Discovery of sustainable drugs for Alzheimer's disease: cardanol-derived cholinesterase inhibitors with antioxidant and anti-amyloid properties

Supplementary Information

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1. *Chemistry*

1.1 General methods

All the commercially available reagents and solvents were used purchased from Sigma-Aldrich® (USA), Tedia® (USA) and PanReac® (Spain). Tetrahydrofuran (THF), dichloromethane (DCM) and triethylamine (TEA) were previously treated with calcium hydride and distillated before use.

The reactions were carried out under magnetic stirring at room temperature or in oil bath heating. For reactions carried out under microwave radiation, the Brastemp® microwave model BMK38ABHNA JetDeFrost with capacity of 38 L and power of 900 W was used without magnetic stirring. For reactions under reflux with reagents and solvents with lower boiling point than the heating temperature, a cooling system of the condenser at temperatures ranging from -8 °C to 10 °C was used.

Thin-layer chromatography (TLC) separations were performed with aluminum-backed sheets with silica gel (Kieselgel 60 F254), and spots were visualized by exposure to UV light (254 nm), or iodine, $FeCl_3$ (ethanolic solution of 5% $FeCl_3$ in 0.5 N HCl), and ninhydrin (0.3 % ninhydrin butanol solution in glacial acetic acid) stains. The compounds were purified using G60 silica gel column chromatography (70-230 mesh) SILICYCLE®, and the flash chromatography system in Isolera Spektra equipment Systems with ACI™ (Biotage®) on SNAP silica cartridge 10 g. The melting points, uncorrected, were determined on MQAPF-302 instrument. The evaporation of the solvents was carried out in a rotary evaporator Tecnal® TE-211, at reduced pressure, ranging from 10 to 0.1 mmHg, and at temperatures between 40 °C and 60 °C. Infrared (IR) spectra were recorded with a Perkin Elmer Spectrum BX spectrophotometer using potassium bromide tablets (KBr). The values for the absorptions (vmax) are referred to in wave numbers, using as unit the reciprocal centimeter (cm^{-1}).

Nuclear magnetic resonance (NMR) spectra of hydrogen (^{1}H-NMR) and carbon (^{13}C-NMR) were registered at 300 MHz and 75 MHz or at 500 MHz and 125 MHz with Bruker Avance DRX300 and DRX500 instruments, respectively. Spectra were acquired using $CDCl_3$ as solvent, and tetramethylsilane (TMS) as an internal reference. The values of chemical shift (δ) are reported in part per million (ppm) relative to TMS and the coupling constants (J) in Hertz (Hz). The areas of the signals were obtained by integration and its multiplicities indicated as: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; dt, doublet of triplets; q, quartet; qi, quintet; m, multiplet; br, broad signal and combinations thereof.

High-resolution mass spectra (HRMS) were recorded on TripleTOF 5600+ system (Sciex). Analyses were performed by flow injection using a liquid chromatographer (Eksigent UltraLC 100, Sciex) at a 0.3 mL min^{-1} flow. Spectra were acquired using a DuoSpray Ion Source (ESI) in positive mode in a 100 – 1,000 Da mass range using external calibration.

Compounds were named following IUPAC rules as applied by ChemDraw Professional 19.1.

*1.2 Synthesis of intermediates **13** and **14***

Obtaining the mixture of acetylated cardanols (LDT12i, **13***)*

To a mixture of unsaturated cardanols (**12**, 2.0 g, 6.57 mmol), acetic anhydride (1.24 mL, 13.13 mmol) and phosphoric acid (4 drops) were added, and the resulting mixture was submitted to microwave irradiation for 3 minutes (3 x 1') with an irradiation power of 270 W. Then, 10% HCl solution (30 mL) was added to the reaction mixture and extracted with ethyl acetate (2 x 30 mL). The combined organic phases were washed with brine (30 mL) and dried over anhydrous sodium sulfate. The solution was concentrated under reduced pressure on a rotary evaporator and the crude product purified on a silica gel column, eluted with dichloromethane, providing 2.1 g of acetylated cardanols as yellow oil (92 % yield); Rf = 0.75 (dichloromethane).

8-(3-hydroxyphenyl)octan-1-ol (LDT71, **14***)*

In a system for ozonolysis reaction, 12 g of the mixture of acetylated cardanols **13** (34.66 mmol) were dissolved in dichloromethane (50 mL) and methanol (50 mL). The reaction system was cooled in dry ice/acetone bath at about -70 °C, and subjected to ozone continuous flow (5 g/mL) in oxygen for 80 minutes (2 x 40"). Next, the excess of ozone was purged with nitrogen. Under ice-cooling bath, 2.62 g of sodium borohydride (69.25 mmol) were added, and the mixture was stirred for 20 hours. Then, the pH was adjusted to 7 with HCl solution. The mixture was poured into water and extracted with ethyl acetate (3 x 40 mL) and the combined organic phases were washed with brine (50 mL). After drying with anhydrous sodium sulfate, the solution was concentrated under reduced pressure and the crude product purified on a silica gel column eluted with a hexane/ethyl acetate gradient (from 95:5 to 50:50), to provide **14** in 60 % yield (4.60 g), as a light-yellow oil. Rf = 0.35 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3351, 2929, 2855, 1589, 1456, 1271, 1156, 1051; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.30 (br, 8H, 3-6), 1.53-1.59 (m, 4H, 2 and 7), 2.54 (t, *J* = 7.6 Hz, 2H, 8), 3.66 (t, *J* = 6.6 Hz, 2H, 1), 4.08 (br, 2H, ArO<u>H</u> and CH_2<u>OH</u>), 6.66 (d, *J* = 6.8 Hz, 1H, 2' and 4'), 6.72 (d, *J* = 7.6 Hz, 1H, 6'), 7.12 (dt, *J* = 6.8 Hz, *J* = 1.2 Hz, 1H, 5'); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 25.8 (<u>C</u>H_2, 3), 29.2 (<u>C</u>H_2, 5), 29.4 (<u>C</u>H_2, 4), 29.4[5] (<u>C</u>H_2, 6), 31.3 (<u>C</u>H_2, 2), 32.7 (<u>C</u>H_2, 7), 35.9 (<u>C</u>H_2, 8), 63.2 (<u>C</u>H_2, 1), 112.8 (<u>C</u>H, 4'), 115.6 (<u>C</u>H, 2'), 120.7 (<u>C</u>H, 6'), 129.4 (<u>C</u>H, 5'), 144.8 (<u>C</u>, 1'), 156.0 (<u>C</u>, 3').

*1.3 Synthesis of target compounds (**2**-**11**)*

*General procedure for obtaining the target ortho-aminomethylphenols (***2-11***)*

A mixture of suitable amine (1 eq) and paraformaldehyde (1 eq) in ethanol (10 mL) was refluxed under magnetic stirring for 1.5 h. Next, 0.2 g of LDT71 (**14**, 0.90 mmol) were added, and the reflux was maintained for about 40 h. Then, the mixture was concentrated under reduced pressure and

purified on silica gel column, eluted using a gradient of hexane and ethyl acetate (from 95:5 to 90:10), providing the corresponding target compound.

2-{[Benzyl(methyl)amino]methyl}-5-(8-hydroxyocthyl)phenol (LDT544, **2***)*

The title compound was obtained according to the general procedure using N-methylbenzylamine, paraformaldehyde and **14**. Compound **2** was obtained as light-yellow oil: 0.2 g (63 %). Rf = 0.48 ($CHCl_3$:EtOH, 19:1); IR (KBr, cm^{-1}) *v* 3390, 2928, 2853, 1581,1453, 1274, 1119, 1071, 755, 699; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ* 1.32 (br, 8H, 3-6), 1.56 (m, 4H, 2 and 7), 2.25 (s, 3H, $NC\underline{H}_3$), 2.54 (t, *J* = 7.7, 2H, 8), 3.61 (s, 2H, $C\underline{H}_2N$, **c**), 3.63 (t, *J* = 6.6 Hz, 2H, 1), 3.73 (s, 2H, $NC\underline{H}_2$, **b**), 6.62 (dd, *J* = 7.6 Hz, *J* = 1.5 Hz 1H, 6'), 6.70 (s, 1H, 2'), 6.90 (d, *J* = 7.6 Hz, 1H, 5'), 7.29-7.38 (m, 5H, 1'', 2'', 3'', 4'' and 5''); ^{13}C-NMR (125 MHz, $CDCl_3$) *δ* 25.9 ($\underline{C}H_2$, 6), 29.4 ($\underline{C}H_2$, 6), 29.4 ($\underline{C}H_2$, 4), 29.4 ($\underline{C}H_2$, 5), 31.4 ($\underline{C}H_2$, 2), 33.0 ($\underline{C}H_2$, 7), 35.9 ($\underline{C}H_2$, 1), 41.4 ($N\underline{C}H_3$), 60.8 (CH_2N, **c**), 61.5 ($N\underline{C}H_2$, **b**), 63.2 ($\underline{C}H_2$, 1), 116.2 (<u>C</u>H, 2'), 119.2 (<u>C</u>, 4'), 119.5 (<u>C</u>H, 6'), 127.8 (<u>C</u>H, 4''), 128.5 (<u>C</u>H, 5'), 128.8 (<u>C</u>H, 2'' and 6''), 129.5 (<u>C</u>H, 3'' and 5''), 137.0 (<u>C</u>, 1''), 144.2 (<u>C</u>, 3'), 157.8 (<u>C</u>, 1'). HRMS (ESI) calcd for $C_{23}H_{23}NO_2$ $[M + H]^+$ 356.259, found 356.2584.

2-[(Diethylamino)methyl]-5-(8-hydroxyoctyl)phenol (LDT636, **3***)*

The title compound was obtained according to the general procedure using diethylamine, paraformaldehyde and **14**. Compound **3** was obtained as light-yellow oil: 0.2 g (72 %). Rf = 0.48 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3401, 2928, 2854, 1582-1457, 1273, 1115, 756; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ* 1.11 (t, *J* = 7.1 Hz, 6H, $C\underline{H}_3CH_2N$, **d**), 1.27-1.32 (m, 8H, 3-6), 1.56 (qi, *J* = 6.9 Hz, 4H, 2 and 7), 2.52 (t, *J* = 7.7, 2H, 8), 2.62 (q, *J* = 7.1 Hz, 4H, $CH_3C\underline{H}_2N$, **c**), 3.62 (t, *J* = 6.6 Hz, 2H, 1), 3.73 (s, 2H, $NC\underline{H}_2$, **b**), 6.59 (d, *J* = 7.5 Hz, 1H, 6'), 6.63 (s, 1H, 2'), 6.80 (d, *J* = 7.5 Hz, 1H, 5'); ^{13}C-NMR (125 MHz, $CDCl_3$) *δ* 11.3 ($C\underline{H}_3CH_2N$, **d**), 25.9 ($\underline{C}H_2$, 3), 29.4 ($\underline{C}H_2$, 6), 29.5 ($\underline{C}H_2$, 5), 29.6 ($\underline{C}H_2$, 4), 31.4 ($\underline{C}H_2$,

7), 32.9 (CH$_2$, 2), 35.8 (CH$_2$, 8), 46.4 (CH$_3$CH$_2$N, **c**), 56.8 (NCH$_2$, **b**), 63.2 (CH$_2$, 1), 116.2 (C, 2'), 119.1 (CH, 6'), 119.4 (CH, 4'), 128.2 (CH, 5'), 143.7 (C, 1'), 158.2 (C, 3'). HRMS (ESI) calcd for $C_{19}H_{33}NO_2$ [M + H]$^+$ 308.259, found 308.2584.

5-(8-Hydroxyoctyl)-2-[(pyrrolidine-1-il)methyl]phenol (LDT637, **4***)*

The title compound was obtained according to the general procedure using pyrrolidine, paraformaldehyde and **14**. Compound **4** was obtained as light-yellow oil: 0.13 g (48 %). Rf = 0.55 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3422, 2931, 2857, 1587, 1473, 1112, 756; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 1.31 (br, 8H, 3-6), 1.55-1.59 (m, 4H, 2 and 7), 1.83 (s, 4H CH$_2$CH$_2$N, **d**), 2.52 (t, *J* = 7.6, 2H, 8), 2.62 (s, 4H, CH$_2$CH$_2$N, **c**), 3.62 (t, *J* = 6.6 Hz, 2H, 1), 3.78 (s, 2H, NCH$_2$, **b**), 6.57 (d, *J* = 7.5 Hz, 1H, 6'), 6.64 (s, 1H, 6'), 6.86 (d, *J* = 7.5 Hz, 1H, 5'); ^{13}C NMR (125 MHz, $CDCl_3$) *δ* 23.8 (CH$_2$CH$_2$N **d**), 25.9 (CH$_2$, 3), 29.4 (CH$_2$, 6), 29.5 (CH$_2$, 4), 29.6 (CH$_2$, 5), 31.4 (CH$_2$, 7), 32.9 (CH$_2$, 2), 35.8 (CH$_2$, 8), 53.6 (CH$_2$CH$_2$N **c**), 58.7 (NCH$_2$, **b**), 63.2 (CH$_2$, 1), 116.0 (CH, 2'), 119.1 (CH, 6'), 119.8 (C, 4'), 127.7 (CH, 5'), 143.8 (C, 1'), 157.9 (C, 1'). HRMS (ESI) calcd for $C_{19}H_{31}NO_2$ [M + H]$^+$ 306.2434, found 306.2428.

5-(8-Hydroxyoctyl)-2-[(piperidin-1-yl)methyl]phenol (LDT638, **5***)*

The title compound was obtained according to the general procedure using piperidine, paraformaldehyde and **14**. Compound **5** was obtained as light-yellow oil: 0.23 g (81 %). Rf = 0.44 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3435, 2930, 2855, 1582, 1455, 1279, 1154, 860, 756; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 1.32 (br, 8H, 3-6), 1.52-1.67 (m, 10H, 2, 7 and CH$_2$CH$_2$CH$_2$N, **d** and **e**), 2.52 (t, *J* = 7.7, 4H, 8), 3.60 (t, *J* = 6.0 Hz, 4H, 1 and CH$_2$CH$_2$CH$_2$N, **c**), 6.58 (dd, *J* = 7.5 Hz, *J* = 1.5 Hz 1H, 6'), 6.65 (s, 1H, 2'), 6.85 (d, *J* = 7.5 Hz, 1H, 5'); ^{13}C NMR (125 MHz, $CDCl_3$) *δ* 24.2 (CH$_2$CH$_2$CH$_2$N, **e**), 25.9 (CH$_2$CH$_2$CH$_2$N, **d**), 26.0 (CH$_2$, 3), 29.4 (CH$_2$, 6), 29.4 (CH$_2$, 4), 29.4 (CH$_2$, 5), 31.4 (CH$_2$, 2), 32.9 (CH$_2$, 7), 35.8 (CH$_2$, 1), 54.0 (CH$_2$CH$_2$CH$_2$N, **c**), 62.0 (NCH$_2$, **b**), 63.2 (CH$_2$, 8), 116.1 (CH, 2'), 118.9 (C, 4'), 119.2

(CH, 6'), 128.4 (CH, 5'), 143.8 (C, 3'), 158.0 (C, 1'). HRMS (ESI) calcd for $C_{20}H_{33}NO_2$ $[M + H]^+$ 320.259, found 320.2593.

5-(8-Hydroxyoctyl)-2-[(thiomorpholine-4-yl)methyl]phenol (LDT639, **6***)*

The title compound was obtained according to the general procedure using thiomorpholine, paraformaldehyde and **14**. Compound **6** was obtained as light-yellow oil: 0.13 g (45 %). Rf = 0.62 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3430, 2928, 2856, 1596, 1526, 1459, 1271, 1151, 757; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ* 1.32 (br, 8H, 3-6), 1.56-1.58 (m, 4H, 2 and 7), 2.53 (t, *J* = 7.5, 2H, 8), 2.73 (sl, 4H, SCH_2CH_2N, **d**), 2.82 (s, 4H, SCH_2CH_2N, **c**), 3.63 (t, *J* = 6.5 Hz, 2H, 1), 3.68 (s, 2H, NCH_2, **b**), 6.60 (d, *J* = 7.4 Hz, 1H, 6'), 6.65 (s, 1H, 2'), 6.86 (d, *J* = 7.5 Hz, 1H, 5'); ^{13}C-NMR (125 MHz, $CDCl_3$) *δ* 25.9 (CH_2, 3), 28.1 (SCH_2CH_2N, **d**), 29.4 (CH_2, 6), 29.5 (CH_2, 4), 29.6 (CH_2, 5), 31.4 (CH_2, 7), 33.0 (CH_2, 2), 35.8 (CH_2, 8), 54.5 (SCH_2CH_2N, **c**), 62.1 (NCH_2, **b**), 63.1 (CH_2, 1), 116.2 (CH, 2'), 118.0 (C, 4'), 119.6 (CH, 6'), 128.8 (CH, 5'), 144.4 (C, 1'), 157.6 (C, 1'). HRMS (ESI) calcd for $C_{19}H_{31}NO_2S$ $[M + H]^+$ 338.2155, found 338.2159.

5-(8-Hydroxyoctyl)-2-[(morpholin-4-yl)methyl]phenol (LDT640, **7***)*

The title compound was obtained according to the general procedure using morpholine, paraformaldehyde and **14**. Compound **7** was obtained as light-yellow oil: 0.16 g (57 %). Rf = 0.47 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3436, 2928, 2854, 1581, 1455, 1274, 1119, 1071, 865, 755; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ* 1.31 (br, 8H, 3-6), 1.53-1.58 (m, 4H, 2 and 7), 2.52 (m, 6H, 8 and OCH_2CH_2N, **c**), 3.61 (t, *J* = 6.6 Hz, 2H, 1), 3.66 (s, 2H, NCH_2, **b**), 3.74 (t, *J* = 4.2 Hz, 4H, OCH_2CH_2N, **d**), 6.60 (dd, *J* = 7.5 Hz, *J* = 1.5 Hz 1H, 6'), 6.65 (s, 1H, 2'), 6.80 (d, *J* = 7.5 Hz, 1H, 5'); ^{13}C-NMR (125 MHz, $CDCl_3$) *δ* 25.9 (CH_2, 3), 29.4 (CH_2, 6), 29.5 (CH_2, 5), 29.6 (CH_2, 4), 31.4 (CH_2, 7), 32.9 (CH_2, 2), 35.8 (CH_2, 8), 53.0 (OCH_2CH_2N, **c**), 61.8 (NCH_2, **b**), 63.1 (CH_2, 1), 66.9 (OCH_2CH_2N, **d**), 116.1 (CH, 2'), 118.0 (C, 4'), 119.6

(CH, 6'), 128.7 (CH, 5'), 144.3 (C, 1'), 157.4 (C, 3'). HRMS (ESI) calcd for $C_{19}H_{31}NO_3$ $[M + H]^+$ 322.2383, found 322.2388.

5-(8-Hydroxyoctyl)-2-[(piperazine-1-yl)methyl]phenol (LDT641, **8***)*

The title compound was obtained according to the general procedure using piperazine, paraformaldehyde and **14**. Compound **8** was obtained as light-yellow oil: 0.14 g (48 %). Rf = 0.48 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3415, 2931, 2856, 1590, 1459, 1285, 1157, 828; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ* 1.30 (br, 8H, 3-6), 1.55-1.57 (m, 4H, 2 and 7), 2.52 (t, *J* = 7.5, 2H, 8), 3.62 (t, *J* = 6.5 Hz, 2H, 1), 3.68 (s, 2H, NCH_2, **b**), 4.04 (s, 1H, NH), 6.61 (d, *J* = 7.3 Hz, 1H, 2'), 6.64 (s, 1H, 5'), 6.87 (d, *J* = 7.5 Hz, 1H, 6'); ^{13}C-NMR (125 MHz, $CDCl_3$) *δ* 25.9 (CH_2, 3), 29.4 (CH_2, 6), 29.5 (CH_2, 4), 29.6 (CH_2, 5), 31.4 (CH_2, 7), 32.9 (CH_2, 2), 35.8 (CH_2, 8), 52.5 (NCH_2CH_2N, **c** and **d**), 61.1 (NCH_2, **b**), 63.2 (CH_2, 1), 116.2 (CH, 2'), 118.2 (C, 4'), 119.6 (CH, 6'), 128.7 (CH, 5'), 144.3 (C, 1'), 157.5 (C, 1'). HRMS (ESI) calcd for $C_{19}H_{32}N_2O_2$ $[M + H]^+$ 321.2543, found 321.2541.

5-(8-Hydroxyoctyl)-2-[(4-methylpiperazine-1-yl)methyl]phenol (LDT642, **9***)*

The title compound was obtained according to the general procedure using 1-methylpiperazine, paraformaldehyde and **14**. Compound **9** was obtained as light-yellow oil: 0.21 g (72 %). Rf = 0.51 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3408, 2933, 2857, 1594, 1458, 1272, 1155, 856; ^{1}H-NMR (500 MHz, $CDCl_3$) *δ* 1.30 (br, 8H, 3-6), 1.52-1.56 (m, 4H, 2 and 7), 2.29 (s, 3H, NCH_3 and NCH_2CH_2N, **d**), 2.51 (t, *J* = 7.6, 3H, 8 and NCH_2CH_2N, **c**), 3.60 (t, *J* = 6.6 Hz, 2H, 1), 3.67 (s, 2H, NCH_2, **b**), 6.58 (d, *J* = 7.1 Hz, 1H, 6'), 6.64 (s, 1H, 2'), 6.85 (d, *J* = 7.5 Hz, 1H, 5'); ^{13}C-NMR (75 MHz, $CDCl_3$) *δ* 25.9 (CH_2, 3), 29.4 (CH_2, 6), 29.5 (CH_2, 4), 29.6 (CH_2, 5), 31.4 (CH_2, 7), 32.9 (CH_2, 2), 35.8 (CH_2, 8), 45.9 (NCH_3), 52.4 (NCH_2CH_2N, **c**), 55.0 (NCH_2CH_2N, **d**), 61.2 (NCH_2, **b**), 62.9 (CH_2, 1), 116.0 (CH, 2'), 118.4 (C, 4'), 119.4

(CH, 6'), 128.6 (CH, 5'), 144.1 (C, 1'), 157.5 (C, 1'). HRMS (ESI) calcd for $C_{20}H_{34}N_2O_2$ $[M + H]^+$ 335.2699, found 335.2704.

1-(4-{[2-Hydroxy-4-(8-hydroxyoctyl)phenyl]methyl}piperazine-1-yl)ethan-1-one (LDT643, **10***)*

The title compound was obtained according to the general procedure using 1-acetylpiperazine, paraformaldehyde and **14**. Compound **10** was obtained as light-yellow oil: 0.25 g (76 %). Rf = 0.51 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3399, 2928, 2854, 1647, 1586, 1472, 1268, 1150, 1112, 830; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ* 1.39 (br, 8H, 3-6), 1.64 (d, *J* = 5.9 Hz, 4H, 2 and 7), 2.17 (s, 3H,NCOCH$_3$), 2.60 (t, *J* = 7.6, 6H, 8 and NCH$_2$CH$_2$N, **d**), 3.60 (s, 4H, NCH$_2$CH$_2$N, **c**), 3.70 (t, *J* = 6.4 Hz, 2H, 1), 3.78 (s, 2H, NCH$_2$Ar), 6.70 (d, *J* = 7.5 Hz, 1H, 2'), 6.76 (s, 1H, 6'), 6.96 (d, *J* = 7.3 Hz, 1H, 5'); ^{13}C-NMR (125 MHz, $CDCl_3$) *δ* 21.4 (NCOCH$_3$), 25.9 (CH$_2$, 3), 29.4 (CH$_2$, 6), 29.5 (CH$_2$, 4), 29.6 (CH$_2$, 5), 31.4 (CH$_2$, 7), 32.9 (CH$_2$, 2), 35.8 (CH$_2$, 8), 41.3 (NCH$_2$CH$_2$N, **c**), 46.1 (NCH$_2$CH$_2$N, **c**), 52.4 (NCH$_2$CH$_2$N, **d**), 52.6 (NCH$_2$CH$_2$N, **d**), 61.1 (NCH$_2$, **b**), 63.2 (CH$_2$, 1), 116.4 (CH, 2'), 117.7 (C, 4'), 119.8 (CH, 6'), 129.0 (CH, 5'), 144.7 (C, 1'), 157.3 (C, 1'), 169.2 (NCOCH$_3$). HRMS (ESI) calcd for $C_{21}H_{34}N_2O_3$ $[M + H]^+$ 363.2648, found 363.2655.

2-[(azepan-1-yl)methyl]-5-(8-hydroxyoctyl)phenol (LDT692, **11***)*

The title compound was obtained according to the general procedure using azepane, paraformaldehyde and **14**. Compound **11** was obtained as light-yellow oil: 0.1 g (32 %). Rf = 0.57 ($CHCl_3$:EtOH, 19:1); IR (film, cm^{-1}) *v* 3421, 2925, 2854, 1581, 1456, 1272, 1149, 865, 748; ^{1}H-NMR (500 MHz, $CDCl_3$) *δ* 1.36 (br, 8H, 3-6), 1.56-1.73 (m, 4H, 2, and 7), 1.67 (s, 4H, CH$_2$CH$_2$CH$_2$N, **e**), 1.73 (sl, 4H, CH$_2$CH$_2$CH$_2$N, **d**), 2.56 (t, *J* = 7.7 Hz, 2H, 8), 2.74 (sl, 4H, CH$_2$CH$_2$CH$_2$N, **c**), 3.66 (t, *J* = 6.6 Hz, 2H, 1), 3.78 (s, 2H, NCH$_2$, **b**), 6.61 (d, *J* = 7.4 Hz, 1H, 6'), 6.68 (s, 1H, 2'), 6.87 (d, *J* = 7.5 Hz, 1H, 5'); ^{13}C-NMR (75 MHz, $CDCl_3$) *δ* 25.9 (CH$_2$, 3), 26.8 (CH$_2$CH$_2$CH$_2$N, **e**), 27.8 (CH$_2$CH$_2$CH$_2$N, **d**), 29.4 (CH$_2$, 6),

29.5 (CH_2, 4), 29.6 (CH_2, 5), 31.4 (CH_2, 7), 32.9 (CH_2, 2), 35.8 (CH_2, 8), 55.4 ($CH_2CH_2CH_2N$, **c**), 62.0 (NCH_2, **b**), 63.2 (CH_2, 1), 116.1 (CH, 2'), 119.0 (C, 6'), 119.7 (CH, 4'), 128.2 (CH, 5'), 143.8 (C, 1'), 158.3 (C, 3'). HRMS (ESI) calcd for $C_{21}H_{35}NO_2$ $[M + H]^+$ 334.2748, found 334.2758.

2. Biological evaluation

Once synthesized and characterized, the target derivatives were transformed into their hydrochloride salts by saturation of solution in dichloromethane with HCl gas, and submitted to the evaluation of the biological activities

2.1 Inhibition of human AChE and BChE activities

The method of Ellman et al. was followed.[1] AChE stock solution was prepared by dissolving human recombinant AChE lyophilized powder (Sigma, Italy) in 100 mM phosphate buffer (pH = 8.0) containing 0.1% Triton X-100. Stock solution of BChE from human serum (Sigma, Italy) was prepared by dissolving the lyophilized powder in an aqueous solution of 0.1% gelatine. Stock solutions of tested compounds (1 mM) were prepared in methanol and serially diluted in methanol to get a set of solutions at decreasing concentrations. The assay solution consisted of a 0.1 M phosphate buffer pH 8.0, with the addition of 340 µM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.02 units of hAChE or hBChE and 550 µM of substrate (acetylthiocoline iodide or butyrylthiocholine iodide, respectively). Final concentration of methanol was 5%. Assays were done with a blank containing all components except the enzyme to account for non-enzymatic substrate hydrolysis. Tested compounds were added to the assay solution and preincubated with the enzyme for 20 min before the addition of substrate. Initial rate assays were performed at 37 °C with a Jasco V-530 double beam Spectrophotometer (Jasco Europe, Italy) equipped thermostated cuvette holder (37°C). Absorbance value at 412 nm was recorded for 240 s and enzyme activity was calculated from the slope of the

obtained linear trend. The reaction rates obtained in the presence and in the absence of the tested compound were compared and the percent inhibition was calculated. Compounds were initially screened at a single concentration of 20 μM. For compounds showing a % inhibition higher than 20% the IC_{50} values were determined. In the latter case, five different concentrations of each compound were used to obtain inhibition of enzyme activity between 20 and 80%. IC_{50} values were determined graphically from log concentration–inhibition curves (GraphPad Prism 4.03 software, GraphPad Software Inc.). Each final value is the mean of at least two independent experiments each performed in triplicate.

2.2 Determination of Aβ antiaggregant activity using transmission electron microscopy (TEM)

Determination of Aβ antiaggregant activity was performed with synthetic Aβ42 peptide – synthesized in the Tanz CRND laboratory, University of Toronto. The peptide was induced to adopt a prevalent unordered secondary structure by treating the peptide as follow: 1 mg/mL solution was freeze-dried, a suitable volume pf a 60 % TFE solution was added to achieve back a 1 mg/mL solution which was freeze-dried. Dried sample was solubilized in 1,1,1,3,3,3,-hexafluoroisopropanol (1 mg/mL) and freeze-dried again. Finally, 20 mL of trifluoroacetic acid solution was added and the obtained solution was freeze-dried.

To start the assay, 500 μL of PBS (saline phosphate buffer) pH 7.4 were added to the dried sample to a final Aβ42 concentration of 60 μM and dispersed by vortexing for ~5 mins. Compounds were evaluated at a 1:5 ratio (Aβ42:binder). A 20 μL aliquot of each sample was collected and transferred over the grid of electron microscopy and left 2 min and exceeding solution was removed with a filter paper. Samples were stained 20 μL of 1 % uranyl acetate solution (negative stain) for 2 min, dried and observed by TEM. Samples were visualized and micrographs obtained on a Hitachi H-7000 operated at 120 kV.

2.3 Determination of the antioxidant activity

The antioxidant activity toward hydroxyl radicals was assessed by using the OxiSelect™ HORAC activity assay kit (Cell Biolabs Inc.). Methanol stock solution (2-4 mM) of ferulic acid and tested compounds were diluted to 50 µM with the assay buffer. Following the manufacturer protocol, assay buffer was used as blank and GA 50 µM as reference antioxidant compound. A 20 µL-aliquot of tested compound solution and 140 µL of fluorescein solution were added in each well of a 96-well clear bottom black plate and incubated for 30 min at room temperature. Finally, 20 µL of hydroxyl radical initiator and 20 µL of Fenton reagent were added, mixed thoroughly and immediately analyzed by a Wallac Victor fluorescence plate reader (PerkinElmer). The fluorescence intensity was measured at excitation wavelength of 480 nm and emission wavelength of 530 nm. The area under the decay curve (AUC) is related to the antioxidant capacity of the tested compound. Results are expressed as GA equivalent (GAE) calculated with the following formula:

$$GAE = \frac{AUC\ (compound) - AUC\ (Blank)}{AUC\ (GA) - AUC\ (Blank)}$$

2.4 PAMPA-BBB assay

For the prediction of **2**-**11** to passively penetrate the brain, a PAMPA-BBB modified protocol was used.[2, 3] Briefly, the filter membrane of the donor plate was coated with PBL (Polar Brain Lipid, Avanti, USA) in dodecane (4 µl of 20 mg/ml PBL in dodecane) and the acceptor well was filled with 300 µl of phosphate buffer saline (PBS pH 7.4; VA). The tested compounds were dissolved first in DMSO and then diluted with PBS pH 7.4 to reach the final concentrations 50 - 500 µM in the donor well. The final concentration of DMSO did not exceed 0.5% (v/v) in the donor solution. 300 µl of the donor solution (VD) was added to the donor wells and the donor filter plate was carefully put on the acceptor plate so that the coated membrane was “in touch” with both donor solution and

acceptor buffer. In principle, test compound diffuses from the donor well through the polar brain lipid membrane (Area = 0.28 cm2) to the acceptor well. The concentration of the tested compound in both donor and the acceptor wells were assessed after 3, 4, 5 and 6 hours of incubation respectively in quadruplicate using the UV plate reader Synergy HT (Biotek, USA) at the maximum absorption wavelength of each compound (n=3). Besides that, solution of theoretical compound concentration, simulating the equilibrium state established if the membrane were ideally permeable was prepared and assessed as well. Concentration of the compounds in the donor and acceptor well and equilibrium concentration were calculated from the standard curve and expressed as the permeability (Pe) according the equation:

$$P_e = C \times ln\left(1 - \frac{[drug]\ acceptor}{[drug]\ equilibrium}\right) \ where\ C = \frac{(V_D \times V_A)}{(V_D \times V_A)\ Area \times Time}$$

2.5 Determination of antioxidant activity in SH-SY5Y cells

To evaluate the antioxidant activity of compounds **5** and **11**, SH-SY5Y cells were seeded in 96-well plates (OptiPlate black, PerkinElmer) at 10x10^3 cells/well and incubated overnight at 37 °C and 5% CO_2 to allow adhesion. After this time, cells were treated for 24 h with 10 µM of compounds **5** and **11**, 100 µM Trolox or vehicle (DMSO). Then, cells were washed with PBS and stained for 30 min with 10 µM H2DCFDA probe (2',7'-dichlorodihydrofluorescein diacetate, ThermoFisher) in DMEM. To induce oxidative stress, cells were carefully washed and treated for 30 min with 100 µM TBH (tert-Butyl hydroperoxide solution, Luperox, Sigma-Aldrich) dissolved in DMEM. Finally, cells were washed three times with PBS supplemented with glucose 5 mM and $CaCl_2$ 1 mM. The DCF fluorescence was measured (λ_{exc} 485 nm; λ_{em} 535 nm) using a microplate reader (Enspire, Perkin Elmer).

2.6 Determination of hepatotoxicity in HepG2 cells

Human hepatocarcinoma HepG2 Cell line was cultured in DMEM supplemented with 10 % FBS, 100 UImL-1 penicillin, 100 mgmL-1 streptomycin, in a 5 % CO_2 atmosphere at 37 °C, with saturating humidity. Cytotoxicity of compounds **5** and **11** was estimated using a MTT-based assay.[4]

Briefly, cells were seeded in 96-well plates at a density of $5x10^3$ cells/well and incubated overnight at 37 °C, 5% CO_2 in humidified atmosphere to allow adhesion. After this time, cells were treated for 24 h with the compounds **5** and **11** or vehicle (DMSO) at different concentration ranging from 1.25 to 10 µM. Then, cells were washed with PBS and treated with 300 µM MTT dissolved in DMEM and incubated for two hours at 37 °C and 5% CO_2. After this time the medium was removed, and the formazan salts were dissolved in DMSO. The formazan absorbance from each well was measured at λ = 570 nm, using a microplate reader (Enspire, Perkin Elmer).

3. Molecular modeling

3.1 Molecular Docking

ChemDraw Ultra version 12.0, CambridgeSoft was used for drawing, displaying and characterizing the ligands' chemical structures. The ligands' ionized form was used when suitable. Before performing molecular docking simulations, we have generated five distinct conformers for each compound, using the Autodock Vina tools.[5] Then, molecular docking simulations were performed using Autodock Vina software.[5] High resolution crystal structures of human butyrylcholinesterase (*h*BChE, 6EQP) and acetylcholinesterase (*h*AChE) in complex with donepezil (4EY7) were retrieved from the Protein Data Bank-PDB and served as a starting point. Docking search space covered the entire cavity of the enzymes and solutions were ranked according to Autodock Vina software score

function (Tables 1 and 2). Molecular visualization of docking poses and putative interactions have been performed with VMD 1.9.3.[6]

Table 1. Binding energies (kcal/mol) for the five distinct conformers of each compound considering AChE as molecular target.

Binding Energies (kcal/mol)											
Conformer	**LDT544**	**LDT636**	**LDT637**	**LDT638**	**LDT639**	**LDT640**	**LDT641**	**LDT642**	**LDT643**	**LDT692**	**LDT161**
1	-9.2	-7.7	-8.1	-8.4	-8.2	-8.2	-8.1	-8.5	-9.1	-8.5	-8.2
2	-9.0	-7.6	-7.8	-8.2	-8.0	-8.2	-8.0	-8.3	-8.8	-8.5	-8.0
3	-8.9	-7.6	-7.8	-8.2	-7.9	-8.1	-7.8	-8.1	-8.6	-8.4	-8.0
4	-8.9	-7.2	-7.8	-8.0	-7.7	-8.0	-7.8	-8.0	-8.2	-8.3	-7.9
5	-8.7	-7.2	-7.8	-8.0	-7.7	-8.0	-7.6	-7.9	-7.9	-8.3	-7.8

Table 2. Binding energies (kcal/mol) for the five distinct conformers of each compound considering BChE as molecular target.

Binding Energies (kcal/mol)											
Conformer	**LDT544**	**LDT636**	**LDT637**	**LDT638**	**LDT639**	**LDT640**	**LDT641**	**LDT642**	**LDT643**	**LDT692**	**LDT161**
1	-8.5	-6.3	-7.4	-7.2	-7.0	-6.2	-6.5	-7.4	-8.0	-7.6	-6.8
2	-8.0	-6.0	-7.1	-7.2	-6.6	-5.9	-6.3	-7.4	-7.6	-7.6	-6.7
3	-8.0	-6.0	-7.1	-7.1	-6.6	-5.9	-6.2	-7.2	-7.6	-7.3	-6.7
4	-7.9	-5.9	-7.0	-7.1	-6.3	-5.8	-6.2	-7.1	-7.5	-7.0	-6.6
5	-7.7	-5.9	-6.8	-7.1	-6.2	-5.8	-6.1	-7.1	-7.3	-7.0	-6.6

4. Plasma stability and kinetic solubility

Stability of compounds **5** and **11** in plasma was assessed by incubating the compounds over the 2 hour range at 37 °C and analysing its decrease with high performance liquid chromatography (HPLC) coupled with mass spectrometer (MS). Pooled human plasma and chemicals used in the analytical part (in LC-MS grade) were purchased from VWR (Stribrna Skalice, Czech Republic). The method was described by Di et al.[7] 5µL of 1 µM solution of compound **5** or **11** in DMSO was added to 500 µL of plasma, vortexed, and incubated at 37 °C for 0, 15, 30, 60, or 120 minutes. After incubation 500 µL of cool acetonitrile with internal standard (IS; 1 µM tacrine-trolox hybrid 7u described in Nepovimova et al.[8]) was added to every sample to stop reaction and samples were vortexed and centrifuged (15610 g, 5 min, Universal 320 R centrifuge, Hettich, Tuttlingen, Germany). 300 µL of supernatant was transferred to vial and analysed by HPLC-MS. Control samples were prepared with 500 µL of potassium phosphate buffer (pH 7.4) instead of plasma. Positive control was prepared using anti-Alzheimer drug rivastigmine.

HPLC-MS analysis was performed using Dionex Ultimate 3000 UHPLC consisting of RS LPG quaternary Pump, RS Column Compartment, RS Autosampler, and Diode Array Detector controlled by Chromeleon (version 7.2.9 build 11323) software (Thermo Fisher Scientific, Germering, Germany) with Q Exactive Plus Orbitrap mass spectrometer with Thermo Xcalibur (version 4.3.73.11) software (Thermo Fisher Scientific, Bremen, Germany). Detection was performed by mass spectrometry in positive mode. Settings of heated electrospray source were: Spray voltage 3.5 kV, Capillary temperature: 262 °C, Sheath gas: 50 arbitrary units, Auxiliary gas: 12.5 arbitrary units, Spare gas: 2.5 arbitrary units, Probe heater temperature: 300 °C, Max spray current: 100 µA, S-lens RF Level: 50. The analysis of compounds **5** and **11** content in samples was performed in reverse phase gradient mode using Kinetex EVO C18 column (2.1x50 mm, 1.7 µm, Phenomenex, Torrance, California, USA) with Kinetex SecurityGuard Ultra C18 guard column (2.1 mm, Phenomenex, Torrance, California, USA). Mobile phase A was ultrapure water of ASTM I type (resistance 18.2 MΩ.cm at 25°C) prepared by Barnstead Smart2Pure 3 UV/UF apparatus (Thermo Fisher Scientific, Bremen, Germany) with 0.1 % (v/v) formic acid (LC-MS grade, VWR, Stribrna Skalice, Czech Republic); mobile phase B was acetonitrile (LC-MS grade, VWR, Stribrna Skalice, Czech Republic) with 0.1 % (v/v) of formic acid. Column was tempered to 35 °C, mobile phase flow was set to 0.5 ml/min and injection volume was 2 µl. Method started with 5 % B and was steady for 0.5 min, then the gradient went from 5 % B to 100 % B in 6 minutes and was kept at 100 % B for 1 min and then went back to 5 % B and equilibrated for 2.5 min. Total runtime of method was 10 min. Compounds and IS were detected with mass spectrometer in total ion current scan in range 105-650 m/z in positive mode. Settings were as follows: retention time for compound **5** was 3.67 min searched mass 320.2582; retention time for compound **11** was 3.68 and searched mass was 334.2737; retention time for IS was 4.72 min and searched mass was 592.3297. Area under curve (AUC) for compounds **5**, **11** or IS was calculated from chromatogram and each sample was characterised as $AUC_{compound}/AUC_{IS}$. Ratio of $sample_{15\text{-}120\ min}/sample_{0\ min}$ in percent was the percentage of undecomposed compound.

5. PAINS behavior assessment

We are aware that **2**-**11** carry a phenolic substructure and feature as Mannich bases, which are recognized as pan assay interference compounds (PAINS) by forming reactive quinone methides or by chelating metal ions.[9] Nevertheless, our purpose in this paper was to synthesize a new set of derivatives, which, with respect to **1**, carry the free phenolic OH of the natural component (i.e., cardanol). Notably, different cholinesterase inhibitory profiles with SAR were delineated within **2**-

11. Different anti-amyloid aggregation abilities (anti-amyloid active compounds **2**, **3**, **5**, **7** and **11**) were shown. This seems to suggested that the activity of the reported series is not caused by pan assay interference.

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