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Fluorescent lactose-derived catanionic aggregates: synthesis, characterisation and potential use as antibacterial

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General procedures

All reactions were performed in oven-dried glassware under a slight positive pressure of nitrogen. ^{1}H-NMR (400 MHz, 500MHz) and ^{13}C NMR (100 MHz, 125MHz) spectra were determined on a Varian INOVA-400 spectrometer, and Varian INOVA-500 spectrometer. Chemical shifts for ^{1}H NMR are reported in parts per million (ppm), calibrated to the residual solvent peak set, with coupling constants reported in Hertz (Hz). The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet. Chemical shifts for ^{13}C NMR are reported in ppm, relative to the central line of a septet at δ = 39.52 ppm for deuterio-dimethylsulfoxide. Infrared (IR) spectra were recorded on a NICOLET 5700 FT-IR spectrophotometer and reported in wavenumbers (cm^{-1}). Microanalytical data were obtained using a Fisons EA CHNS-O instrument (T = 1000 °C). Fluorescence spectra were recorded on a Cary Eclypse spectrofluorimeter.

Cryo-Transmission Electron Microscopy

Aliquots (4 µL) of **Coum18** particles (2 mg/mL in water) were deposited on EM grids coated with a perforated carbon film. After draining the excess liquid with a filter paper, grids were quickly plunged into liquid ethane and mounted onto a Gatan 626 cryoholder. Cryo-TEM observation was performed with a Tecnai F20 (FEI) microscope operated at 200 kV. The images were recorded with an USC1000-SSCCD camera (Gatan).

Cell Culture and Live Cell Imaging

Human cervical cell line (HeLa cells) and chinese hamster ovary cell line (CHO cells) were grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with high glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U mL−1), and streptomycin (100 µg mL−1) (Invitrogen) in a 5% CO2 incubator at 37 °C. Cells were seeded in number of 10^5 cells/cm^2 in 35 mm dishes and experiments were carried out 2 days after seeding. Vesicles were added to the cells at 5 µM concentration and incubated at 37 °C for 2 h. For live cell imaging, after replacing the particle suspension with fresh serum-free medium, cells were stained for 30 min. with 650 nM Hoechst 33258, a nuclear probe used to evaluate chromatin condensation and distinguish apoptotic nuclei from healthy ones. Microscopy observations were made with 40×/0.75 NA water-immersion objectives, using a Zeiss (Axioskop) upright fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with an HBO 50 W L-2 mercury lamp (Osram, Berlin, Germany). Twelve-bit-deep images were acquired with a monochrome-cooled CCD camera (QICAM, Qimaging, Canada). Cells were visualized in contrast and fluorescence microscopy. The vesicle and Hoechst fluorescence was observed with standard fluorescein (ex 470 ± 20, em 535 ± 40) and blue (ex 360 ± 20, em 460 ± 25) filter sets, respectively. Image alignments and fluorescence measurements were obtained with Image Pro Plus software (Media Cybernetics, Silver Springs, MD).

Statistics

Statistical analysis was carried out with Excel (Microsoft Co., Redmond, WA). Results were expressed as a mean ± standard deviation (SD) of three independent experiments. Statistically significant difference was evaluated by two sample t test with $p < 0.05$ as a minimal level of significance.

Antibacterial activity

Test-bacteria

The antibacterial activity of natural products was assessed against four bacterial strains belonging to the American Type Culture Collection (Maryland, USA): *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853.

Determination of Minimal Inhibitory Concentration (MIC)

A micro-dilution broth method, according to the EUCAST (VER 4.0 – June 2014, and ISO Standard 20776-1 2006 e ISO 20776-2 2007) was employed for the determination of antimicrobial activity of compound **Coum 18** and his sub-units, **2d** and **3** on 96 microwells plates;
each well contains 125 µl of Mueller-Hinton Broth Cation-Adjusted with serial two fold dilution in concentration range 0,008 - 512 µg/ml of the compound and then inoculated with a bacterial suspension in logarithmic phase of growth to reach a final concentration of $2x10^4$ CFU/mL.
The MIC value of each compound was determined as the lowest concentration that completely inhibited bacterial growth after 18 h of incubation at 37°C.

Determination of Minimal Bactericidal Concentration (MBC)

For the determination of MBC, a portion of liquid (10 µl) from each plates well that exhibited no growth were taken and then incubating on Columbia 5% sheep agar at 37°C for 24 hr. The lowest concentration that revealed no visible bacterial growth after sub-culturing was taken as MBC.

Experimental

General procedure for the synthesis of N-alkyllactosylamines **1a**, **1b, 1c** and **1d.**

Appropriate alkylamine (4.70 mmol) suspended in propan-2-ol (40 mL) was added to a solution of D-lactose monohydrate (1 g, 2.77 mmol) in 20 mL of distilled water. The suspension was stirred for 24 h, at RT, then heated at 65°C and left under stirring for 30 min. The solvent was evaporated to

dryness. The product was purified by recrystallization in boiling EtOH and the desired product was isolated as a white powder.

N-dodecyllactosylamine (**1a**)

Yield 58%; ^{1}H NMR (400 MHz, $DMSO_{-d6}$) δ (ppm): 0.85 (t, 3H, CH_3), 1.23 (m, 18H, CH_2), 1.38 (m, 2H, CH_2), 2.19 (sBr, 1H, NH), 2.75 and 2.92 (s, 2H, $\underline{CH_2}$NH), 3.20-3.71 (m, 14H, $\underline{CH}$OH and $\underline{CH_2}$OH), 4.19-5.09 (m, 7H, OH)

N-tetradecyllactosylamine (**1b**)

Yield 59%; ^{1}H NMR (400 MHz, $DMSO_{-d6}$) δ (ppm): 0.85 (t, 3H, CH_3), 1.24 (m, 22H, CH_2), 1.38 (m, 2H, CH_2), 2.23 (sBr, 1H, NH), 2.75 and 2.92 (s, 2H, $\underline{CH_2}$NH), 3.19-3.71 (m, 14H, $\underline{CH}$OH and $\underline{CH_2}$OH), 4.19-5.08 (m, 7H, OH)

N-hexadecyllactosylamine (**1c**)

Yield 51%; ^{1}H NMR (400 MHz, $DMSO_{-d6}$) δ (ppm): 0.85 (t, 3H, CH_3), 1.24 (m, 26H, CH_2), 1.37 (m, 2H, CH_2), 2.20 (sBr, 1H, NH), 2.78 and 2.93 (s, 2H, $\underline{CH_2}$NH), 3.24-3.71 (m, 14H, $\underline{CH}$OH and $\underline{CH_2}$OH), 4.19-5.09 (m, 7H, OH)

N-octadecyllactosylamine (**1d**)

Yield 53%; ^{1}H NMR (400 MHz, $DMSO_{-d6}$) δ (ppm): 0.85 (t, 3H, CH_3), 1.23 (m, 30H, CH_2), 1.38 (m, 2H, CH_2), 2.22 (sBr, 1H, NH), 2.75 and 2.92 (s, 2H, $\underline{CH_2}$NH), 3.19-3.71 (m, 14H, $\underline{CH}$OH and $\underline{CH_2}$OH), 4.19-5.08 (m, 7H, OH)

General procedure for the synthesis of N-alkyllactylamines **2a**, **2b, 2c** and **2d.**

$NaBH_4$ (0.075 g, 1.90 mmol) was added to a stirred suspension of N-alkyllactosylamines **1a**, **1b, 1c** and **1d** (1.30 mmol) in MeOH (50 mL). The suspension was stirred for 2 hours, and then filtered to remove the unreacted N-alkyllactylamines. The filtrate was evaporated under reduced pressure to dryness to get the desired N-alkyllactylamines as white solids.

N-dodecyllactylamines (**2a**)

Yield 96%; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.90 (t, 3H, CH_3), 1.29 (m, 18H, CH_2), 1.57 (m, 2H, CH_2), 2.67 (m, 4H, $\underline{CH_2}$-NH-$\underline{CH_2}$), 3.35-4.27 (m, 13H, $\underline{CH}$OH and $\underline{CH_2}$OH)

N-tetradecyllactylamines (**2b**)

Yield 87%; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.90 (t, 3H, CH_3), 1.29 (m, 22H, CH_2), 1.56 (m, 2H, CH_2), 2.65 (m, 4H, $\underline{CH_2}$-NH-$\underline{CH_2}$), 3.35-4.27 (m, 13H, $\underline{CH}$OH and $\underline{CH_2}$OH)

N-hexadecyllactylamines (**2c**)

Yield 86%; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.89 (t, 3H, CH_3), 1.29 (m, 26H, CH_2), 1.56 (m, 2H, CH_2), 2.65 (m, 4H, $\underline{CH_2}$-NH-$\underline{CH_2}$), 3.35-4.27 (m, 13H, $\underline{CH}$OH and $\underline{CH_2}$OH)

N-octadecyllactylamines (**2d**)

Yield 99%; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.89 (t, 3H, CH_3), 1.29 (m, 30H, CH_2), 1.54 (m, 2H, CH_2), 2.65 (m, 4H, $\underline{CH_2}$-NH-$\underline{CH_2}$), 3.35-4.27 (m, 13H, $\underline{CH}$OH and $\underline{CH_2}$OH)

Synthesis of 11-((2-oxo-2H-chromen-7-yl)oxy) undecanoic acid (**3)**

7-hydroxycoumarin (0.518 g, 3.19 mmol) was dissolve in dry EtOH (40 mL) under N_2 atmosphere. K_2CO_3 (1.325 g, 9.59 mmol), and KI (0.106 g, 0.639 mmol) were then added to the solution. A solution of 11-bromoundecanoic acid (1.101 g, 4.15 mmol) in dry EtOH (15 mL) was added dropwise and the resulting suspension was refluxed for 48 hours, then cooled down and the mixture poured it in distilled water (100 mL). HCl 5% v/v was added to the solution until pH 5.0. EtOH was evaporated under reduced pressure and the residue was filtered. The resulting solid was washed with H_2O (3x10 mL) and with hexane (3x10 mL) to obtain 11-((2-oxo-2H-chromen-7-yl)oxy) undecanoic acid (**3**) as an off white solid (0.762 g, 2.20 mmol, yield 69 %).

^{1}H NMR (400 MHz, $DMSO_{-d6}$) δ (ppm): 1.25 (m, 10H, CH_2), 1.40 (m, 2H, CH_2), 1.48 (m, 2H, CH_2), 1.71 (m, 2H, CH_2), 2.18 (t, 2H, CH_2), 4.05 (t, 2H, CH_2), 6.27 (d, 1H, H_{Arom}), 6.93 (d, 1H, H_{Arom}), 6.96 (s, 1H, H_{Arom}), 7.61 (d, 1H, H_{Arom}), 7.98 (d, 1H, H_{Arom}), 11.95 (m, 1H, COOH)

IR (KBr) *v* (cm^{-1}): 2915, 2849 (C-H), 1720, 1701, 1619, 1610, 1557 (C=O)

General procedure for the synthesis of the ion-pairs **Coum12-Coum 18**.

N-alkyllactylamines (**2a**, **2b, 2c** and **2d**) (0.29 mmol) and 11-((2-oxo-2H-chromen-7-yl)oxy) undecanoic acid (**3**) (0.100 g, 0.29 mmol) were mixed in H_2O (22 mL) and leave under stirring at RT for 48 hours. The suspension is then freeze dried to obtain a white powder.

Coum 12 (4a)

Yield 95%; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.90 (t, 3H, CH_3), 1.29-1.33 (m, 30H, CH_2), 1.49 (m, 2H, CH_2), 1.60 (m, 2H, CH_2), 1.81 (p, 2H, CH_2), 2.15 (t, 2H, CH_2), 2.94-3.07 (m, 4H, $\underline{CH_2}$-NH-$\underline{CH_2}$), 3.31 (CD_3OD), 3.40-4.00 (m, 11H, $\underline{CH}$OH and $\underline{CH_2}$OH), 4.07 (t, 2H, CH_2), 4.25-4.60 (m, 2H, $\underline{CH}$OH), 4.84 (H_2O), 6.23 (d, 1H, H_{Arom}), 6.89 (d, 1H, H_{Arom}), 6.93 (d, 1H, H_{Arom}), 7.53 (d, 1H, H_{Arom}), 7.88 (d, 1H, H_{Arom});

^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 14.42, 23.72, 26.04, 27.07, 27.73, 30.16, 30.25, 30.30, 30.37, 30.45, 30.50, 30.55, 30.63, 30.66, 30.74, 30.82, 33.06, 35.11, 39.13, 49.00 (CD_3OD), 62.07, 62.51, 69.84, 70.32, 71.42, 72.58, 74.84, 75.95, 76.72, 77.09, 98.10, 102.19, 106.12, 113.17, 113.88, 114.23, 130.42, 145.84, 158.76, 163.46, 164.19, 168.64, 170.44

IR (KBr) *v* (cm^{-1}): 3390 (N-H), 2925, 2851 (C-H), 1729, 1709, 1621, 1558 (C=O)

Decomposition point : 110°C

Coum 14 (4b)

Yield 93%; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.90 (t, 3H, CH_3), 1.29-1.33 (m, 34H, CH_2), 1.49 (p, 2H, CH_2), 1.60 (m, 2H, CH_2), 1.81 (p, 2H, CH_2), 2.16 (t, 2H, CH_2), 2.92-3.08 (m, 4H, $\underline{CH_2}$-NH-$\underline{CH_2}$), 3.31 (CD_3OD), 3.40-4.00 (m, 11H, $\underline{CH}$OH and $\underline{CH_2}$OH), 4.07 (t, 2H, CH_2), 4.25-4.60 (m, 2H, $\underline{CH}$OH), 4.84 (H_2O), 6.23 (d, 1H, H_{Arom}), 6.89 (d, 1H, H_{Arom}), 6.93 (d, 1H, H_{Arom}), 7.53 (d, 1H, H_{Arom}), 7.88 (d, 1H, H_{Arom})

^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 14.43, 23.72, 26.04, 27.07, 27.77, 30.16, 30.26, 30.30, 30.37, 30.45, 30.50, 30.55, 30.64, 30.66, 30.74, 30.84, 33.06, 35.11, 39.22, 49.00 (CD_3OD), 62.07, 62.57, 69.84, 70.33, 71.42, 72.58, 74.84, 75.95, 76.70, 77.08, 98.09, 102.19, 106.13, 113.17, 113.88, 114.23, 130.42, 145.84, 158.75, 163.50, 164.17, 168.73, 169.91

IR (KBr) *v* (cm^{-1}): 3400 (N-H), 2921, 2852 (C-H), 1731, 1713, 1618, 1561 (C=O)

Decomposition point : 130°C

Coum 16 (4c)

Yield 92%; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.90 (t, 3H, CH_3), 1.29-1.33 (m, 38H, CH_2), 1.49 (p, 2H, CH_2), 1.60 (m, 2H, CH_2), 1.81 (p, 2H, CH_2), 2.16 (t, 2H, CH_2), 2.85-3.09 (m, 4H, $\underline{CH_2}$-NH-$\underline{CH_2}$), 3.31 (CD_3OD), 3.40-4.00 (m, 11H, $\underline{CH}$OH and $\underline{CH_2}$OH), 4.07 (t, 2H, CH_2), 4.25-4.60 (m, 2H, $\underline{CH}$OH), 4.84 (H_2O), 6.23 (d, 1H, H_{Arom}), 6.89 (d, 1H, H_{Arom}), 6.93 (d, 1H, H_{Arom}), 7.53 (d, 1H, H_{Arom}), 7.88 (d, 1H, H_{Arom})

^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 14.43, 23.72, 26.03, 27.07, 27.79, 30.15, 30.26, 30.30, 30.36, 30.45, 30.54, 30.63, 30.65, 30.76, 30.84, 33.06, 35.10, 39.29, 49.00 (CD_3OD), 61.37, 62.50,

69.84, 70.34, 72.57, 74.84, 76.71, 102.18, 106.12, 113.16, 113.88, 114.23, 130.41, 145.83, 158.86, 163.45, 164.18, 168.60, 169.93

IR (KBr) *v* (cm^{-1}): 3406 (N-H), 2920, 2851 (C-H), 1732, 1711, 1618, 1561 (C=O)

Decomposition point : 160°C

Coum 18 (4d)

Yield 88%; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.90 (t, 3H, CH_3), 1.29-1.33 (m, 42H, CH_2), 1.49 (p, 2H, CH_2), 1.60 (m, 2H, CH_2), 1.81 (p, 2H, CH_2), 2.16 (t, 2H, CH_2), 2.77-3.10 (m, 4H, $\underline{CH_2}$-NH-$\underline{CH_2}$), 3.31 (CD_3OD), 3.40-4.00 (m, 11H, $\underline{CH}OH$ and $\underline{CH_2}OH$), 4.07 (t, 2H, CH_2), 4.25-4.60 (m, 2H, $\underline{CH}OH$), 4.84 (H_2O), 6.23 (d, 1H, H_{Arom}), 6.90 (d, 1H, H_{Arom}), 6.93 (d, 1H, H_{Arom}), 7.53 (d, 1H, H_{Arom}), 7.88 (d, 1H, H_{Arom})

^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 14.43, 23.72, 26.04, 27.07, 27.81, 30.15, 30.31, 30.36, 30.45, 30.54, 30.65, 30.75, 30.85, 33.06, 39.32, 49.00 (CD_3OD), 61.38, 62.55, 69.84, 72.70, 74.99, 76.68, 102.18, 113.15, 114.23, 130.42, 145.84, 158.75, 163.50, 164.17, 168.80, 169.86

IR (KBr) *v* (cm^{-1}): 3423 (N-H), 2919, 2850 (C-H), 1731, 1711, 1618, 1562 (C=O)

Decomposition point : 200°C

IR characterization

The formation of the desired compounds was demonstrated by means of infrared spectroscopy by comparing the spectrum of the carboxylic coumarin derivative **3** with that of the **Coum 12 - Coum 18** in the 1750-1550 cm^{-1} region (Figure 1). The shift of the band of the carboxylic acid at 1720 cm^{-1} and 1701 cm^{-1} and the disappearance of the band at 1610 cm^{-1} confirmed the formation of the salt.[14]

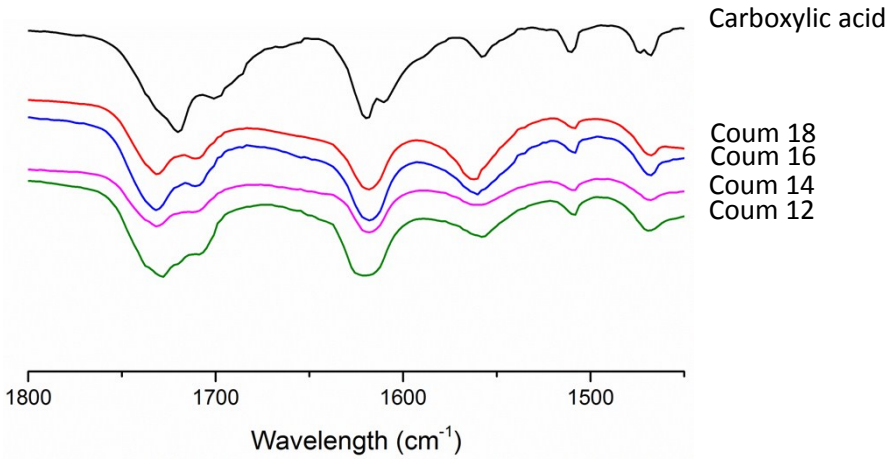

Figure S1: Comparison of the IR spectra of the C=O stretching region of the carboxylic acid **3**, and of **Coum12**, **Coum14**, **Coum16**, and **Coum18**.

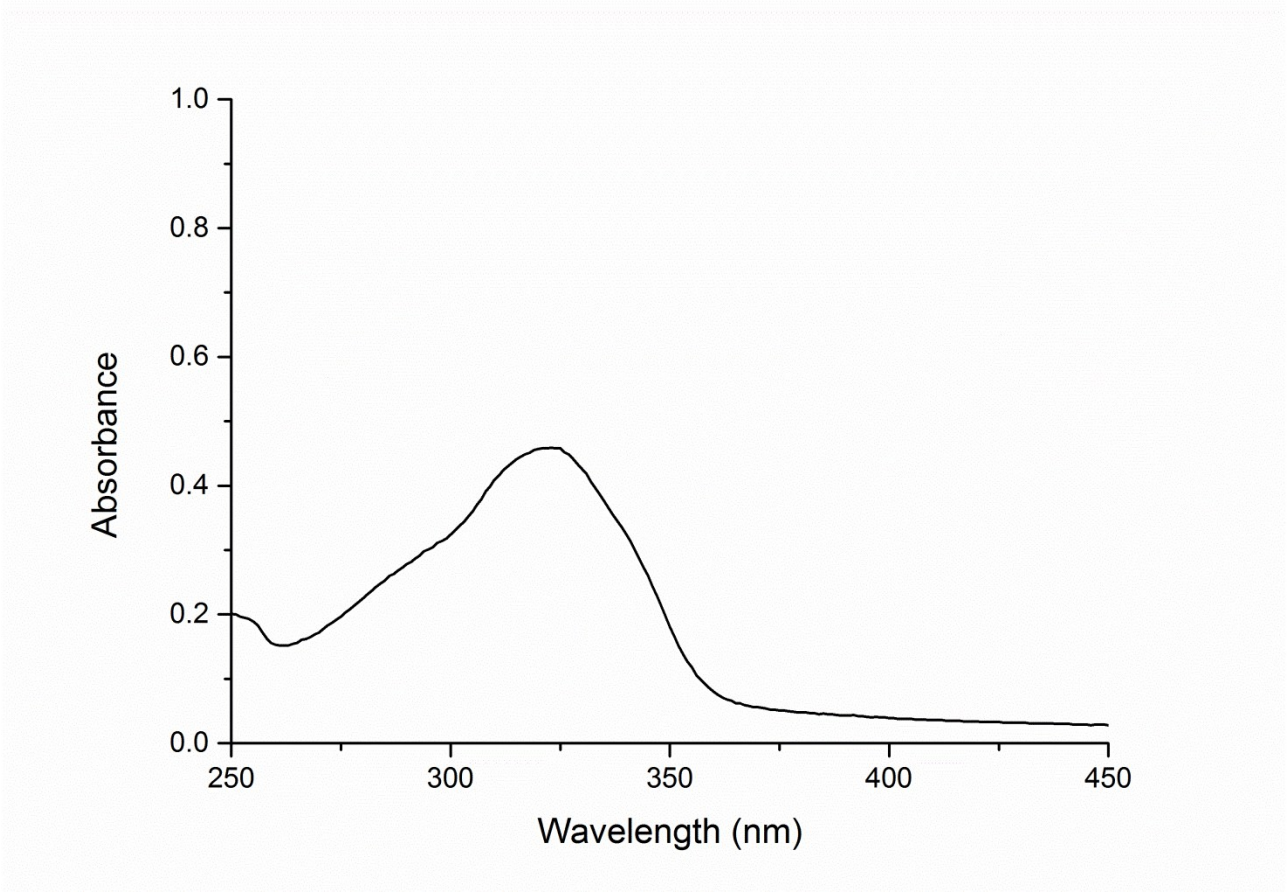

Figure S2 UV-Vis spectrum of **Coum 18** ($3.5\ 10^{-5}$ M) in water at pH 7.5.

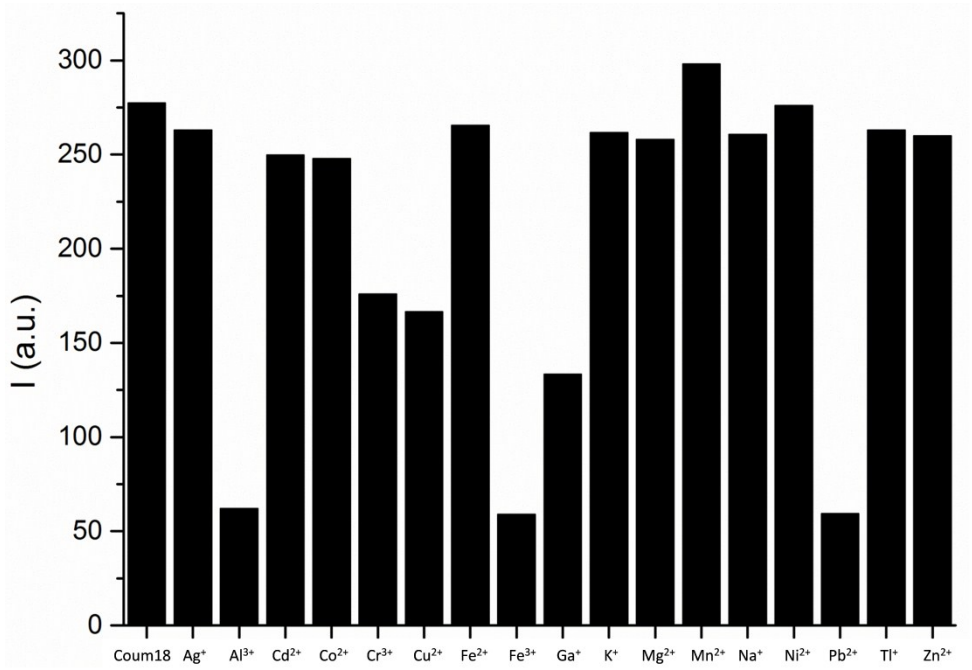

Figure S3 Changes in the emission intensity of **Coum18** ($1.0 \cdot 10^{-7}$ M) in water at pH 7.5 upon addition of one equivalent of metal ions (Ag^{+}, Al^{3+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ga^{+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, Pb^{2+}, Tl^{+} and Zn^{2+}) at (λ_{exc} = 324 nm, λ_{exc} = 394 nm).

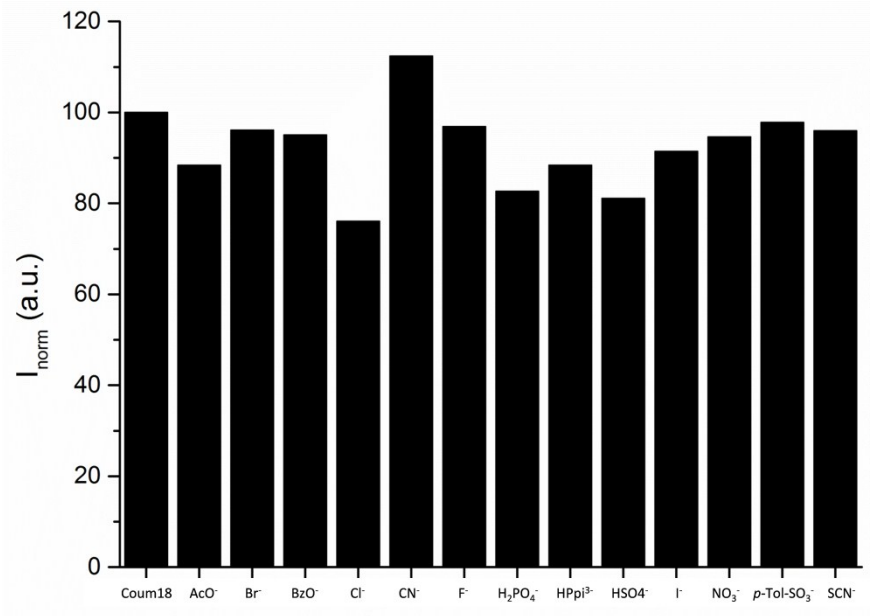

Figure S4 Changes in the emission intensity of **Coum18** in water ($1.0 \cdot 10^{-7}$ M) in water at pH 7.5 upon addition of one equivalent of anions (AcO^{-}, Br^{-}, BzO^{-}, Cl^{-}, CN^{-}, F^{-}, $H_2PO_4^{-}$, $HPpi^{-}$, HSO_4^{-}, I^{-}, NO_3^{-}, SCN^{-}, p-Tol-SO_3^{-}) (λ_{exc} = 324 nm, λ_{exc} = 394 nm).