6).

Mobile phases containing high portions of alcohols and/or water tend to accelerate the elution of molecular species with low unsaturation, giving an elution order by increasing unsaturation within each PN group. This is the case in the system used for the intact DGalDAG as described earlier (6).

Mobile phases containing significant amounts of acetonitrile or propionitrile and low or no amounts of water will, however, reverse this internal order, and molecular species with high unsaturation will be eluted first in the PN group (21). The molecular species present in oats have previously been identified (6). Since the mobile phase used in this study has a high content of acetonitrile, the elution order is tentatively given as 18:3/18:2, 18:2/18:2, 18:2/18:1, 18:2/16:0, 18:1/18:1, 18:1/16:0, and 16:0/16:0. The chromatogram is shown in Figure 3.

When a comparison is made between intact DGalDAG and their peracetylated analogs (Table 2), an improvement is noticed both with respect to peak width and peak resolution, important features when analyzing complex mixtures such as molecular species of lipids. The fact that the peak widths for most molecular species are almost halved enables better means of quantification and improves the limit of detection. Unfavorable adsorption effects between underivatized sites

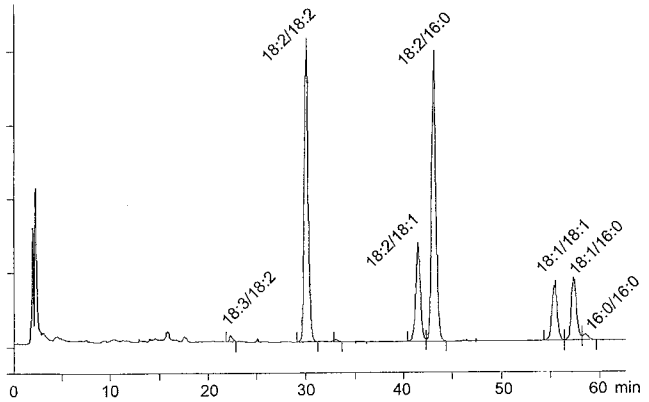

FIG. 3. Reversed-phase high-performance liquid chromatography of peracetylated DGalDAG with tentative assignments of molecular species. See Figure 1 for abbreviation.

on the stationary phase and the polar headgroups on the elutes have thus been reduced.

ACKNOWLEDGMENTS

Part of this work has previously been presented at the AOCS meeting in San Antonio, Texas, May 1995.

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TABLE 2
Chromatographic Characteristics in the HPLC Analysis of DGalDAG and Peracetylated DGalDAG

	DGalDAG	Peracetylated DGalDAG
Average peak width[a]	1.61	0.86
Average resolution factor	2.7	7.0
Total analysis time	54 min	58 min
Effective analysis time	27–54 min	26–58 min

[a]Obtained from the tangents at the inflection points of the peaks. HPLC, high-performance liquid chromatography. See Table 1 for other abbreviation.

[Received November 5, 1999; accepted June 3, 2000]