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# Topological connection between vesicles and nanotubes in single component lipid membranes driven by head-tail interactions

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#### 18 Abstract

Lipid nanotube-vesicle networks are important channels for intercellular communication and 19 20 transport of matter. Experimentally observed in neighboring mammalian cells, but also 21 reproduced in model membrane systems, a broad consensus exists on their formation and 22 stability. Lipid membranes must be composed of at least two components, each stabilizing low 23 (generally a phospholipid) and high curvatures. Strong anisotropy or enhanced conical shape 24 of the second amphiphilic component is crucial for the formation of nanotunnels. Anisotropic 25 driving forces generally favor nanotube protrusions from vesicles. In the present work, we 26 report the unique case of topologically-connected nanotubes-vesicles obtained in the absence 27 of directional forces, in single-component membranes, composed of an anisotropic bolaform glucolipid, above its melting temperature, T<sub>m</sub>. Cryo-TEM and fluorescence confocal 28 29 microscopy show the interconnection between vesicles and nanotubes in a single-phase region, 30 between 60° and 90°C under diluted conditions. Solid-state NMR, necessarily recorded on the 31 dry powder, yet demonstrates that the glucolipid can simultaneously assume two distinct 32 configurations, head-head and head-tail. These arrangements, seemingly of comparable energy above the  $T_{\rm m}$ , could explain the existence and stability of the topologically-connected vesicles 33 34 and nanotubes, which are generally not observed for classical single-component phospholipid-35 based membranes above their  $T_{\rm m}$ .

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*Keywords:* Nanotube vesicle networks; Tunnelling nanotubes; Block liposomes; Liposomes;
 Lipid nanotubes; Biosurfactants; Microbial glycolipids;

39

#### 40 Introduction

41 Topological connections between closed lipidic compartments through nanotubes<sup>1-3</sup> 42 have been shown to play a crucial role in the transfer of matter and communication in neighboring mammalian cells.<sup>4</sup> These singular nanosystems, observed since the '90s as 43 spontaneous non-equilibrium structures in electroformed model liposome membranes.<sup>5,6</sup> have 44 since been largely studied, both experimentally and theoretically.<sup>7–9</sup> Addressed in the literature 45 by different terms, tunnelling nanotubes (TNT),<sup>1,4,9</sup> block liposomes<sup>10–12</sup> or nanotube-vesicle 46 networks,<sup>13–16</sup> (instead of tubes, some work speak of tethers<sup>6,17</sup>) all refer to a similar 47 48 phenomenon, driven by various internal or external forces. The latter must overcome the energy 49 barrier needed to bend a phospholipid bilayer from low positive mean and gaussian curvatures 50 (vesicle) to a high mean and zero Gaussian (tube) curvatures.

51 A large body of both experimental and theoretical work has shown that budding and 52 eventual nanotube formation from an existing membrane can only occur spontaneously for membranes of at least two-components<sup>9,10,24,25,11,12,18–23</sup> and below a vesicle-nanotube phase 53 transition event.<sup>26,27</sup> Otherwise, external anisotropic driving forces like electroformation,<sup>5–</sup> 54 <sup>8,28,29</sup> osmotic pressure,<sup>6</sup> laser "tweezers"<sup>30</sup> or electrodynamics<sup>13–16</sup> must be employed to obtain 55 56 similar results with, in some cases, an impressive degree of 2D and 3D organization.<sup>13–16</sup> The 57 origin of spontaneous nanotube formation has been shown to be related to a nanoscale phase 58 separation between two membrane components, stabilizing low and high curvatures respectively,<sup>10–12,31</sup> with at least one component being highly anisotropic.<sup>20,32,33</sup> Theoretically, 59 this behavior has been explained by deviations in the elastic properties of membranes due to in-60 61 plane orientational ordering of membrane inclusions composed of anisotropic amphiphiles, these referring to a non-symmetrical shape upon a 90° tilt along the amphiphile axis.<sup>8,9,19,24,25,33</sup> 62

63 In this work, we show unexpected nanotubing of membranes prepared from a singlecomponent lipid, in the absence of external directional forces and above the lipid's  $T_{\rm m}$ . This 64 65 phenomenon is observed for a novel anisotropic double amphiphile (bolaform amphiphile, or bolaamphiphile), a glucolipid composed of β-D-glucose and a C18:1-cis fatty alcohol (G-66 67 C18:1-OH, Figure 1). This compound is obtained by microbial fermentation of a geneticallymodified S. bombicola yeast in the presence of oleyl alcohol<sup>34</sup> and is developed in the broader 68 69 context of extending the library of new biobased surfactants and lipids, in view of replacing petrochemical low molecular weight amphiphiles.<sup>35–41</sup> The structure of G-C18:1-OH is 70 71 analogous to that of other microbial glycolipids developed through genetic engineering.<sup>42,43</sup>

72 Topological connections between nanotubes and vesicles are observed by means of 73 cryogenic transmission electron microscopy (cryo-TEM), fluorescence microscopy and wide angle X-ray scattering (WAXS) above the melting temperatures,  $T_m$  = 48.3°C under strong 74 75 dilution (0.5 wt%). Spin diffusion and <sup>1</sup>H double-quantum single-quantum (DQ-SQ) 2D solid-76 state nuclear magnetic resonance (ssNMR) spectroscopy under magic angle spinning (MAS), 77 necessarily performed on the dry glucolipid powder, provides a spectroscopic ground to 78 understand the vesicle-nanotube coexistence. The bolaform glucolipids could be in a head-79 head/tail-tail configuration in the vesicles, but rather in a head-tail configuration in the 80 nanotubes. In the dry glucolipid, both configurations are energetically comparable in the singlephase region above  $T_{\rm m}$  and their coexistence is evidenced by the splitting of some <sup>13</sup>C NMR 81 82 signals. These facts could explain the stability of nanotubes, while the following hypotheses are formulated for their formation: membrane inclusions with different orientational ordering<sup>13–16</sup> 83 84 possibly driven by inter-vesicle collisions.

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90 Figure 1 – Non acetylated C18:1 alcohol glucoside, G-C18:1-OH, is obtained by a bioprocess performed

- 91 with modified *S. bombicola* yeast.
- 92

#### 93 Experimental Section

94 Synthesis of non acetylated C18:1 Alcohol Glucosides (G-C18:1-OH). G-C18:1-OH (Mw= 95 418.56 g.mol<sup>-1</sup>) was produced by aerobic whole cell bioprocess with a modified *S. bombicola* strain as described by Van Renterghem et al. (Fig. S4 in Ref. <sup>34</sup>). The molecule was purchased 96 97 from the Bio Base Europe Pilot Plant (Gent, Belgium) and has the generalized chemical 98 structure given in figure Figure 1. The HPLC and <sup>1</sup>H NMR spectrum (MeOD-d4) with peak 99 assignment are shown in Figure S 1. High purity levels (99%) and high degree of uniformity 100 were obtained, as can be derived from HPLC-ELSD chromatogram, <sup>1</sup>H NMR and table of 101 contaminant given in Figure S 1.

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<sup>1</sup>*H* solution Nuclear Magnetic Resonance (NMR). <sup>1</sup>H solution NMR experiments were performed on a Bruker Avance III 300 spectrometer using a 5 mm <sup>1</sup>H-X BBFO probe using methanol-d4 as solvent. The number of transients is 8 with 3 s recycling delay, an acquisition time of 5.46 s and a receiver gain of 362. The <sup>1</sup>H NMR spectrum and relative assignment are shown in Figure S 1 and Table S 1. <sup>13</sup>C solution NMR were performed on the same probe using DMSO-d6. Corresponding chemical shifts are given in Table 1. Referencing is done with respect to TMS.  $\delta_{1H}=0$  ppm,  $\delta_{13C}=0$  ppm.

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Sample preparation. The sample was dissolved in milliQ-grade water at the concentration of 5 mg/mL (0.5 wt%). Lack of pH-sensitive probes (e.g., COOH groups) in G-C18:1-OH, as otherwise found in other microbial amphiphiles,<sup>44</sup> but also the will to avoid ion-specific effects,<sup>45</sup> exclude the use of buffer. The solution was heat at the given temperature using a CH3-150 Combitherm-2 dry block heating device. For the cryo-TEM experiments, the vials were heat at the given temperature next to the cryofixation device and plunged into liquid ethane as fast as possible. For temperatures of 25°C and 70°C, the sample was heat at 90°C and then 118 cooled at to the desired temperature. For the SAXS experiment, the sample was heat at 90°C in 119 the block heating device and cooled at to 25°C before analysis. For the SANS experiment, the 120 sample was heat at 90°C directly in front of the beam using the thermalized sample holder 121 available.

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123 *Differential Scanning Calorimetry (DSC)*. DSC was performed using a DSC Q20 apparatus 124 from TA Instruments equipped with the Advantage for Q Series Version acquisition software 125 (v5.4.0). Acquisition was performed on both hydrated and dry powder sample (~ 3-5 mg) sealed 126 in a classical aluminium cup and using an immediate sequence of heating and cooling ramps at 127 a rate of 10°C.min<sup>-1</sup>. Melting temperatures,  $T_m$ , 1 and 2,  $T_{m1}$  and  $T_{m2}$ , were taken at the minimum 128 of the endothermic peak.

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130 Small and Wide Angle Scattering experiments. Small angle neutron scattering (SANS) 131 experiments were performed at the D11 beamline of Institut Laue Langevin (Grenoble, France) 132 during the run No. 9-13-778. Four q-ranges have been explored and merged using the following 133 wavelengths,  $\lambda$ , and sample-to-detector (StD) distances. 1) ultra-low q:  $\lambda$ = 13.5Å, StD= 39 m; 134 2) low-q:  $\lambda = 5.3$ Å, StD= 39 m; 3) mid-q:  $\lambda = 5.3$ Å, StD= 8 m; 4) high-q:  $\lambda = 5.3$ Å, StD= 1.4 m. The sample (C= 5 mg.mL<sup>-1</sup>) was prepared in 99.9% D<sub>2</sub>O to limit the incoherent background 135 136 scattering. The sample solution was analyzed in standard 1 mm quartz cells. Direct beam, empty 137 quartz cell, H<sub>2</sub>O (incoherent scatterer) within the quartz cell were recorded and boron carbide 138 (B4C) was used as neutron absorber. The sample acquisition was measured at 90°C, where 139 temperature was controlled through the controller thermalized sample holder available at the 140 beamline. The background sample  $(D_2O)$  signal was subtracted from the experimental data. 141 Absolute values of the scattering intensity were obtained from the direct determination of the 142 number of neutrons in the incident beam and the detector cell solid angle. The 2D raw data were 143 corrected for the ambient background and empty cell scattering and normalized to yield an 144 absolute scale (cross section per unit volume) by the neutron flux on the samples. The data were 145 then circularly averaged to yield the 1D intensity distribution, I(q). The software package Grasp 146 (developed at ILL and available free of charge) was used to integrate the data, while the 147 software package SAXSUtilities (developed at ESRF and available free of charge) was used to 148 merge the data acquired at all configurations and subtract the background.

Wide-angle X-ray scattering (WAXS) was performed under temperature control at the
 SWING beamline of SOLEIL synchrotron facility (Saint-Aubin, France) during the run
 20201747 (energy: 14 keV, sample-to-detector distance: 0.5 m). Two sample concentrations in

 $H_2O$  were probed, C= 5 mg.mL<sup>-1</sup> and C= 25 mg.mL<sup>-1</sup>. The 2D data were integrated azimuthally 152 153 at the beamline using the software Foxtrot and in order to obtain the I(q) vs. q spectrum after masking the beam stop shadow. Silver behenate  $(d_{(100)} = 58.38 \text{ Å})$  was used as a standard to 154 155 calibrate the q-scale. Sample solutions were inserted in borosilicate capillaries of 1.5 mm in 156 diameter. Capillaries were flame-sealed. A capillary oven with controlled temperature ( $\pm 0.5^{\circ}$ C), 157 provided at the beamline, was used to control the sample temperature between 25°C and 111°C. 158 Data were normalized by the transmission and calibrated to the SAXS signal of H<sub>2</sub>O at large q-159 values (I=  $0.0163 \text{ cm}^{-1}$ ) in order to obtain an absolute intensity scale. The water signal was 160 measured by subtracting the signal of the empty capillary from the signal of a water-filled capillary. The signal of (water + capillary) was used as background for the samples and it was 161 162 subtracted after integration of the 2D data.

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164 Cryogenic transmission electron microscopy (cryo-TEM). Cryo-TEM experiments were carried 165 out on an FEI Tecnai 120 twin microscope operating at 120 kV and equipped with a Gatan 166 Orius CCD numeric camera. The sample holder was a Gatan Cryoholder (Gatan 626DH, 167 Gatan). Digital Micrograph software was used for image acquisition. Cryofixation was 168 performed using a homemade cryofixation device. The solutions were deposited on a glow-169 discharged holey carbon coated TEM copper grid (Quantifoil R2/2, Germany). Excess solution 170 was removed and the grid was immediately plunged into liquid ethane at -180°C before 171 transferring them into liquid nitrogen. All grids were kept at liquid nitrogen temperature 172 throughout all experimentation. Cryo-TEM images have been treated and analyzed using Fiji 173 (is just ImageJ) software, available free of charge at the developer's website.<sup>46</sup>

174 The sample's concentration was  $C = 5 \text{ mg.mL}^{-1}$ . For technical reasons, the temperature 175 during cryofixation could not be controlled. For this reason, the temperature associated with 176 each cryo-TEM image in the text was related to the equilibration temperature just before 177 cryofixation, the device being as close as possible to the dry bath used for temperature control. 178 Since temperature control during cryofixation was not possible in our device, and practically 179 impossible at high temperatures, one should take the  $T^*$  values as indicative of the given value. 180 Although the sample transfer between the dry bath and the cryofixation device was performed 181 as fast as possible, it is more than likely that the actual temperature of the sample at the moment 182 of cryofixation was lower than the one indicated here, although impossible to know precisely.

184 Fluorescence microscopy. Images were recorded using a 40x objective on a Leica SP5 upright 185 confocal microscope with 405 nm laser excitation. The sample was marked with 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan, Mw= 353.54 g.mol<sup>-1</sup>) fluorophore as follows. A 186 187 stock solution of 3 mg of Laurdan in 100  $\mu$ L of acetone was initially prepared (C= 30 mg.mL<sup>-</sup> <sup>1</sup>). A volume of 0.28 µL of the stock Laurdan solution was diluted in 1 mL of a 5 mg.mL<sup>-1</sup> G-188 189 C18:1-OH water solution temporary set at 110°C in a sealed Eppendorf. The latter was 190 important so to enhance inclusion of Laurdan in the membrane constituted by the glucolipid, 191 while in its vesicle phase. The final Laurdan concentration was approximately 24 µmol, with a 192 molar Laurdan-to-G-C18:1-OH molar ratio of approximately 1-to-500. Fluorophore-to-lipid 193 ratios above 1:200 were generally considered as non-interfering with the lipid phase behavior, 194 as also verified in this work.

195 Laurdan can be excited between 365 nm and 410 nm and its emission wavelength can 196 vary according to the rigidity of the membrane: intercalation within a rigid or flexible membrane generates emissions at 440 nm or 490 nm, respectively.<sup>47</sup> In the present experiment, 197 we have flame-sealed a solution of G-C18:1-OH at 5 mg.mL<sup>-1</sup> containing Laurdan (1-to-500 in 198 199 lipid-to-Laurdan ratio) in flat optical capillaries of 0.1 mm thickness. Capillaries were set on a 200 Linkam support for temperature control, eventually placed under the microscope. The sample 201 was excited at 405 nm and detection was made in spectral mode with a 5 nm window sliding 202 every 5 nm from 412 nm to 602 nm in 16 bits, which allows quantify the signal and compare the different conditions. Quantitative analysis was performed according to the literature:<sup>48,49</sup> 203 204 several ROI (range of interest) have been drawn on several images using the ROI manager of 205 Fiji (is just ImageJ) software. The integrated intensity of each ROI at 440 nm and 490 nm was 206 eventually recorded and used to calculate the value of the generalized polarization, GP, as 207 follows of

208  $GP = \frac{(\lambda_{440} - \lambda_{490})}{\lambda_{440} + \lambda_{490}}$ 

A negative GP indicates a flexible membrane while a positive GP indicates a rigid membrane.

Solid-state Nuclear Magnetic Resonance (ssNMR): <sup>1</sup>H and <sup>13</sup>C ssNMR experiments have been performed using an Avance III HD Bruker 7.05 T ( $v_{1H}$ = 300 MHz) spectrometer and a 4 mm magic angle spinning (MAS) probe, except for the BAck-to-BAck (BABA) experiment, which was performed on an Avance III Bruker 16.44 T ( $v_{1H}$ = 700 MHz) spectrometer equipped with 2.5 mm MAS probe. Temperature was controlled through a Smart cooler BCUII/a BCU-xtreme unit, using 10 min of equilibration time after each temperature variation. Temperature was 217 always varied from high (110°C) to low (70°C) before analysis. Due to combined use of fast 218 MAS rates, necessary to achieve high resolution spectra, and heating close to the boiling point 219 of water, neither diluted nor concentrated samples could be used for ssNMR experiments. All 220 tests employing up to 50 wt% G-C18:1-OH in water resulted in sample centrifugation, drying 221 but also expulsion from the rotor, causing sudden stop of rotor spinning. For these reasons, all 222 attempts to study hydrated G-C18:1-OH failed and only the dry powder could be analyzed. The 223 dry sample powder was analyzed as such in zirconia rotors (4 mm or 2.5 mm) spinning at MAS= 224 10 kHz (4 mm) or 22 kHz (2.5 mm). and Chemical shifts were calibrated with respect to 225 adamantane (<sup>13</sup>C:  $\delta_{13C}$ (CH2)= 38.48 ppm;  $\delta_{1H}$ = 1.91 ppm) and glycine alpha ( $\delta_{13C}$ (CO)= 176.03 ppm). Details on the acquisition parameters are given in the Supporting Information. 226

227

#### 228 Results and discussion

#### 229 Topologically-connected nanotubes and vesicles

230 G-C18:1-OH is a biobased glucolipid, water-insoluble at room temperature, and of 231 which the physicochemical properties are poorly known. The DSC thermogram performed at 232 10°C/min of G-C18:1-OH as a function of the water content (Figure 2a) shows two endothermic 233 peaks on the dry powder (99.6 wt% glucolipid). They are attributed to two melting phenomena 234 occurring at the following temperatures,  $T_{\rm m}$ : 48.3°C ( $T_{\rm m1}$ ) and 95.7°C ( $T_{\rm m2}$ ), the enthalpy of the 235 former being lower than that of the latter. Interestingly, DSC also shows that  $T_{m1}$  is hydration 236 independent and reversible (the associated exothermic peak is systematically observed), while 237 the transition at  $T_{m2}$  is simply suppressed (here tested up to 130°C) on hydrated powders and 238 never observed on the cooling profile, even at slow cooling rates (1°C/min). The thermogram 239 recorded on the dry powder is typical of single-component lipids having an intermediate,<sup>50</sup> 240 metastable, phase (often referred to the ripple phase,  $P_{\beta'}$ , in reference to the periodic undulation of the bilaver), 50-54 between the classical lamellar gel (L<sub>β</sub>) and liquid crystalline (L<sub>α</sub>) phases 50-241 242 <sup>54</sup> and characterized by regions of both liquid order and disorder.<sup>54,55</sup> The effect of water does 243 not influence the low-temperature crystallization at  $T_{m1}$ , but it contributes to disrupt the ordered 244 array of hydrocarbon chains in the metastable phase.

On the basis of the DSC thermogram, the aqueous phase behavior of G-C18:1-OH under diluted conditions (5 mg.mL<sup>-1</sup>) was studied at four different temperatures (red marks on Figure 2a): below and above  $T_{m1}$ , but also between  $T_{m1}$  and  $T_{m2}$ , corresponding to the thermogram of the dry powder.



- 249
- Figure 2 a) DSC thermogram of G-C18:1-OH under dry (0.4 wt% water, estimated by thermogravimetric analysis) and hydrated conditions recorded at a heating rate of 10°C/min. Red marks indicate the temperatures at which cryo-TEM experiments are performed on the corresponding 5 mg/mL aqueous solutions. b) Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at  $T^*=130^{\circ}$ C, 90°C, 70°C and 25°C.  $T^*$  indicates the temperature of the sample solution before cryofixation.  $T^* \neq$  T, T being the temperature of the sample upon plunging in liquid ethane. Please refer to the materials and
- 256 methods section for more information.

258 The series of cryo-TEM images in Figure 2b show an overview of the most relevant 259 structures observed on G-C18:1-OH solution from  $T^*= 130^{\circ}$ C to  $T^*= 25^{\circ}$ C, whereas  $T^*$  is 260 indicative of the equilibrium temperature before cryofixation (refer to materials and methods section for more information on the sense and interpretation of  $T^*$ ). At  $T^*>100^{\circ}$ C, the sample 261 262 is essentially composed of polydisperse single wall vesicles, massively shown by the 263 complementary pictures presented in Figure S 2. In between  $T_{m1}$  and  $T_{m2}$ , at  $T^*=90^{\circ}$ C and  $T^*=$ 264 70°C (Figure 2b, Figure 3, Figure 4, complemented by Figure S 3 and Figure S 4), vesicles are 265 connected by nanotubes. At  $T^* < T_{m1}$ , flat crystals are otherwise observed (Figure 2b and Figure 266 S 5).

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Figure 3 – Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at *T*\*= 90°C.

A deeper insight at  $T^*=90^{\circ}$ C is given in Figure 3 (more images are provided in Figure S 3). Arrows 1 through 6 mark very clearly the typical continuity in the glucolipid membrane between the vesicle and nanotubes. Arrow N° 7 shows that a single nanotube connects the outer membrane of two vesicles several hundred nanometers apart. The connection is shown by 275 arrows N°5 and N°6. The corresponding profiles, A through D, confirm the tubular nature of 276 the elongated objects. Profiles B and D are both characterized by typical symmetrical thick 277 walls (low intensity depths highlighted by shaded rectangles) surrounding a hollow region (high 278 intensity peak, between the shaded rectangles). The wall thickness varies between 3.5 and 4.0 279 nm for profile B and between 4.1 and 6.1 nm for profile D, while the total nanotube diameter 280 varies between 14 nm at B and 20 nm at D. At the same time, the thickness of the vesicle wall 281 is about 6 nm at A and 13.5 nm at C. Considerations about the bilayer or interdigitated structure 282 of the membrane will be presented below.

283 The connection between nanotubes and vesicles seems to be multiple. For instance, 284 arrow N°1 (Figure S 3) and N°4 (Figure 3) show a single-wall vesicle, of which the membrane 285 is extruded into a nanotube. Similarly, a closer look at arrows N°1, 2 and 3 in Figure 3 show 286 that the vesicle is single-wall and fully closed, while the nanotube nucleates at the vesicle outer 287 surface, with no apparent topological continuity. In many regions of the sample at  $T^*=90^{\circ}$ C, one can find exvaginations or  $buds^{23,25,31}$  in the nanotube wall. This is shown, for instance, by 288 289 arrows N°7 and particularly put in evidence in Figure 4 by arrows N°1 through N°4, where the 290 amplitude of the oscillation varies between 20-25 nm (arrow N°1) and 6-8 nm (arrows N°3-4).



Figure 4 - Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heated at *T*\*= 90°C.

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294 Still between  $T_{m2}$  and  $T_{m1}$ , but at lower temperature ( $T^*=70^{\circ}C$ ), the nanotubes seem to 295 vary from single threads (arrow N°1, Figure S 4) to nanotubes of diameter below 15 nm, both 296 connecting nanotubes of diameter ranging between 50 nm and 100 nm (arrows N°2, Figure S 297 4). The larger tubes are most likely formed by the inflation and flattening of the nanotubes, 298 probably after assimilation of surrounding vesicles and fusion of the exvagination discussed 299 above. The transition between vesicles, tubes and flat membranes is certainly more accentuated. 300 Arrow N°3 in Figure S 4 shows distinct vesicles while arrow N°4 identifies a flat structure 301 evidently formed from fused vesicles. In particular, arrow N°5 points at the fusion of two vesicles while arrow N°6 points at two vesicles fusing into the flat membrane. Additional areas
where vesicle fusion is prominent are indicated by the oscillations at the outer skirt of broad
lamellae and pointed by arrow N°7.

Finally, below  $T_{m1}$ , at  $T^*= 25^{\circ}C$  for instance, mainly flat crystals, with no visible exvaginations or tubes, are visible (Figure S 5) by cryo-TEM and the material turns into an insoluble precipitate. Massive presence of flat crystalline structures is also clear from the bulk iridescent behavior of a G-C18:1-OH solution under manual shear.

309 In summary, despite the uncertainty related to the actual state of equilibrium of the 310 glucolipids at the moment of cryofixation, cryo-TEM depicts a vesicle phase at  $T > 100^{\circ}$ C, a 311 flat lamellar phase below  $T_{m1}$  and an interconnected vesicle-nanotube network between  $T_{m1}$  and 312 about 100°C. The combination of statistically-relevant fluorescence microscopy and 313 scattering/diffraction experiments provides an additional proof of the coexistence of 314 vesicle/nanotube systems between the two transition temperatures. Please note that the 315 experiments reported below have been performed under controlled conditions of temperature, 316 which is then indicated by the use of T, instead of  $T^*$ .



Figure 5 – a-c) Confocal fluorescence microscopy images recorded at 90°C and 70°C for a 5 mg/mL G-C18:1-OH aqueous solution. d-g) Typical fluorescence microscopy images recorded at T= 80°C using Laurdan (refer to materials and methods section for more details on this fluorophore). Excitation wavelength is 405 nm for all the images, while emission wavelength is 490 nm in (d,f) and 440 nm in (e,g). h) Full emission spectra obtained from the integration of various fluorescence images recorded from 412 nm to 600 nm (step: 5 nm) at 90°C, 80°C and 70°C. i) Box plot of GP, defined as  $GP = \frac{(\lambda_{440} - \lambda_{490})}{\lambda_{440} + \lambda_{490}}$  with  $\lambda_{440}$ and  $\lambda_{490}$  being the emission wavelengths at 440 nm and 490 nm, at 90°C, 80°C and 70°C. Each data point

corresponds to a set of regions of interest (ROIs) (54 at 90°C, 65 at 80°C and 75 at 70°C) selected in the corresponding fluorescence images. ROIs were representatively selected so to include the entire image but 327 also vesicles alone, tubes alone and mixtures of vesicles and tubes.

328

329 Figure 5a,b shows two typical confocal fluorescence microscopy images recorded on a 330 G-C18:1-OH solution (5 mg/mL) in a flame-sealed capillary heat at  $T = 90^{\circ}$ C and  $T = 70^{\circ}$ C. 331 Although the resolution of optical microscopy is not comparable with cryo-TEM, the sample is 332 broadly composed of vesicles coexisting with nanotubes, whereas in some cases the nanotube 333 starts from the vesicle itself (arrow N°1), in good agreement with cryo-TEM data. Furthermore, 334 SANS recorded at  $T = 90^{\circ}$ C (Figure S 6a) shows a scattering profile with a pronounced -2 335 dependence of the scattering intensity against the wavevector q in the log-log scale. This 336 indicates the massive presence of a flat interface and it is expected typical of bilayers in 337 vesicular morphologies.<sup>56</sup> However, the typical signature of nanotubes cannot be observed in 338 the SANS profile, and this can be explained by both an argument related to the relative lower 339 amount of nanotubes with respect to vesicles at 90°C but also to the steeper slope of flat 340 morphologies (-2) compared to tubes (-1), the signal of the former masking the tube signal at 341 low wavevector values. The SANS profile is characterized by a diffraction peak at q = 0.167 Å<sup>-</sup> 342 <sup>1</sup>, of which the assignment is refined by WAXS data.

343 Temperature-driven WAXS experiments in Figure S 6b,c (5 mg.mL<sup>-1</sup> and 25 mg.mL<sup>-1</sup>, 344 respectively) show a lamellar order, possibly attributed to a multilamellar structure of the 345 vesicles (d<sub>v</sub>, Figure S 6d) at high temperature and to the interplanar arrangement of the 346 crystalline flat structures (d<sub>L</sub>, Figure S 6d) at  $T = 25^{\circ}$ C. The sharp (001) reflection indicates 347 typical interlamellar distance of 35.3 Å at 25°C, although this value varies with temperature 348 (40.0 Å at 111°C, 37.2 Å at 88°C), thus confirming its assignment to a hydration-dependent interlamellar order. More intriguing is the second, broader, peak at q= 0.403 Å<sup>-1</sup> (d= 15.6 Å) 349 350 and its reflection, both clearly visible at 88°C, below  $T_{m2}$ . We attribute it to the inter-glucolipid 351 arrangement inside the tubular phase (dt, Figure S 6d), coexisting with the vesicle phase, 352 between  $T_{m1}$  and  $T_{m2}$  and to the planar lipid arrangement (d<sub>i</sub>, Figure S 6d) in the rigid flat lamellar structure, below  $T_{m2}$ . Its temperature-, hence hydration-, independent position also 353 354 confirms its structural origin. Finally, besides the water-structure peak at about 2 Å<sup>-1</sup>, we also observe a broad peak at 1.44 Å<sup>-1</sup>, characteristics of the lateral packing (d\*, Figure S 6d) of lipids 355 356 in bilayers. While its position could be consistent with an  $L_{\alpha}$  phase<sup>57,58</sup> above  $T_{m2}$ , its broadness 357 and positional invariance as well as the coexistence between vesicles and tubes do not allow a 358 precise assignment.

Does G-C18:1-OH assume a bilayer or interdigitated structure, the latter being the one 359 found for other similar bolaform glucolipids?<sup>42,43</sup> The size of G-C18:1-OH can be estimated to 360 be at the most 32 Å, given by the length, L, of the C16 tail (L = 22 Å, from the Tanford formula, 361 L=  $1.54 + 1.265 \times n$ , (n= 16)<sup>59</sup>) and the sugar (about 10 Å).<sup>42,43</sup> However, the *cis* conformation 362 of the C=C bond imposes a "kink" of 60° in the aliphatic chain, which reduces the effective 363 364 length of the oleic backbone to about 17 Å, for an effective total G-C18:1-OH length of about 27 Å. Cryo-TEM provides a tube thickness varying from 35 Å to 60 Å and a vesicle membrane 365 thickness of at least 60 Å. WAXS suggests an interlamellar thickness (membrane and water) 366 between 40 Å and 37 Å and an inter-lipid distance in the tube of about 16 Å. In the absence of 367 a clear form factor oscillation profile, as found for similar glucolipids,<sup>42,43</sup> one must 368 369 acknowledge that these data pledge neither in favor of fully interdigitated nor a double layer 370 structure and might suggest a tilted lipid arrangement with partial interdigitation.<sup>60</sup>

371

## <sup>13</sup>C ssNMR demonstrates that G-C18:1-OH can assume two coexisting, head-head and head-tail, configurations

374 Vesicles are characterized by low positive mean and gaussian curvatures, while 375 nanotubes are described by a combination of a high positive mean and zero gaussian curvatures. 376 In this regard, the coexistence of vesicles and nanotubes requires glucolipids to adapt 377 themselves to both environments at the same time. For this reason, the formation of nanotube-378 vesicle networks generally requires a specific driving force, able to impose a high mean and 379 zero gaussian curvature to the membrane. In many systems, insertion of at least a second 380 constituent (lipid, surfactant, protein) in the membrane becomes a necessary internal driving 381 force for the nucleation and growth of so-called membrane inclusions, from which topologically-connected nanotubes can form,<sup>10–12,32,61</sup> as explained by orientational ordering 382 arguments.62 It has also been shown that external driving forces, such as use of laser 383 "tweezers",<sup>30</sup> osmotic pressure,<sup>6</sup> electroformation<sup>5-8,28,29</sup> or controlled suction,<sup>13-16</sup> are 384 385 otherwise necessary, as also supported by theoretical studies.<sup>19</sup>

The bending energy of tubes is higher than that of a vesicle membrane,<sup>8</sup> due to the dependence of the membrane free energy from the principal curvatures. For this reason, isotropic (rotational symmetry along the main axis), but also anisotropic,<sup>33</sup> lipids generally stabilize low-curvature shapes, like flat membranes or vesicles. Lipid nanotubes are also a common phase formed by amphiphiles, but generally for lipids with a certain complexity in their molecular structures, as exampled by diacetylenic derivatives of phospholipids, among others.<sup>26,27,63–65</sup> Negative contributions to the bending energy favoring the simultaneous 393 presence of interconnected vesicles and tubes was shown to be driven by the presence of 394 anisotropic lipids in a membrane continuum. Spontaneous deviations to the elastic theory in the 395 presence of surfactants, lipids and proteins with anisotropic structures favor the formation of 396 protrusions with radius of the order of the fraction of a micron.<sup>9–12,19–23,62</sup>

In the absence of both internal and external driving forces mentioned above, G-C18:1-397 398 OH should behave as a standard lipid. Its DSC profile (Figure 2)<sup>53,66,67</sup> under both hydrated and 399 dry conditions identifies a hydration-independent transition  $(T_{m1})$  slightly above room 400 temperature; this is followed by one main transition at higher temperature  $(T_{m2})$  in the dry 401 sample.<sup>50</sup> For such a profile, one expects a gel phase (L<sub> $\beta$ </sub>) below  $T_{m1}$  and a liquid crystalline 402 structure, possibly in the morphology of vesicles (L<sub>4</sub> phase), possibly above  $T_{m1}$ , and most likely above  $T_{m2}$ <sup>31</sup> as experimentally found. On the basis of the thermogram recorded on the dry 403 powder, one may also expect a "ripple" (P<sub> $\beta$ </sub>) phase between  $T_{m1}$  and  $T_{m2}$  characterized by 404 405 periodic undulations of the bilayer (generally addressed to as regions with both liquid order and disorder).54,55 406

407 In the present work, instead of a  $L_{\beta}$  phase, we observe flat crystals below  $T_{m1}$ , and a 408 vesicle-nanotube network instead of the "ripple" phase. The latter is particularly unexpected 409 because the membrane is composed of a single-component (G-C18:1-OH) and no anisotropic 410 external force (osmotic, hydrodynamic, electric) is used to pull and stabilize the tubes. In 411 addition, the nanotubes are observed at fairly high temperatures, above  $T_{m1}$  (between about 60°C and 90°C), while this morphology is generally favored at low temperature (proportionality 412 with 1/kT, figure 5 in Ref. <sup>62</sup>), commonly below a given transition temperature, characteristics 413 for each specific amphiphile.<sup>27</sup> Furthermore, nanotubes of G-C18:1-OH seem to be stable in 414 time, thus differing from what is found in electroformed vesicles.<sup>7,8</sup> They are also observed 415 away from phase transition events (may them be at T<sub>m1</sub> or T<sub>m2</sub>), as otherwise reported before 416 417 for a diacetylenic derivative of phosphatidylcholine<sup>26</sup> and many other systems.<sup>27</sup>

A stable nanotube-vesicle network for a single-component phospholipid in the absence of external driving forces is not expected and has never been reported before. It could be explained by the coexistence of two micro-separated phases of different rigidity. This hypothesis is excluded by confocal microscopy on Laurdan-labelled membranes and magic angle spinning (MAS) solid-state NMR (ssNMR) experiments.

423 Laurdan is a well-known fluorophore used to put in evidence the coexistence of rigid 424 and fluid domains in both model and biological membranes.<sup>47–49</sup> Laurdan was used here to label 425 the G-C18:1-OH membranes between  $T_{m1}$  and  $T_{m2}$  and to verify the possible micro-segregation 426 and coexistence of rigid (e.g.,  $L_{\beta}$ ,  $\lambda_{em}$ = 440 nm) and fluid (e.g.,  $L_{\alpha}$ ,  $\lambda_{em}$ = 490 nm) domains, in 427 tubes and vesicles, respectively. A set of quantitative experiments performed on a 5 mg.mL<sup>-1</sup> 428 solution, summarized in Figure 5, shows an excess of fluorescence at only  $\lambda_{em}$  = 490 nm for 429 temperature between 90°C and 70°C (Figure 5h). The GP value, which quantifies the rigid/fluid 430 character of the membrane, is systematically negative (Figure 5i), indicating an overall fluid 431 membrane at all temperatures. GP is quantified on more than 50 regions of interest (ROIs) at 432 each temperature, including vesicles and tubes alone as well as connected nanotube-vesicles (Figure 5d-g). The dispersion of GP at 80°C and 70°C between -0.8 and -0.2 could suggest the 433 434 spurious presence of membranes with various degrees of flexibility, although fluidity is always 435 predominant. The combination of WAXS (broad peak,  $d^*= 1.44$  Å<sup>-1</sup>, invariant with temperature, Figure S 6b,c) and confocal fluorescence microscopy using Laurdan confirms the 436 437 single-phase of the membrane composed of G-C18:1-OH, both in vesicles and nanotubes. This 438 is also consistent with the lack of phase transitions in DSC experiments between ~85°C and 439  $\sim$ 60°C, especially in hydrated samples. We conclude that the vesicle-nanotube transition is not 440 driven by the local segregation of gel phase  $(L_{\beta})$  microdomains, nor that the nanotubes are 441 composed of such phase.

442 ssNMR is the best-suited approach to investigate the origin of the local driving force for 443 the nucleation of anisotropic inclusions, of which the average orientation depends on the local 444 membrane and inclusion curvatures and was shown to promote tubing.<sup>62</sup> ssNMR can provide 445 information on the local molecular mobility, e.g. through the nuclear spin relaxation behavior, 446 but also on intermolecular interactions and stereochemistry, e.g. through variations in the 447 chemical shift.<sup>68–70</sup>

448 Ideally, ssNMR should be performed on diluted G-C18:1-OH samples above  $T_{m1}$ . 449 However, fast MAS, necessary for high resolution, centrifuges the sample. As a first 450 approximation, hydrated G-C18:1-OH samples, for instance at 50 wt% as classically done for 451 phospholipids,<sup>71</sup> could be studied. Unfortunately, permeability of the sample holder combined 452 to heating (close to the boiling point of water) and MAS provoke dehydration and even sample 453 ejection. After experiencing both of the above, this approach was abandoned. As a second 454 approximation, the study of the dehydrated G-C18:1-OH powder avoids centrifugation and 455 evaporation. If studying dried lipids may not reflect their exact behaviour under diluted 456 conditions, valuable structural information can still be obtained in dehydrated molecular 457 systems, like proteins, thus illustrating that this approach is not unreasonable. Furthermore, if 458 DSC (Figure 2a) shows that hydration suppresses the  $T_{m2}$ , it also shows that the value of  $T_{m1}$  is 459 not affected by water at concentrations as low as 10 wt%. For this reason, performing a 460 structural analysis of dehydrated G-C18:1-OH by ssNMR above  $T_{m1}$  may help understanding

the local arrangement of G-C18:1-OH in two morphologies, vesicles and nanotubes, havingsensibly different curvatures.

To explore the local mobility of the most relevant chemical groups of G-C18:1-OH above  $T_{m2}$  (T= 110°C) and between  $T_{m2}$  and  $T_{m1}$  (70 < T / °C < 90), we combine a series of <sup>1</sup>H and <sup>13</sup>C experiments. Single-pulse (SP) MAS experiments (<sup>1</sup>H and <sup>13</sup>C), when recorded under quantitative conditions, allow the detection of all chemical species, while cross-polarization (CP) MAS (<sup>13</sup>C only) experiments are sensitive only to constrained groups, for which the <sup>1</sup>H-<sup>13</sup>C dipolar coupling is not averaged to zero by the molecular motions.



<sup>Figure 6 – a) <sup>1</sup>H MAS (10 kHz) ssNMR spectra recorded from 110°C to 70°C. b) <sup>13</sup>C MAS (10 kHz) ssNMR
spectra recorded using high power decoupling single pulse (SP) and cross-polarization (CP) pulse sequences.
Contact time for CP experiments and corresponding temperatures are given on each spectrum. Full spectra
are given in Figure S 7.</sup> 

The most relevant <sup>1</sup>H and <sup>13</sup>C spectra are given in Figure 6 (full range in Figure 7 and 474 475 Figure S 7). As expected, the <sup>1</sup>H solid-state SP/MAS spectra show broader peaks compared to 476 the solution-state NMR spectrum (Figure 6a), reflecting the obvious solid-state character of the 477 sample at any temperature. The sugar group and the C=C bond resonate in the 2.5-6 ppm range 478 and 5.35 ppm, respectively, while the OH groups of the sugar moiety are found below 4.5 ppm 479 (Figure 6a, full assignment in Table S 1). Very broad (~1 ppm) at 100°C, the position of the 480 OH peak varies with the temperature value and becomes narrower (<~0.5 ppm) and visible at 481  $\delta_{\rm H}$ = 4.64 ppm at T= 70°C. Line narrowing and chemical shift variations of the OH resonance 482 in ssNMR is always a direct sign of H-bonding interactions, whereas the strength of the H-483 bonding, hence its OH...H distance, is correlated to a dispersion in chemical shifts.<sup>72–75</sup> All other

resonances broaden from 110°C to 70°C, thus indicating slower and/or more anisotropic reorientational motions of all chemical groups. The specific dynamic behaviour of the sugar headgroup is also reflected by its <sup>1</sup>H transverse relaxation time,  $T_2$ , whereas the shorter the  $T_2$ , the higher the rigidity. Experiments performed between 110°C and 70°C, shown and commented in Figure S 8 in the Supporting Information, globally show a single, short (< 1 ms below 100°C),  $T_2$  relaxation component for H1', H5' and H6' and two, both short and long (between 4 ms and 12 ms)  $T_2$  for the acyl chain (CH<sub>2</sub>, H9,10, H8,11), even below 100°C.

At 110°C, <sup>13</sup>C SP/MAS experiments (Figure 6b, full range in Figure 7 and Figure S 7, 491 492 assignments in Table 1) show a single peak at  $\delta_{C}$ = 103.35 ppm for C1' and at  $\delta_{C}$ = 130.03 ppm 493 for C9,10, as expected for a symmetrical conformation with two chemically-equivalent carbons.<sup>76</sup> At 70°C, the C1' peak disappears, while C9,10 now shows two peaks around 128 494 495 ppm and 130 ppm (Table 1; full temperature series in Figure S 7b), attributed to C9<sub>2</sub> ( $\delta_{C}$ = 128.69 496 ppm) and C10 in a non-symmetric configuration respectively, for which these two carbons are chemically inequivalent.<sup>76</sup> More precisely, the resonance at 130 ppm is actually the 497 498 superposition of two close resonances, C9,10<sub>1</sub> ( $\delta_{C}$ = 130.03 ppm) and C10<sub>2</sub> ( $\delta_{C}$ = 130.24 ppm), 499 where subscripts 1 and 2 stand for two different conformations of the *cis*-olefin group, as reported for various phases of oleic acid in the vicinity of 0°C.76 500

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502 Table 1 – <sup>13</sup>C Chemical shifts of G-C18:1-OH in solution and solid-state. Solid-state experiments are 503 recorded at MAS= 10 kHz. All spectra are reported in Figure 7 and Figure S 7. The contact time for cross 504 polarization is 3 ms. In all ssNMR experiments, temperature is first raised at 110°C and then lowered to 505 either 90°C or 70°C. Attribution of the <sup>13</sup>C resonances in Table 1 is performed on the basis of the solution 506 spectrum (Figure S 1b). Additional peaks observed in both single pulse and cross polarization ssNMR 507 experiments are attributed, when possible, to a configuration effect of the given atom, e.g., C9,101 and C92 508 indicate two configurations of C9. All new peaks observed in solid-state and not detected in solution are 509 highlighted in red for convenience.



- 510
- 511

		δ / ppm			
Peak	Liquid (DMSO-d6)	Solid Single Pulse T= 110°C	Solid Single Pulse T= 90°C	Solid Single Pulse T= 70°C	Solid Cross Polarization T= 70°C
C102		-	-	130.24	130.24
C9,10/C9,101	130.09	130.03	130.03	130.03	130.03

C9 <sub>2</sub>	-	-	-	128.69	128.69
C1′/C1'1	103.29	103.35	103.35	-	104.43
C1'2	-	-	-	-	103.82
C3'	77.24	77.09	77.02	-	78.29
?	-	76.34	76.29	-	76.21
?					75.25
C2'	73.88	73.98	73.90	73.70	73.70
C4'	70.52	70.77	70.54	70.41	70.98
C16	69.00	70.16	-	-	70.29
?	-	-	-	-	67.93
C1,C5',C6'	61.53	62.41	62.36	62.39	62.62
C1,C5',C6'	61.17	-	-	-	59.56
C2'	33.00	32.90	32.90	32.83	32.79
CH2(trans)	-	-	-	31.98	31.96
CH2(gauche),C15	29.41	29.81	29.87	30.04	-
C8,11	27.06	27.47	27.47	28.32	28.17
CH2	25.97	26.25	26.28	26.30	28.17

<sup>13</sup>C CP/MAS spectra at 70°C (Figure 6b, full range in Figure 7 and Figure S 7d) at 513 514 increasing contact times confirm the presence of two cis-olefin environments: one for which C9,10 are equivalent, labelled here C9,10 $_I$ , and one for which they are inequivalent, labelled 515 516 C9<sub>2</sub>, C10<sub>2</sub>. Both environments are rigid enough to be observed at short contact time ( $t_c = 50 \mu s$ ). 517 A similar double environment is also observed for the sugar C1': CP/MAS at 70°C shows two 518 peaks (C1'<sub>1</sub> at  $\delta_{C}$ = 104.43 ppm, C1'<sub>2</sub> at  $\delta_{C}$ = 103.82 ppm, Figure 6b, Figure S 7d). These are 519 significantly shifted downfield from the C1' peak observed at 110°C and are detected at contact 520 times as short as 50 µs, thus indicating their rigid character. One also finds that CP/MAS is 521 totally inefficient at 100°C for short contact times (50 µs) and poorly efficient even at 3 ms, 522 typical of fast-tumbling molecules (Figure S 7c); also, both gauche ( $\delta_{C}$ = 30.04 ppm) and all*trans* ( $\delta_{C}$ = 31.98 ppm) conformations of the CH<sub>2</sub> carbons coexist at 70°C (Figure S 7b).<sup>77</sup> 523

524 According to ssNMR, one main, mobile, molecular environment exists at 110°C, as 525 shown by the well-resolved <sup>1</sup>H spectrum, single C=C (C9,10<sub>1</sub>) and anomeric (C1') resonances, 526 gauche conformation of the aliphatic  $CH_2$  and single  $T_2$  of the aliphatic  $CH_2$  chain and sugar protons. The sugar group is certainly mobile (inefficient  ${}^{1}H \rightarrow {}^{13}C$  CP/MAS) but still more 527 constrainted than the acyl chain (shorter  $T_2$  (<sup>1</sup>H)). As the temperature is lowered, the overall 528 529 rigidity increases (broadening of <sup>1</sup>H NMR peaks, faster <sup>1</sup>H transverse relaxation and efficient  $^{1}\text{H}\rightarrow^{13}\text{C}$  magnetization transfer during CP/MAS), presumably driven by H-bonding 530 531 interactions (downfield shift and line narrowing of the OH <sup>1</sup>H signal, very short OH  $T_2$ , loss of 532 the C1' signal in SP/MAS). At the same time, a number of spectroscopic data (two concomitant resonances for: anomeric C1'<sub>1</sub> and C1'<sub>2</sub>, *cis*-olefin C9,10<sub>1</sub> and C9<sub>2</sub>, C10<sub>2</sub>, and CH<sub>2</sub>, *gauche* and all-*trans*, environments) and relaxation behaviour (fast and slow  $T_2$  relaxation components) strongly suggests the coexistence of two molecular environments. Further proofs of the coexistence of two distinct molecular domains are given by a number of chemical shift variations in the <sup>13</sup>C CP/MAS spectrum at 70°C compared to the one at 110°C, 90°C and solution. This is shown in Figure 7 and in the list of <sup>13</sup>C chemical shifts in Table 1.





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Figure 7 - Comparison between solution- and solid-state <sup>13</sup>C NMR spectra of G-C18:1-OH. Solid-state experiments are recorded directly on the sample powder at a MAS of 10 kHz (4 mm zirconia rotor). SP: Single Pulse; CP: Cross Polarization. In all the ssNMR experiments, temperature is first raised at 110°C and then lowered to either 90°C or 70°C. Segmented lines are meant to highlight the known peak position in solution, thus stressing the strong chemical shift variations in the solid-state.

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547 NMR experiments above  $T_{m2}$  suggest that vesicles could contain G-C18:1-OH with a 548 single family of mobile conformations, characterized by chemically-equivalent C=C (red, 549 Figure 8b) and a single C1' conformation (green, Figure 8b). Experiments between  $T_{m1}$  and  $T_{m2}$ 550 otherwise suggest that two coexisting conformations, characterized by chemically-equivalent 551 (red, Figure 8b) and inequivalent (magenta, Figure 8b) C=C, could be associated to nanotubes. 552 Two distinct C1' sites (grey, Figure 8b) also indicate two configurations of the sugar headgroup. 553 A comment on this point will be given in the following paragraphs.

The local (Å-scale) variation of molecular mobility below  $T_{m2}$  drives the coexistence of two distinct molecular environments of the same G-C18:1-OH molecule. This occurs within a single-phase domain (as deduced by WAXS) of equivalent membrane rigidity (as derived from Laurdan probe in confocal microscopy). Although never reported before, the difference in the local mobility of the same amphiphile (here, G-C18:1-OH) could satisfy the theoreticallyrequired conditions, that induce local lipid segregation and formation of membrane inclusions (Figure 8c). In case of their non-zero average orientation, the local membrane curvature increases,<sup>62</sup> and nanotubes can spontaneously form (Figure 8b,c). More intriguing, this mechanism occurs at least 10°C above  $T_{m1}$ , although it should not be favored above the T<sub>m</sub>.<sup>27</sup>

From both experimental and theoretical backgrounds, the conditions of topologicallyconnected vesicles-nanotubes systems are met only when either internal (two components), or external, driving forces are applied.<sup>19</sup> In the present single-lipid system, of which the phase behavior is summarized in Figure 8a, the most plausible driving force could be explained by the NMR results collected on the dry sample. G-C18:1-OH concomitantly occupies two molecular environments, characterized by: 1) two distinct conformations of the sugar displaying equivalent mobility and 2) two conformations of the acyl chain unsaturation.





572 Figure 8 – a) Thermotropic phase behavior of G-C18:1-OH at 5 mg.mL<sup>-1</sup>. Note that  $T_{m2}$  is observed on the 573 dry powder, only. b) Possible relationship between the molecular environment, as derived from NMR, and 574 membrane curvatures. All the molecular drawings refer to the same G-C18:1-OH molecule (bulky ellipsoid 575 ≡ sugar headgroup), while each color corresponds to a given configuration. Grey of C1'<sub>1</sub> and C1'<sub>2</sub> indicates 576 the uncertainty about the assignment of the specific NMR peak. The curvature, C, is defined as 1/R, R being 577 the curvature radius. R<sub>1</sub>, R<sub>2</sub>: radius of curvature; C<sub>G</sub>: Gauss curvature; C<sub>M</sub>: Mean curvature. c) Free 578 interpretation of the anisotropic, high-curvature ( $C_M > 0$ ;  $C_G = 0$ ), inclusions in the membrane (low  $C_M$  and 579 C<sub>G</sub>) continuum driven by inter-sugar H-bonding and reduced intramembrane diffusion for a G-C18:1-OH 580 glucolipid. 581

582 Two related questions are unanswered at the moment. What actually drives nanotube 583 formation and is the organization of G-C18:1-OH within the vesicles and tubes the same? Lipid 584 nanotubes have been studied for at least three decades, over which broad experimental and 585 theoretical insight has been generated. Despite this fact, the actual understanding is still matter 586 of debate.<sup>27</sup> Micelle-to-nanotube and vesicle-to-nanotube phase transitions are generally 587 reported for single amphiphiles below a given  $T_{\rm m}$  and intermediate phase composed of twisted 588 and flat fibers is concomitantly reported. Initially thought to be a chirality-driven phenomenon, 589 recent studies propose a more general mechanism involving crystallization and symmetry breaking in the lipid packing driven by optimal packing.<sup>27</sup> If twisted ribbons were observed 590 before for a number of microbial glycolipid bolaamphiphiles,<sup>44</sup> they are not found for G-C18:1-591 592 OH. This fact, as well as the large body of cryo-TEM images and the observation of nanotubes 593 above  $T_{m1}$ , rather pledge in favour of a direct topological change from positive (vesicles) to 594 zero (nanotubes) Gaussian curvatures. In this case, vesicle-to-nanotube transitions could be 595 driven by external forces. 5,6,30,7,8,13-16,28,29

596 Considering the absence of external forces in the present system, there are only two 597 possibilities left, spontaneous budding of the membrane or nanotube pulling after inter-vesicle 598 collisions due to Brownian motion. Both are possible, as inclusions composed of a different arrangement of the same molecule in the membrane continuum can theoretically explain 599 spontaneous nanotube formation in an isotropic medium.<sup>6,7,22,23,62,9–12,18–21</sup> However, the second 600 601 one is probably more likely to prevail, because it could explain the large number of nanotubes 602 connecting two distant vesicles. Interestingly, similar events occur for any phospholipid-based 603 vesicular solution, but nanotubes are extremely rare unless at least two lipids of different 604 molecular structure are mixed together. The spectroscopic, <sup>1</sup>H and <sup>13</sup>C, and relaxation (<sup>1</sup>H) 605 ssNMR data, even if collected on a dry powder, demonstrate that the coexistence of two G-606 C18:1-OH configurations, involving both the sugar headgroup and fatty alcohol chain, are 607 possible for this molecule. As a matter of fact, this is not so surprising, as more than one energy 608 minima, each corresponding to a given conformation, are known for glucose, its non-reducing derivative, methylglucose, and carbohydrates in general.<sup>78-80</sup> 609

610 The presence of two configurations could explain the coexistence between two 611 structures of different curvature, but the data presented so far do not explain the origin of these 612 two configurations. In other words, what drives the change in the local structure of the sugar 613 headgroup and fatty alcohol within the membrane?

The association of known self-assembled structures to spectroscopic <sup>13</sup>C ssNMR data is
 known for years, in particular for gluconamides, lipids having a linear sugar headgroup.<sup>81–84</sup> It

616 was found that *gauche* effects, in some cases up to the  $\gamma$  position, in the sugar chain induce 617 upfield chemical shifts, while anti and trans conformations result in downfield shifts. Each 618 structure (monolayer crystal, bilayer crystal or micellar fiber) was then characterized by a given 619 organization of the gluconamide and a characteristic <sup>13</sup>C ssNMR fingerprint, which could be 620 explained by gauche,  $\gamma$ -gauche, anti and trans conformations of the headgroup. Similar 621 structural-spectroscopic correlations have been collected over the years for much simpler cyclic sugars,<sup>85</sup> whereas pyranoses can undergo important conformational changes (chair, half-chair, 622 boat, skew), when the appropriate energy barriers are crossed.<sup>78–80,86</sup> A quantitative 623 conformational study based on the <sup>13</sup>C chemical shift variations shown in Table 1 would be 624 certainly helpful, but very delicate in the context of the present state of the art, because a 625 rigorous approach requires at least one known crystal structure,<sup>81,82</sup> which is currently missing 626 627 for this compound. A qualitative approach will then be adopted.

628 G-C18:1-OH is a bolaform amphiphile and flip-flop could be a possible mechanism 629 involved in the nanotube stabilization, whereas head-tail interactions have long been considered as important in nanotube-forming bolaform amphiphiles.<sup>60,63</sup> From mere considerations based 630 on  $T_2$  (<sup>1</sup>H) relaxation arguments, one could exclude head-tail configurations: if the R-CH<sub>2</sub>OH 631 632 endgroup (H1) was involved in a direct interaction with the sugar group, its corresponding  $T_2$ 633 would probably be comparable to the sugar's one (OH and H1'), but this does not seem to be 634 the case. However, this hypothesis would be based on the comparison of the relaxation from 635 CH and CH<sub>2</sub> protons, additionally involved in different kind of motions. Besides, the relaxation 636 of H1 is influenced by the strong overlap between H1 and H5'. For these reasons, relaxation 637 arguments are not solid enough and will not be used.

638 We then address this question by exploring intermolecular interactions in a direct 639 manner, using 2D <sup>1</sup>H-<sup>1</sup>H homonuclear ssNMR correlation spectroscopy. At 90°C, in the fast-640 tumbling domain of molecular motion (narrow <sup>1</sup>H NM**R** peaks, Figure 6a) and corresponding to the vesicle-rich region, spin diffusion (NOESY) experiments are preferred over dipolar 641 642 coupling based experiments, because such interaction is averaged out by molecular motion. 643 However, when the molecular motion is more constrained, in the domain of nanotube stability 644 at 70°C (broad <sup>1</sup>H spectra, Figure 6a), Double-Quantum Single-Quantum (DQ-SQ) correlation 645 experiments are best suited to probe interaction between close molecular neighbors. These experiments are reported in Figure 9 and the related interpretation is only briefly reported 646 647 below, while a more detailed discussion can be found on Page 12 in the Supporting Information.



649 Figure 9 – a) 2D <sup>1</sup>H-<sup>1</sup>H NOESY NMR experiment performed at MAS= 10 kHz (4 mm probe) on a Bruker 650 300 MHz Avance III HD spectrometer using 300 ms of mixing time. Sample temperature is regulated to 651 90°C, reached by cooling from 110°C. b) 2D <sup>1</sup>H-<sup>1</sup>H DQ-SQ (BAck-to-BAck, BABA, pulse sequence used for 652 the DQ excitation scheme) NMR experiment performed at MAS= 22 kHz (2.5 mm probe) on a Bruker 700 653 MHz Avance III spectrometer using a DQ excitation time equal to 2 rotor periods. Sample temperature is 654 regulated to 70°C, reached by cooling from 110°C. The typical schemes illustrating how to interpret these 655 2D experiments are reported above each experimental map. Zooms of the sugar region and configurational 656 interpretation are given below each 2D map. More information are given in the text below.

657 Head-tail or head-head configurations could be probed by the existence of H1'-H1 658 interactions, whereas H1 ( $\delta_{H}$ = 3.56 ppm) is associated to the glucolipid tail and H1' ( $\delta_{H}$ = 4.32 ppm) to the sugar headgroup. These atoms are more than 15 Å apart, and a direct interaction 659 660 could not be observed in both NOESY and DQ-SQ experiments. NOESY experiments (Figure 661 9a) show that H1'-H1 correlations are not observed. Meanwhile, clear-cut, direct, H1'-H1 662 interactions are indeed observed are detected at  $T = 70^{\circ}$ C (DQ cross-peaks related to H1'-H1 663 correlation in DQ-SQ experiment shown in Figure 9b). Interestingly, the absence of auto peaks at  $\delta_{H}$  = 4.32 ppm and  $\delta_{H}$  = 3.35 ppm in the SQ dimension excludes H1'-H1' and Hx'-Hx' (x= 2, 664 3, 4) interactions respectively, strengthening the idea that sugar headgroups from two nearest-665 666 neighbor G-C18:1-OH molecules are not adjacent in the nanotube-vesicle region.

667 Homonuclear 2D ssNMR supports the idea, according to which G-C18:1-OH can 668 undergo molecular flip-flops and a head-tail arrangement in the dry product. Extrapolating these 669 conclusions in the lower concentration range, lack of H1'-H1 proximity at T= 90°C suggests a head-head configuration in the vesicle-rich region and head-tail configuration within the 670 671 nanotubes. It is however still unclear whether flip-flops are driven by spontaneous, temperature-672 induced, conformational changes in the glucolipid packing, stimulating inclusions within the membrane itself,<sup>6,7,22,23,62,9–12,18–21</sup> or by defects generated by inter-vesicle collisions. In fact, a 673 674 combination of both is not unlikely. Nanotubes are not stable when temperature is 20 to 30 675 degrees above  $T_{m1}$ , indicating that inter-vesicle collisions alone cannot explain the unusual phenomenon. In an energy landscape approach,<sup>87</sup> the head-head configuration is certainly 676 677 favored at high temperature, while both head-head and head-tail configurations could be 678 favored in the mid-temperature region, corresponding to the range between  $T_{m1}$  and  $T_{m2}$  in the 679 dry powder. Both statistical and defect-induced flip-flop events contribute to form high mean, 680 zero Gaussian, curvature morphologies (nanotubes), for which the head-tail conformation, 681 limiting the proximity between bulky sugar headgroups, is probably more adapted (Figure 8c). 682 The flip-flop interpretation now reasonably explains the strong differences in the sugar and fatty 683 alcohol conformations, deduced by the multiple NMR signals attributed to chemically-684 equivalent groups. The existence of various low-energy conformations, each having specific NMR signature, for sugars in solution<sup>78-80,86</sup> and solid-state<sup>81-84</sup> further supports our 685 686 interpretation.

687 One last point should be clarified. Why, between  $T_{m1}$  and  $T_{m2}$ , are two molecular 688 environments able to coexist, instead of a single one of average rigidity, as found in the case of 689 "ripple" phases?<sup>50–54</sup> We believe that this question can be answered based on the NMR results: 690 the relatively short  $T_2$  values of the H1' and OH protons shows the constrained mobility of the 691 sugar headgroup, despite a certain flexibility of the chain (long  $T_2$  component). This trend may 692 suggest restrictions in translational mobility, probably due to the sugar-OH hydrogen bonding 693 (Figure 8c), meaning that lipids are not able to diffuse within the membrane, thus keeping two 694 different configurations stable.

695

#### 696 Conclusions

697 Topologically-connected nanotube-vesicle lipidic systems are commonly observed for 698 self-assembled membranes composed of at least two amphiphiles, and spontaneously driven by 699 anisotropic inclusions and/or by external directional forces. This work shows that strong 700 differences in membrane curvature can spontaneously occur in single-lipid systems without 701 external anisotropic forces. Coexistence and interconnection between nanotubes and vesicles 702 are evidenced via cryo-TEM and fluorescence confocal microscopy in a single-phase region at 703 temperature above a transition temperature,  $T_{m1}$  = 48.3°C, determined by DSC on the hydrated 704 sample, but below the second transition temperature,  $T_{m2}$  (95.7°C) determined by DSC on the 705 dry powder. X-ray diffraction (WAXS) also shows the coexistence of two lattice periods, one 706 attributed to an inter-membrane repeating distance and the other possibly related to the intra-707 nanotube glucolipid arrangement. Labelling the systems with Laurdan, a fluorophore of which 708 the emission strongly varies with the local membrane rigidity, excludes the presence of rigid 709 micro-separated phases (e.g.,  $L_{\beta}$ ).

<sup>1</sup>H and <sup>13</sup>C ssNMR, despite the fact that it can only be performed on the dry powder, confirm the presence of one main fluid G-C18:1-OH environment above  $T_{m2}$ . Below  $T_{m2}$ , the splitting of some <sup>13</sup>C peaks and the coexistence of short and long <sup>1</sup>H relaxation components demonstrate that G-C18:1-OH can simultaneously pack into structurally and dynamically inequivalent environments. Combining advanced 2D <sup>1</sup>H-<sup>1</sup>H correlation experiments based on spin diffusion or double-quanta excitation suggests that vesicles could contain a preferred headhead configuration of the bolaform lipid, while nanotubes a preferred head-tail assembly.

The presence of two environments composed of the same molecule characterized by different molecular configurations and rigidity fulfills the theoretical conditions necessary to spontaneously nucleate a membrane inclusion of non-zero average orientation, described as necessary to observe topologically-connected nanotube-vesicle structures in the absence of external forces. This hypothesis could also be enriched by the possibility of nanotubes to be pulled away between two vesicles after a collision event.

This work expands the actual theoretical and experimental framework on lipid networks stabilized by a different radius of curvature. It could also motivate the synthesis, or isolation, of new lipids providing a higher level of 3D architectural control in soft networks, possibly inthe range of room temperature.

727

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739

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904	Supporting Information
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906	Topological connection between vesicles and nanotubes in single-
907	component lipid membranes driven by head-tail interactions
908	
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Parameter		Method
Dry Matter (DM %)	99.6	Infrared balance 105°C
Glucose (%)	0.12	HPLC-Metacarb
Glycerol (%)	n.d.*	HPLC-Metacarb
Eres Estty Asid contant (%)	<0.01	Internal method BBEPP:
Flee Fatty Acid content (%)	<0.01	GC with FID Detector
O(1/0/)	<0.01	Internal method BBEPP:
011 (%)	<0.01	GC with FID Detector
Moulds (CFU/g)	<10	3M-Nordval n°16 B
Yeasts (CFU/g)	<10	3M-Nordval n°16 B
Anaerobic count (CFU/g)	<10	SP-VG M005 B
Protein (%)	t.b.d.*	BCA protein assay

929 Figure S 1 - a) HPLC-ELSD chromatogram and composition table.





Group	N°	δ / ppm
CH₂	1	3.54
CH₂	2	1.53
CH₂	3-7/12-14	1.32
CH₂	8,11	2.03
СН	9,10	5.34
CH₂	15	1.62
CH₂	16	3.88
СН	1'	4.24
СН	2'	3.16
СН	3'	3.34
СН	4'	3.29
СН	5'	3.54
CH <sub>2</sub>	6'	3.66

936 Figure S 1 (next) – b) <sup>1</sup>H NMR spectrum (MeOD-d4) recorded on the purified G-C18:1-OH compounds

#### 937 and related assignment



941 Figure S 2 – Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at *T*\*= 130°C.





944 Figure S 3 - Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at  $T^* = 90^{\circ}$ C.



Figure S 4 – Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at  $T^*$ = 70°C.





955 Figure S 5 – Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at  $T^*= 25^{\circ}$ C.



Figure S 6 – a) SANS experiment recorded at 90°C for a 5 mg/mL G-C18:1-OH aqueous solution. b-c)
WAXS experiments recorded on aqueous solutions of G-C18:1-OH at b) 5 mg/mL and c) 25 mg/mL. d)
Structural interpretation of the repeating distances pointed out in c).



965



Figure S 7 – ssNMR MAS (10 kHz) experiments recorded on a G-C18:1-OH dry powder. a) <sup>1</sup>H spectra recorded from 110°C to 70°C. b) <sup>13</sup>C single pulse (SP) spectra recorded with high power <sup>1</sup>H decoupling from 110°C to 70°C. c) <sup>13</sup>C cross polarization (CP) spectra recorded at 110°C and contact time, t<sub>c</sub>, of 50 µs and 3000 µs. d) <sup>13</sup>C CP spectra recorded at 70°C and for  $20 < t_c / \mu s < 3000$ 





973Figure S 8 – a) Short and d) long  $T_2$  components displayed by the <sup>1</sup>H transverse relaxation signal. c) <sup>1</sup>H974transverse relaxation signals recorded on a G-C18:1-OH dry powder from 110°C to 70°C. The area of each975given resonance is plotted (log-lin) as a function of the echo time (CPMG pulse sequence) for each976temperature and fitted using the equation given above.  $T_{2s}$  and  $T_{21}$  indicate the short and long  $T_2$  relaxation977times respectively obtained by describing the <sup>1</sup>H transverse relaxation signal using a biexponential decay.978For H1', H5' and H6', the <sup>1</sup>H relaxation function could be described by a monoexponential decay.

979 - Protons from the sugar group (H1', H5', H6') show a single  $T_2$  relaxation component (Figure 980 6c and Figure S 8), while protons of the acyl chain (CH<sub>2</sub>, H9,10, H8,11) show both short (Figure 981 6c) and long (Figure 6d)  $T_2$  relaxation component.

- The short  $T_2$  relaxation time systematically decreases from 110°C to 70°C for all species by a factor ranging from two to seven, depending on the specific chemical group. Very long  $T_2$  are observed at 110°C for all acyl species, while nearly unchanged values are measured below 90°C, without any clear trend.

986 - The sugar protons (H1', H5', H6') have the shortest set of  $T_2$  values (< 1 ms) below 100°C.

987 The long component of  $T_2$  generally ranges between 4 ms and 12 ms.

- 988 Table S 1 <sup>1</sup>H chemical shifts of G-C18:1-OH in solution and in the solid-state. Solid-state spectra are
- 989 recorded at a MAS frequency of 10 kHz. \*: in DMSO-d6 ; #: broad peak.



		δ <sub>н</sub> / ppm			
Group	N°	Liquid (MeOD-d4)	Solid ( <i>T</i> = 110°C)	Solid ( <i>T</i> = 70°C)	
CH₂	H1	3.54	3.	57	
CH₂	H2	1.53	1.	56	
CH₂	H3-7/12-14	1.32	1.	35	
CH2	H8,11	2.03	2.03		
СН	H9,10	5.34	5.34		
CH2	H15	1.62	1.64		
CH <sub>2</sub>	H16	3.88	3.83		
СН	H1'	4.24	4.32		
СН	H2'	3.16	3.35		
СН	H3'	3.34	3.35		
СН	H4'	3.29	3.35		
СН	H5'	3.54	3.51 3.57		
CH <sub>2</sub>	H6'	3.66	3.83		
OH*	OH1	4.32			
OH*	OH2'	4.92			
OH*	OH3'	4.90	4.5 <sup>#</sup> 4.63		
OH*	OH4'	4.87			
OH*	OH6'	4.45			

993 Detailed discussion about the NOESY and DQ-SQ 2D <sup>1</sup>H-<sup>1</sup>H ssNMR experiments presented in
994 Figure 9

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2D<sup>1</sup>H-<sup>1</sup>H ssNMR experiments are a unique tool to explore through-space proximities between <sup>1</sup>H nuclei. ssNMR offers a large number of possibilities, each adapted to a given system. The advantage of 2D maps with respect to a dynamics approach is their direct readability; in clear, two nuclei which exchange their magnetization through-space (whichever mechanism may be responsible for this exchange) will display a specific cross-peak. However, the way cross-peaks are presented and interpreted strictly depends on the nature of the pulse program, which in turn is chosen according to the physical nature of the sample.

1D, temperature-dependent, <sup>1</sup>H NMR experiments performed on G-C18:1-OH (Figure 1003 1004 4a in the main text) indicate sharp peaks at 90°C and broad peaks at 70°C. Narrow peaks 1005 indicate fast and/or large-amplitude molecular tumbling, averaging the strong <sup>1</sup>H-<sup>1</sup>H dipolar 1006 interactions. This excludes the use of pulse sequences, like DQ-SQ, which is not efficient under 1007 these conditions. For this reason, we employ spin-diffusion-based pulse sequences, like 1008 NOESY, which is more adapted for samples with high molecular mobility. When mixing times 1009 are chosen to be short (< 1 s), one generally explores through-space proximities within about 5 10 Å. On the contrary, when molecular motions are restricted, dipolar coupling becomes 1010 1011 significant, <sup>1</sup>H NMR peaks are broader and pulse sequences, like DQ-SQ, can now be used, with a spatial resolution of less than 5 Å. 1012

1013 Whenever possible, DQ-SQ experiments are preferred over spin diffusion-based pulse 1014 sequences, because they are more selective, in particular for likewise interactions, which are 1015 not discriminated in NOESY-type experiments. This is illustrated by the typical interaction 1016 schemes for NOESY (top Figure 9a) and DQ-SQ (top Figure 9b). For two given A and B 1017 protons, 2D NOESY maps will display two on-diagonal and two square-shaped symmetrical 1018 off-diagonal cross-peaks. Off-diagonal cross-peaks illustrate the mutual A-B and B-A 1019 interaction, but the on-diagonal peaks are systematic and do not prove any A-A or B-B 1020 interaction. Similarly to NOESY, 2D DQ-SQ maps display off-diagonal peaks (symmetric 1021 across the diagonal, top Figure 9b) upon A-B and B-A interaction, but in contrast they will 1022 display on-diagonal peaks only in the presence of self-interaction. In the tutorial example of 1023 Figure 9b top, the on-diagonal A-A peak indicates mutual A-A interaction while lack of on-1024 diagonal B-B peak evidences the absence of B-B proximities. Combining NOESY and DQ-SQ 1025 experiments is capital to study the local arrangement of G-C18:1-OH and its evolution with 1026 temperature.

1027 At  $T=90^{\circ}$ C, the H1 and H5' contributions are very close but possible to discriminate 1028 them ( $\delta(H1)$ = 3.54 ppm,  $\delta(H5')$ = 3.51 ppm) according to <sup>1</sup>H NMR chemical shifts in Table S 1. 2D <sup>1</sup>H-<sup>1</sup>H NOESY in Figure 9a also discriminates between the two components, attributed 1029 1030 on the basis of the interactions with their mutual neighbors (green H1-H2 and H5'-H1' squares 1031 in Figure 9a). On this basis, 2D NOESY does not show a direct H1'-H1 correlation (blue, red-1032 crossed), but rather an extensive network of intra-sugar interactions. These features exclude 1033 suggest intra- and inter-sugar interactions and they exclude head-tail arrangements as a major 1034 molecular configuration at 90°C.

At 70°C, the <sup>1</sup>H NMR peaks are broader, indicating a reduction of the  $T_2$  (<sup>1</sup>H) relaxation 1035 1036 times and/or residual homonuclear dipolar interactions, excluding the use of NOESY. In this case, <sup>1</sup>H-<sup>1</sup>H DQ-SQ correlation experiments are best suited. Figure 9b presents the <sup>1</sup>H-<sup>1</sup>H DQ-1037 SQ experiment on G-C18:1-OH at 70°C. Peaks are broader than in NOESY and attribution may 1038 1039 be cumbersome. However, the experiment is performed at 700 MHz and MAS= 22 kHz, which 1040 improves spectral resolution with respect to the same experiment performed at 300 MHz and 1041 MAS= 10 kHz. The auto-peak at  $\delta_{\rm H}$ = 3.56 ppm in the SQ dimension is attributed to H1-H1 spin 1042 pairs, mainly resulting from intra CH<sub>2</sub> interaction, while the DQ cross-peaks at  $\delta_{H}$ = 1.56-3.56 1043 ppm and  $\delta_{\text{H}}$ = 4.32-3.56 ppm respectively origin from H2-H1 and H1'-H1 interactions (Figure 9b). DQ-SQ map does not show any auto-peak corresponding to H1'-H1' or Hx'-Hx' (x=2', 1044 1045 3', 4') interactions (red-crossed, blue, Figure 9b), thus excluding any proximity (< 5 Å) between close glucose neighbors. On the contrary, DQ-SQ maps (recorded both at 700 MHz, Figure 9b, 1046 1047 or at 300 MHz, not shown) systematically show DQ cross-peaks, which can only be attributed 1048 to H1'-H1 interactions ( $\delta_{H}$ = 4.32-3.56). The presence of these H1'-H1 interactions can only be 1049 explained by a head-tail arrangement of the G-C18:1-OH molecules, as illustrated below the 1050 DQ-SQ map in Figure 9b.