Supporting Information

Codrugs linking L-Dopa and sulphur-containing antioxidants: new pharmacological tools against Parkinson’s disease.

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A series of multifunctional codrugs (1-6) were synthesised to overcome the pro-oxidant effect associated with L-Dopa (LD) therapy. The antioxidant properties and pharmacological “in vivo” behaviour of new compounds were evaluated. Codrug 4 emerged as the derivative with the best overall dopaminergic and antioxidant activity: this multifunctional codrug has the potential to provide an alternative to LD therapy in order to avoid nigrostriatal oxidative degeneration.

\begin{align*}
1: & \quad R = \text{N-acetylCys}; R_1 = \text{OMe} \\
2: & \quad R = \text{Ac}; R_1 = \text{Cys-OMe} \\
3: & \quad R = \text{N-acetylMet}; R_1 = \text{OMe} \\
4: & \quad R = \text{Ac}; R_1 = \text{Met-OMe} \\
5: & \quad R = \text{N-acetylCysCys}; R_1 = \text{OMe} \\
6: & \quad R = \text{Bucillamine}; R_1 = \text{OMe}
\end{align*}
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Chemistry

Ac-Cys(SBu')-OH (7), H-LD(Ac)₂-OMe HCl, H-Cys(SBu')-OMe HBr (8), H-Cys(SBu')-OBu' were synthesized as previous reported.¹⁻⁴ The H-LD(Ac)₂-OH HCl was N-acetylated as described by Chenault et al. to obtain Ac-LD(Ac)₂-OH.⁵ Ac-Cys(SBu')-Cys(SBu')-OBu' (15) was synthesized by coupling of Ac-Cys(SBu')-OH (7) and H-Cys(SBu')-OBu' with DCC and HOBt in CH₂Cl₂ for 3 h at rt and 16 h at 5 °C. Subsequent removal of O-tertbutyl protection gave the desired dipeptide Ac-Cys(SBu')-Cys(SBu')-OH (16) in quantitative yield. Codrugs 1-6 were synthesized as outlined in Schemes 1-4 employing solution phase procedures by elongation of the suitably protected aminoacid or dipeptide in the C- (compounds 1, 3 and 5-6) or N- (compounds 2 and 4) direction with the LD derivative. Simultaneous deprotection of the cysteine -SH and the catecholic hydroxy-groups to give the desired codrugs 1-2 and 5-6 was obtained when the corresponding protected di- or tripeptide precursor was treated for 1.5 h at room temperature with a small excess (1.2 equiv.) of tri-n-butylphosphine in a water/n-propanol solution, made slightly alkaline (pH 8.5) by aqueous ammonia. Deprotection of the catecholic hydroxy-groups to give 3 and 4 was obtained when the corresponding protected dipeptide precursor was treated for 1 h at room temperature with saturated aqueous NaHCO₃ in a water/methanol solution.⁶
Scheme 1

Reagents and conditions: a) H-LD(Ac)₃-OMe·HCl, DCC, TEA, HOBr, DMF, 3 h, 0 °C then 16 h, 5 °C; b) Ac-LD(Ac)₃-OH, DCC, TEA, HOBr, DMF, 3 h, 0 °C then 16 h, 5 °C; c) (n-Bu)₃P, n-PrOH·H₂O (2:1), 1.5 h, rt, pH 8.5.

Scheme 2

Reagents and conditions: a) H-LD(Ac)₃-OMe·HCl, DCC, TEA, HOBr, DMF, 3 h, 0 °C then 16 h, 5 °C; b) Ac-LD(Ac)₃-OH, DCC, TEA, HOBr, DMF, 3 h, 0 °C then 16 h, 5 °C; c) NaHCO₃  ss, MeOH·H₂O (2:1), 1 h, rt.
Scheme 3

Reagents and conditions: a) H-Cys(SBu')-OBu', DCC, HOBr, CH₂Cl₂, 3 h, 0 °C then 16 h, 5 °C; b) TFA, 2 h, rt; c) H-LD(Ac)₂-OMe HCl, DCC, TEA, HOBr, DMF, 3 h, 0 °C then 16 h, 5 °C; d) (n-Bu)₃P, n-PrOH:H₂O (2:1), 1.5 h, rt, pH 8.5.

Scheme 4

Reagents and conditions: a) H-LD(Ac)₂-OMe HCl, DCC, TEA, HOBr, DMF, 3 h, 0 °C then 16 h, 5 °C; b) (n-Bu)₃P, n-PrOH:H₂O (2:1), 1.5 h, rt, pH 8.5.
Experimental Section

Microanalyses were performed on a 1106 Carlo Erba CHN analyzer, with results within (0.4%) of the calculated values. Optical rotations were taken at 25 °C with a Perkin-Elmer 241 polarimeter. $^1$H- and $^{13}$C-NMR spectra were recorded on a Varian VXR 300-MHz spectrometer. Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me$_4$Si).

The LC-MS/MS system employed consisted of an LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300 °C and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N$_2$) as both the sheath gas and the auxiliary gas. The identity of all new compounds was confirmed by elemental analysis, NMR data, and LC-MS/MS system; homogeneity was confirmed by TLC on silica gel Merck 60 F$_{254}$. Solutions were routinely dried over anhydrous sodium sulphate prior to evaporation. Chromatographic purifications were performed by Merck 60 70-230 mesh ASTM silica gel column.

Chemicals. Ac-Met-OH (11), H-Met-OMeHCl (12) were obtained by Fluka. Oxidized form of bucillamine (18) was a kind gift from Santen Pharmaceutical Co, Ltd. All other chemicals used were of the highest purity commercially available. Lucigenin, luminol, xanthine and xanthine oxidase were purchased from Sigma.

Ac-Cys(SBu$^t$)-Cys(SBu$^t$)-OH (16).

H-Cys(SBu$^t$)-OBu$^t$ (15 mmol) in dry CH$_2$Cl$_2$ (20 mL) was added at 0 °C to an ice-cold solution of 7 (15 mmol) in dry CH$_2$Cl$_2$ (20 mL), followed by portion-wise addition of a solution of DCC (15 mmol) in dry CH$_2$Cl$_2$ (20 mL) and then of HOBT (15 mmol) in dry CH$_2$Cl$_2$ (20 mL) with stirring. After 3 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum. The residue was taken up in AcOEt and the organic layer washed with citric acid 5%, saturated aqueous NaHCO$_3$ and brine. The residue obtained after drying and
evaporation was chromatographed on silica gel using CHCl₃:Et₂O (1:1) to give the corresponding pure full protected dipeptide 15 (yield 65%). This dipeptide 15 (8 mmol) was dissolved in TFA (10.4 mL), and after 2 h at room temperature the solution was evaporated to dryness and the residue repeatedly evaporated with ether to give Ac-Cys(SBu')-Cys(SBu')-OH (16) in quantitative yield, which was used without further purification. Rф = 0.34, CHCl₃:Et₂O (1:1); ¹H-NMR (DMSO-d₆) δ: 1.27 and 1.28 (18H, 2 x s, SBu'), 1.83 (3H, s, AcNH), 2.81-3.14 (4H, m, 2 x Cys β-CH₂), 4.40 and 4.53 (2H, m, 2 x Cys α-CH), 8.20 (1H, d, J = 8.40 Hz, Cys NH), 8.38 (1H, d, J = 7.8 Hz, Cys NH); ¹³C-NMR (DMSO-d₆) δ: 23.21 (Cys Ac), 29.97 and 30.21 (2 x SBu'), 41.72 and 43.55 (2 x Cys β-CH₂), 48.36 and 48.47 (2 x SBu'), 52.45 and 52.60 (2 x Cys α-CH), 170.04, 170.78 and 172.18 (3 x CO).

**General method for coupling with L-Dopa (compounds 9, 10, 13, 14, 17 and 19).**

A reaction mixture was formed with an ice cold solution of Cys or Met, to which CysTCys or oxidized bucillamine derivatives (12.1 mmol) in dry DMF (20 mL), L-Dopa (12.1 mmol) in TEA (1.7 mL, 12.1 mmol) and DMF (20 mL) were added at 0 °C, followed by portion-wise addition of a solution of DCC (12.1 mmol) in dry DMF (20 mL) and HOBt (12.1 mmol) in dry DMF (15 mL) with stirring. After 3 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum. The residue was taken up in CHCl₃ and the organic layer washed with citric acid 5% and brine. The residue obtained after drying and evaporation was chromatographed on silica gel using CHCl₃:Et₂O or CHCl₃:MeOH as eluant to give the corresponding pure full protected di- or tripeptide methyl ester.

**Ac-Cys(SBu')-LD(Ac)₂-OMe (9).** Yield: 70%; Rф = 0.41, CHCl₃:Et₂O (1:1); ¹H-NMR (CDCl₃) δ: 1.26 (9H, s, SBu'), 1.88 (3H, s, Cys Ac), 2.21 and 2.23 (6H, 2 x s, LD Ac), 2.88-3.01 (2H, m, Cys β-CH₂), 3.09-3.21 (2H, m, LD β-CH₂), 3.69 (3H, s, OMe), 4.71 (1H, m, Cys α-CH), 4.83 (1H, m, LD α-CH), 6.45 (1H, d, J = 8.80 Hz, Cys NH), 6.80 (1H, d, J = 7.48 Hz, LD NH), 6.91-7.02 (3H,
m, ArH); $^1^3$C-NMR (CDCl$_3$) δ: 20.80 and 21.10 (2 x LD Ac), 23.07 (Cys Ac), 30.03 (SBu'), 37.12 (LD β-CH$_2$), 42.46 (Cys β-CH$_2$), 48.51 (SBu'), 52.78 (Cys α-CH), 52.80 (OMe), 52.84 (LD α-CH), 123.53-141.94 (LD Ar), 168.53, 169.11, 170.03, 171.30 and 171.32 (5 x CO).

**Ac-LD(Ac)$_2$-Cys(SBu')-OMe (10).** Yield: 86%; R$_f$ = 0.40, CHCl$_3$:MeOH (95:5); $^1$H-NMR (CDCl$_3$) δ: 1.28 (9H, s, SBu'), 1.95 (3H, s, LD Ac), 2.23 and 2.24 (6H, 2 x s, LD Ac), 3.04-3.15 (4H, m, Cys β-CH$_2$ and LD β-CH$_2$), 3.71 (3H, s, OMe), 4.74-4.77 (2H, m, Cys α-CH and LD α-CH), 6.48 (1H, d, J = 7.91 Hz, Cys NH), 7.03 (4H, m, LD NH and ArH); $^1^3$C-NMR (CDCl$_3$) δ: 20.86 and 20.88 (2 x LD Ac), 23.38 (LD Ac), 29.96 (SBu'), 37.44 (LD β-CH$_2$), 41.83 (Cys β-CH$_2$), 48.51 (SBu'), 52.39 (Cys α-CH), 52.87 (OMe), 54.11 (LD α-CH), 123.70-142.11 (LD Ar), 170.47, 170.58, 170.61, 170.67 and 170.81 (5 x CO).

**Ac-Met-LD(Ac)$_2$-OMe (13).** Yield: 57%; R$_f$ = 0.59, CHCl$_3$:MeOH (95:5); $^1$H-NMR (CDCl$_3$) δ: 1.81-2.06 (2H, m, Met β-CH$_2$), 1.88 (3H, s, Met Ac), 2.05 (3H, s, Met CH$_3$), 2.10-2.28 (6H, 2 x s, LD Ac), 2.48-2.52 (2H, m, Met γ-CH$_2$), 3.01-3.23 (2H, m, LD β-CH$_2$), 3.74 (3H, s, OMe), 4.57 (1H, m, Met α-CH), 4.89 (1H, m, LD α-CH), 6.39 (1H, d, J = 8.40 Hz, Met NH), 6.84 (1H, d, J = 8.10 Hz, LD NH), 6.95-7.09 (3H, m, ArH); $^1^3$C-NMR (CDCl$_3$) δ: 15.40 (Met CH$_3$), 20.85 and 21.04 (2 x LD Ac), 23.17 (Met Ac), 30.37 and 30.86 (Met β- and γ-CH$_2$), 37.19 (LD β-CH$_2$), 51.98 (Met α-CH), 52.52 (OMe), 52.87 (LD α-CH), 123.65-142.01 (LD Ar), 168.56, 169.03, 170.92, 171.16 and 171.55 (5 x CO).

**Ac-LD(Ac)$_2$-Met-OMe (14).** Yield: 74%; R$_f$ = 0.63, CHCl$_3$:MeOH (95:5); $^1$H-NMR (CDCl$_3$) δ: 1.92-2.08 (2H, m, Met β-CH$_2$), 1.98 (3H, s, LD Ac), 2.05 (3H, s, Met CH$_3$), 2.25-2.26 (6H, 2 x s, LD Ac), 2.40-2.45 (2H, m, Met γ-CH$_2$), 3.02-3.09 (2H, m, LD β-CH$_2$), 3.71 (3H, s, OMe), 4.60 (1H, m, Met α-CH), 4.69 (1H, m, LD α-CH), 6.31 (1H, d, J = 7.97 Hz, LD NH), 6.71 (1H, d, J =
7.69 Hz, Met NH), 7.04-7.10 (3H, m, ArH); $^{13}$C-NMR (CDCl$_3$) δ: 15.59 (Met CH$_3$), 20.85 and 20.87 (2 x LD Ac), 23.37 (LD Ac), 30.07 and 31.29 (Met β- and γ-CH$_2$), 37.60 (LD β-CH$_2$), 51.93 (Met α-CH), 52.77 (OMe), 54.28 (LD α-CH), 123.73-142.15 (LD Ar), 168.49, 169.77, 170.45, 170.70, and 171.89 (5 x CO).

Ac-Cys(SBu')-Cys(SBu')-LD(Ac)$_2$-OMe (17). Yield: 54%; R$_f$ = 0.55, CHCl$_3$:MeOH (97:3); $^{1}$H-NMR (CDCl$_3$) δ: 1.29 and 1.30 (18H, 2 x s, SBu'), 1.94 (3H, s, Cys Ac), 2.10 and 2.15 (6H, 2 x s, LD Ac), 2.99-3.15 (6H, m, 2 x Cys β-CH$_2$ and LD β-CH$_2$), 3.77 (3H, s, OMe), 4.65-4.98 (3H, m, 2 x Cys α-CH and LD α-CH), 6.45 (1H, d, J = 7.45 Hz, Cys NH), 7.15-7.23 (5H, m, Cys NH, LD NH and ArH); $^{13}$C-NMR (CDCl$_3$) δ: 20.86 and 21.25 (LD Ac), 23.33 (Cys Ac), 29.9 and 30.06 (2 x SBu'), 37.09 (LD β-CH$_2$), 41.42 and 41.69 (2 x Cys β-CH$_2$), 48.57 and 48.84 (2 x SBu'), 52.81 (OMe), 53.29 and 53.58 (2 x Cys α-CH), 53.68 (LD α-CH), 123.61-141.74 (LD Ar), 168.56, 169.29, 169.50, 170.64, 170.70 and 171.20 (6 x CO).

4-((S)-2-((R)-7,7-dimethyl-6-oxo-1,2,5-dithiazepane-4-carboxamido)-3-methoxy-3-oxopropyl)-1,2-phenylene diacetate (19). Yield: 78%; R$_f$ = 0.75, CHCl$_3$:MeOH (95:5); $^{1}$H-NMR (CDCl$_3$) δ: 1.55 and 1.65 (6H, 2 x s, Buc CH$_3$), 2.26 and 2.28 (6H, 2 x s, LD Ac), 3.13-3.19 (4H, m, Buc β-CH$_2$ and LD β-CH$_2$), 3.76 (3H, s, OMe), 4.87 (1H, m, LD α-CH), 5.10 (1H, m, Buc α-CH), 6.36 (1H, d, J = 8.40 Hz, LD NH), 6.55 (1H, d, J = 7.8 Hz, Buc NH), 6.95-7.25 (3H, m, ArH); $^{13}$C-NMR (CDCl$_3$) δ: 20.86 and 20.97 (LD Ac), 26.76 (Buc CH$_3$), 30.56 (Buc CH$_3$), 34.14 (Buc β-CH$_2$), 36.94 (LD β-CH$_2$), 53.08 (Buc α-CH), 53.60 (OMe), 53.95 (LD α-CH), 55.97 (Buc C(CH$_3$)$_2$), 123.86-141.38 (LD Ar), 168.56, 168.92, 171.33, 173.58 and 173.69 (5 x CO).

Chemiluminescence (CL) measurements
Chemiluminescence measurements were performed in an AutoLumat LB 953 (Berthold Co., Wilbad, Germany). Lucigenin-amplified chemiluminescence was measured in a reaction mixture containing 0.9 U/mL xanthine oxidase, 150 µM lucigenin in 1 mL of 50 mM PBS, pH 7.4, and 10 µg/mL of NAC and codrugs 1-6. The reaction was started by injecting xanthine at a final concentration of 50 µM. Data, expressed as percent inhibition of CL reaction, were calculated by subtracting from 100 the differences in the area of the sample with respect to the blank solution considered as 100% of superoxide anion present in the sample as follows:

\[
\text{\% inhibition} = 100 - \left( \frac{\text{Area}_{\text{sample solution}} \times 100}{\text{Area}_{\text{blank solution}}} \right)
\]

Luminol amplified-chemiluminescence was measured in the presence of 100 µM luminol in 1 ml of 50 mM PBS, pH 7.4, and 10 µg/mL of NAC and compounds 1-6. The reaction was started by injecting hydrogen peroxide at a final concentration of 50 mM. Data were expressed as percent inhibition of CL and were calculated as described for lucigenin amplified-chemiluminescence.

**Animals**

Male Wistar rats (n=104) (Harlan, UD, Italy) that weighed 275-325 g at the beginning of the experiments were used. The animals were individually housed in a room on a 12 h light/dark cycle (lights off at 7:00 A.M.) at constant temperature (20-22 °C) and humidity (45-55%). Rats were offered food pellets (4RF; Mucedola, Settimo Milanese, Italy) and tap water ad libitum and were handled once a day for 5 min during the first week after arrival. All procedures were conducted in adherence to the European Community Council Directive for Care and Use of Laboratory Animals.

**Drug administration**

Benserazide hydrochloride, a peripheral dopa-decarboxylase inhibitor, was dissolved in water whereas the LD and codrugs 1-6 were dissolved in dimethyl sulfoxide. All animals (n = 8 per group) received a dose of benserazide (16.36 mg/kg) combined with LD (65.46 mg/kg) or 1-6 in equimolar doses (0.332 mmol/kg). A control group receiving dimethyl sulfoxide (5 mL/kg) was
included in the experiment for behavioral study. The drugs were given at a volume of 5 mL/kg in a single oral administration by intragastric tube. For intracerebroventricular injection, drugs were dissolved in sterile isotonic saline and injected into the lateral cerebroventricle in a volume of 3 µL per rat and the doses were 0.1 or 1 µmol/kg.

**Intracerebroventricular cannulation and injection**

For intracranial surgery, rats were anesthetized by intramuscular injection of 100-150 µL of a solution containing ketamine (86.2 mg/mL) and acepromazine (1.3 mg/mL) per 100 g body weight. A guide cannula (0.65 mm outside diameter) for injections of drugs was stereotaxically implanted and cemented to the skull. For intracerebroventricular cannula placements, the coordinates in millimeters with reference to bregma were as follows: anteroposterior (AP), 1.0; lateral (L), 1.8; ventral (V), 2.0. A single cannula was aimed at the right lateral cerebroventricle and the drugs were injected through a stainless-steel injector protruding beyond the cannula tip 2.5 mm. Coordinates were taken from Paxinos and Watson (Paxinos G, Watson C. 1986. The rat brain in stereotaxic coordinates, Ed 2. North Ryde, Australia: Academic) and adjusted for the body weight of the animals. Experiments began 1 week after surgery. Before the experiment, rats received one vehicle microinjection to habituate the animals to the drug administration procedure. To evaluate the dose-response curve for the locomotor effect of drugs, eight groups of rats (n = 8) were injected intracerebroventricularly with LD, 3, 4 (0.1 or 1 µmol/kg) or its vehicle. The drugs were administered daily during 1 week. After completion of the experiments, rats were sacrificed with CO₂ and cannula placements were verified histologically.

**Pharmacokinetic analysis**

After slight anaesthesia with carbon monoxide, the blood of rats was collected for the determination of LD metabolites by cardiac puncture from five rats of each group and then collected in vials containing heparin (250 I.U.). The blood sampling schedules were 1, 2, 4, 6, 12, and 24 h after
treatment with drugs. All samples were centrifuged at 2000 x g for 10 min and the plasma samples were kept at -80 °C until analysis. Aliquots (400 µL) were taken at various times and deproteinized by mixing with 40 µL of 4 M perchloric acid. After centrifugation for 10 min at 4000 x g and filtration (Millipore 0.45 µm), 10 µL of the layer supernatant were chromatographed as described below. The amounts of LD, DA; DOPAC and HVA were plotted as a function of incubation time.

**Open-field studies**

Automated locomotor activity boxes (MedAssociates, VT 05478) were used to quantify behavioural activity. Each animal was placed in the activity box, a square plastic box measuring 43cm×43cm×30 cm, and spontaneous locomotor activity parameters were monitored during the dark phase (from 9:00 A.M). Activity was recorded for 10 min, starting 2 min after placing the animal in the test cage. Movements from each rat were automatically recorded by interruption of two orthogonal light beams (3.5 and 13 cm above the activity box floor), which were connected to automatic softwares (Activity Monitor, MedAssociates). The behavioural tests were performed 1.5 h after oral treatment with drugs and before blood collection. In the i.c.v. administration, behavioural tests were performed immediately after injection. The behavioural parameters observed were locomotion (numbers of ambulatory episodes), rearings (numbers of rears), stereotype counts (numbers of grooming movements) and the numbers of entries into the central square of the arena. Locomotion counts were recorded when the low row of photocells was interrupted, while rearing counts were recorded by interruptions in the higher row of photocells. The open field is divided into two squares, and the numbers of entries into the central area (25cm×25 cm), which can be considered an unprotected area for rats, were recorded. The number of entries into the central squares of the arena provided a measure of emotionality, fear and anxiety. The numbers of ambulatory and rearing episodes in the 25cm×25cm central zone of the field are expressed as percentage of total ambulatory and rearing episodes, respectively. Low percentage of ambulatory
and rearing episodes in the center is considered a sign of high emotionality/fear. Between each test session, the apparatus was cleaned with alcohol (10%) and dried with a cloth.

**HPLC UV assays**

All analyses were carried out on a Waters 1525 Binary HPLC pump, equipped with a Waters 2996 photodiode array detector, a 20-µL Rheodyne injector and a computer integrating apparatus. The column was a Waters X-Terra RP

18 (5 µm, 3.0 x 150 mm); the mobile phase was a mixture of water/methanol (60:40). The flow rate was 0.6 mL/min.

**HPLC-EC assays**

For the measurements of the LD and DA, striatum from each animal (treated as described above) were individually homogenized for 2 min with a Dyna-Mix homogenizer (Fisher Scientific) in 500 µL of 0.05 N perchloric acid solution containing (w/v) 0.064% 1-octanesulphonic acid sodium salt, 0.060% heptanesulphonic acid sodium salt, 0.004% sodium EDTA, 0.010% sodium metabisulphite and 25 ng/mL DHBA as an internal standard. The whole procedure was carried out on ice. The resulting homogenate was then centrifuged at 4500×g for 10 min and the supernatant was filtered using 0.45 µ Millipore filters. The filtrate was set in a low volume insert vial and a portion was injected directly into the liquid chromatography equipment (10 µL). The HPLC system consisted of a Waters 600 controller pump, a Rheodyne 7295 injector with a 10 µL loop and an Antec Leyden Decade II detector; the operating potential was 0.75 V. Separation was achieved on a Waters Symmetry RP-C18 column (4.6mm×150 mm, 5 µm). The mobile phase consisted of 0.045 M monobasic sodium phosphate, 0.001 M 1-octanesulphonic acid sodium salt, 0.006% triethylamine, 0.015% 100 µM sodium EDTA and 6% acetonitrile. The pH of the mobile phase was adjusted to 3.0 by o-phosphoric acid. The mobile phase was filtered and degassed by vacuum. A flow rate of 1 mL/min was used in all experiments.

Monoamine stock solutions were prepared at a concentration of 1 mg/mL (as a free base) in 0.05 N
perchloric acid containing 0.064% 1-octanesulphonic acid sodium salt, 0.060% heptanesulphonic acid sodium salt, 0.004% sodium EDTA, and 0.010% sodium metabisulphite. These standard solutions were freshly prepared every week and stored at 4 °C for use right away.

The monoamine and their metabolites were identified on the basis of retention time. Final values were expressed in terms of picomoles per gram of tissue. Measurements were performed in triplicate for each original sample.

**Aqueous solubility**

Compound (50 mg) was placed in a microtube containing 1 mL of deionized water and was shaken at 25 °C for 1 h to ensure the solubility equilibrium. After centrifugation, a 20 µL portion of the supernatant was analyzed by HPLC as described in the previous section. The solubilities of codrugs 1-6 were also determined in 0.02 M phosphate buffer of pH 7.4, 0.02 M sodium acetate buffer of pH 5.0 and 0.02 M hydrochloridric acid buffer of pH 1.3.

**Lipophilicity**

*Log P*

A mixture was prepared with a 20 mL portion of each compound solution (1.0 mM) in 0.1 M, pH 7.4 phosphate buffer saturated with 1-octanol, to which 20 mL of 1-octanol saturated with 0.1 M, pH 7.4 phosphate buffer solution was added. The mixture was shaken at 37 °C for 1 h to ensure equilibrium, and centrifuged. The concentration of each compound in aqueous phase was analyzed by HPLC as described previously. Apparent partition coefficient was calculated by employing the equation (Co - Cw)/Cw, where Co and Cw represent the initial and equilibrium concentration of the drug in aqueous phase, respectively.  

*clogP*
The calculated clogP was determined using Advanced Chemistry Development (ACD/Labs), version 4.55.

**LogK**

Oil/water partition coefficients can be also estimated using reverse-phase chromatographic retention times (RT) due to the good relationship between log octanol/water partition coefficients and log capacity factor (logK) values determined using octadecyl silica columns.

Each compound was dissolved in methanol (concentration 10 mg/mL). Aliquots of each solution were filtered and analyzed by the HPLC method as previously described.

logK values were calculated according to the following equation:

\[
\text{logK} = \log(\frac{t_r - t_0}{t_0})
\]

where \(t_r\) was the retention time of the sample peak and \(t_0\) was the retention time of a non-retained solvent peak.

**Kinetics of hydrolysis in aqueous solutions**

For studies realized at pH 7.4, a solution of 70 ml of phosphate buffer (pH 7.4; 0.02 M) and 30 mL of acetonitrile was prepared. For each compound analyzed, 5 mg were dissolved in this solution. The resulting mixture was sonicated for 3 min, and then stirred at 37±0.5 °C. At different intervals of time, an aliquot (20 µL) of this solution was collected and injected into the analytical column. The concentration of the product was determined according to the difference of the amount of the product in the starting solution \((t = 0)\) and in the solution at the time of analysis. Pseudo-first-order rate constants (K_{obs}) for the hydrolysis of the codrugs were then calculated from the slopes of the linear plots of log (% residual codrugs) against time. The experiments were run in triplicate for each codrug and the mean values of the rate constants were calculated. For studies realized at pH 1.3 the same protocol was used, but phosphate buffer was replaced by 0.02 M hydrochloridric acid buffer.\(^{13}\)
Kinetics of hydrolysis in plasma

The enzymatic hydrolysis of compounds 1-6 was evaluated in human and rat plasma at 37 °C. Stock solutions were prepared by dissolving a weighed amount of each compound in methanol to give a concentration 3 mg/mL. A volume of 0.04 mL of this solution was added with 3.96 mL of prewarmed (37 °C) plasma previously diluted to 80% with 50mM phosphate buffer, pH 7.4, prethermostated at 37 °C. The resulting solution was kept at 37 °C and 0.2 mL samples were withdrawn at intervals and added to 0.4 mL of cold (4 °C) acetonitrile to precipitate serum proteins. After centrifugation for 10 min at 10,000 rpm and at 5 °C, the supernatant was assayed by the HPLC method previously described in order to determine the amount of each compound. Pseudo-first-order hydrolysis rate constants for the enzymatic hydrolysis were calculated from the slopes of linear plots of the logarithm of residual compound against time.14

Statistical analysis

The experimental data are expressed as mean values ± S.E of five rats used. The significance of differences among different treatment groups was calculated using the analysis of variance (ANOVA) followed by the Newman-Keuls test. P values < 0.05 were considered statistically significant.
Supporting Information references


**Table S1. Elemental analyses of codrugs 1-6**

<table>
<thead>
<tr>
<th>Compd</th>
<th>Formula</th>
<th>Calc. %</th>
<th>Found %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>1</td>
<td>C₁₅H₂₀N₂O₅S</td>
<td>50.55</td>
<td>5.66</td>
</tr>
<tr>
<td>2</td>
<td>C₁₅H₂₀N₂O₅S</td>
<td>50.55</td>
<td>5.66</td>
</tr>
<tr>
<td>3</td>
<td>C₁₅H₂₀N₂O₅S</td>
<td>53.11</td>
<td>6.29</td>
</tr>
<tr>
<td>4</td>
<td>C₁₅H₂₀N₂O₅S</td>
<td>53.11</td>
<td>6.29</td>
</tr>
<tr>
<td>5</td>
<td>C₁₅H₂₀N₂O₅S</td>
<td>47.05</td>
<td>5.48</td>
</tr>
<tr>
<td>6</td>
<td>C₁₅H₂₀N₂O₅S</td>
<td>49.02</td>
<td>5.81</td>
</tr>
</tbody>
</table>

**Table S2. Lipophilicity of codrugs 1-6**

<table>
<thead>
<tr>
<th>Compd</th>
<th>logP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>clogP</th>
<th>logk&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N.D.</td>
<td>0.52</td>
<td>-0.30 (± 9.0x10⁻³)</td>
</tr>
<tr>
<td>2</td>
<td>N.D.</td>
<td>0.79</td>
<td>-0.26 (± 6.5x10⁻³)</td>
</tr>
<tr>
<td>3</td>
<td>0.38 (± 0.01)</td>
<td>0.68</td>
<td>-0.22 (± 7.7x10⁻³)</td>
</tr>
<tr>
<td>4</td>
<td>-0.19 (± 0.3x10⁻³)</td>
<td>0.95</td>
<td>-0.25 (± 7.5x10⁻³)</td>
</tr>
<tr>
<td>5</td>
<td>-0.14 (± 0.55x10⁻³)</td>
<td>0.93</td>
<td>-0.11 (± 2.0x10⁻³)</td>
</tr>
<tr>
<td>6</td>
<td>0.92 (± 0.08)</td>
<td>1.95</td>
<td>0.20 (± 8.0x10⁻³)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means of three experiments, standard deviation is given in parentheses.

<sup>b</sup> N.D. = not determined

**Table S3. Solubility of codrugs 1-6**

<table>
<thead>
<tr>
<th>Compd</th>
<th>Solubility in water&lt;sup&gt;a&lt;/sup&gt; (mg/mL)</th>
<th>Solubility at pH 1.3&lt;sup&gt;a&lt;/sup&gt; (mg/mL)</th>
<th>Solubility at pH 7.4&lt;sup&gt;a&lt;/sup&gt; (mg/mL)</th>
<th>Solubility at pH 5.0&lt;sup&gt;a&lt;/sup&gt; (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>210.32 (± 10.52)</td>
<td>208.06 (± 10.40)</td>
<td>200.78 (± 10.04)</td>
<td>227.64 (± 11.38)</td>
</tr>
<tr>
<td>2</td>
<td>153.71 (± 7.69)</td>
<td>147.43 (± 7.37)</td>
<td>159.26 (± 7.96)</td>
<td>151.98 (± 7.59)</td>
</tr>
<tr>
<td>3</td>
<td>100.61 (± 13.91)</td>
<td>154.14 (± 10.68)</td>
<td>174.32 (± 3.19)</td>
<td>237.44 (± 0.01)</td>
</tr>
<tr>
<td>4</td>
<td>103.15 (± 13.07)</td>
<td>255.64 (± 10.25)</td>
<td>100.79 (± 12.56)</td>
<td>246.77 (± 11.14)</td>
</tr>
<tr>
<td>5</td>
<td>15.00 (± 1.67)</td>
<td>19.97 (± 2.04)</td>
<td>9.08 (± 0.85)</td>
<td>15.05 (± 1.66)</td>
</tr>
<tr>
<td>6</td>
<td>14.33 (± 0.08)</td>
<td>7.07 (± 0.18)</td>
<td>13.47 (± 0.59)</td>
<td>3.82 (± 0.06)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means of three experiments, standard deviation is given in parentheses.
### Table S4. Kinetic data for chemical hydrolysis of codrugs 1-6 at 37 °C

<table>
<thead>
<tr>
<th>Compd</th>
<th>pH 1.3&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pH 7.4&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t½ (h)</td>
<td>K&lt;sub&gt;obs&lt;/sub&gt; (h&lt;sup&gt;1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>1</td>
<td>301.0 (±10.5)</td>
<td>0.002 (±0.07x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>2</td>
<td>290.6 (±5.8)</td>
<td>0.002 (±0.04x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>3</td>
<td>296.3 (±11.8)</td>
<td>0.002 (±0.08x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>4</td>
<td>292.1 (±4.4)</td>
<td>0.002 (±0.03x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>5</td>
<td>292.8 (±8.8)</td>
<td>0.002 (±0.06x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>6</td>
<td>292.4 (±14.7)</td>
<td>0.002 (±0.10x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means of three experiments, standard deviation is given in parentheses.

### Table S5. Rate constants for the hydrolysis of codrugs 1-6 in 80% rat plasma and 80% human plasma at 37 °C

<table>
<thead>
<tr>
<th>Compd</th>
<th>Rat plasma&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Human plasma&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t½ (min)</td>
<td>K&lt;sub&gt;obs&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>1</td>
<td>46.8 (±1.4)</td>
<td>0.010 (±0.20x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>2</td>
<td>36.6 (±1.6)</td>
<td>0.019 (±0.85x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>3</td>
<td>115.2 (±11.0)</td>
<td>0.002 (±0.07x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>4</td>
<td>93.0 (±10.2)</td>
<td>0.003 (±0.17x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>5</td>
<td>55.8 (±10.5)</td>
<td>0.003 (±0.11x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>6</td>
<td>69.6 (±3.5)</td>
<td>0.010 (±0.50x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means of three experiments, standard deviation is given in parentheses.
Legend to Figure S1

Inhibition (%) of lucigenin amplified-CL in presence of 10 μg/mL of NAC and 1-6. CL was measured in the presence of 0.9 U/mL xanthine oxidase and 150 μM lucigenin; the reaction was started by injecting xanthine at a final concentration of 50 μM in 50 mM PBS, pH 7.4. Inhibition values refer to percent inhibition of CL reaction. Data are expressed as mean ± S.D. Each experiment was performed in triplicate. *P<0.05: each sample vs the others except 3 vs 4.

Figure S1
Inhibition (%) of luminol amplified-CL in presence of 10 μg/mL of NAC and 1-6. CL was measured in the presence of 100 μM luminol; the reaction was started by injecting hydrogen peroxide at a final concentration of 50 mM in 50 mM PBS, pH 7.4. Inhibition value refer to percent inhibition of CL reaction. Data are expressed as mean ± S.D. Each experiment was performed in triplicate. *P<0.05: each sample vs the others except 3 vs 4 and 6.
Legend to Figure S3

Locomotion, rearing, and grooming, 1.5 h after oral administration of LD, and 1-6 in rats (n=8 per group). Data are expressed as mean ± S.E. \(^aP<0.05\) compared to control group. \(^bP<0.05\) compared to LD- and 2-treated groups.

Figure S3
Legend to Figure S4

Numbers of entries in the center, 1.5 h after oral administration of LD, and 1-6 in rats (n=8 per group). Data are expressed as mean ± S.E. *P<0.05 compared to control group.

Figure S4
Legend to Figure S5

Effect of i.c.v. LD, 3, 4 or its vehicle on locomotion, rearing, % ambulatory episodes and % rearing episodes during 1 week. Rats (n=8) were injected daily into the lateral cerebroventricle with 1 umol/kg of LD, 3, 4 or its vehicle (control) and immediately monitored. Ambulatory episodes and rearing episodes in the central zone of the open field are expressed as percentage of total ambulatory and rearing episodes, respectively. Data are expressed as mean ± S.E. *P<0.05 compared to control groups; b P<0.05 compared to LD-treated group.

Figure S5
Legend to Figure S6

Effect of i.c.v. LD, 3, 4 or its vehicle on locomotion, rearing, % ambulatory episodes and % rearing episodes during 1 week. Rats (n=8) were injected daily into the lateral cerebroventricle with 0.1 µmol/kg of LD, 3, 4 or its vehicle (control) and immediately monitored. Ambulatory episodes and rearing episodes in the central zone of the open field are expressed as percentage of total ambulatory and rearing episodes, respectively. Data are expressed as mean ± S.E. *P<0.05 compared to control groups; **P<0.05 compared to 3-treated group; ***P<0.05 compared to LD-treated group.

Figure S6

Locomotion (dose 0.1 µmol/kg)  % ambulatory episodes (dose 0.1 µmol/kg)

Rearing (dose 0.1 µmol/kg)  % rearing episodes (dose 0.1 µmol/kg)