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Research paper

Unsaturated fatty acids lactose esters: cytotoxicity, permeability enhancement and antimicrobial activity

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Chemical compounds studied in this article:
(*Z*)-6′-*O*-Hexadec-9-enoyl lactose (lactose palmitoleate, URB1076)
(*Z*)-6′-*O*-Tetracos-15-enoyl lactose (lactose nervonate, URB1077)

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ABSTRACT

Sugar based surfactants conjugated with fatty acid chains are an emerging broad group of highly biocompatible and biodegradable compounds with established and potential future applications in the pharmaceutical, cosmetic and food industries. In this work, we investigated absorption enhancing and antimicrobial properties of disaccharide lactose, monoesterified with unsaturated fatty acids through an enzymatic synthetic approach. After chemical and cytotoxicity characterizations, their permeability enhancing activity was demonstrated using intestinal Caco-2 monolayers through transepithelial electrical resistance (TEER) and permeability studies. The synthesized compounds, namely lactose palmitoleate (URB1076) and lactose nervonate (URB1077), were shown to exhibit antimicrobial activity versus eight pathogenic species belonging to Gram-positive, Gram-negative microorganisms and fungi.

1. Introduction

Over the past few decades there has been a growing interest on sugar-based surfactants due to the large range of applications, from the biomedical field to cosmetics and food industries [1,2]. This class of molecules is generally classified as biocompatible and biodegradable non-ionic surfactants with emulsifying and antimicrobial abilities [3,4]. Their surface-active properties and applications are mainly influenced by the nature of the sugar headgroup (e.g. mono-, di- or polysaccharides), the carbon chain length and the degree of substitution [5].

The increasing demand for healthy and non-toxic additives has intensified the need for, and research on, novel compounds for food, medical and pharmaceutical applications. In this context, the development of sugar-fatty acid esters is becoming increasingly attractive. Among their possible applications, absorption-enhancing potential for biologics delivery has been recently evaluated [6,7].

Biological therapeutics (biologics) have and will continue to have a major impact on the management of a number of diseases. While their therapeutic potential is often unmatched by small drug molecules, biologics suffer from injection-only administration. Non-invasive delivery of this class of therapeutics is highly attractive. However, drug delivery technologies, which offer the possibility to achieve safe and clinically relevant non-invasive delivery of biologics, are currently lacking. The key challenge to achieving this is a poor permeation of therapeutic macromolecules across the mucosal surfaces [8], which have evolved as biological structures presenting a barrier to the movement of material from the external environment into the systemic circulation.

The use of absorption enhancing agents is a common approach utilized to improve mucosal absorption (and hence the resulting bioavailability) of biologics following mucosal administration [8–11]. While the use of absorption enhancing agents offers significant potential in enabling non invasive delivery of biologics, ‘absorption enhancers’, which are chemically diverse compounds

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exerting their absorption-enhancing effect through different mechanism(s), have often been associated with unacceptable toxicity profile [12]. Absorption enhancers that are capable of improving the mucosal absorption of biotherapeutics in a safe and therapeutically-effective manner are highly desirable, but the search for these continues [13–15].

In this study we synthesized and characterized lactose palmitoleate and lactose nervonate, two new biodegradable lactose esters based on unsaturated fatty acids, namely palmitoleic (C16:1ω7) and nervonic (C24:1ω9) acids. The cytotoxicity of these compounds was evaluated in vitro and associated with the capacity to act as oral absorption enhancers of biotherapeutics as tested on the intestinal Caco-2 monolayers. Additionally, the compounds were also evaluated for antimicrobial activity by testing minimum inhibitory concentration (MIC) and effect on the growth inhibition of several pathogenic microorganisms.

2. Experimental section

2.1. Chemicals, materials and methods

Palmitoleic acid and nervonic acid were purchased from TCI, lactose monohydrate from Carlo Erba, while Lipozyme® (immobilized from *Mucor miehei*), *p*-toluenesulfonic acid, 2,2-dimethoxypropane, tetrafluoroboric acid diethyl ether complex and all organic solvents used in this study were purchased from Sigma. Prior to use, acetonitrile was dried with molecular sieves with an effective pore diameter of 4 Å and toluene was saturated with water. Caco-2 cells were obtained from the European Collection of Cell Cultures. Dulbecco's Modified Eagles Medium (DMEM), Hank's Balanced Salt Solution (HBSS, with sodium bicarbonate and without phenol red), non-essential amino acids (100%), L-glutamine (200 mM), fetal bovine serum (FBS), antibiotic/antimycotic solution (10–12,000 U/mL penicillin, 10–12 mg/mL streptomycin, 25–30 μg/mL amphotericin B), trypsin–EDTA solution (2.5 mg/mL trypsin, 0.2 mg/mL EDTA) and fluorescein isothiocyanate-labeled ovalbumin (FITC-OVA) were supplied by Sigma (Poole, UK). MTS reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (commercially known as CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay) was purchased from Promega (USA). Tissue culture flasks (75 cm^3 with ventilated caps), black 96-well plates and Transwell® inserts (12 mm diameter, 0.4 μm pore size, were purchased from Corning (USA). All other chemicals (reagent grade) were purchased from Sigma. Ultrapure chitosan chloride of 213 kDa average molecular weight ('Protasan UP CL 213') was obtained from Novamatrix (Denmark). Thermal analysis was carried out using differential scanning calorimetry (DSC). DSC analysis was performed using a DSC 8500 (Perkin-Elmer, Norwalk, USA) equipped with an intracooler (Intracooler 2, Perkin-Elmer, Norwalk, USA) and analyzed in an inert N_2 atmosphere. The structures of compounds were unambiguously assessed by MS, ^{1}H NMR, ^{13}C NMR, and IR. ESI-MS spectra were recorded with a Waters Micromass ZQ spectrometer in a negative or positive mode using a nebulizing nitrogen gas at 400 L/min and a temperature of 250 °C, cone flow 40 mL/min, capillary 3.5 kV and cone voltage 60 V; only molecular ions $[M-H]^-$ or $[M+NH_4]^+$ are given. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 400 or 101, respectively, spectrometer and analyzed using the TopSpin software package. Chemical shifts were measured by using the central peak of the solvent. IR spectra were obtained on a Nicolet Atavar 360 FT spectrometer. Column chromatography purifications were performed under "flash" conditions using Merck 230–400 mesh silica gel. TLC was carried out on Merck silica gel 60 F254 plates, which were visualized by exposure to ultraviolet light and by exposure to an aqueous solution of ceric ammonium molybdate.

2.2. Synthesis of lactose-based surfactants

2.2.1. General procedure for the synthesis of lactose tetra acetate esters (Z)-6′-O-hexadec-9-enoyl- and (Z)-6′-O-tetracos-15-enoyl-4-O-(3′,4′-O-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-O-isopropylidene-1,1-di-O-methyl-D-glucopyranose (**3a,b**)

Lipozyme® (0.078 g) was added to a solution of palmitoleic acid (**1a**) or nervonic acid (**1b**) (0.79 mmol) and 4-*O*-(3′,4′-*O*-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucopyranose (lactose tetra acetate, LTA) [16] (**2**) (0.401 g, 0.79 mmol) in water-saturated toluene at 25 °C. The mixture was stirred at 75 °C for 12 h, cooled, diluted with acetone, then filtered, and the filtrate was concentrated. The purification of the residue by column chromatography (petroleum ether/EtOAc 7:3) gave **3a,b** as pale yellow oils.

3a. Yield: 70% (0.413 g). ESI-MS: *m/z* 744 $(M-H)^-$, 763 $(M+NH_4)^+$. ^{1}H NMR (CD_3OD) δ: 0.93 (t, 3H, J = 6.7 Hz, CH_3), 1.30–1.38 (m, 22H), 1.39 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.59–1.70 (m, 2H, CH_2CH_2COOR), 2.03–2.06 (m, 4H, $CH_2CH{=}CHCH_2$), 2.40 (t, 2H, J = 7.0 Hz, CH_2COOR), 3.45–3.47 (m, 6H, 2-OCH_3), 3.47 (dd, 1H, $J_{8\text{-}9}$ = 7.1 Hz, $J_{8\text{-}7}$ = 8.0 Hz, H^8), 3.91 (dd, 1H, $J_{4\text{-}3}$ = 1.2 Hz, $J_{4\text{-}5}$ = 5.0 Hz, H^4), 4.04 (ddd, 1H, $J_{11\text{-}12a}$ = 1.5 Hz, $J_{11\text{-}10}$ = 2.2 Hz, $J_{11\text{-}12b}$ = 6.8 Hz, H^{11}), 4.05 (dd, 1H, $J_{6b\text{-}5}$ = 6.0 Hz, $J_{6b\text{-}6a}$ = 8.7 Hz, H^{6b}), 4.08 (dd, 1H, $J_{9\text{-}10}$ = 5.5 Hz, $J_{9\text{-}8}$ = 7.1 Hz, H^9), 4.14 (dd, 1H, $J_{3\text{-}4}$ = 1.2 Hz, $J_{3\text{-}2}$ = 7.5 Hz, H^3), 4.17 (dd, 1H, $J_{6a\text{-}5}$ = 6.0 Hz, $J_{6a\text{-}6b}$ = 8.7 Hz, H^{6a}), 4.22 (dd, 1H, $J_{10\text{-}11}$ = 2.2 Hz, $J_{10\text{-}9}$ = 5.5 Hz, H^{10}), 4.27 (dd, 1H, $J_{12b\text{-}11}$ = 6.8 Hz, $J_{12b\text{-}12a}$ = 11.5 Hz, H^{12b}), 4.30 (dd, 1H, $J_{12a\text{-}11}$ = 1.5 Hz, $J_{12a\text{-}12b}$ = 11.5 Hz, H^{12a}), 4.31 (ddd, $J_{5\text{-}4}$ = 5.0 Hz, $J_{5\text{-}6a} \cong J_{5\text{-}6b}$ = 6.0 Hz, H^5), 4.41 (d, 1H, $J_{1\text{-}2}$ = 6.2 Hz, H^1), 4.51 (d, 1H, $J_{7\text{-}8}$ = 8.0 Hz, H^7), 4.51 (dd, 1H, $J_{2\text{-}1}$ = 6.2 Hz, $J_{2\text{-}3}$ = 7.5 Hz, H^2), 5.35 (ddd, 1H, $J_{22\text{-}23a} \cong J_{22\text{-}23b}$ = 6.0 Hz, $J_{22\text{-}21}$ = 11.0 Hz, CH=CH), 5.39 (ddd, 1H, $J_{21\text{-}20a} \cong J_{21\text{-}20b}$ = 6.0 Hz, $J_{21\text{-}22}$ = 11.0 Hz, CH=CH) ppm. ^{13}C NMR (CD_3OD) δ: 13.0, 22.3, 24.2, 24.6, 25.1, 25.5, 25.7, 26.2, 26.7, 26.8, 27.0, 28.6, 28.76, 28.81, 28.9, 29.39, 29.43, 31.5, 33.5, 53.0, 55.1, 63.1, 65.5, 70.8, 73.3, 73.5, 75.4, 76.4, 76.8, 77.5, 79.4, 103.1, 105.7, 108.5, 109.7, 109.8, 129.4, 129.5, 173.8 ppm. IR (Nujol): 2952, 1729, 1712 cm^{-1}.

3b. Yield: 47% (0.222 g). ESI-MS: *m/z* 856 $(M-H)^-$, 875 $(M+NH_4)^+$. ^{1}H NMR (CD_3OD) δ: 0.93 (t, 3H, J = 6.7 Hz, CH_3), 1.30–1.38 (m, 38H), 1.39 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.59–1.70 (m, 2H, CH_2CH_2COOR), 2.03–2.08 (m, 4H, $CH_2CH{=}CHCH_2$), 2.40 (t, 2H, J = 7.0 Hz, CH_2COOR), 3.45–3.47 (m, 6H, 2-OCH_3), 3.48 (dd, 1H, $J_{8\text{-}9}$ = 7.1 Hz, $J_{8\text{-}7}$ = 8.0 Hz, H^8), 3.91 (dd, 1H, $J_{4\text{-}3}$ = 1.2 Hz, $J_{4\text{-}5}$ = 5.0 Hz, H^4), 4.04 (ddd, 1H, $J_{11\text{-}12a}$ = 1.5 Hz, $J_{11\text{-}10}$ = 2.2 Hz, $J_{11\text{-}12b}$ = 6.9 Hz, H^{11}), 4.05 (dd, 1H, $J_{6b\text{-}5}$ = 6.0 Hz, $J_{6b\text{-}6a}$ = 8.7 Hz, H^{6b}), 4.08 (dd, 1H, $J_{9\text{-}10}$ = 5.6 Hz, $J_{9\text{-}8}$ = 7.1 Hz, H^9), 4.14 (dd, 1H, $J_{3\text{-}4}$ = 1.2 Hz, $J_{3\text{-}2}$ = 7.5 Hz, H^3), 4.17 (dd, 1H, $J_{6a\text{-}5}$ = 6.0 Hz, $J_{6a\text{-}6b}$ = 8.7 Hz, H^{6a}), 4.21 (dd, 1H, $J_{10\text{-}11}$ = 2.2 Hz, $J_{10\text{-}9}$ = 5.5 Hz, H^{10}), 4.27 (dd, 1H, $J_{12b\text{-}11}$ = 6.9 Hz, $J_{12b\text{-}12a}$ = 11.5 Hz, H^{12b}), 4.29–4.33 (m, 2H, H^5, H^{12a}), 4.41 (d, 1H, $J_{1\text{-}2}$ = 6.2 Hz, H^1), 4.51 (d, 1H, $J_{7\text{-}8}$ = 8.0 Hz, H^7), 4.51 (dd, 1H, $J_{2\text{-}1}$ = 6.2 Hz, $J_{2\text{-}3}$ = 7.5 Hz, H^2), 5.35 (ddd, 1H, $J_{28\text{-}29a} \cong J_{28\text{-}29b}$ = 6.0 Hz, $J_{28\text{-}27}$ = 11.0 Hz, CH=CH), 5.39 (ddd, 1H, $J_{27\text{-}26a} \cong J_{27\text{-}26b}$ = 6.0 Hz, $J_{27\text{-}28}$ = 11.0 Hz, CH=CH) ppm. ^{13}C NMR (CD_3OD) δ: 13.1, 22.3, 24.2, 24.6, 25.1, 25.5, 25.7, 26.2, 26.7, 26.7, 26.9, 28.8, 28.9, 28.9, 29.0, 29.1, 29.20, 29.22, 29.33, 29.34, 29.35, 29.4, 29.4, 31.7, 33.5, 53.0, 55.1, 63.1, 65.5, 70.8, 73.3, 73.6, 75.4, 76.4, 76.9, 77.6, 79.4, 103.1, 105.7, 108.4, 109.7, 109.9, 129.5, 129.5, 173.8 ppm. IR (Nujol): 2965, 1731, 1713 cm^{-1}.

2.2.2. General procedure for the synthesis of lactose fatty acid esters (Z)-6′-O-hexadec-9-enoyl- and (Z)-6′-O-tetracos-15-enoyl-4-O-(β-D-galactopyranosyl)-D-glucopyranose (**4a,b**)

Compounds **3a** or **3b** (0.43 mmol) were dissolved in tetrafluoroboric diethylether/water/acetonitrile (1:5:500) and the mixture