Glycerol Derivatives of Cutin and Suberin Monomers: Synthesis and Self-Assembly

Jean-Paul Douliez*
INRA, rue de la Géraudie, 44316 Nantes, France

Joël Barrault and François Jerome
Laboratoire de Catalyze en Chimie Organique, CNRS, 40 av. du recteur Pineau, 86022 Poitiers, France

Antonio Heredia
Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, Campus Teatinos, E-29071 Málaga, Spain

Laurence Navailles and Frédéric Nallet
Centre de Recherche Paul-Pascal/CNRS, 115 Avenue Albert-Schweitzer, 33600 Pessac, France

Received October 25, 2004; Revised Manuscript Received November 18, 2004

Glycerol derivatives of cutin and suberin monomers were synthesized by acid catalysis. Their dispersion in an aqueous solution was examined by phase contrast microscopy, neutron scattering, and solid state NMR. It is shown that the phase behavior strongly depends on the nature of the derivatives forming either lumps of aggregated membranes or well dispersed membranes.

Introduction

Cutin and suberin are insoluble plant biopolymers located at the periphery of organs, the so-called "plant skin".1 They are constituted by inter-esterified hydroxyalkanoic acids with palmitic and stearic chain length and glycerol which contribute to the polyester structure.2,3 Whereas the role of these polymers as plant protecting actors is rather well defined,1 several basic questions remain to be elucidated. This includes the understanding of the routing of the monomers from the cell where they are synthesized to the extracellular location where they are polymerized and the effect of monomers which are released by hydrolysis upon pathogen attacks. To date, the use of fatty acids for in vivo and in vitro experiments required to answer these questions is hampered by their weak solubility in water, that is their bio-availability. It then appears of strong importance to determine the phase behavior of these monomers in aqueous solution and which are the relevant physicochemical parameters involved for their full dispersion. The phase behavior of non-hydroxyl fatty acids has already been studied, and it was shown that they can form vesicles depending on the pH and the concentration.4–7 Bilayer formation occurs at a pH around the pKₐ of the fatty acid carboxylic group suggesting that hydrogen bonds between the carboxylic and carboxylate groups are determinant. Incorporation of alcohol,5 alkylamine,8 or cholesterol9,10 has been shown to promote the formation of membranes. Of particular interest, monoglycerides which are known to form lumps in solution11 were also shown to form bilayers and vesicles in mixture with fatty acids.12 We expect from those recent results obtained on monoglycerides/fatty acid systems12 that full dispersions of cutin and suberin monomers can be achieved at basic pH and upon mixing with their monoglyceride derivatives. Showing this essential property is the aim of the present communication in which phase contrast microscopy, Fourier transform infrared spectroscopy (FTIR), small angle neutron scattering (SANS), and deuterium solid-state NMR are used to study the phase behavior of glycerol derivatives of cutin and suberin monomers, pure as well as in mixtures.

Materials and Methods

Lipids. Hexadecanoic acid (MW: 256), sn-1-monoheptanoyl-rac-glycerol (MW: 330), ω-hydroxyl hexadecanoic acid (MW: 272), α, ω hexadecadienoic acid (MW: 286) and glycerol (MW: 92) were from Sigma-Aldrich. Cutin monomers (dihydroxy hexadecanoic acid) were extracted as reported elsewhere.13

Synthesis. Glycerol derivatives were obtained by acid catalysis. Fatty acids were dissolved in tetrahydrofuran (THF) at a concentration of 50 mg/mL under magnetic stirring for 10 min at 60 °C. Glycerol or d₅-glycerol (Sigma-Aldrich) at a 10 molar excess and 0.1% of methyl sulfonic acid (msa, Sigma-Aldrich) were added, and the reaction was allowed to proceed at 60 °C. The progress of the glycerol ester formation was followed by reverse phase liquid chromatography (RP-HPLC) as a function of time. The monoglyceride derivatives were further purified by phase partitioning. Ten volumes of water, chloroform, and methanol were added to the product of the reaction. After vigorous agitation and an overnight period of rest, the subphase was
recovered, dried under a stream of nitrogen, hydrated with a minimum of water, and then freeze-dried.

**Lipid Dispersions.** For lipid mixtures, each of the components were weighed exactly and dissolved in methanol/chloroform (1/1 v/v). The solvent was then dried with a stream of nitrogen, and the lipid mixture was hydrated with a minimum of water and then freeze-dried. Then, lipids were dispersed in a Tris pH 7.5 20 mM buffer solution at a total lipid concentration of 10 mg/mL. Because of the weak sensibility of NMR, samples with d5-glycerol were prepared at 50 mg/mL. All mixtures were submitted to 3 cycles of heating plus vigorous vortexing (60 °C, 10 min) and cooling (−20 °C, 30 min) and were stored at −20 °C. Prior to use, each sample was heated at 60 °C for 10 min.

**HPLC.** The chromatography analysis was performed using a solvent delivery system (Waters, Milford, MA) equipped with a 717 plus auto-sampler controlled by the Waters-Breeze software. The column was a Symmetry C18 (5 μm, 4.5*150 mm) from Waters. It was connected to a light scattering detector (Sedex55, SEDERE, France) and a refractive index detector 133 (Gilson). According to ref 14, the mobile phase was constituted by acetonitrile (ACN)—water (0.1% trifluoro acetic acid (TFA)) in various proportions depending on the lipid mixtures to be analyzed. The elution was done at a constant flow rate of 1 mL/min.

**Mass Spectrometry.** Mass measurements were performed with an ion trap mass spectrometer (LCQ Advantage, Thermo-Finnigan, San Jose, USA) equipped with an electrospray ionization source (ESI). Samples were dissolved in 500 μL of ethanol:dichloromethane (1:1, vol/vol), and 10 μL of a 25% NH4OH solution (Carlo Erba reagents) was added prior the injection. They were infused into the mass spectrometer at a continuous flow rate of 5 μL/min. The instrument was operated in the positive ion mode. Mass data were recorded on the range 100–1000 M/z using the X-Calibur v. 1.3 software.

**Phase Contrast Microscopy.** Observations were made at 20× magnification using an optical microscope in the phase contrast or interferential contrast mode (Nikon Eclipse E−400, Tokyo, Japan) equipped with a 3-CCD JVC camera allowing digital images (768 × 512 pixels) to be collected. Unless otherwise mentioned, a drop of the lipid dispersion (about 5 μL) was deposited on a glass slide surface (76 × 26 × 1.1 mm, RS France) and immediately covered with a homemade cover slide (22 × 22 mm, Menzel-Glaser, Germany). The cover slides were cleaned with ethanol and acetone.

**Solid-State NMR.** Deuterium solid-state NMR experiments were performed at several temperatures from 90 to 350 K on a 400 MHz Bruker spectrometer operating at 61 MHz for deuterium using a static double channel probe, the sample coil being adapted to load a 7 mm rotor such as those used for magic angle spinning probes equipped with a stretched stator. Typically, lipid dispersions were previously heated at 60 °C and 700 μL was transferred in the rotor which was sealed and then end-capped. A Hahn quadrupolar echo sequence15 was used with an inter pulse delay of 40 μs. Eight k points in 10k accumulations (every 2 s) were done with a 90° pulse and spectral width of 8 μs and 250 kHz, respectively. Free induction decay signal were zero-filled to 16k points prior to Fourier transform after a broad line exponential multiplication of 200 Hz.

**Fourier Transform Infrared Spectroscopy.** Fourier transformed infrared spectra (250 scans) were recorded as a function of increasing temperature at a resolution of 1 cm−1 on a Nicolet Magna IR 550 spectrometer. After heating to 70 °C, a drop (100 μL) or a lump of the sample was deposited on the crystal and then covered with a homemade cup allowing the hydration to be controlled. Signals were obtained by attenuated total reflection (ATR) using a single reflection accessory fitted with a thermostated diamond crystal.

**Small Angle Neutron Scattering (SANS).** Experiments were performed at Laboratoire Léon-Brillouin (laboratoire mixte CEA/CNRS) (Saclay, France) on spectrometer PAXY. The neutron beam was collimated by appropriately chosen neutron guides and circular apertures, with a beam diameter at the sample of 7.6 mm. The neutron wavelength was set to 4 or 8 Å with a mechanical velocity selector (Δλ/λ ≈ 0.1), the 2D detector (128 × 128 pixels, pixel size 5 × 5 mm2) being positioned at 1.4 or 6.7 m, respectively. The scattering wave vector, Q, then ranges from typically 0.006 to 0.5 Å−1, with a significant overlap between the two configurations. The samples, prepared with deuterated water, were held in flat quartz cells with a 2 mm optical path and temperature controlled by a circulating fluid to within ±0.2 °C. The isotropically averaged spectra were corrected for solvent, cell and incoherent scattering, as well as for background noise.

**Results and Discussion**

**Synthesis and Characterization.** Glycerol derivatives of ω-hydroxy hexadecanoic acid, 1, and α,ω-hexadecanedioc acid, 2, were synthesized. The chemical structure of the compounds and the products of the reaction are displayed Figure 1 together with the chromatograms as obtained by HPLC. Compound 1 was eluted at 16 min in ACN/water (60/40 v/v). The product of the reaction, 3, produced two peaks (Figure 1) in a similar elution pattern than for palmitin.14 The first peak (8%) was assigned to the sn-2-omegaxyhexadeconol-glycerol, whereas the second to the rac-sn-1 and sn-3 isomers.14 In the present case, both isomers are equivalent. There was no peak at a time corresponding to the elution of pure 1 indicating that all of the initial fatty acids reacted to give the glycerol derivatives. The reaction time did not exceed 2 h after which all the initial compounds were transformed in compound 3. This reaction time is markedly lower than in the case of the carbodiimide activated reaction.14 This reveals that acid catalysis is more appropriate for an eventual further valorization of such glycerol derivatives. The product of the reaction was submitted to mass spectrometry (Figure 1). The expected molecular weight, M, for 3 is 272 + 92 – 18 = 346 g/mol, where 272 and 92 are the molecular weights of the fatty acid and glycerol, respectively, and 18 stands for the loss of water upon the esterification reaction. The spectrum is composed of 2 main peaks at 347 and 364 which correspond to the masses M + 1 and M + 18. This latter peak stands from the ammonium ion of product 3.
In the same way, compound 2 was eluted in ACN/water (60/40, v/v) as a single peak at 13 min (Figure 1). This retention time is lower than for 1 as expected because of the higher polarity induced by the second carboxylic group compared to the ω-hydroxyl. In this case, there exists three possible isomers for the product of the reaction, 4, because two glycerol units are esterified. These are the sn-1,sn-1-monohexadecanediol-rac-glycerol (1/1), sn-2,sn-2-monohexadecanediol-glycerol (2/2), and the sn-1,sn-2-monohexadecanediol-rac-glycerol (1/2). The product of this reaction yielded two peaks at 6.5 and 7 min (Figure 1) and were assigned to the 1/1 and 1/2 isomers since the intensity ratio is similar to the one previously obtained with product 3. The third isomer 2/2 has not been produced during the reaction in agreement with the lower probability of esterification on position 2 of the glycerol. Mass spectrometry of the total product provided a main peak (Figure 1) with a mass of 452 corresponding to the mass M + 18.

Cutin monomers were eluted in ACN/water (40/60, v/v) as a single peak at 14 min (not shown). A more polar mobile phase was used compared to compounds 1 to 4 since cutin monomers are mainly composed of dihydroxyl-fatty acids. This was confirmed by mass spectrometry using the negative mode. Cutiglycerides were eluted by HPLC in a similar pattern than for compound 3 and masses corresponding to M + 1 and M + 18 were measured (not shown). Other derivatives using deuterated glycerol-d5, sorbitol, deuterated palmitic acid (d31) and palmitoleic acid were also successfully synthesized together with methyl esters of cutin monomers by using this procedure (not shown).

**Dispersion in an Aqueous Solution.** Whereas pure fatty acids and monoglycerides are known to be insoluble in water, their binary mixtures can be fully dispersed at basic pH. Then, we realized various equimolar mixtures of compounds 1–4 in an attempt to obtain fully dispersed systems. As observed by phase contrast microscopy, neither pure commercial fatty acids 1 and 2 nor the equimolar binary mixture were well dispersed in solution and were present as aggregated crystals (not shown). In the latter case, this means that the hydrogen bond network which can form between both bola fatty acids is not sufficient for the formation of membranes as for mixtures of monopolar fatty acids and alcohol. Product 3 was no more dispersed and remained as lumps of aggregated membranes (Figure 2) as in the case of monopolar monoglyceride. Product 4, however, was perfectly dispersed as a milky viscous solution of polydisperse vesicles (Figure 2), a behavior which contrasts with that of other monoglycerides (see above and ref 11). Equimolar binary mixtures 1 + 4, 2 + 4, and 3 + 4 were also well dispersed as turbid viscous solutions of membranes (not shown). As in the case of monopolar fatty acid/monoglyceride mixtures, product 4 helps for the dispersion of bola-fatty acids 1 and 2 and even product 3, which remains as aggregated membranes when in pure form. This feature should be helpful to improve the bio-availability of these lipids. For instance, the effect of these monomers on cell cultures or on plants could be studied by using such binary mixtures. Moreover, these systems appear as being among the most simple than can be used for encapsulating bioactive molecules. Other binary mixtures including product 3 and the bola fatty acids remained precipitated as lumps of aggregated membranes in a similar way than for pure product 3. Then, the presence of the ω-hydroxyl group in product 4 induces a strong modification of the behavior in solution. One observed similar lumps of aggregated membranes for cutiglycerides and the binary mixture with the monomers and lipid
Fourier Transform Infrared Spectroscopy. The shift of the \( v_{\text{CH}} \) band as a function of temperature is a nice probe to follow the dynamic of the lipid alkyl chains.\(^{17} \) It was presently applied to pure products and the binary mixtures (not shown). Pure fatty acids 1 and 2 exhibited a \( v_{\text{CH}} \) of 2849 cm\(^{-1} \) in the temperature range observed. This value is typical for alkyl chains in an all trans conformation and confirms that compounds 1 and 2 remain as crystals in aqueous solution. On the contrary, cutin monomers displayed a \( v_{\text{CH}} \) of 2854 cm\(^{-1} \) at room temperature and higher showing that alkyl chains are in a fluid regime. This feature was also shown in the cutin polymer.\(^{18} \) Pure product 3 exhibited a \( v_{\text{CH}} \) of 2850 cm\(^{-1} \) from 27 to 62 °C after which the value increased to 2853 cm\(^{-1} \) which was reached at 72 °C. These values are typical of a phase transition from a gel to a fluid phase for which alkyl chains melt.\(^{9,12,17} \) Similar observations were made with product 4 or the binary mixture 3 + 4, except that the transition occurs at 57 °C. For all other binary mixtures, the transition was shifted to higher temperature by about 5 °C.

Deuterium Solid-State NMR. NMR was applied to aqueous solutions of pure products 3 and 4 which were also synthesized with deuterated glycerol-d5. This allows for the identification of the various phases as a function of the temperature. In that case, the spectrum corresponds to the superimposition of three signal originating from the three labeled positions on the glycerol.\(^{19} \) At low temperature, the spectra exhibited a broad powder line shape typical for lipids embedded in a gel phase\(^{15} \) (Figure 3), which is in agreement with the FTIR observations. This indicates that the glycerol undergoes a low degree of mobility similar to that observed for conventional monoglycerides in their gel phase.\(^{19,20} \) Upon increasing the temperature, the spectrum became axially symmetric with well resolved signal and quadrupolar splittings. The isotropic line centered in the middle of the spectrum stands for the signal of deuterated water in natural abundance and that of one deuteron from the glycerol sn-3 position. The two deuterons at that position are known to be nonequivalent; that is, they exhibit two different quadrupolar splittings.\(^{19} \) In the direction from the sn-1 to the sn-3 position in the glycerol moiety, the quadrupolar splitting decreased from 23.6 to 9.2 and 1.6 kHz accounting for the internal glycerol motions, values which are very close to those obtained for mono-olein.\(^{19} \) This result confirms that lipids are embedded in a fluid phase at that temperature.

Small Angle Neutron Scattering. To get a deeper view of the structure of the membranes, we performed SANS experiments on the mixture 2 + 4, both below (30 °C) and above (60 °C) the phase transition. In both cases, the 2D spectra (not shown) are somewhat anisotropic, presumably owing to the wall or flow-induced orientation effects on long-range organized phases. The spectra, see Figure 4 for the sample at 30 °C, mainly feature a strong small-angle scattering signal but, indeed, both distinctly display two sharp Bragg peaks in the ratio 2:1, located at about \( Q_0 = 0.065 \) Å\(^{-1} \) (30 °C) and \( Q_0 = 0.072 \) Å\(^{-1} \) (60 °C). This is characteristic for a 1D, lamellar packing of flat membranes, the repeat distance \( 2 \pi / Q_0 \) being 97 Å (at 30 °C) or 87 Å (at 60 °C). Moreover, it is possible to describe the main features of the spectra in terms of the form factor of a randomly oriented, planar membrane of well-defined thickness \( \delta \), namely the function\(^{21} \)

\[
P(Q) = \frac{\sin^2(\delta Q/2)}{Q^2}
\]

in order to estimate the value of the membrane thickness \( \delta \). The corresponding fits are displayed in Figure 4, as well as in the Porod representation in Figure 5. Such fits can obviously not describes faithfully the data in \( Q \) ranges where structure factor effects are important, namely near the Bragg peaks. Besides, the model function does not take into account possible fluctuations in the parameter \( \delta \). Such fits have nevertheless been shown to be useful for a wealth of lamellar systems.\(^{22} \) In the present case, they yielded \( \delta \approx 18 \) and 13 Å for membranes in the gel (\( T = 30 \) °C) and fluid phase (60 °C), respectively. It must here be noticed that the length for an extended alkane chain bearing 16 carbons is 18.6 Å (from \( C_1 \) to \( C_{16} \)). Then, our SANS results are consistent with bola-fatty acids adopting a membrane spanning orientation, a common feature for bolalipids.\(^{23,24} \) In the gel phase, the alkyl chain is nearly all-trans in agreement with our FTIR data. In the same way, the smaller value of the thickness in the fluid phase confirms that a significant amount of gauche defects occurs, decreasing the average length.

In summary, it is shown that glycerol modification of cutin and suberin monomers by acid catalysis is fast and easy to
perform. It clearly appears that synthesizing glycerol derivatives allows for a better dispersion of these fatty acids in aqueous solution. It allows for the formation of membranes rather than crystals in aqueous solution, gel and even full dispersions in the case of the \( \alpha,\omega \)-diglycerol derivative (compound 4). This is expected to improve the bioavailability of these lipids for a biological use, for instance, as substrate for enzymes. Studies are under progress for the midchain hydroxylation of fatty acids and their glycerol derivatives by cytochrome P450 by using such fatty acid dispersions.

Acknowledgment. We thank Hélène Rogniaux for the mass spectrometry analysis. We are also especially grateful to Laurence Noirez, our local contact for the SANS experiments at Laboratoire Léon-Brillouin.

References and Notes


BM049325O