Supplementary Information - New Journal of Chemistry

Novel mono, di and tri- fatty acid esters bearing secondary amino acid ester head group as transdermal permeation enhancers

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1. Synthesis and characterisation

*General procedure for mono Michael addition **I***

To a solution of *tert*-butyl acrylate **2** in alcohol**,** an amine **1, 5 or 8** was added at room temperature and stirred for 4 - 30 h at 25 to 45 °C. Alcohol and excess *tert*-butyl acrylate were evaporated in vacuo and the resulting residue was recrystallized or column purified using hexane and ethyl acetate (3:1) to yield the mono Michael addition product (**3, 6 and 9)**.

*General procedure (esterification) for synthesis of amino ester derivatives **II***

Fatty acid was added to a stirred mixture of mono Michael adduct (compound **3**, **6** or **9**), DCC, and DMAP in dry DCM under a nitrogen atmosphere at room temperature (RT). The resulting reaction mixture was further stirred RT for 18 - 24 h. From the reaction mass, precipitated dicyclohexylurea was removed by filtration. The organic layer (filtrate) was evaporated under reduced pressure and the obtained residue was purified by column chromatography (silica gel #70-230 and 10-15% ethyl acetate in hexane as an eluent) to yield the ester derivative.

FT-IR spectra of all the compounds were recorded on a Bruker Alpha-*p* spectrometer with diamond ATR (Germany). ^{1}H NMR and ^{13}C NMR measurements were performed on a Bruker NMR spectrometer (United Kingdom) at 400 and 100 MHz respectively. HRMS was performed on a Waters Micromass LCT Premier TOF-MS (United Kingdom).

*Synthesis of tert-butyl 3-((2-hydroxyethyl) amino) propanoate (compound **3**)*

To a solution of *tert*-butyl acrylate **2** (135.0 g, 1.05 mol) in methanol (500 ml**),** 2-aminoethanol **1** (61.0 g, 1.0 mol) was added at room temperature and stirred for 24 h at the same temperature. Methanol and excess *tert*-butyl acrylate were evaporated in vacuo and the resulting residue was purified by column chromatography using hexane and ethyl acetate

(3:1) to yield compound **3** as a thick oil (151.0 g, 80%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.38 (s; 9H; -C(<u>CH_3</u>)$_3$), 2.30 (t; 2H; -NHCH$_2$<u>CH_2</u>-), 2.39-2.43 (m; 4H; –<u>CH_2</u>NH<u>CH_2</u>-), 3.59 (m; 2H; -<u>CH_2</u>OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.08, 35.46, 44.65, 50.90, 60.43, 80.73, 172.01.

*Synthesis of tert-butyl 3-((1,3-dihydroxypropan-2-yl) amino) propanoate (compound **6**)*

To a solution of *tert*-butyl acrylate **2** (141.0 g, 1.10 mol) in ethanol (300 ml**),** 2-amino-1,3-propanediol **5** (91.11 g, 1.0 mol) was added at room temperature and stirred for 4 h at the same temperature. Ethanol and excess *tert*-butyl acrylate were evaporated in vacuo and the resulting residue was recrystallized using hexane and ethyl acetate (3:1) to yield compound **6** as a white solid (201.74 g, 92%). M.p. 69 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.39 (s; 9H; -C(<u>CH_3</u>)$_3$), 2.30 (t; 2H; -NHCH$_2$<u>CH_2</u>-), 2.47 (m; 1H; –NH<u>CH</u>(CH_2-)$_2$), 2.74 (t; 2H; -NH<u>CH_2</u>CH_2;), 3.25 - 3.38 (m; 4H; -<u>CH_2</u>OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.09, 35.84, 36.41, 60.18, 62.83, 81.40, 172.84. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 242.1367 found 242.1368.

*Synthesis of tert-butyl 3-((1, 3-dihydroxy-2-(hydroxymethyl) propan-2-yl) amino) propanoate (compound **9**)*

To a solution of *tert*-butyl acrylate **2** (128.17 g, 1.0 mol) in ethanol (250 ml**),** Trizma **8** (60.57 g, 0.5 mol) was added at 45 °C and stirred for 30 h at the same temperature. Ethanol and excess *tert*-butyl acrylate were evaporated in vacuo and the resulting residue was recrystallized using hexane and ethyl acetate (3:1) to yield compound **9** as a white solid (112.19 g, 90%). M.p. 69 °C; Rf = 0.55 ($CHCl_3$: MeOH; 9:1); FTIR: 3322.08, 3285.98, 2872.02, 1707.18, 1171.35, 1020.30 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.46 (s; 9H; -C(<u>CH_3</u>)$_3$), 2.44 (t; 2H; -NHCH$_2$<u>CH_2</u>-), 2.83 (t; 2H; -NH<u>CH_2</u>CH_2;), 3.18 (bs; 3H; -*OH*), 3.56 (s; 6H; -<u>CH_2</u>OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.09, 35.84, 36.41, 60.18, 62.83, 81.40, 172.84. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 272.15 found 272.10.

*2-((3-(tert-butoxy)-3-oxopropyl) amino) ethyl stearate (MSAPE) **4** (**I**)*

Stearic acid (14.51g, 0.051 mol) in DCM (50 ml) was added to a solution of compound **3** (9.50 g, 0.05 mol), DCC (10.52 g, 0.051 mol) and DMAP (1.22g, 0.01 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 18 h and worked up as per the given general procedure II to obtain MSAPE **4** (I) as thick liquid (20.280 g, 89% yield) after column chromatographic purification. FTIR: 2915.37, 2847.87, 1727.59, 1642.58, 1461.33, 1155.84 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.81 (t; 3H; -*CH*$_3$), 1.18 (m; 28H; -*CH*$_2$-), 1.38 (s; 9H; -C(*CH*$_3$)$_3$), 1.55 (m; 2H; -*CH*$_2$$CH_2COO$-), 2.29 (t; 2H; -*CH*$_2$$COO$-), 2.45 (t; 2H; -$NHCH_2$*CH*$_2$-), 3.51-3.57 (m; 4H; –*CH*$_2$$NH$*CH*$_2$-), 4.12 (m; 2H; -*CH*$_2$$OOC$-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.11, 22.69, 24.83, 25.43, 29.66, 31.93, 33.91, 34.12, 34.23, 35.18, 42.87, 44.70, 45.06, 47.27, 61.94, 81.46, 170.21, 173.50. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 478.3872 found 478.3870.

*2-((3-(tert-butoxy)-3-oxopropyl) amino) ethyl oleate, (MOAPE) **4** (**II**)*

A solution of oleic acid (14.41g, 0.051 mol) in DCM (50 ml) was added to a mixture of compound **3** (9.50 g, 0.05 mol), DCC (10.52g, 0.051 mol) and DMAP (1.22g, 0.01 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 18 h and worked up as per the general procedure II to obtain MOAPE **4** (II) as thick liquid (20.73g, 91% yield) after column chromatographic purification. FTIR: 2922.54, 2852.98, 1730.35, 1653.61, 1457.99, 1151.46 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.81 (t; 3H; -*CH*$_3$), 1.19 (m; 20H; -*CH*$_2$-), 1.36 (s; 9H; -C(*CH*$_3$)$_3$), 1.55 (m; 2H; -*CH*$_2$$CH_2COO$-), 1.98 (m; 4H; -*CH*$_2$$CH$=$CH$*CH*$_2$-), 2.22 (t; 2H; -*CH*$_2$$COO$-), 2.45 (t; 2H; -$NHCH_2$*CH*$_2$-), 3.49-3.54 (m; 4H; –*CH*$_2$$NH$*CH*$_2$-), 4.12 (m; 2H; -*CH*$_2$$OOC$-), 5.28 (m; 4H; -*CH*=*CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.68, 25.29, 27.22, 28.05, 28.06, 29.76, 33.09, 33.98, 34.09, 34.20, 35.23,

42.71, 44.60, 44.90, 47.08, 61.73, 62.05, 81.36, 129.96, 129.99, 170.28, 171.48, 173.35, 173.45. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 476.3716 found 476.3728.

2-((3-(tert-butoxy)-3-oxopropyl) amino) ethyl (9Z,12Z)-octadeca-9,12-dienoate, (MLAPE) ***4*** ***(III)***

A solution of linoleic acid (12.0 g, 0.043 mol) in DCM (25 ml) was added to a mixture of compound **3** (7.94 g, 0.042 mol), DCC (8.83 g, 0.043 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 22 h and worked up as per the given general procedure II to obtain MLAPE **4** (III) as thick liquid (17.05 g, 90% yield) after purification. FTIR: 2923.36, 2853.52, 1730.13, 1653.24, 1457.99, 1151.44 cm^{-1}. ^1H NMR ($CDCl_3$) δ (ppm): 0.82 (t; 3H; -CH_3), 1.23 (m; 16H; -CH_2-), 1.38 (s; 9H; -C(CH_3)$_3$), 1.55 (m; 2H; -CH_2CH_2COO-), 1.98 (m; 4H; -$CH_2CH{=}CHCH_2$-), 2.25 (t; 2H; -CH_2COO-), 2.45 (t; 2H; -$NHCH_2CH_2$-), 3.49-3.54 (m; 4H; –CH_2NHCH_2-), 4.12 (m; 2H; -CH_2OOC-), 5.28 (m; 4H; -$CH{=}CH$-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.05, 22.55, 24.84, 25.28, 27.18, 28.04, 29.09-29.75, 31.50, 31.88, 33.072, 33.12, 34.08, 35.21, 47.08, 61.71, 62.03, 80.64.05, 81.35, 127.88, 130.18, 170.27, 171.47, 173.36, 173.43. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 474.3559 found 474.3557.

2-((3-(tert-butoxy)-3-oxopropyl) amino) ethyl (9Z,12Z,15Z)-octadeca-9,12,15-trienoate (MLLAPE) ***4*** ***(IV)***

A solution of linolenic acid (7.50 g, 0.0269 mol) in DCM (25 ml) was added to a mixture of compound **3** (5.0 g, 0.0264 mol), DCC (5.45 g, 0.0269 mol) and DMAP (1.22g, 0.01mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and worked up as per the given general procedure II to obtain MLLAPE **4** (IV) as thick liquid (10.095 g, 85% yield) after column chromatographic purification. FTIR: 2928.52, 2856.01, 1727.82, 1629.20, 1457.72, 1152.66 cm^{-1}. ^1H NMR ($CDCl_3$) δ (ppm): 0.89 (t; 3H; -CH_3),

1.23 (m; 12H; -CH_2-), 1.36 (s; 9H; -C(CH_3)$_3$), 1.53 (m; 2H; -$CH_2$$CH_2$COO-), 1.98 (m; 4H; -$CH_2$CH=CH$CH_2$-), 2.24 (t; 2H; -$CH_2$COO-), 2.44 (t; 2H; -NHCH$_2$$CH_2$-), 3.49-3.53 (m; 4H; –$CH_2NHCH_2$-), 4.10 (m; 2H; -$CH_2$OOC-), 5.29 (m; 4H; -CH=CH-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.26, 20.53, 22.55, 25.28, 25.60, 31.50, 33.08, 33.12, 33.97, 34.08, 34.19, 42.71, 44.91, 47.78, 61.73, 62.04, 81.37, 127.09, 128.22, 130.20, 131.92, 170.28, 171.48, 173.37.

*2-((3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl distearate (DSAPE) **7** (**I**)*

Stearic acid (13.10 g, 0.0461 mol) in DCM (50 ml) was added to a solution of compound 3 (5.0 g, 0.0228 mol), DCC (9.50 g, 0.0461 mol) and DMAP (2.44 g, 0.02 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 22 h and worked up following general procedure II to obtain DSAPE **7** (I) as thick liquid (15.78g, 92% yield) after purification by using column chromatography. FTIR: 2914.26, 2849.09, 1732.04, 1470.43, 1152.05 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 81 (t; 6H; -CH_3), 1.19-1.23 (m; 56H; -CH_2-), 1.38 (s; 9H; -C(CH3)$_3$), 1.55 (q; 4H; -$CH_2$$CH_2$COO-), 1.95 (m; 8H; -$CH_2$CH=CH$CH_2$-), 2.25 (t; 4H; -$CH_2$COO-), 2.34 (t; 2H; -NHCH$_2$$CH_2$-), 2.83 (t; 2H; -NH$CH_2$$CH_2$-), 2.96 (m; 1H –NH$CH$($CH_2$-)$_2$), 4.03 (s; 4H; -$CH_2$OOC-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.67, 24.87, 27.16, 27.21, 28.10, 29.11 - 29.76, 31.90, 34.16, 43.02, 55.38, 63.38, 80.58, 171.84, 173.57. ESI-TOF MS, *m/z*: [M + Na]$^+$ - calculated 774.6588, found 774.6595.

*2-((3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl dioleate (DOAPE) **7** (**II**)*

A solution of oleic acid (13.0 g, 0.0461 mol) in DCM (50 ml) was added to a solution of compound **3** (5.0 g, 0.0228 mol), DCC (9.50 g, 0.0461 mol) and DMAP (2.44 g, 0.02 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 22 h and worked up as per the given general procedure II to obtain DOAPE **7** (II) as thick liquid (15.87g, 93% yield) after column purification. FTIR: 2923.54, 2853.64, 1734.07, 1662.78,

1459.33, 1367.17, 1156.55 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 81 (t; 6H; -*CH*$_3$), 1.19-1.23 (m; 40H; -*CH*$_2$-), 1.38 (s; 9H; -C*(CH3)*$_3$), 1.55 (q; 4H; -*CH*$_2$$CH_2COO$-), 1.95 (m; 8H; -*CH*$_2$CH=CH*CH*$_2$-), 2.25 (t; 4H; -*CH*$_2$COO-), 2.34 (t; 2H; -$NHCH_2$*CH*$_2$-), 2.83 (t; 2H; -NH*CH*$_2$$CH_2$-), 2.96 (m; 1H –NH*CH*($CH_2$-)$_2$), 4.03 (s; 4H; -*CH*$_2$OOC-), 5.27 (m; 4H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.67, 24.87, 27.16, 27.21, 28.10, 29.11 - 29.76, 31.90, 34.16, 43.02, 55.38, 63.38, 80.58, 129.72, 129.98, 171.84, 173.57. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 770.6275, found 770.6281.

2-((3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl (9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate) (DLAPE) ***7 (III)***

A solution of linoleic acid (6.46g, 0.023mol) in DCM (25ml) was added to a mixture of compound **6** (2.5 g, 0.0114 mol), DCC (4.75g, 0.023 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mass was stirred at room temperature for 24 h and worked up following general procedure II to obtain DLAPE **7** (III) as thick liquid (7.64 g, 90% yield) after column chromatographic purification. FTIR: 2925.68, 2854.28, 1735.04, 1454.93, 1156.54 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 82 (t; 6H; -*CH*$_3$), 1.23 (m; 32H; -*CH*$_2$-), 1.38 (s; 9H; -C*(CH3)*$_3$), 1.55 (q; 4H; -*CH*$_2$$CH_2COO$-), 1.98 (m; 8H; -*CH*$_2$CH=CH*CH*$_2$-), 2.25 (t; 4H; -*CH*$_2$COO-), 2.33 (t; 2H; -$NHCH_2$*CH*$_2$-), 2.70 (t; 2H; -NH*CH*$_2$$CH_2$-), 2.83 (m; 1H –NH*CH*($CH_2$-)$_2$), 4.03 (s; 4H; -*CH*$_2$OOC-), 5.28 (m; 8H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.57, 24.88, 25.45, 25.62, 27.19, 28.11, 29.12 - 29.61, 31.52, 34.16, 34.92, 36.10, 43.05, 55.36, 63.46, 80.54, 127.90, 128.04, 130.02, 130.20, 171.84, 173.59. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 766.5962, found 766.5976.

2-((3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl (9Z,9'Z,12Z,12'Z,15Z,15'Z)-bis(octadeca-9,12,15-trienoate) (DLLAPE) ***7 (IV)***

A solution of linolenic acid (6.41 g, 0.023 mol) in DCM (25 ml) was added to a mixture of compound 6 (2.5 g, 0.011 mol), DCC (4.75g, 0.023 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and worked up as per the given general procedure II to give DLLAPE **7** (IV) as thick liquid (7.26g, 86% yield) after purification. FTIR: 2926.91, 2854.49, 1734.86, 1456.85, 1154.96 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 90 (t; 6H; -*CH*$_3$), 1.24 (m; 24H; -*CH*$_2$-), 1.38 (s; 9H; -C(*CH3*)$_3$), 1.55 (q; 4H; -*CH*$_2$$CH_2COO$-), 1.98 (m; 8H; -*CH*$_2$CH=CH*CH*$_2$-), 2.25 (t; 4H; -*CH*$_2$COO-), 2.33 (t; 2H; -$NHCH_2$*CH*$_2$-), 2.73 (t; 2H; -NH*CH*$_2$$CH_2$-), 2.84 (m; 1H – NH*CH*(CH_2-)$_2$), 4.03 (s; 4H; -*CH*$_2$OOC-), 5.29 (m; 12H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.27, 20.54, 22.57, 24.86, 25.52, 25.61, 27.20, 28.10, 29.11 - 29.58, 31.52, 34.15, 43.00, 55.39, 63.33, 80.64, 127.11, 127.73, 128.24, 128.28, 130.20, 130.24, 131.94, 171.85, 173.56. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 762.5649, found 762.5663.

2-((3-(tert-butoxy)-3-oxopropyl) amino)-2-((stearoyloxy)methyl) propane-1,3-diyl distearate, (TSAPE) ***10 (I)***

A solution of stearic acid (14.37 g, 0.051 mol) in DCM (50 ml) was added to a mixture of compound 9 (2.0 g, 0.016 mol), DCC (10.42 g, 0.051 mol) and DMAP (2.44 g, 0.02 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and worked up as per the given general procedure II to obtain TSAPE **10** (I) as thick liquid (15.22 g, 88% yield) after column chromatographic purification. M.p. 58 °C; FTIR: 2916.07, 2849.18, 1752.83, 1718.20, 1609.02, 1468.45, 1195.72, 1154.03 - 1020.30 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 81 (t; 9H; -*CH*$_3$), 1.19 (m; 84H; -*CH*$_2$-), 1.39 (s; 9H; -C(*CH*$_3$)$_3$), 1.54 (q; 6H; -*CH*$_2$$CH_2COO$-), 2.25 (t; 6H; -*CH*$_2$COO-), 2.32 (t; 2H; -$NHCH_2$*CH*$_2$-), 2.77 (t; 2H; -NH*CH*$_2$$CH_2$-), 4.03 (s; 6H; -*CH*$_2$OOC-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.11, 22.67, 24.90, 28.11, 29.16, 29.26, 29.35, 29.69, 29.70, 31.92, 34.20, 36.10, 36.76, 60.51, 63.07, 81.06, 172.22, 173.45; ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 1070.93, found 1070.79.

2-((3-(tert-butoxy)-3-oxopropyl) amino)-2-((((Z)-octadec-9-enoyl) oxy) methyl) propane-1,3-diyl(9Z,9'Z)-bis(octadec-9-enoate) (TOAPE) ***10 (II)***

A solution of oleic acid (17.84 g, 0.063 mol) in DCM (50 ml) was added to a mixture of compound **9** (2.5 g, 0.021 mol), DCC (13.03 g, 0.063 mol) and DMAP (2.44 g, 0.02 mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and general procedure II was followed for work up to obtain TOAPE **10** (II) as thick liquid (19.37 g, 90% yield) after column chromatographic purification. FTIR: 2923.46, 2855, 1746.15, 1662.78, 1459.29, 1379.17, 1157.32 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 81 (t; 9H; -*CH_3*), 1.19-1.23 (m; 60H; -*CH_2*-), 1.37 (s; 9H; -C*$(CH3)_3$*), 1.54 (q; 6H; -*CH_2*CH_2COO-), 1.93 (m; 12H; -*CH_2*CH=CH*CH_2*-), 2.25 (t; 6H; -*CH_2*COO-), 2.28 (t; 2H; -NHCH$_2$*CH_2*-), 2.75 (t; 2H; -NH*CH_2*CH_2-), 4.04 (s; 6H; -*CH_2*OOC-), 5.27 (m; 6H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.11, 22.67, 24.86, 27.18, 27.22, 28.10, 29.14 - 29.77, 31.90, 34.16, 37.71, 57.27, 63.22, 80.57, 129.71, 129.98, 171.79, 173.26. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 1064.88, found 1064.76.

2-((3-(tert-butoxy)-3-oxopropyl) amino)-2-((((9Z,12Z)-octadeca-9,12-dienoyl) oxy) methyl) propane-1,3-diyl (9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate) (TLAPE) ***10 (III)***

Linoleic acid (7.08 g, 0.025 mol) in DCM (25 ml) was added to a mixture of compound **9** (1.0 g, 0.008 mol), DCC (5.12 g, 0.025 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mass was stirred at room temperature for 24 h and worked up as per the given general procedure II to obtain TLAPE **10** (III) as thick liquid (7.36g, 86% yield) after column chromatographic purification. FTIR: 2922.84, 2854.77, 1744.70, 1666.39, 1457.92, 1378.45, 1150.55 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 90 (t; 9H; -*CH_3*), 1.31 (m; 48H; -*CH_2*-), 1.45 (s; 9H; -C*$(CH_3)_3$*), 1.62 (m; 6H; -*CH_2*CH_2COO-), 2.04 (m; 12H; -*CH_2*CH=CH*CH_2*-), 2.33 (t; 6H; -*CH_2*COO-), 2.37 (t; 2H; -NHCH$_2$*CH_2*-), 2.83 (t; 2H; -

NH*CH*$_2$-), 4.12 (s; 6H; -*CH*$_2$OOC-), 5.38 (m; 12H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.57, 24.84, 25.53, 27.19, 27.52, 28.11, 29.14 - 29.62, 31.52, 34.15, 57.27, 63.21, 80.57, 127.90, 128.04, 130.21, 171.80, 173.27. ESI-TOF MS, *m/z*: $[M+Na]^+$ - calculated 1058.84, found 1058.77.

2-((3-(tert-butoxy)-3-oxopropyl) amino)-2-((((9Z,12Z,15Z)-octa -dec-9,12,15-trienoyl) oxy) methyl) propane-1,3-diyl (9Z,9'Z,12Z,12'Z,15Z, 15'Z) -bis(octadeca-9,12,15-trienoate) (TLLAPE) ***10*** *(**IV**)*

Linolenic acid (7.03 g, 0.025 mol) in DCM (25 ml) was added to a mixture of compound **9** (1.0 g, 0.008 mol), DCC (5.12 g, 0.025 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and worked up as per the given general procedure II to obtain TLLAPE **10** (IV) as thick liquid (7.15 g, 84% yield) after column chromatographic purification. FTIR: 2927.36, 2858.37, 1746.03, 1663.52, 1458.04, 1388.94, 1157.18 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 90 (t; 9H; -*CH*$_3$), 1.23 (m; 36H; -*CH*$_2$-), 1.37 (s; 9H; -C*(CH3)*$_3$), 1.54 (m; 6H; -*CH*$_2$$CH_2$COO-), 1.99 (m; 12H; -*CH*$_2$CH=CH*CH*$_2$-), 2.25 (t; 6H; (-*CH*$_2$COO-), 2.29 (t; 2H; -NHCH$_2$*CH*$_2$-), 2.75 (t; 2H; -NH*CH*$_2$), 4.04 (s; 6H; -*CH*$_2$OOC-), 5.29 (m; 18H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.27, 22.58, 24.84, 25.60, 27.20, 28.10, 29.13, 29.33, 29.59, 31.52, 34.14, 37.69, 57.25, 63.21, 80.55, 127.11, 127.73, 128.23, 128.27, 130.22, 131.93, 171.79, 173.25. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 1052.79, found 1052.68.

2. *In vitro* cytotoxicity

Cell culture

Complete growth medium was prepared by supplementing EMEM cell culture media with streptomycin (100 mg/ml), 10 % bovine calf serum (10 %) and penicillin (100 units/ml). The resultant complete growth medium was used to sustain the growth of human breast

adenocarcinoma (MCF 7), liver hepatocellular carcinoma (Hep G2), and human lung carcinoma (A549) cells using standard aseptic cell culture protocols. The cells were incubated at the following conditions (a temperature of 37 °C and humidified atmosphere of 5 % CO_2).

Solutions

Stock solutions of the different series were prepared by dissolving in appropriate volumes of DMSO and purified water. These stock solutions were further diluted in the growth media to attain the final concentrations of 20, 40, 60, 80 and 100 µg/ml (Rambharose et al., 2015).

MTT assay

MCF 7, A549 and Hep G2 cells were seeded in 96 well plates at an equivalent cell density of 4.4 $x10^3$ cells per well. Following 24 h incubation the initial culture medium was removed and each well was replaced with 100 µL of fresh growth medium containing the ester derivatives with final concentrations of 20, 40, 60, 80 and 100 µg/ml. The control consisted of cells and culture medium only, whilst wells containing medium only was used as the blank control. The treatments were incubated with the cells for 48 h. Subsequent to the 48 h incubation with the derivatives, the treatment mediums were removed and each well was supplemented with MTT reagent (100 µL of 5 mg/ml in PBS) and 100 µL of complete medium, then incubated for a further 4 h. Afterwards the media and MTT solution was removed and 100 µL of DMSO was added to each well, allowing the solubilisation the MTT formazan crystals. A microplate spectrophotometer (Spectrostar Nano) at a wavelength of 540 nm was used to detect the optical density of each well[1]. All the cytotoxicity experiments were carried out with n = 6. The quantity of viable cells was calculated with the following equation:

$$\%\ Cell\ Viability\ = \frac{A_{540\ nm\ treated\ cells}}{A_{540\ nm\ untreated\ cells}}\ x100 \qquad eq.(1)$$

3. *Harvesting rat skin tissue*

The University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee granted ethical clearance for this study in 2014 (054/14/Animal), and was renewed (015/15/Animal). The animals used for experiments were housed at the Biomedical Resource Unit (BRU) of UKZN under controlled 12-h light/dark cycles, temperature and humidity conditions. The rats were fed and received water *ad libitum* on a daily basis. After CO_2 euthanasia, the body fur was carefully removed using standard shaving procedures. The skin located around the abdominal region was harvested and excised to remove any subcutaneous fat. Skin samples were stored (-20˚C) and utilized within 3 months[1,2]. The skin was thawed using a solution of pH 7.4 PBS at room temperature prior to permeation experiments.

4. Transepithelial electrical resistance (TEER) evaluations

TEER reading taken before the experiment (0 h) was representative of 100% skin integrity i.e. before exposure to the gel treatments to the skin. The rebound effect of the skin post drug treatment was measured by removing the drug loaded gel from the donor compartment after a period of 6 hours and replacing it with fresh PBS for a period of 2 hours followed by subsequent TEER measurements[1,3]

5. Light Microscopy (LM)

The control tissues used in this study originated from histological evaluations of freshly harvested excised skin tissue sections. After careful tissue removal, the control skin tissue was fixed in 10 % buffered formalin, whereas treated tissue samples comprised of skin tissues that were exposed to either OA or MOAPE at a concentration of 1% w/w. The treated

skin tissues were removed from the Franz diffusion cells at the end of the permeation study, and fixed in 10 % buffered formalin. The control and enhancer treated skin samples were fixed using formalin for seven days at room temperature. Thereafter, an ethanol gradient ranging from 50 % up to 96 % was used to gradually dehydrate the skin tissue sections. Subsequently, the skin tissue sections were embedded within paraffin wax blocks. The tissue samples were sectioned using a microtome (Leica RM2235, Leica Biosystems Germany) and skin sections were collected on slides, dried and stained with hematoxylin and eosin (H&E). The images from the stained slides were captured using a Leica slide scanner (SCN 400, Leica Biosystems Germany). All experiments were performed in triplicate[1,3,4].

6. Statistical Analysis

The data was analysed using GraphPad Prism® (Graph Pad Software Inc., Version 5., USA) and presented as mean ± standard deviation (SD). The results were analysed by one-way analysis of variance (ANOVA) followed by the non-parametric Kruskal-Wallis test or t tests followed by the non-parametric Mann-Whitney test. $p < 0.05$ was considered statistically significant.

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