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Original article

Synthesis and properties of isocannabinoid and cholesterol derivatized rhamnosurfactants: application to liposomal targeting of keratinocytes and skin

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Abstract

The usefulness of vesicles to cargo material depends on the design of new ligands able to incorporate easily inside the bilayer and also to direct the vesicles to the targeted site. Therefore, the synthesis of two new rhamnose-bearing surfactants is described. The hydrophobic part consists of cholesterol (in compound **3**) and citrylidene phloroglucinol (in compound **6**). The ability of these two rhamnolipids to incorporate into a DPPC membrane and to form aggregates is investigated, respectively, by differential scanning calorimetry and by surface tension measurements. Those two new surfactants were incorporated in fluorescent liposomes to study their interactions with keratinocytes and skin sections. Intraliposomal delivery to keratinocytes was observed in both cases, even if the kinetics of delivery were different according to the rhamnosurfactant used. Skin sections were stained by both liposomal formulations, and different interactions between the liposomes and skin cells according to the surfactant used were noted.

Keywords: Rhamnosurfactant; Cannabinoid; Cholesterol; Liposomes; Skin

1. Introduction

The past 30 years have seen new vesicles prepared with synthetic lipids for drug delivery purposes [1,2]. These closed vesicles can entrap water-soluble substances in the inner aqueous phase or lipophilic substances in the bilayer. Liposomal drugs [3] have proven their clinical utility, but tremendous efforts have still to be made to improve their efficiency, particularly in cell targeting. A liposome designed to serve as a vehicle for bioactive compounds has to be stable under physiological conditions, non-damaging for the environment and, in order to be totally efficient, able to target specific cells or tissues. Targeting can enhance drug uptake by the selected cells and reduce drug concentration into the other cells. The development of new liposomes which can increase drug accumulation in specific cells or tissues involves the presence of surface-associated targeting molecules known as ligands [4]. The ligands are natural or synthetic surfactants that are able (a) to recognize specific cells [5]; (b) to incorporate easily inside the liposome bilayer [6]. For example, α-L-rhamnose cell surface receptors are present on the surface of keratinocytes [7]. Therefore α-L-rhamnose bearing surfactants can be used to target liposomes toward these cells. To our knowledge, no publication dealing with such synthetic rhamnosurfactants [8] except alkylrhamnosides [9] can be found in the open literature. In contrast, biological produced rhamnolipids have been largely reported [10].

The ability of cholesterol to bind with phospholipids in a membrane [11] prompted us to synthesize a cholesteryl–rhamnose surfactant (rhamnosurfactant **3**). In order to enlarge the investigation on interactions between the liposome bilayer and the ligand, we also focused our attention on the use of a new hydrophobic moiety and a citrylidene phloroglucinol (in

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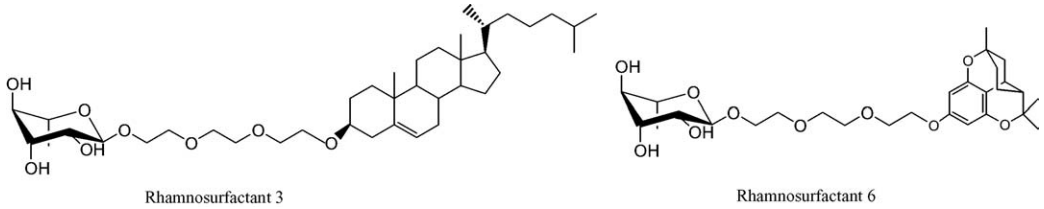


Fig. 1. Rhamnosurfactants **3** and **6**.

rhamnosurfactant **6**) was prepared as isocannabinoid derivatives are also believed to interact with lipids in membranes [12] (Fig. 1).

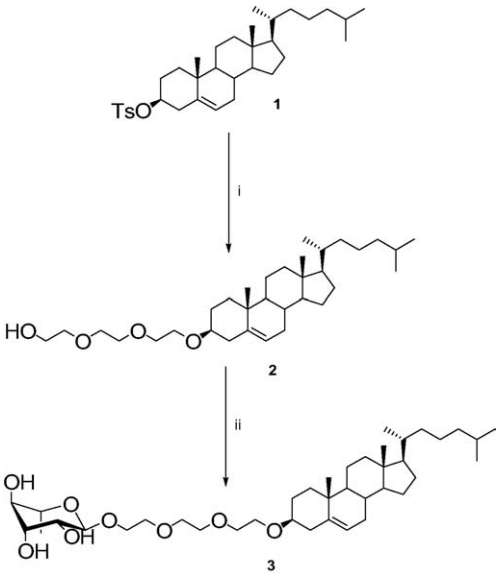
How these variations in the structure would influence the surfactant properties had to be investigated. Therefore, the amphiphilic properties of surfactants **3** and **6** were studied using two different methods: (a) tensiometric determination of the critical micelle concentration (CMC) and (b) thermotropic phase transitions of 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine (DPPC) membranes containing **3** and **6** by differential scanning calorimetry (DSC). Those two new surfactants were incorporated in liposomes to study their interaction with keratinocytes and skin sections. Liposomal delivery to keratinocytes and skin section was done using three types of palmitoyl-oleoylphosphatidylcholine/palmitoyl-oleoylphosphatidylglycerol (POPC/POPG) liposomes: a) liposomes loaded with rhamnosurfactant **3**, (b) liposomes loaded with rhamnosurfactant **6**, and (c) for comparison purposes, liposomes loaded with a cholesteryl succinate [7] instead of one of the rhamnose ligand.

2. Chemistry

The synthesis of rhamnosurfactant **3** was easily accomplished as outlined in Scheme 1. Starting from the cholesteryl toluenesulfonate **1** [13], compound **2** was prepared by refluxing with triethyleneglycol in dioxane [14]. A Fisher reaction [15] with α-L-rhamnose led to the α anomer of rhamnosurfactant **3** in 30% yield Scheme 1.

Under acidic conditions, citrylidene phloroglucinol leads to a new isocannabinoid due to the opening of the pyrane ring [16,17]. For this reason, the glycosylation cannot be done in the presence of citrylidene phloroglucinol and another strategy had to be followed for the synthesis of compound **6** (Scheme 2). The glycosylation was therefore carried out starting from α-L-rhamnose and triethyleneglycol under acidic conditions [14] to give compound **4** in 52% yield. In the second step, selective bromination of the primary alcohol with triphenylphosphine and carbon tetrabromide [18] gave the expected compound **5** in 55% yield. This compound was then condensed with citrylidene phloroglucinol [19] under basic conditions [20] to afford surfactant **6** in 65% yield (α/β anomer 90/10) Scheme 2.

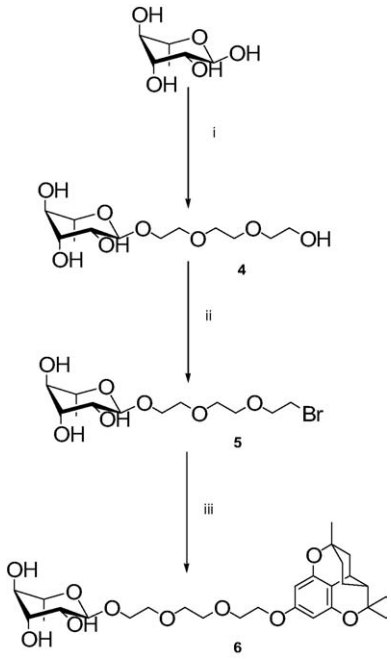
The different compounds were fully characterized using ^{1}H, ^{13}C NMR and mass spectroscopy.



Scheme 1. Synthesis of rhamnosurfactant **3**. Reagents and conditions: (i) triethyleneglycol, dioxane, Δ; (ii) *p*-toluenesulfonic acid, THF, Δ.

3. Results and discussion

The capacity of each surfactant to form aggregates was studied by CMC determinations. The surface tension method



Scheme 2. Synthesis of rhamnosurfactant **6**. Reagents and conditions: (i) triethyleneglycol, *p*-toluenesulfonic acid Δ; (ii) PPh_3, CBr_4; (iii) Cs_2CO_3, DMF, Δ.

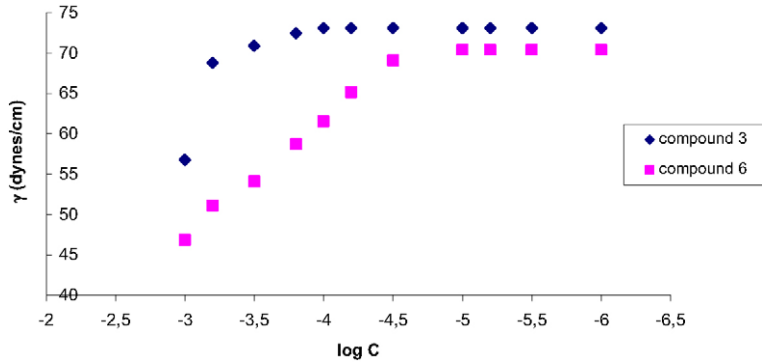


Fig. 2. Plot of surface tension vs. the log of the concentration of **3** and **6**.

[21] was used to measure the CMC from the plots of the surface tension (σ) against the log of the surfactant concentration, log(C). Clear break points were observed at 0.1 mM for rhamnosurfactant **3** and 0.025 mM for rhamnosurfactant **6**. Fig. 2 gives the data graphically.

The structures of the two lipids are similar in many ways. Although they both have a terpene-derived cyclic structure, the hydrophobic moieties differ appreciably. Micellization is driven, among others, by hydrophobic interactions between the surfactants. Changing the hydrophobic chain from cholesterol to citrylidene phloroglucinol decreases the CMC about fourfold, which can reflect a more hydrophobic behavior of citrylidene phloroglucinol with respect to the cholesterol moiety. This observation is reasonable as the tremendous affinity of cannabinoids for membranes was the main reason for the difficulty to detect their receptors [22].

The use of liposomes depends on their chemical composition [23]. The nature of lipids has considerable influence on the properties of the liposomes. For this reason, it is necessary to characterize the thermal behavior of liposomes prepared with our synthetic lipids. The influence of rhamnosurfactants **3** and **6** on the thermotropic behavior of a membrane was assayed by DSC with DPPC as the model membrane. The calorimetric scans were made on DPPC multilamellar vesicles in the presence of several concentrations of our synthetic lipids. The hydrated DPPC lipids undergo a low enthalpy pretransition prior to a chain-melting transition [24] which were detected at 33 and 41 °C, respectively. The adsorption of surfactants **3** and **6** within the DPPC bilayer had a pronounced effect upon the thermotropic properties of the DPPC membrane (Fig. 3a,b).

In both cases, the endothermic adsorption shifts regularly to lower temperature as the amount of synthetic lipids was increased. However, the cholesteryl surfactant **3** broadens and shifts more significantly the phase transition of DPPC in comparison to the citrylidene phloroglucinol surfactant. The decrease in phase transition temperature indicates that the glycolipids we have synthesized can indeed bind to the phospholipids bilayer.

3.1. Intraliposomal delivery to cultured human keratinocytes (HK)

To follow the intraliposomal delivery to keratinocytes, liposomes were formed in presence of propidium iodide (IP), intensively washed and then incubated to the cells. The tracer, namely IP, was observed after 10 min of incubation in all the conditions tested. However, liposomes loaded with rhamnosurfactants **3** and **6** showed a strong nuclear staining to 10% or 27% of the cells, respectively, when liposomes without

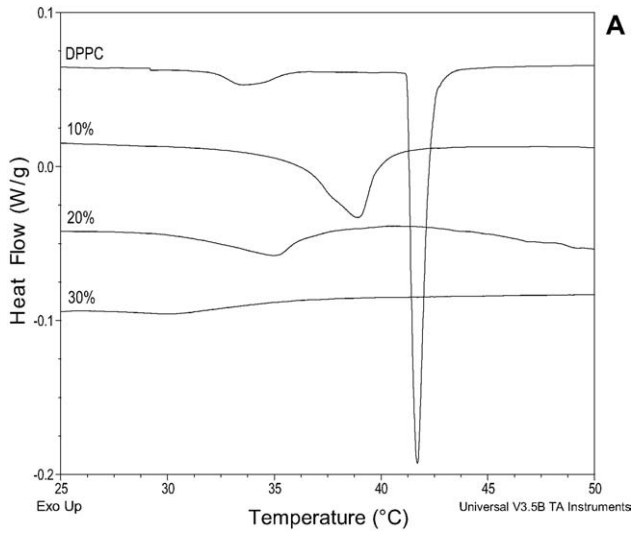


Fig. 3a. DSC thermogram for aqueous dispersions of **3** with DPPC.

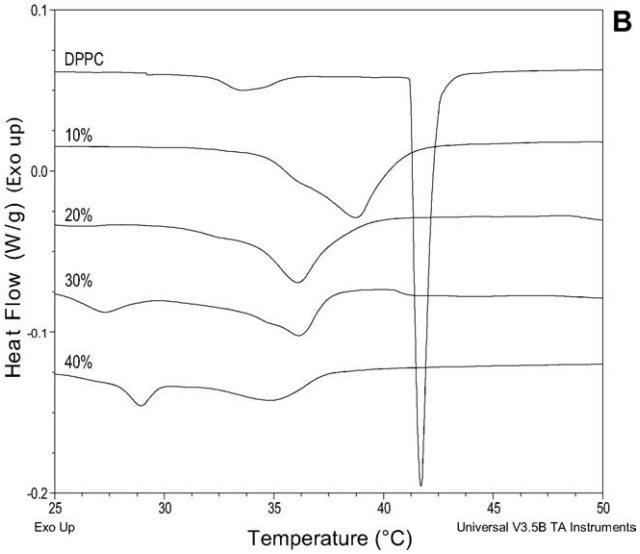


Fig. 3b. DSC thermogram for aqueous dispersions of **6** with DPPC.

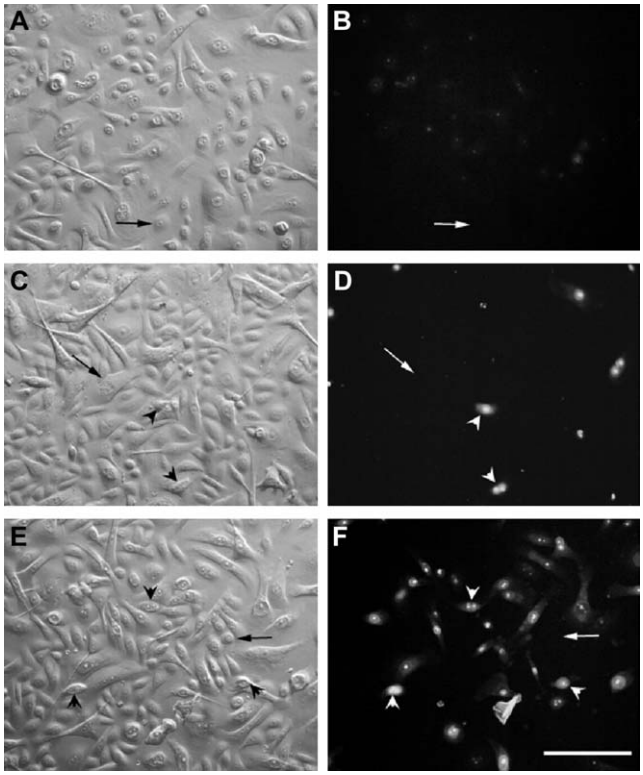


Fig. 4. Liposome delivery to cultured HK. Liposomes (cholesterylsuccinate (A, B), compound **3** (C, D), compound **6** (E, F)) containing propidium iodide (IP) were loaded onto cultured keratinocytes. After 40 min of incubation, cultures were observed by Hoffman contrast (A, C, E) and by epifluorescence (B, D, F). Note that IP staining was weak and mainly nucleolar when cells are incubated with cholesterylsuccinate-liposome whereas a strong nuclear staining was observed fort both rhamnosurfactant 3- and rhamnosurfactant 6-liposomes. Arrows point to negative cells, arrowheads to positive cells. Bar represents 50 μm.

ligand showed only a weak nucleolar staining (Fig. 4). After 40 min of incubation the same profile was observed except for liposomes loaded with rhamnosurfactant **3** which stained cells up to 23%.

These results showed that both ligands increased the intraliposomal delivery to the cells suggesting that a physical interaction could occur between the L-rhamnose and the cells. Two kinetics were observed according the compound tested, that also suggested either that the lipophilic part influenced the transfer or that different receptors existed at the surface of the cells.

3.2. Liposomal adhesion to human skin cells

In order to characterize the interactions between the liposomes and human skin cells, liposomal solutions were formed in presence of Oregon Green® 488 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DHPE-fluoresceine, Interchim, France). This tag allowed us to trace the liposomes fixed on the skin sections.

Cholesterylsuccinate-liposomes did not fix skin cells as shown in Fig. 5 whereas rhamnosurfactant **3**- and rhamnosurfactant **6**-liposomes stained the skin section. Their staining

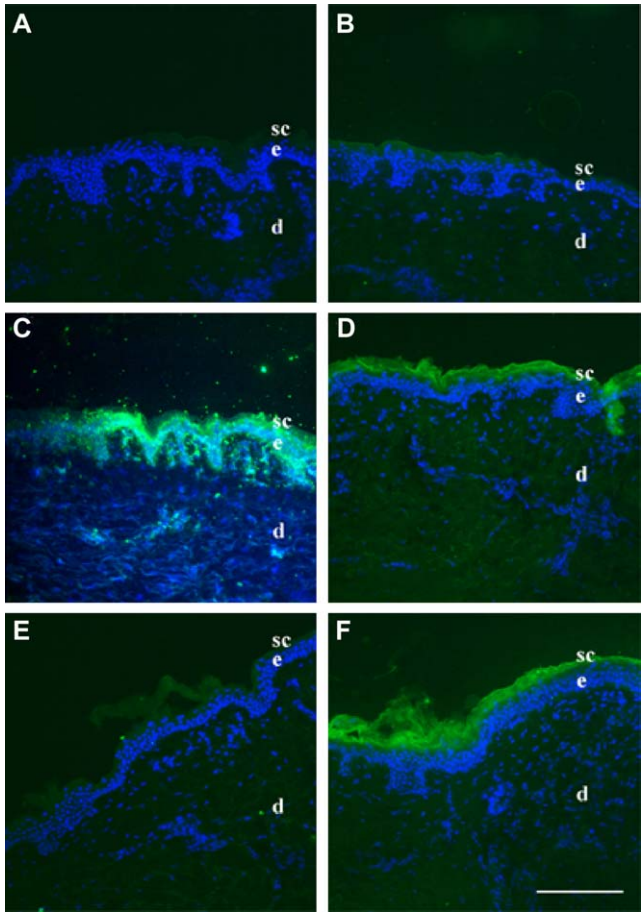


Fig. 5. Liposome fixation to skin sections. Liposomes were tagged with a fluorescent lipid (green) and then applied to the skin section counterstained with Hoescht 33258 (blue). The nuclei staining (blue) allowed us to visualize the different compartment of the skin, namely the epidermis (e) and the dermis (d). No staining was observed in the mock control (A) as in the skin section incubated with chol-liposome (B). Rhamnosurfactant 3-liposome strongly stained all the layers of the epidermis (C) whereas rhamnosurfactant 6-liposome staining was restricted to the anuclei layers of the epidermis i.e. the cornified layers (sc, D). This staining was inhibited when the section was incubated with L-rhamnose prior rhamnosurfactant 6-liposome incubation (E), but not when both incubations were preformed simultaneously (F). Bar represents 50 μm.

was different, through out the epidermis for rhamnosurfactant **3** and the uppermost layers of the epidermis for rhamnosurfactant **6**. No clear staining has been noticed in the dermis.

To further test this interaction, free L-rhamnose was used in competition with the rhamnosurfactant **6**-liposome. When free L-rhamnose was incubated prior to the liposome, a complete inhibition of the liposome–skin cell interaction was observed. However, no difference was shown when L-rhamnose and rhamnosurfactant **6**-liposome were incubated simultaneously. The same result was obtained with the rhamnosurfactant **3**-liposome (data not shown).

These observations could fit with what was observed previously with cultured keratinocytes. Indeed, two different kinetics according to the compound used were obtained for the intraliposomal delivery and two different staining were detected on human skin sections. However, rhamno-

surfactant-**6** liposome stained the uppermost layer of the epidermis that is known to produce high background interactions and also that is composed of terminally differentiated cells that were not present in cultures. Hence, even if results concordate between the two set of experiments, one might be cautious with their interpretations. Nonetheless, in the open literature two rhamnose-receptors have been characterized in steelhead trout. They are also characterized by different affinity to L-rhamnose and different activity in hemagglutination assay. The search for L-rhamnose receptors in human deserves full attention.

4. Conclusion

The synthesis of two rhamnose-bearing surfactants has been described. The ease of their synthesis, their ability to incorporate liposome bilayers, and the lack of toxicity [25] of their natural constituents make these sugar surfactants appropriate tools for the studies of liposomes–cell interactions and further development to human. Thus, liposome bearing ligands reported there-in have been successfully used to deliver material to HK. Moreover, experiments on skin sections have shown specific interaction with epidermis. Considering these results, such ligands are interesting candidates for the development of new tagged liposomes which can find applications in medicine as well as in cosmetic fields.

5. Experimental section

5.1. Materials and methods

5.1.1. Chemistry

Melting points were determined on a Büchi apparatus and are uncorrected. TLC analysis were performed on aluminum sheets coated with Merck silica gel 60 F_{254}. Compounds were visualized by spraying the TLC plates with dilute 10% aqueous sulfuric acid solution or anisaldehyde solution, followed by charring at 150 °C. ^{1}H and ^{13}C NMR spectra were recorded on Brüker AC-200 and AC-400 spectrometers, respectively. Chemical shifts (δ) are reported in parts per million from internal standard tetramethylsilane. Coupling constants (J) are measured in Hertz. Multiplicity is reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet) and combination of these signals. Optical rotations were recorded on a Perkin Elmer 241 polarimeter. Fast atom bombardment mass spectra (FAB-MS) were recorded in positive mode on a JEOL DX 300 spectrometer using NOBA (nitro-benzylic alcohol) as matrix. Column chromatographies were performed on silica gel 60. All solvents used for reactions were anhydrous and all reactions were carried out under inert atmosphere. Compounds are named according to IUPAC rules.

*5.1.2. Cholest-5-en-3β-ol-p-toluenesulfonate (**1**)*

A solution of cholest-5-en-3β-ol (5 g, 12.93 mmol) and *p*-toluenesulfonylchloride (2.71 g, 14.22 mmol) in dry pyridin (12.5 ml) was stirred for 12 h at room temperature. Following addition of water (25 ml), the mixture was extracted with diethyl ether (3 × 25 ml). The organic phases were dried over anhydrous sodium sulfate, and concentrated in vacuo to give the expected compound as a white powder in 90% yield. The compound was recrystallized in petroleum ether. R*f* = 0.82 (silica, methylene chloride); m.p. 130–132 °C (Litt. 132 °C); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.82 (d, 2H, J = 8.3 Hz); 7.36 (d, 2H, J = 8.6 Hz); 5.32 (d, 1H, J = 5.3 Hz); 4.29–4.40 (m, 1H); 1.62 (s, 3H); 0.99 (s, 3H); 0.92 (d, 3H, J = 7.6 Hz); 0.88 (d, 6H, J = 7 Hz); 0.68 (s, 3H).

*5.1.3. 8-(Cholest-5-en-3β-yloxy)-3,6-dioxaoctan-1-ol (**2**)*

To a solution of cholesteryl tosylate (1 g, 1.85 mmol) in dry dioxane (40 ml) was added tri(ethyleneglycol) (10 ml, 74 mmol). The mixture was refluxed for 5 h under a nitrogen atmosphere. Then the reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 5% aqueous solution of $NaHCO_3$, water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give an yellow oil which was chromatographed on silica gel (petroleum ether/ethyl acetate 8:2). The expected compound was obtained as a white oily solid in 78% yield. R*f* = 0.17 (silica, petroleum ether/ethyl acetate 6:4); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.34 (d, 1H, J = 5.16 Hz); 3.59–3.73 (m, 12H); 3.17–3.21 (m, 1H); 2.79 (m, 1H); 1.02 (s, 3H); 0.91 (d, 3H, J = 6.45 Hz); 0.87 (d, 6H, J = 6.55 Hz); 0.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.3; 122.0; 80.0; 73–67.6; 62.2; 57.2; 56.6; 50.0; 42.7; 40.2; 39.9; 39.4; 37.6; 37.3; 36.6; 36.2; 32.4; 32.3; 28.7; 28.6; 28.4; 24.7; 24.2; 23.2; 23.0; 21.5; 19.8; 19.1; 12.3 . FAB-MS: *m/z*: 541 [M + Na^+].

5.1.4. 8-(Cholest-5-en-3β-yloxy)-3,6-dioxaoctanyl α-L-rhamnopyranoside (3)

To a solution of L-(+)-rhamnose (60.6 mg, 0.33 mmol) and 8-(cholest-5-en-3β-yloxy)-3,6-dioxaoctan-1-ol (85 mg, 0.166 mmol) in dry tetrahydrofuran (4 ml) was added *p*-toluenesulfonic acid (72.4 mg, 0.38 mmol). The reaction mixture was stirred at 60 °C for 2 days. After addition of anhydrous sodium carbonate, the mixture was filtered, and the filtrate concentrated in vacuo. The residue was chromatographed on silica gel (methylene chloride with gradient of methanol 1–5%) to give in 30% yield the expected compound as a white oily solid. R*f* = 0.47 (silica, methylene chloride/methanol 95:5); $[\alpha]^{25}_D$ = –33 (c = 1; $CHCl_3$); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.35 (d, 1H, J = 5.13 Hz); 4.84 (d, 1H, J = 1.32 Hz); 3.97 (br, 2H); 3.73–3.84 (m, 2H); 3.65–3.67 (m, 12H); 3.46–3.52 (m, 2H); 3.15–3.23 (m, 1H); 2.66 (br.s, 1H); 1.32 (d, 3H, J = 6.17 Hz); 1 (s, 3H); 0.93 (d, 3H, J = 6.44 Hz); 0.88 (d, 6H, J = 6.54 Hz), 0.69 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 141.2; 122.1; 100.3; 80.0; 73.5; 72.1; 71.2; 71.2; 71.0; 70.7; 68.7; 67.6; 66.9; 57.2; 56.6; 50.6; 42.7; 40.2; 39.9; 39.4; 37.6; 37.3; 36.6; 36.2; 32.4; 32.3; 28.7; 28.7; 28.4; 24.7; 24.3; 23.2; 23.0; 21.5; 19.8; 19.1; 18.0; 12.3. FAB-MS: *m/z*: 665 [M + H^+], 687 [M + Na^+], 703 [M + K^+].

5.1.5. 1,3,6-Trioxanonanyl L-rhamnopyranoside (4)

To a solution of L-(+)-rhamnose (5 g, 27.4 mmol) and tri-(ethyleneglycol) (11 ml, 82.34 mmol) in dry tetrahydrofurane (100 ml) was added *p*-toluenesulfonic acid (6.78 g, 35.68 mmol). The reaction mixture was stirred for 2 days at 60 °C. After addition of anhydrous sodium carbonate, the solution was filtered and concentrated in vacuo to give a yellow oil which was chromatographed on silica gel (methylene chloride with methanol gradient 1–10%). The expected compound was obtained as a colorless oil in 51% yield (α/β anomer 95/5). R*f* = 0.55 (silica, methylene chloride/methanol 8:2); $[\alpha]^{25}{}_{D} = -38$ (*c* = 1; $CHCl_3$); ^{1}H NMR (CD_3OD, 200 MHz) α anomer: δ 4.74 (d, 1H, *J* = 1.65 Hz); 3.77–3.85 (m, 2H); 3.58–3.71 (m, 12H); 3.32–3.43 (m, 2H); 1.28 (d, 3H, *J* = 6.2 Hz). β Anomer: δ 4.62 (s, 1H); 3.77–3.85 (m, 2H); 3.58–3.71 (m, 12H); 3.32–3.43 (m, 2H); 1.28 (d, 3H, *J* = 6.2 Hz). ^{13}C NMR (100 MHz, CD_3OD) δ 100.8; 73.0; 72.7; 72.7; 71.2; 70.7; 70.4; 70.4; 68.8; 66.7; 61.2; 17.0. FAB-MS: *m*/*z*: 297 [*M* + H^+], 319 [*M* + Na^+], 335 [*M* + K^+].

5.1.6. 1-Bromo-3,6-dioxaoctanyl L-rhamnopyranoside (5)

To a solution of 1,3,6-trioxanonanyl-L-rhamnopyranoside (2.4 g, 7.66 mmol) and tetrabromomethane (5.1 g, 15.32 mmol) in dry tetrahydrofurane (100 ml) was added triphenylphosphine (4.03 g, 15.32 mmol). The mixture was stirred at room temperature for 12 h. Then the mixture was filtered and the filtrate was concentrated in vacuo. The residue was chromatographed on silica gel (methylene chloride with gradient methanol 1–5%) to give in 55% yield the expected compound as a colorless oil (α/β anomer 90/10). R*f* = 0.42 (silica, methylene chloride/methanol 9:1); $[\alpha]^{25}{}_{D} = -42$ (*c* = 1; $CHCl_3$); ^{1}H NMR ($CDCl_3$, 200 MHz) α anomer: δ 4.83 (s, 1H); 3.99–4.13 (m, 2H); 3.68–3.87 (m, 13H); 3.51 (m, 4H); 1.33 (d, 3H, *J* = 6.13 Hz). β Anomer: δ 4.55 (s, 1H); 3.99–4.13 (m, 2H); 3.68–3.87 (m, 13H); 3.51 (m, 4H); 1.33 (d, 3H, *J* = 6.13 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 100.4; 73.1; 72.9; 72.0; 71.6; 71.2; 70.9; 70.6; 68.6; 66.9; 30.8; 18.0. FAB-MS: *m*/*z*: 359 [M^{79}Br + H^+], 361 [M^{81}Br + H^+], 381 [M^{79}Br + Na^+], 383 [M^{81}Br + Na^+], 739 [2M^{79}Br + Na^+], 743 [2M^{81}Br + Na^+].

5.1.7. Citrylidene phloroglucinol

A solution of phloroglucin (6 g, 37 mmol) and freshly distilled citral (7 ml, 40.7 mmol) in dry pyridin (3.6 ml, 44.4 mmol) was refluxed for 6 h. After cooling, chloroform was added to the mixture and the solution was washed with 10% aqueous solution of HCl and with water. The organic layers were dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was chromatographed on silica gel (petroleum ether/diethyl ether 9:1) to give a white solid (40% yield) which was crystallized from a mixture petroleum ether/methylene chloride. m.p = 151–152 °C. (Ref. [19], 150–152 °C); R*f* = 0.44 (silica, petroleum ether/diethyl ether 6:4); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.04 (s, 2H); 4.77 (s, 1H); 2.84 (ddd, 1H, *J* = 3.16, 2.69, 1.70 Hz); 2.23 (ddd, 1H, *J* = 13.25, 3.16, 1.55 Hz); 2.03 (ddd, 1H, *J* = 11.38, 5.05, 2.69 Hz); 1.83 (dd, 1H, *J* = 13.22, 1.70 Hz); 1.74 (ddd, 1H, *J* = 13.54, 5.69, 5.05 Hz); 1.54 (s, 3H); 1.45 (dt, 1H, *J* = 13.54, 13.28, 5.25 Hz); 1.39 (s, 3H); 1.24 (dt, 1H, *J* = 13.28, 5.25, 5.05 Hz); 1.05 (s, 3H); 0.69 (dddd, 1H, *J* = 13.54, 13.28, 11.38, 5.69 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 157.8; 157.5; 156.0; 109.8; 98.9; 97.3; 85.0; 75.5; 47.1; 37.7; 35.7; 30.0; 29.4; 28.1; 24.1; 22.4. FAB-MS: *m*/*z*: 261 [*M* + H^+].

5.1.8. 1-Citrylidenephloroglucinyl-3,6-dioxaoctanyl L-rhamnopyranoside (6)

A solution of citrylidene phloroglucinol (75 mg, 0.289 mmol) and cesium carbonate (282 mg, 0.867 mmol) in dry DMF (3 ml) was stirred at 70 °C for 1 h. 1-Bromo-3,6-dioxaoctanyl-L-rhamnopyranoside (217.4 mg, 0.577 mmol) was then added and the mixture was stirred at 50 °C for 2 h. The mixture was diluted with methylene chloride and washed three times with brine. The organic phase was dried over anhydrous sodium sulfate, filtered and concentrated in vacuo. The residue was chromatographed on silica gel (methylene chloride with methanol gradient 1–5%) to give the expected compound in 65% yield (α/β anomer 90/10) as a white oily solid. R*f* = 0.39 (silica, methylene chloride/methanol 95:5); $[\alpha]^{25}{}_{D} = -31$ (*c* = 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 6.04 (s, 2H); 4.74 (d, 1H, *J* = 1.7 Hz); 4.62 (s, 1H); 3.85–3.77 (m, 2H); 3.71–3.58 (m, 12H); 3.43–3.32 (m, 2H); 2.84 (ddd, 1H, *J* = 3.2, 2.7, 1.70 Hz); 2.23 (ddd, 1H, *J* = 13.3, 3.2, 1.6 Hz); 2.03 (ddd, 1H, *J* = 11.4, 2.7, 5.1 Hz); 1.83 (dd, 1H, *J* = 13.2, 1.70 Hz); 1.74 (ddd, 1H, *J* = 13.5, 5.7, 3.1 Hz); 1.54 (s, 3H); 1.45 (dt, 1H, *J* = 13.5, 13.3, 5.3 Hz); 1.39 (s, 3H); 1.28 (d, 3H, *J* = 6.2 Hz); 1.24 (dt, 1H, *J* = 13.3, 5.3, 5.1 Hz); 1.05 (s, 3H); 0.639 (dddd, 1H, *J* = 13.5, 13.3, 11.4, 5.7 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.2; 157.9; 157.5; 110.1; 100.3; 98.2; 98.2; 96.1; 84.6; 75.3; 73.4; 72.1; 71.3; 71.2; 71; 70.6; 70.1; 68.5; 68.0; 66.9; 47.1; 37.7; 35.7; 30.1; 29.4; 28.2; 24.2; 22.4; 18.0. FAB-MS: *m*/*z*: 539 [*M* + H^+], 561 [*M* + Na^+].

5.2. Critical micelle concentration (CMC)

5.2.1. Materials

CMCs were determined at 24 °C with a Du Noüy tensiometer. The apparatus was calibrated before measurements.

5.2.2. Method

Deionized water and dioxane (1%) were added to the appropriate amount of surfactant to a final volume of 20 ml and a concentration of 10^{-3} M. Several solutions of decreasing concentrations were prepared by diluting the primary one until a 10^{-6} M solution was obtained. Surface tensions (mN/m) measurements were determined for each solution. The CMC values were given by the intersection of the curve slopes. The reproducibility of the experiments was checked by, at least, three consecutive measurements for each concentration.

5.3. Differential scanning calorimetry (DSC)

5.3.1. Materials

DPPC was purchased from Sigma-Aldrich. The DSC experiments were performed on a T.A. Instrument Q 100.

5.3.2. Method

Appropriate amounts of DPPC and surfactant were dissolved in a small volume of chloroform. The solution was then evaporated under vacuum with a bath temperature around 50 °C (temperature above the phase transition temperature of DPPC) and the last traces of solvent were removed with high vacuum for 2 h. Deionized water was added to the dried samples to a final concentration of lipids around 0.5 mg/ml. The mixtures were heated with a water bath to 50 °C for 30 min then vortexed for 1 min. The operation was repeated three times. An equivalent volume of each sample was placed into small aluminium pans which were then sealed. Prior to scanning, samples were held at 50 °C for 5 min to ensure complete equilibrium. During scanning, a reference pan containing an identical volume of water was used. The heating and cooling rates were 10 °C/min in all the experiments. The measurements were performed in the temperature interval from 10 to 60 °C. The reproducibility of the DSC experiments was checked by three consecutive scans for each sample.

5.4. Liposome preparation, cell culture and liposome delivery

Large vesicles were prepared by hydration of a film comprised of 80% of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Sigma-Aldrich), succinate derivative of cholesterol or compound **3** or compound **6** (15%), and 5% of 1-palmitoyl-2-oleoyl-3-phosphoglycerol (POPG, Sigma-Aldrich)) (5×10^{-7} mol total lipid) with a 0.5 ml phosphate buffer saline (PBS) solution (1 μM) of propidium iodide, followed by 19 extrusions through a 100 nm polycarbonate filter (LiposoFast, Avestin). The lipid mixture allowed, at room temperature, to prepare the liposomes above the phase transition temperature.

After centrifugation the supernatant was discarded and the pellet resuspended in PBS. This operation was repeated three times, the final volume of the liposome suspension being 30 μl.

HK were isolated from foreskin following the Rheinwald and Green [26] method. They were cultured in serum free medium (SFM) supplemented with epidermal growth factor (EGF, 5 ng/ml) and bovine pituitary extract (BPE, 50 μg/ml, Invitrogen Europe) at 37 °C, 5% CO_2 in a humidified atmosphere.

Two to three days before the liposome delivery assay, cells were split on a coverslip at 1,000 cells per cm^2. Then, 20 μl of liposome solutions (average density 50,000 per ml) were loaded onto the cells and incubated for 10 and 40 min. At each time point, cells were observed using an inverted microscope (Nikon Eclipse TE300) equipped with Hoffmann contrast, epifluorescence and a digital camera (Nikon DXM1200) for picture recording.

5.5. Liposome preparation, skin section and liposome adhesion

Large vesicles comprised of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, 75%, Sigma), succinate derivative of cholesterol or compound **3** or compound **6** (15%), 1-palmitoyl-2-oleoyl-3-phosphoglycerol (POPG, 5%, Sigma) and Oregon Green® 488 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Oregon Green® 488 DHPE, 5%, Molecular Probes), were prepared by the hydration method with Dulbecco's phosphate buffer saline (DPBS, Eurobio) as described above.

Skin biopsies obtained from plastic surgery after collecting informed patient consent, were embedded into OCT-compound (Labonord, Templemars, France), snap-freezed in liquid nitrogen and store at –80 °C until used. Six μm section were obtained using the microcryotome (Slee, Mainz, Germany) and incubated with a saturating solution (0.1% gelatin in PBS (Sigma) for 2 hr in a humidified chamber. Ten microliters of a liposomal solution (average density 50,000 per ml) were then loaded onto the section and incubated for 2 hr in a dark humidified chamber, washed twice in 0.1% gelatin in PBS for 15 min and finally washed once in 0.1% gelatin in PBS containing Hoescht 33258 to stain cell nuclei and once with PBS. Observations were performed using the microscope described above. For the L-rhamnose competition, sections were either preincubated in L-rhamnose (5×10^{-5} mol/l final concentration) and then with the liposomal solution or incubated simultaneously with both solutions. Sections were then treated as described above.

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