Supporting Information

Versatile fluorophores for bioimaging applications: π-expanded naphthalimide derivatives with skeletal and appendage diversity

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1. Synthesis

1.1. Measurements and Materials

All chemicals and solvents were purchased from commercial suppliers and used without further purification. The NMR spectra were recorded on a 500 MHz instrument at 27 °C. The chemical shifts (δ) are presented in ppm followed by integral intensity, multiplicity and corresponding coupling constants (J) in Hz, and by the signal assignment, which is based on analyses of 2D COSY, HSQC, HMBC and ROESY correlation spectra. Both ¹H and ¹³C chemical shifts are referenced to TMS (using the solvent signals CHCl₃ 7.26 ppm, CDCl₃ 77.16 ppm, CHD₂SOCD₃ 2.50 ppm, CD₃SOCD₃ 39.52 ppm). Mass spectra were obtained using electrospray ionisation (ESI) with a LTQ Orbitrap spectrometer. Silica (40-63 D, 60 Å) was used to separate the compounds via flash chromatography.

1.2 Preparation of starting aldehydes 3 and 8

Imide 2: Anhydride 1 (1.07 g, 5.0 mmol) was treated with hexylamine (0.7 mL, 0.54 g, 5.3 mmol) in ethanol (60 mL) at reflux temperature overnight. Hydrochloric acid (2M, 20 mL) was added and solid was filtered off, washed by water and dried in vacuo to obtain 1.40 g (94%) of imide 2. ¹H NMR (500 MHz, CDCl₃): 8.45 (1H, d, 2.5, H₄), 8.41 (1H, dd, 7.3, 0.7, H₁₀), 8.04 (1H, dd, 8.3, 0.7, H₈), 7.66 (1H, dd, 8.3, 7.3, H₉), 7.59 (1H, d, 2.5, H₆), 7.02 (1H, vbr s, OH), 4.18 (2H, ~t, 7.7, H₁₄), 1.73 (2H, ~kv, 7.6, H₁₅), 1.38-1.27 (4H, m, H₁₇ and H₁₈), 0.88 (3H, t, 7.0, H₁₉). ¹³C APT NMR (126 MHz, CDCl₃): 164.61 (C₂), 164.42 (C₁₂), 155.10 (C₅), 133.57 (C₇), 132.75 (C₈), 129.05 (C₁₀), 127.63 (C₉), 124.08 (C₃), 123.67 (C₁₃), 122.62 (C₁₁), 122.51 (C₄), 116.64
Aldehyde 3: Imide 2 (1.5 g, 5.0 mmol) was treated with hexamethylenetetramine (1.4 g, 10.0 mmol) in trifluoroacetic acid (40 mL) at reflux for 24h. The reaction mixture was diluted with ice-water and product was extracted into dichloromethane. The organic solution was washed sequentially with water, saturated solution of sodium bicarbonate, water and brine, then it was dried with anhydrous sodium sulphate, and the solvent was evaporated to dryness in vacuo. The residue was purified by flash chromatography (dichloromethane) to give 1.6 g (37%) of aldehyde 3. 

**1H NMR (500 MHz, CDCl3):** 12.87 (1H, s, OH), 10.937 (1H, s, major H20), 10.943 (1H, s, minor H20), 8.674 (1H, dd, 8.5, 0.9, major H8), 8.78 (1H, dd, 8.5, 7.3, H9), 4.16 (2H, ~t, 7.7, H14), 1.72 (2H, ~kv, 7.6, H15), 1.41 (2H, ~t, 7.7, H16), 1.27 (6H, m, H17 and H18), 0.89 (3H, t, 7.1, H19).

**13C NMR (126 MHz, CDCl3):** 193.574 (major C20), 193.528 (minor C20), 163.91 (C5), 163.75 (C12), 162.61 (C2), 132.12 (C7), 130.57 (C3), 129.84 (C9), 128.62 (C10), 124.76 (minor C8), 124.72 (major C8), 124.36 (major C4), 124.26 (minor C4), 123.77 (C11), 122.81 (C13), 114.13 (C6), 41.05 (C14), 31.66 (C17), 28.12 (C15), 26.90 (C16), 22.70 (C18), 14.18 (C19). Two sets of signals were observed for several 1H and 13C atoms as a consequence of the hindrance rotation of the CH=O group due to a hydrogen bond with the OH group. 

**HRMS (ESI+):** calcd. for C19H18N2O4 [M - H] - 324.12413; found 324.12420.

Imide 5: Anhydride 4 (5.54 g, 20 mmol) was treated with hexylamine (3.3 mL, 25 mmol) in ethanol (130 mL) at reflux temperature overnight. Hydrochloric acid (2M, 50 mL) was added and solid was filtered off, washed by water and dried in vacuo to obtain 7.10 g (98%) of imide 5.

**1H NMR (500 MHz, CD3SOCD3:CDCl3 3:1):** 8.54 (1H, dd, 7.3, 1.1, H10), 8.49 (1H, dd, 8.5, 1.1, H8), 8.31 (1H, d, 7.8, H4), 8.08 (1H, d, 7.8, H5), 7.85 (1H, dd, 8.5, 7.3, H9), 4.03 (2H, ~t, 7.6, H14), 1.62 (2H, ~kv, 7.5, H15), 1.34 (2H, m, H16), 1.29 (2H, m, H17), 0.84 (3H, t, 7.2, H19).

**13C NMR (126 MHz, CD3SOCD3:CDCl3 3:1):** 162.58 (C12), 162.54 (C2), 130.57 (C3), 129.84 (C9), 128.62 (C10), 124.72 (major C8), 124.36 (major C4), 124.26 (minor C4), 123.77 (C11), 122.81 (C13), 114.13 (C6), 41.05 (C14), 31.66 (C17), 28.12 (C15), 26.90 (C16), 22.70 (C18), 14.18 (C19). Two sets of signals were observed for several 1H and 13C atoms as a consequence of the hindrance rotation of the CH=O group due to a hydrogen bond with the OH group. 

**HRMS (ESI+):** calcd. for C19H18N2O4 [M - H] - 360.05937, 362.05732; found 360.05949, 362.05743.

Imide 6: Imide 5 (7.1 g, 19.7 mmol) was treated with sodium methoxide (5.4 g, 100 mmol) and CuSO4*5H2O (catalytic amount, 0.1 g) in methanol (100 mL) at reflux temperature for 2 days. After evaporation, water (150 mL) and hydrochloric acid (2M, 10 mL) was added and product was extracted into ethyl acetate (5x 100 mL). Organic fractions were dried with anhydrous sodium sulphate and evaporated to dryness in vacuo and the residue was purified by flash chromatography (dichloromethane) to give 5.4 g (88%) of Imide 6.

**1H NMR (500 MHz, CDCl3):** 8.45 (1H, dd, 7.3, 1.2, H10), 8.40 (1H, d, 8.3, H4), 8.40 (1H, dd, 8.4, 1.2, H8), 7.56 (1H, dd, 8.4, 7.3, H9), 6.90 (1H, d, 8.3, H5), 4.05 (2H, ~t, 7.7, H14), 4.02 (3H, s, H20), 1.63 (2H, ~kv, 7.7, H15), 1.34 (2H, ~kv, 7.5, H16), 1.29-1.21 (4H, m, H17 and H18), 0.80 (2H, ~t, 7.1, H19). 

**13C NMR:**
NMR (126 MHz, CDCl₃): 164.53 (C12), 163.95 (C2), 160.77 (C6), 133.38 (C4), 131.49 (C10), 129.37 (C13), 128.55 (C8), 125.94 (C9), 123.50 (C7), 122.53 (C11), 115.24 (C3), 105.21 (C5), 56.25 (C20), 40.42 (C14), 31.69 (C17), 28.22 (C15), 26.94 (C16), 22.68 (C18), 14.16 (C19).
HRMS (ESI⁺): calcd. for C₁₉H₂₂N₃O₃ [M + H]⁺ 312.15942; found 312.16011.

Imide 7: Imide 6 (5.6 g, 18 mmol) was treated with hydriodic acid (57%, 60 mL) at 130°C overnight. After cooling down, water (150 mL) was added and solid was filtered off, washed by water and dried in vacuo. The residue was purified by flash chromatography (dichloromethane/methanol 97:3) to give 4.5 g (84%) of imide 7. ¹H NMR (500 MHz, CDCl₃): 8.63 (1H, dd, 7.3, 1.2, H10), 8.56 (1H, dd, 8.4, 1.2, H8), 8.48 (1H, d, 8.1, H4), 7.72 (1H, dd, 8.4, 7.3, H9), 7.07 (1H, d, 8.1, H5), 7.06 (1H, br s, OH), 4.17 (2H, ~t, 7.7, H14), 1.73 (2H, ~kv, 7.7, H15), 1.43 (2H, ~kv, 7.5, H16), 1.33 (2H, m, H17), 1.32 (2H, m, H18), 1.32 (2H, m, H17), 1.32 (2H, m, H18), 0.88 (3H, ~t, 7.1, H19). ¹³C NMR (126 MHz, CDCl₃): 164.74 (C12), 164.21 (C2), 158.13 (C6), 133.41 (C4), 132.05 (C10), 129.99 (C13), 128.80 (C8), 126.06 (C9), 122.74 (C11), 122.57 (C7), 115.54 (C3), 110.25 (C5), 40.61 (C14), 31.74 (C17), 28.29 (C15), 26.99 (C16), 22.73 (C18), 14.20 (C19). HRMS (ESI⁺): calcd. for C₁₈H₂₀N₂O₃ [M + H]⁺ 298.14377; found 298.14397.

Aldehyde 8: Imide 7 (1.9 g, 6.4 mmol) was treated with hexamethylenetetramine (1.9 g, 13.6 mmol) in trifluoroacetic acid (40 mL) at reflux for 24h. The reaction mixture was diluted with ice-water and product was extracted into dichloromethane. The organic solution was washed sequentially with water, saturated solution of sodium bicarbonate, water and brine, then it was dried with anhydrous sodium sulphate, and the solvent was evaporated to dryness in vacuo. The residue was purified by flash chromatography (dichloromethane) to give 1.6 g (77%) of aldehyde 8. ¹H NMR (500 MHz, CDCl₃): 13.16 (1H, s, OH), 10.11 (1H, s, H20), 8.72 (1H, dd, 7.3, 1.2, H10), 8.71 (1H, dd, 8.3, 1.2, H8), 8.70 (1H, s, H4), 7.79 (1H, dd, 8.3, 7.3, H9), 4.15 (2H, ~t, 7.7, H14), 1.71 (2H, ~kv, 7.6, H15), 1.42 (2H, m, H16), 1.38-1.29 (4H, m, H17 and H18), 0.89 (3H, ~t, 7.1, H19). ¹³C NMR (126 MHz, CDCl₃): 196.58 (C20), 165.87 (C6), 163.84 (C12), 163.18 (C2), 135.03 (C10), 134.26 (C4), 131.98 (C13), 130.48 (C8), 127.18 (C9), 123.06 (C7 or C11), 123.04 (C7 or C11), 115.33 (C5), 115.11 (C3), 40.70 (C14), 31.68 (C17), 28.20 (C15), 26.91 (C16), 22.69 (C18), 14.18 (C19). HRMS (ESI⁻): calcd. for C₁₉H₁₈N₂O₄ [M - H]⁻ 324.12413; found 324.12457.
1.3. Preparation of π-expanded naphthalimides 9-12

Probe 9: Aldehyde 3 (650 mg, 2.0 mmol) was treated with diethyl malonate (0.4 mL, 420 mg, 2.6 mmol) and piperidine (5 drops) in ethanol (15 mL) at reflux temperature overnight. The reaction mixture was evaporated to dryness in vacuo. The residue was purified by flash chromatography (dichloromethane/methanol 97:3) to give 620 mg (74%) of probe 9. $^1$H NMR (500 MHz, CDCl$_3$): 9.28 (1H, d, 0.8, H$_2$O), 8.67 (1H, dd, 7.3, 1.0, H10), 8.64 (1H, dd, 8.5, 1.0, H8), 8.53 (1H, d, 0.8, H4), 8.00 (1H, dd, 8.5, 7.3, 7.3, H9), 4.50 (2H, q, 7.1, H24), 4.18 (2H, t, 7.7, H14), 1.73 (2H, ~kv, 7.7, H15), 1.47 (3H, t, 7.1, H25), 1.43 (2H, m, H16), 1.38-1.29 (4H, m, H17 and H18), 0.89 (3H, ~t, 7.2, H19). $^{13}$C NMR (126 MHz, CDCl$_3$): 163.40 (C12), 163.10 (C23), 162.58 (C2), 155.66 (C22), 155.34 (C5), 142.79 (C20), 130.83 (C10), 129.80 (C9), 128.74 (C7), 128.19 (C3), 127.43 (C8), 125.23 (C13), 123.83 (C11), 121.07 (C4), 120.61 (C21), 116.46 (C6), 62.79 (C24), 41.15 (C14), 31.64 (C17), 28.14 (C15), 26.90 (C16), 22.69 (C18), 14.41 (C25), 14.17 (C19). HRMS (ESI$^+$): calcd. for C$_{24}$H$_{23}$N$_1$O$_6$Na$_1$ [M + Na]$^+$ 444.14176; found 444.14185.

Probe 10: Aldehyde 3 (650 mg, 2.0 mmol) was treated with ethyl bromoacetate (0.3 mL, 453 mg, 2.7 mmol) and potassium carbonate (830 mg, 6.0 mmol) in acetonitrile (15 mL) at reflux temperature for 2 days. The reaction mixture was evaporated to dryness in vacuo. The residue was purified by flash chromatography (dichloromethane) to give 450 mg (57%) of probe 10. $^1$H NMR (500 MHz, CDCl$_3$): 8.83 (1H, d, 1.0, H4), 8.65 (1H, dd, 7.4, 1.2, H10), 8.49 (1H, dd, 8.2, 1.2, H8), 8.07 (1H, d, 1.0, H20), 7.90 (1H, dd, 8.2, 7.4, H9), 4.53 (2H, q, 7.1, H23), 4.20 (2H, t, 7.7, H14), 1.75 (2H, ~kv, 7.6, H15), 1.49 (3H, t, 7.1, H24), 1.44 (2H, ~kv, 7.5, H16), 1.36 (2H, m, H17), 1.35 (2H, m, H18), 0.90 (3H, t, 7.1, H19). $^{13}$C NMR (126 MHz, CDCl$_3$): 164.10 (C12), 163.88 (C2), 158.88 (C22), 153.43 (C5), 148.82 (C21), 130.06 (C10), 129.34 (C8), 128.14 (C9), 128.07 (C6), 126.71 (C7), 125.71 (C13), 123.83 (C11), 122.92 (C3), 117.97 (C4), 112.27 (C20), 62.30 (C23), 41.00 (C14), 31.70 (C17), 28.22 (C15), 26.96 (C16), 22.72 (C18), 14.49 (C24), 14.20 (C19). HRMS (ESI$^+$): calcd. for C$_{24}$H$_{23}$N$_1$O$_5$ [M + H]$^+$ 394.16490; found 394.16513.
Probe 11: Aldehyde 8 (650 mg, 2.0 mmol) was treated with diethyl malonate (0.4 mL, 420 mg, 2.6 mmol) and piperidine (5 drops) in ethanol (15 mL) at reflux temperature overnight. The reaction mixture was evaporated to dryness in vacuo. The residue was purified by flash chromatography (dichloromethane/methanol 97:3) to give 590 mg (70%) of probe 11. $^1$H NMR (500 MHz, CDCl$_3$): 8.87 (1H, dd, 8.4, 1.2, H8), 8.77 (1H, dd, 7.4, 1.2, H10), 8.71 (1H, s, H4), 7.94 (1H, dd, 8.4, 7.4, H9), 4.47 (2H, q, 7.2, H24), 4.17 (2H, ~t, 7.7, H14), 1.73 (2H, m, H15), 1.45 (3H, t, 7.2, H25), 1.45-1.30 (6H, m, H16, H17 and H18), 0.89 (3H, ~t, 7.1, H19). $^{13}$C NMR (126 MHz, CDCl$_3$): 163.56 (C12), 162.90 (C2), 162.54 (C23), 156.44 (C6), 155.35 (C22), 148.78 (C20), 134.18 (C10), 130.89 (C4), 130.45 (C13), 129.12 (C8), 128.57 (C9), 123.26 (C11), 121.31 (C7), 119.87 (C3), 119.08 (C21), 114.26 (C5), 62.55 (C24), 40.92 (C14), 31.66 (C17), 28.18 (C15), 26.91 (C16), 22.70 (C18), 14.38 (C25), 14.18 (C19). HRMS (ESI$^+$): calcd. for C$_{24}$H$_{23}$N$_1$O$_6$Na [M + Na$^+$] 444.14176; found 444.14184.

Probe 12: Aldehyde 8 (650 mg, 2.0 mmol) was treated with ethyl bromoacetate (0.3 mL, 453 mg, 2.7 mmol) and potassium carbonate (830 mg, 6.0 mmol) in acetonitrile (15 mL) at reflux temperature for 2 days. The reaction mixture was evaporated to dryness in vacuo. The residue was purified by flash chromatography (dichloromethane) to give 320 mg (41%) of probe 12. $^1$H NMR (500 MHz, CDCl$_3$): 8.87 (1H, s, H4), 8.76 (1H, dd, 8.2, 1.2, H8), 8.66 (1H, dd, 7.4, 1.2, H10), 7.89 (1H, dd, 3.0 to H4, H9), 7.78 (1H, s, H20), 4.50 (2H, q, 7.1, H23), 4.18 (2H, ~t, 7.7, H14), 1.74 (2H, ~kv, 7.7, H15), 1.47 (3H, t, 7.1, H24), 1.44 (2H, ~kv, 7.4, H16), 1.38-1.29 (4H, m, H17 and H18), 0.89 (3H, ~t, 7.2, H19). $^{13}$C NMR (126 MHz, CDCl$_3$): 164.18 (C12), 163.95 (C2), 158.89 (C22), 154.59 (C6), 147.25 (C21), 131.08 (C3), 127.82 (C9), 127.78 (C13), 127.47 (C4), 126.99 (C8), 123.86 (C5), 123.51 (C11), 119.92 (C3), 119.54 (C7), 115.21 (C20), 62.07 (C23), 40.86 (C14), 31.70 (C17), 28.22 (C15), 26.95 (C16), 22.71 (C18), 14.49 (C19), 14.19 (C24). HRMS (ESI$^+$): calcd. for C$_{23}$H$_{22}$N$_1$O$_5$ [M + H]$^+$ 394.16490; found 394.16459.

1.4. Preparation of LysoSers 13-16

Scheme S4. Preparation of LysoSers 13 and 14.
LysoSer 13. Probe 9 (100 mg, 0.24 mmol) was treated with 2M aqua solution of sodium hydroxide (5 mL) in ethanol (5 mL) at reflux temperature for 2h. Acetic acid was added until pH of solution was not slightly acidic. Formed solid was filtered off, washed by water and dried in vacuo to obtain 90 mg (96%) of corresponding acid. Acid was used to next step without next purification. Acid (85 mg, 0.22 mmol) was treated with 2M solution of oxalyl chloride (3 mL) in dichloromethane with N,N-dimethylformamide (2 drops) as catalyst in dichloromethane (5 mL) at room temperature for 2h. The reaction mixture was evaporated to dryness in vacuo to obtain 89 mg (100%) of corresponding chloride of acid. Prepared chloride acid (89 mg, 0.22 mmol) was treated with N,N-dimethylethylenediamine (25 mg, 0.28 mmol) and with triethylamine (36 mg, 0.36 mmol) in dichloromethane (5 mL) at room temperature overnight. The reaction mixture was evaporated to dryness in vacuo and the residue was purified by flash chromatography (dichloromethane/methanol 9:1) to give 88 mg (88%, 84% overall yield) of LysoSer 13. \(^1\)H NMR (500 MHz, CDCl\(_3\)): 9.61 (1H, s, H20), 9.19 (1H, br t, 5.9, NH), 8.73 (1H, dd, 8.4, 1.0, H8), 8.67 (1H, dd, 7.4, 1.0, H10), 8.51 (1H, d, 0.6, H4), 8.00 (1H, dd, 8.4, 7.4, H9), 4.17 (2H, ~t, 7.7, H14), 4.02 (2H, br q, 5.9, H24), 3.30 (2H, br s, H25), 2.89 (6H, br s, H26), 1.73 (2H, ~kv, 7.6, H15), 1.43 (2H, ~kv, 7.5, H16), 1.39-1.29 (4H, m, H17 and H18), 0.90 (3H, t, 7.1, H19). \(^13\)C APT NMR (126 MHz, CDCl\(_3\)): 163.33 (C12), 162.51 (C2 or C23), 162.45 (C2 or C23), 160.22 (C22), 154.46 (C5), 142.68 (C20), 131.06 (C10), 129.91 (C9), 128.82 (C7), 128.14 (C3), 127.93 (C8), 125.35 (C13), 123.71 (C11), 120.75 (C4), 120.33 (C21), 117.28 (C6), 56.89 (C25), 43.88 (C26), 41.16 (C14), 35.51 (br, C24), 31.64 (C17), 28.13 (C15), 26.90 (C16), 22.70 (C18), 14.18 (C19). HRMS (ESI\(^+\)): calcd. for C\(_{26}\)H\(_{30}\)N\(_3\)O\(_5\) [M + H]\(^+\) 464.21800; found 464.21829.

LysoSer 14. Probe 11 (130 mg, 0.30 mmol) was treated with 2M aqua solution of sodium hydroxide (5 mL) in ethanol (5 mL) at reflux temperature for 2h. Acetic acid was added until pH of solution was not slightly acidic. Formed solid was filtered off, washed by water and dried in vacuo to obtain 115 mg (95%) of corresponding acid. Acid was used to next step without next purification. Acid (110 mg, 0.28 mmol) was treated with 2M solution of oxalyl chloride (3 mL) in dichloromethane with N,N-dimethylformamide (2 drops) as catalyst in dichloromethane (5 mL) at room temperature for 2h. The reaction mixture was evaporated to dryness in vacuo to obtain 115 mg (100%) of corresponding chloride of acid. Prepared chloride acid (115 mg, 0.28 mmol) was treated with N,N-dimethylethylenediamine (27 mg, 0.31 mmol) and with triethylamine (40 mg, 0.40 mmol)
in dichloromethane (5 mL) at room temperature overnight. The reaction mixture was evaporated to dryness in vacuo and the residue was purified by flash chromatography (dichloromethane/methanol 9:1) to give 110 mg (85%, 81% overall yield) of LysoSer 14. $^1$H NMR (500 MHz, CDCl$_3$): 9.11 (1H, d, 0.4, H$_2$O), 8.93 (1H, br t, 5.3, NH), 8.84 (1H, dd, 8.4, 1.2, H8), 8.77 (1H, dd, 0.4, 0.3, H4), 8.76 (1H, dd, 7.4, 1.2, H10), 7.94 (1H, dd, 8.4, 7.4, 0.3, H9), 4.18 (2H, ~t, 7.7, H14), 3.69 (2H, q, 5.9, H24), 2.72 (2H, br t, 6.5, H25), 2.44 (6H, s, H26), 1.73 (2H, ~kv, 7.6, H15), 1.43 (2H, ~kv, 7.5, H16), 1.38-1.27 (2H, m, H17 and H18), 0.89 (3H, t, 7.2, H19). $^{13}$C NMR (126 MHz, CDCl$_3$): 163.52 (C12), 162.83 (C2), 161.18 (C23), 160.06 (C22), 155.28 (C6), 148.38 (C20), 133.99 (C10), 131.26 (C4), 131.14 (C13), 128.77 (C8), 128.62 (C9), 123.31 (C11), 121.21 (C7), 120.24 (C3), 119.65 (C21), 115.18 (C5), 57.75 (C25), 40.94 (C14), 37.51 (C24), 31.66 (C17), 28.17 (C15), 26.90 (C16), 14.18 (C19). HRMS (ESI$^+$): calcd. for C$_{26}$H$_{30}$N$_3$O$_5$ [M + H]$^+$ 464.21800; found 464.21837.

LysoSer 15. Probe 10 (118 mg, 0.3 mmol) was treated with N,N-dimethylethylenediamine (1 mL) in ethanol (10 mL) at reflux temperature overnight. The reaction mixture was evaporated to dryness in vacuo. The residue was purified by flash chromatography (dichloromethane/methanol 9:1) to obtain 112 mg (86%) of LysoSer 15. $^1$H NMR (500 MHz, CD$_3$SOCD$_3$): 8.86 (1H, dd, 8.2, 1.2, H8), 8.85 (1H, br t, 5.8, NH), 8.67 (1H, d, 1.0, H4), 8.53 (1H, dd, 7.4, 1.2, H10), 8.39 (1H, d, 1.0, H20), 8.00 (1H, ddd, 8.2, 7.4, 0.3, H9), 4.06 (1H, ~t, 7.5, H14), 3.45 (2H, td, 6.7, 5.8, H23), 2.47 (2H, covered, H24), 2.24 (6H, s, H25), 1.65 (1H, ~kv, 7.4, H15), 1.36 (2H, m, H16), 1.35-1.26 (4H, m, H17 and H18), 0.87 (3H, ~t, 7.1, H19). $^{13}$C NMR (126 MHz, CD$_3$SOCD$_3$): 163.30 (C12), 163.12 (C2), 157.37 (C22), 152.23 (C21), 151.45 (C5), 130.26 (C8), 129.37 (C10), 128.56 (C6), 128.08 (C9), 126.13 (C7), 124.78 (C13), 122.81 (C11), 116.58 (C4), 109.13 (C20), 57.84 (C24), 45.07 (C25), 39.94 (C14), 36.86 (C23), 30.93 (C17), 27.39 (C15), 26.17 (C16), 21.94 (C18), 13.89 (C19). HRMS (ESI$^+$): calcd. for C$_{25}$H$_{30}$N$_3$O$_4$ [M + H]$^+$ 436.22308; found 436.22327.

LysoSer 16. Probe 12 (118 mg, 0.3 mmol) was treated with N,N-dimethylethylenediamine (1 mL) in ethanol (10 mL) at reflux temperature overnight. The reaction mixture was evaporated to dryness in vacuo. The residue was purified by flash chromatography (dichloromethane/methanol 9:1) to obtain 108 mg (83%) of LysoSer 16. $^1$H NMR (500 MHz, CD$_3$SOCD$_3$): 8.88 (1H, br t, 5.7, NH), 8.86 (1H, s, H4), 8.77 (1H, dd, 8.2, 1.2, H8), 8.53 (1H, dd, 7.4, 1.2, H10), 8.02 (1H, dd, 8.2, 7.4, H9), 7.86 (1H, s, H20), 4.04 (2H, ~t, 7.5, H14), 3.46 (2H, td, 6.7, 5.7, H23), 2.52 (2H, t, 6.7, H24), 2.25 (6H, s, H25), 1.63 (2H, ~kv, 7.4, H15), 1.35 (2H, m, H16), 1.36-1.25 (4H, m, H17 and H18), 0.86 (3H, ~t, 7.1, H19). $^{13}$C NMR (126 MHz, CD$_3$SOCD$_3$): 163.35 (C2), 163.10 (C12), 157.40 (C), 152.50 (C6), 150.26 (C21), 130.03 (C10), 127.97 (C9), 127.31 (C4), 126.45 (C13), 126.35 (C8), 124.11 (C5), 122.79 (C11), 118.76 (C3), 118.63 (C7), 111.08 (C20), 58.01 (C24), 45.09 (C25), 39.80 (C14), 36.82 (C23), 30.94 (C17), 27.41 (C15), 26.17 (C16), 21.96 (C18), 13.89 (C19). HRMS (ESI$^+$): calcd. for C$_{25}$H$_{30}$N$_3$O$_4$ [M + H]$^+$ 436.22308; found 436.22343.
1.5. Copies of $^1$H and $^{13}$C NMR spectra of compounds 2-16
\(^1\)H NMR spectrum of compound 3

\(^{13}\)C NMR spectrum of compound 3
\(^1\text{H NMR spectrum of compound 5}\)

\(^{13}\text{C NMR spectrum of compound 5}\)
$^1$H NMR spectrum of compound 6

$^{13}$C NMR spectrum of compound 6
$^{1}H$ NMR spectrum of compound 8

$^{13}C$ NMR spectrum of compound 8
$^1$H NMR spectrum of compound 9

$^{13}$C NMR spectrum of compound 9
$^1$H NMR spectrum of compound 10

$^{13}$C NMR spectrum of compound 10
$^1$H NMR spectrum of compound 11

$^{13}$C NMR spectrum of compound 11
$^1$H NMR spectrum of compound 12

$^{13}$C NMR spectrum of compound 12
$^1$H NMR spectrum of LysoSer 13

$^{13}$C NMR spectrum of LysoSer 13
\(^1\)H NMR spectrum of LysoSer 14

\(^{13}\)C NMR spectrum of LysoSer 14
$^1$H NMR spectrum of LysoSer 15

$^{13}$C NMR spectrum of LysoSer 15
$^{1}H$ NMR spectrum of LysoSer 16

$^{13}C$ NMR spectrum of LysoSer 16
2. Analytical studies of LysoSers 13-16

2.1. Spectral characteristics

Spectral characteristics in the range of 300-800 nm were obtained by using a UV/VIS spectrometer Cintra 404 GBC. The LysoSers were measured in four solvents (DMSO, methanol, ethanol and phosphate buffer). Each LysoSers was at first dissolved in DMSO 1mM stock solution which was diluted to a final concentration of 10 or 50 μM and measured in a classical 1 cm PMMA cuvette. The absorption values of the LysoSers 13-16 are summarized in Table S1.

2.2. Fluorescence properties

Fluorescence properties of the compounds were measured using a SCINCO FluoroMate FS-2 fluorescence spectrometer. The substances were dissolved in DMSO and diluted to a final concentration in the range of 0.1 μM to 50 μM. The measurement was carried out in Fluorescence Quartz Cuvette Cell and the length of the absorbent layer was 1 cm. Obtained emission spectra of the individual fluorescent probes are shown in Figure S1. The absorption and emission values of the LysoSers 13-16 are summarized in Table S1.

Table S1. Absorption and emission values of the LysoSers 13-16.

<table>
<thead>
<tr>
<th>LysoSers</th>
<th>Solvent</th>
<th>Absorbance maxima (nm)</th>
<th>Emission maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>DMSO</td>
<td>379</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>EtOH</td>
<td>388</td>
<td>448</td>
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<td>442</td>
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<tr>
<td></td>
<td>PBS</td>
<td>389</td>
<td>448</td>
</tr>
<tr>
<td>14</td>
<td>DMSO</td>
<td>368</td>
<td>432</td>
</tr>
<tr>
<td></td>
<td>EtOH</td>
<td>319</td>
<td>434</td>
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<tr>
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<td>MeOH</td>
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<td>435</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>320</td>
<td>437</td>
</tr>
<tr>
<td>15</td>
<td>DMSO</td>
<td>373</td>
<td>424</td>
</tr>
<tr>
<td></td>
<td>EtOH</td>
<td>376</td>
<td>422</td>
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<tr>
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<td>MeOH</td>
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<td></td>
<td>PBS</td>
<td>373</td>
<td>429</td>
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<tr>
<td>16</td>
<td>DMSO</td>
<td>368</td>
<td>416</td>
</tr>
<tr>
<td></td>
<td>EtOH</td>
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<td>415</td>
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<tr>
<td></td>
<td>MeOH</td>
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<td>416</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>360</td>
<td>428</td>
</tr>
</tbody>
</table>
Figure S1. Fluorescence intensity of emission spectra of the LysoSers 13-16 that were measured in four solvents.
2.3. Absolute fluorescence quantum yields

Absolute fluorescence quantum yields ($\Phi_F$) of LysoSers 13-16 and LysoTracker Blue DND-22 (LT-B) were obtained by using the absolute method by detecting the whole fluorescence of the sample through the use of an integrating sphere (FS5 Spectrofluorometer - SC-30 Integrating Sphere, Edinburgh Instruments). The LysoSers and LT-B were measured in water solution with an absorbance of measured samples between 0.1 - 0.2. The LysoSers were dissolved in DMSO and a stock solution (1 mM) was diluted by water to a final concentration 5 μM for all samples. Resulting values of $\Phi_F$ are summarized in Table S2.

Table S2. Fluorescence quantum yields of LysoSers 13-16 and LysoTracker Blue DND-22 (LT-B).

<table>
<thead>
<tr>
<th>LysoSers</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>LT - B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantum Yields ($\Phi_F$)</td>
<td>15.49 %</td>
<td>4.36 %</td>
<td>16.48 %</td>
<td>23.54 %</td>
<td>2.31 %</td>
</tr>
</tbody>
</table>

3. Biological studies of LysoSers 13-16

3.1 Cell cultures

Human dermal fibroblast cell lines were used for intracellular localization (and for MTT assay) of LysoSers 13-16. Human fibroblast cells HF-P4 (human fibroblasts – patient 4) are normal dermal fibroblasts which were obtained from the residual skin of a healthy patient (with informed consent of the patient). HF-P4 cell lines were obtained from the Departments of Dermatovenerology of the First and Second Faculties of Medicine and from the Department of Otorhinolaryngology, Head and Neck Surgery of the First Faculty of Medicine (both faculties of the Charles University, Prague, Czech Republic) with the consent of donors. The HF-P4 cell line has been obtained and processed in accordance with the relevant local ethics committee, with respect to the Declaration of Helsinki protection of patient’s rights and benefits with signed written informed consent of each patient. Cells were cultivated under standard conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, streptomycin (100 μg / ml) at 37 °C at 0.05 tension of CO₂.

Two different human melanoma cell lines were used for MTT cell proliferation, cytotoxicity test and for intracellular studies. Highly invasive cell line A-2058, which is a human skin melanoma cell line derived from the metastatic site of lymph nodes. The line was purchased from ATCC (A2058 - (ATCC® CRL-11147 ™), Virgina, USA). As a second cell line, highly metastatic BLM melanoma cell line was used, which was obtained as a friendly gift from L. van Kempen and J.H.J.M. van Krieken, Department of Pathology, Radboud University Nijmegen Medical Center, The Netherlands. The BLM cell line has been obtained and processed in accordance with the relevant local ethics committee, with respect to the Declaration of Helsinki protection of patient’s rights and benefits with signed written informed consent of each patient. Cells were cultivated under standard conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, streptomycin (100 μg / ml) at 37 °C at 0.05 tension of CO₂.

3.2. Intracellular studies

Intracellular localization of the LysoSers 13-16 in living cells was studied on a confocal microscope Leica TCS SP8 WLL SMD-FLIM (equipped with 405 nm pulsed laser) at 37 °C and...
0.05 tension of CO₂ using an HC PL APO CS2 63x / 1.2W watertight lens. The cover glasses of thickness 22x22 mm were placed in Petri dishes. Into such prepared Petri dishes were applied human dermal fibroblast cells (HF-P4) at a density of 10 000 cells per well. The cells (HF-P4, BLM or A-2058) were stored under standard culture conditions in a culture medium (DMEM with 10% FBS). When the cells (HF-P4, BLM or A-2058) have reached the desired confluency (after 24 hours), media was removed from the dish and the cells were rinsed twice with PBS and further incubated in complete culture medium with the LysoSers 13, 14 at 1 μM and LysoSers 15, 16 at 200 nM concentration for next 30 minutes. As co-localizing agent was used the commercially available organic fluorescent probe. As a co-label mark of lysosomes, it was chosen green fluorescent dye LysoTracker Green DND-26 (LT-G). LT-G was added to cells at 200 nM concentration and incubated with the cells for 30 minutes under standard growth conditions. After removal from the incubator, the cells were again washed twice with PBS and analyzed (Fig. S2-S4). Excitation wavelength for the tested substances was 405 nm and the emission was measured in 415 - 500 nm range. LT-G was excited at 504 nm and the emission was collected in 510-550 nm range.

<table>
<thead>
<tr>
<th>A phase contrast</th>
<th>fluorescence</th>
<th>B fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-2058</td>
<td>13 - 1 μM</td>
<td>LT-G - 200 nM</td>
</tr>
<tr>
<td></td>
<td>13 - 1 μM</td>
<td>merge, r= 0.76</td>
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<tr>
<td>A-2058</td>
<td>15 - 200 nM</td>
<td>LT-G - 200 nM</td>
</tr>
<tr>
<td></td>
<td>15 - 200 nM</td>
<td>merge, r= 0.90</td>
</tr>
<tr>
<td>A-2058</td>
<td>15 - 200 nM</td>
<td>LT-G - 200 nM</td>
</tr>
<tr>
<td></td>
<td>15 - 200 nM</td>
<td>merge, r= 0.78</td>
</tr>
</tbody>
</table>

**Figure S2.** Fluorescence and intracellular localization of fluorescent probes 13-16 for the lysosomal targeting. (A) Images of the same field of cells showing phase contrast (left column) and cellular fluorescence (right column). (B) Lysosomal localization of LysoSers 13-16. LysoSers were co-labeled by the commercial specific lysosomal probe LysoTracker Green DND-26 (LT-G) in the A-2058 cell line. Images were captured with Leica TCS SP8 WLL SMD-FLIM confocal microscope using an HC PL APO CS2 63x / 1.2W watertight lens. The parameter r is Pearson’s correlation coefficient for the co-localization.
Figure S3. Fluorescence and intracellular localization of fluorescent probes 13-16 for the lysosomal targeting. (A) Images of the same field of cells showing phase contrast (left column) and cellular fluorescence (right column). (B) Lysosomal localization of LysoSers 13-16. LysoSers were co-labeled by the commercial specific lysosomal probe LysoTracker Green DND-26 (LT-G) in the BLM cell line. Images were captured with Leica TCS SP8 WLL SMD-FLIM confocal microscope using an HC PL APO CS2 63x / 1.2W watertight lens. The parameter r is Pearson’s correlation coefficient for the co-localization.
3.3. Photostability in the HF-P4 cell line

The photostability of the prepared LysoSers 13-16 is shown as a photobleaching in the HF-P4 cell line. HF-P4 cells (10 000 cells per well) were seeded on 35 mm glass-bottom dishes for live-cell imaging in complete culture media and allowed to adhere for 24 hours. After this time, when the cells reached the desired confluency, the media was removed and the cells were washed twice with PBS and incubated in DMEM (Dulbecco’s Modified Eagle Medium) without phenol red containing the tested LysoSers 13-16 (500 nM) or LysoTracker Green DND-26 (LT-G, 200 nM) or LysoTracker Blue DND-22 (LT-B, 500 nM) for 30 minutes under the standard growth conditions. Excitation wavelength for the LysoSers and LT-B was 405 nm and the emission was measured in 415 - 500 nm range. LT-G was excited at 504 nm and the emission was collected in 510 - 550 nm range. Exposure time was 15 minutes and laser power was set on 20%. Figures S5 and S6 show normalized bleaching LysoSers 13-16, LT-G and LT-B.
Figure S5. Graph shows normalized bleaching curves of LysoSers 13-16 and commercial lysosomal probes LysoTracker Green DND 26 (LT-G) and LysoTracker Blue DND 22 (LT-B).

Figure S6. Photobleaching of LysoSers 13-16, LysoTracker Green DND 26 (LT-G) and LysoTracker Blue DND 22 (LT-B) in the HF-P4 cell line. Pictures show the initial intensity of the compounds (0 min) and the photobleaching that occurs after 5 min of constant illumination.
3.4. Pearson's correlation coefficient

Values of Pearson's correlation coefficient were observed by comparing LysoSers 13-16 with specific commercial lysosomal probe LysoTracker Green DND-26 (LT-G). Fiji – Image J software was used for evaluation. Resulting values are summarized in table S3 and scatter plots are shown on figure S7.

Table S3. Values of Pearson’s correlation coefficient on three cell lines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pearson’s correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>HF-P4</td>
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<td>BLM</td>
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<td>15</td>
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<tr>
<td>16</td>
<td>HF-P4</td>
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<tr>
<td></td>
<td>BLM</td>
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<tr>
<td></td>
<td>A-2058</td>
</tr>
</tbody>
</table>

Figure S7. 2-D scatter plots of Pearson’s correlation coefficient of LysoSers 13-16.
4. MTT cell proliferation and cytotoxicity assays of LysoSers 13-16

A colorimetric cell metabolic activity assay was used for the cytotoxicity test of the LysoSers (Fig. S8-S9). In our experiments, (HF-P4, BLM and A-2058) cells were cultured under standard conditions in a culture medium (DMEM with 10% FBS).\cite{1,2} After 24 hours of cultivation, cells were plated on 96-well plates at a density of 5000 cells per well. The cells were stored under standard culture conditions for next 24h. After this time, the medium was removed and medium with tested compounds was added and cultivated for 48h. Afterwards, the medium was exchanged for tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, purchased from Sigma-Aldrich) and incubated for 2h (37°C). After a double hour incubation, the yellow tetrazole dye was removed and DMSO was added, which reduced to purple formazan in living cells. This was followed by testing with the (NanoQuant microplate reader by Tecan) An absorbance of converted dye was measured at a wavelength of 570 nm with the reference of 630 nm.

Figure S8. Cytotoxicity assay (showing IC\textsubscript{50} values) of LysoSers 13-16 on three cell lines.
Figure S9. IC<sub>50</sub> curves of the LysoSers 13-16.

5. References