Wavelength-dependent optoacoustic imaging probes for NMDA receptor visualization

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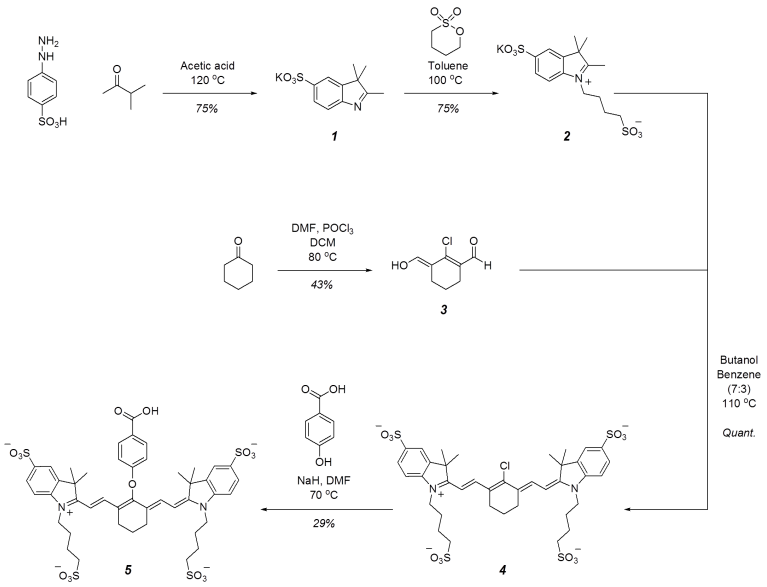
1. Synthesis

1.1 Synthesis of the heptamethine cyanine dye precursor. The synthesis of the heptamethine cyanine dye began with construction of the hetrocyclic indole ring, using standard Fischer indole synthesis conditions described in *Scheme S1*.[1] Hydrazinobenzene sulfonic acid and 3-methyl-2-butanone were stirred in acetic acid at 120 °C for three hours, at which point the solvent was removed to give the crude indole. Purification by precipitation of the potassium salt gave ***1*** in a 75% yield. This was then subject to an *N*-alkylation through reaction with 1,4-butanesultone in anhydrous toluene at 100 °C for twelve hours.[2] Upon completion of the reaction, as confirmed by ESI-MS, the solvent was decanted from the purple precipitate. Trituration with cold propan-2-ol yielded the quaternary ammonium salt, ***2***, as a free flowing pink powder.

Classical syntheses of heptamethine cyanine dyes involve condensation of the 2-methyl substituted quaternary indole with a bis-aldehyde, and this strategy was employed here. The bis-aldehyde was synthesised according to a literature procedure.[3] Initially, anhydrous DMF and $POCl_3$ were stirred as a solution in anhydrous DCM at 0 °C to allow for *in-situ* formation of the Vilsmeier-Haak reagent. To this solution was added cyclohexanone and the resulting mixture was heated to 80 °C and reaction progress monitored by TLC. Complete conversion was achieved after 3 hours, at which point the reaction was cooled to room temperature and poured onto ice and left overnight.



Scheme S1.

Complete hydrolysis of the imine intermediate gave the bis-aldehyde, ***3***, as bright yellow crystals. Next, condensation of ***3*** with 2.2 equivalents of ***2*** gave the conjugated heptamethine cyanine dye. This reaction was undertaken by stirring the two compounds in a mixture of anhydrous butan-1-ol and anhydrous benzene at 110 °C, overnight. After this time, a green solution and a green precipitate formed. The presence of the desired compound was confirmed through observation of an absorption band centered around 784 nm, which is consistent with heptamethine cyanine dyes of this nature. Removal of the solvent and washing with diethyl ether gave the chloro-substituted heptamethine cyanine dye, ***4***, as a green powder.[4]

Due to the electron-deficient nature of the π-system, the chlorine group at the meso-position of the cyclohexyl ring in heptamethine dyes is known to be susceptible to nucleophillic substitution. Compound ***4*** was subject to nucleophillic displacement by reaction with 4-hydroxy benzoic acid in the presence of an excess of NaH in anhydrous DMF at 70 °C. The likely mechanism of this reaction involves an $S_{NR}1$ pathway, initiated by a single electron transfer from the nucleophile to the cationic π-system.[5] The progress of the reaction was monitored by analytical RP-HPLC and upon completion of the reaction, the solvent was removed and the crude residue purified by preparative RP-HPLC (mobile phase comprising 25 mM triethylammonium acetate buffer and acetonitrile), to give the heptamethine cyanine dye, ***5***, as a green powder. Confirmation of the substitution of the chlorine atom was achieved by comparison of the maximum absorption wavelength of ***5*** and ***4***. A hypsochromic shift of 12 nm was observed for ***5***, consistent with substitution by an electron rich ether group.[6]

1.2 Synthesis of the NMDA Receptor-Binding Antagonists. The synthesis of the NMDA receptor-binding moiety of $\mathbf{L}^{\mathbf{1}}$ followed the reported literature method (*Scheme S2*).[7] Initially, the benzyl-protected 4-hydroxypropiophenone underwent α-bromination, via reaction with molecular bromine in anhydrous diethyl ether. After stirring at room temperature for 30 minutes, a colour change was observed and the reaction was quenched by the addition of water. After work-up and subsequent purification by column chromatography, compound ***6*** was isolated in an 82% yield. Reaction of ***6*** with 4-benzylpiperidine was carried out in refluxing ethanol overnight.[8] The progress of the reaction was monitored by TLC and upon completion, the solvent was removed to give the crude product. Purification by column chromatography afforded ***7*** as a yellow oil, which was then reduced to the corresponding alcohol, ***8***. Ketone reduction was carried out using two equivalents of $LiAlH_4$ in anhydrous THF at 0°C. These conditions had previously been reported to give only the *threo*-

diastereoisomer of ifenprodil, whereas $NaBH_4$ reduction often led to diastereoisomeric mixtures.[8] Upon completion of the reaction, as verified by ESI-MS, the reaction was quenched and the lithium salts removed. Compound ***8*** was isolated as a white solid in a 76% yield. Confirmation of the presence of only the *threo*-diastereoisomer was based on ^{1}H NMR analysis, by comparison to the reported literature data (*Figure S1*).[8] The proton next to the hydroxyl group resonated at 4.28 ppm, and split into a doublet through coupling to the adjacent proton, with a coupling constant of 10 Hz. If the *erythro* diastereoisomer had been present, the doublet would have been shifted to higher frequency by approximately 0.90 ppm, with a much smaller coupling constant due to the relative position of the nearest proton.[8]

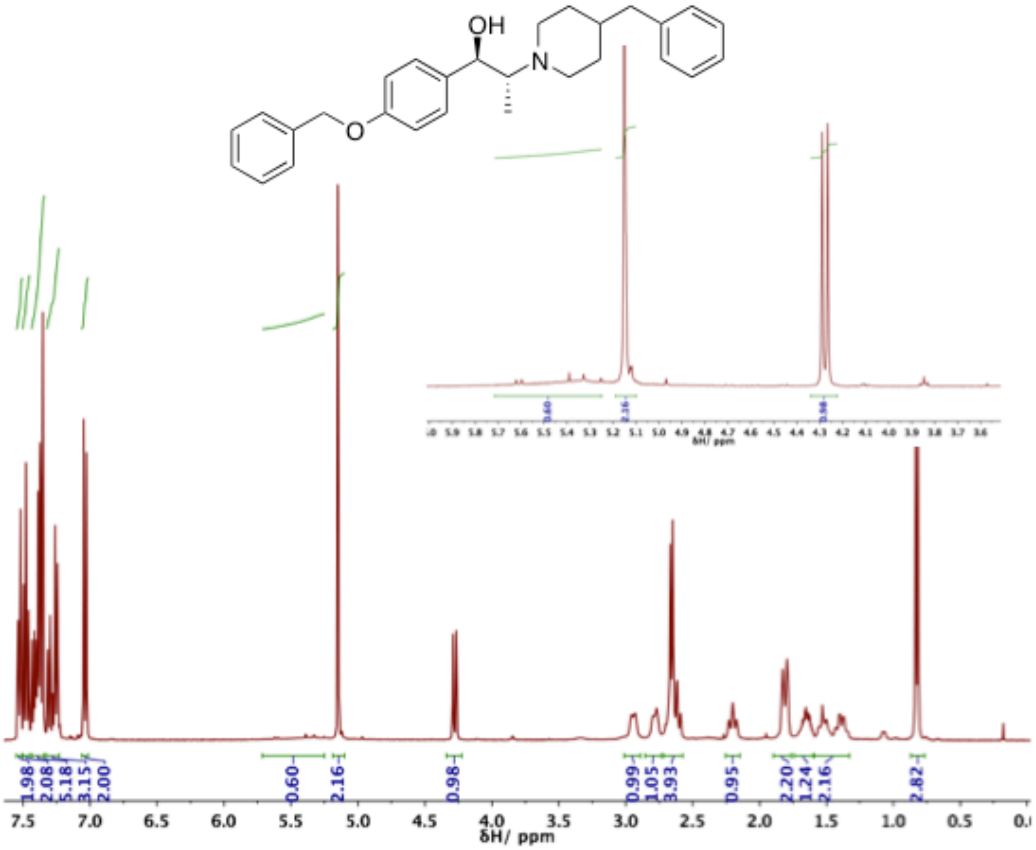
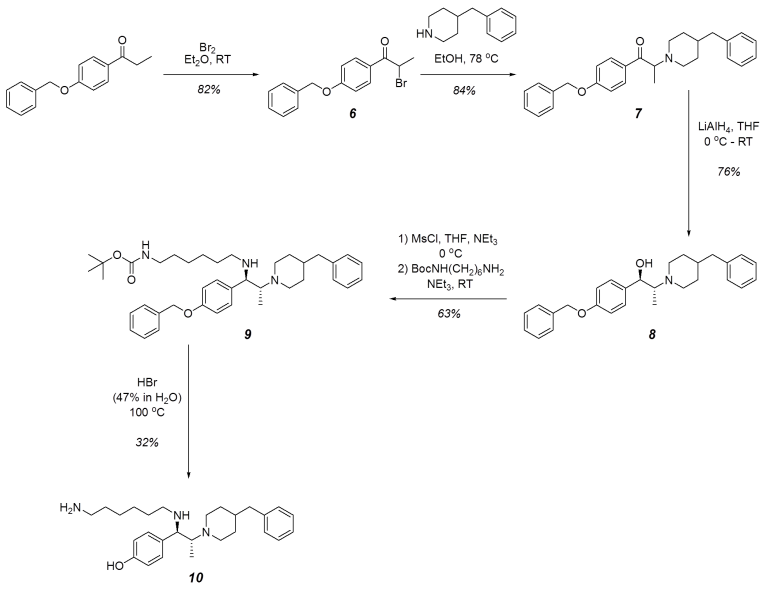


Figure S1. ^{1}H NMR spectrum of compound **8** showing the presence of only one diastereoisomer. (*Inset*) Expanded region between 3.50-6.00 ppm showing the presence of the doublet at 4.28 ppm (J = 10 Hz) assigned to the proton adjacent to the hydroxyl group in benzyl-protected *threo* ifenprodil, **8**. (Literature data for ***8***: δH 4.1-4.2 ppm, J = 8.5-10 Hz).[8]

Racemic benzyl-protected *threo*-ifenprodil, ***8***, was subjected to a stereospecific mesylation-amination sequence to form ***9***.[9] The alcohol, ***8***, was first activated towards nucleophilic substitution via *in-situ* formation of the reactive mesylate, by reaction with methanesulfonylchloride in anhydrous THF under basic conditions. The presence of the mesylate was confirmed by TLC analysis after 30 minutes. After this time, *N*-Boc-1,6-hexyldiamine and triethylamine were added and the resulting solution stirred at room

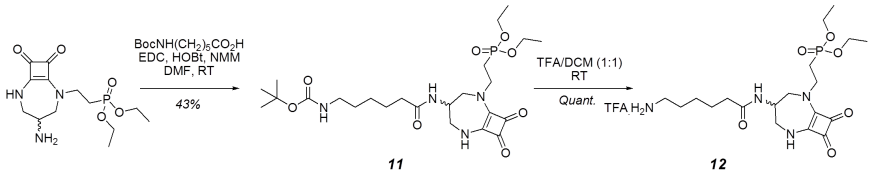
temperature overnight. Work-up and subsequent purification by column chromatography gave ***9*** in 63% yield. Confirmation of a stereospecific reaction was again obtained through analysis of the proton resonances at the asymmetric centres, using ^{1}H NMR spectrometry, similar to the conformational analysis of ***8***. Finally, compound ***9*** was subject to acidic double deprotection to remove the benzyl and carbamate protecting groups. This was achieved by stirring ***9*** in aqueous HBr at 100 °C overnight, which gave the free amine, ***10***, as a pale yellow solid after purification by column chromatography.[9]



Scheme S2.

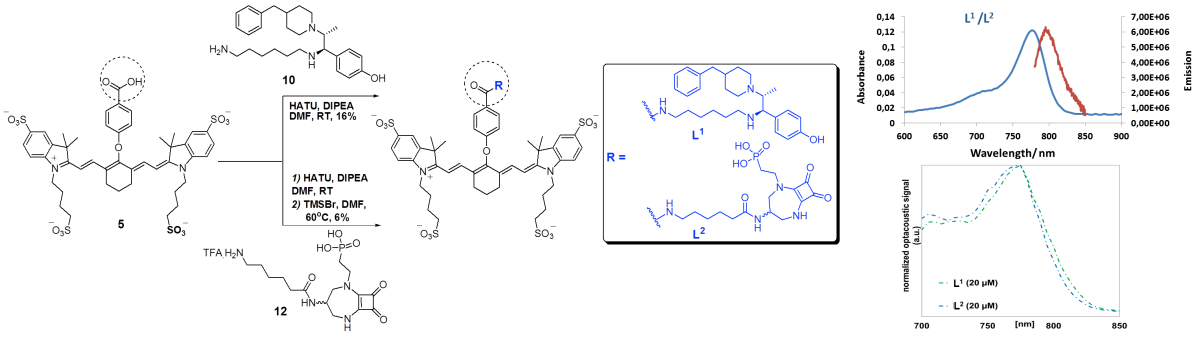
The synthesis of the NMDA receptor-binding moiety of **L**^{2} followed the sequence shown in *Scheme S3*. First, the primary amine, [Diethyl-2-(4-amino-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)ethyl)phosphonate[10]; **11]**, underwent amide bond formation with *N*-Boc-6-aminohexanoic acid to form **12**. This reaction was undertaken by stirring the acid as a solution in anhydrous DMF with EDC.HCl and HOBt for 20 minutes. After this time, a solution of the amine, and NMM was added dropwise and the reaction progress monitored by ESI-MS. Upon consumption of the starting materials after five hours, the solvent was removed to give the crude product. After work-up and purification by column chromatography, the carbamate, **12**, was isolated as a yellow oil in a 43% yield. The final step before conjugation to the heptamethine cyanine dye, **5**, involved hydrolysis of the carbamate protecting group. This was achieved using a 1:1 mixture of trifluoroacetic acid and DCM at room temperature for 30 minutes, at which point the solvent was removed under reduced pressure. Excess TFA was removed via repeated addition and removal of DCM under reduced

pressure. This yielded the TFA salt of the amine, which was used directly in the coupling to **5**, without further purification.



Scheme S3.

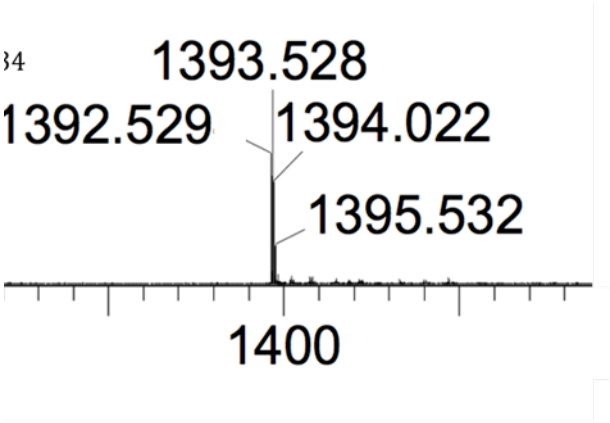
1.3 Synthesis of final NIR probes L^1 and L^2. With each component in hand, the final step in the synthesis of the NMDA receptor-targeted contrast agents, L^1 and L^2, involved conjugation of the receptor-binding moiety, **12**, to the heptamethine cyanine dye, ***5*** (*Scheme S4*). In each case, conjugation was undertaken through amide bond formation, utilising HATU as a coupling reagent in the presence of diisopropylethylamine in anhydrous DMF. The reactions were stirred at room temperature and the progress monitored by analytical RP-HPLC. Once no further reaction of the starting materials was observed, the solvent was removed and the crude residue that remained purified by preparative RP-HPLC (mobile phase comprising 25 mM triethylammonium acetate buffer and acetonitrile). In the case of L^2, one final step was carried out in which hydrolysis of the phosphonate ethyl esters occurred by reaction with an excess of bromotrimethylsilane in anhydrous DMF at 60 °C. This step was conducted prior to the final HPLC purification. Accordingly, the compounds, L^1 and L^2, were obtained as green solids.



Scheme S4. ***(Left)*** OA imaging probes for NMDAR-tagging. ***(Right)*** Absorption (blue) and emission (red) spectra of L^1/L^2 (H_2O, $\lambda_{ex}/\lambda_{em}$ = 776/801 nm, 298 K) and optoacoustic signal of L^1/L^2.

The presence of the desired conjugates was confirmed through analysis of their high-resolution mass spectra, with comparison of the theoretical and observed mass distributions of **L**[1] shown below (*Figure S2*).

L[1]



L[2]

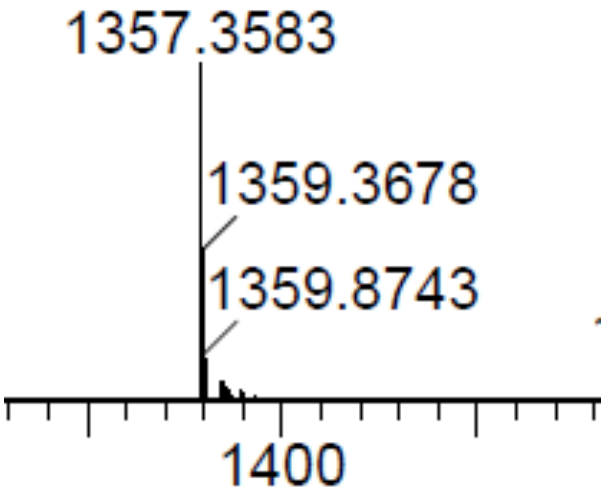


Figure S2. Observed high-resolution electrospray mass spectra of **L**[1and 2]; TOF negative mode.

2 Experimental Procedures

2.1 General Procedures

All solvents used were laboratory grade and anhydrous solvents, when required, were freshly distilled over the appropriate drying agent. Water was purified by the 'PuriteSTILLplus' system, with conductivity of ≤ 4 μS cm^{-1}. All reagents used were purchased from commercial suppliers (Acros, Aldrich, Fluka, Merck, life technology and Strem) and were used without further purification unless otherwise stated. Reactions requiring anhydrous conditions were carried out using Schlenk line techniques under an atmosphere of argon.

Thin layer chromatography was performed on neutral aluminium sheet silica gel plates (Merck Art 5554) and visualised under UV irradiation (254 nm), or using specific reagent staining. Preparative column chromatography was performed using silica gel (Merck Silica Gel 60, 230-400 mesh).

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded in commercially available deuterated solvents on a Varian Mercury-400 (^{1}H 399.960, ^{13}C 100.572), Bruker Avance-400 (^{1}H 400.052, ^{13}C 100.603 and ^{31}P 161.91), Varian Inova-500 (^{1}H 499.722, ^{13}C 125.671,), Appleby VNMRS-600 (^{1}H 599.832, ^{13}C 150.828), or Varian VNMRA-700 (^{1}H 699.731, ^{13}C 175.948 and ^{31}P 283.26) spectrometer. All chemical shifts are given in ppm and all coupling constants are three-bond, unless otherwise stated, and are reported in Hz.

Low-resolution electrospray mass spectra (LR-MS) were recorded on a Fisons VG Platform II, Waters Micromass LCT or Thermo-Finnigan LTQ FT instrument operating in positive or negative ion mode as stated, with MeOH or MeCN as the carrier solvent. High-resolution electrospray mass spectra (HR-MS) were recorded using the Thermo-Finnigan LTQ FT mass spectrometer.

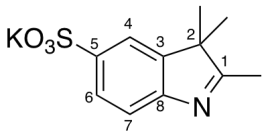
Reverse phase HPLC was performed at 298 K on a Perkin Elmer system, comprising a Perkin Elmer Series 200 pump, Perkin Elmer Series 200 auto-sampler, Perkin Elmer Series 200 UV/Vis detector and Perkin Elmer Series 200 fluorescence detector. Either an XBridge C18 column, 4.6 x 100 mm, i.d. 5 μm (flow rate 2 mL/minute) or an XBridge C18 column, 19 x 100 mm, i.d. 5 μm (flow rate 17 mL/ minute) was used with run times varying between 20 and 30 minutes. Solvent systems comprising gradient elusions of H_2O (0.1% FA)/ MeOH (0.1% FA) or 25 mM triethylammonium acetate buffer (pH 7)/ MeCN were used.

Melting points were recorded using a Gallenkamp (Sanyo) apparatus and are uncorrected.

All samples for optical analyses were contained in quartz cuvettes with a path length of 1 cm and a polished base. Measurements were recorded at 298 K. UV/Vis absorbance spectra were measured on a Perkin Elmer Lambda 900UV/Vis/NIR spectrometer using FL Winlab software. Samples were measured relative to a reference of pure solvent contained in a matched cell. Emission spectra were measured on a ISA Joblin-Yvon Spex Fluorolog-3 luminescent spectrometer using DataMax v2.20 software. Excitation wavelengths were selected according to the specific measurement. An integration time of 0.5 seconds, increment of 0.5 nm and scitation and emission slit widths of 2.5 and 1.5 nm, respectively, were used throughout. Lifetime measurements were carried out on a Perkin Elmer LS55 luminescence spectrometer.

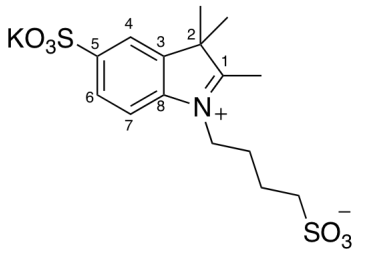
2.2 Synthesis

Potassium-2,3,3-trimethyl-3*H*-indole-5-sulfonate, 1[1]



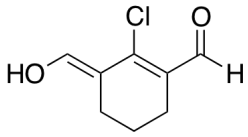
Hydrazinobenzene sulfonic acid (15.0 g, 79.5 mmol) and 3-methyl-2-butanone (25.2 mL, 240 mmol) were dissolved in acetic acid (45 mL) and the mixture heated to reflux for 3 h. After this time, the acetic acid was removed under reduced pressure and the crude residue obtained dissolved in MeOH. This was stirred with a saturated solution of potassium hydroxide in propan-2-ol. The alkaline solution turned yellow and the potassium salt of the sulfoindole (**1)** precipitated as a pale yellow solid (16.5 g, 75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.24 (6H, s, (C**H**$_3$)$_2$), 2.21 (3H, s, C**H**$_3$), 7.33 (1H, d, J = 8, **H**^{7}), 7.54 (1H, dd, J = 8, 2, **H**^{6}), 7.62 (1H, d, J = 2, H^4). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.2, 22.51 (**C**H$_3$), 53.2 (**C**(CH$_3$)$_2$), 118.1, 119.2, 125.1, 145.1, 145.2, 153.6 (Ar-**C**), 188.8 (**C**=N). MS (ES$^+$) *m/z* 240.5 [M+H]$^+$; $C_{11}H_{14}NO_3S$ requires 240.0694; found 240.0696. M.Pt > 250 °C.

Potassium 2,3,3-trimethyl-1-(4-sulfonatobutyl)-3*H*-indol-1-ium-5-sulfonate, 2[2]



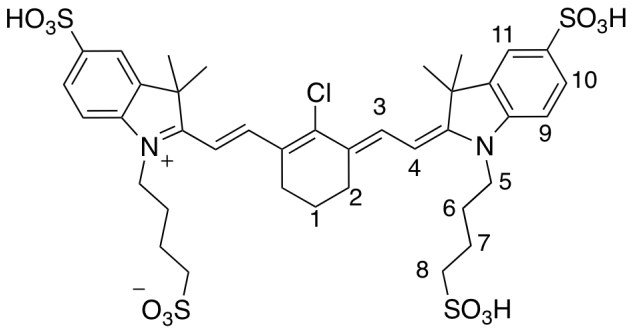
Potassium-2,3,3-trimethyl-3*H*-indole-5-sulfonate (**1**) (1.68 g, 7.0 mmol) and 1,4-butanesultone were stirred as a solution in toluene (12 mL) under an atmosphere of argon at 100 °C for 12 hours. After this period, the solvent was decanted and the residue triturated with cold propan-2-ol to yield **2** as pink powder (1.73 g, 66%). ^{1}H NMR (400 MHz, D_2O) δ 1.62 (6H, s, (C**H**$_3$)$_2$), 1.92 (2H, quin, J = 7, NCH$_2$C**H**$_2$) 2.13 (2H, quin, J = 7, C**H**$_2$CH$_2$SO$_3$), 2.99 (2H, t, J = 7, NC**H**$_2$CH$_2$), 4.56, (2H, t, J = 7, CH$_2$C**H**$_2$SO$_3$), 7.93 (1H, d, J = 8, **H**^{7}), 8.05 (1H, dd, J = 8, 2, **H**^{6}), 8.14 (1H, d, J = 2, H^4). ^{13}C NMR (101 MHz, D_2O) δ 21.4, 21.5 (**C**H$_3$), 25.8 (NCH$_2$**C**H$_2$), 47.8 (**C**H$_2$CH$_2$SO$_3$), 50.0 (N**C**H$_2$CH$_2$), 55.0 (CH$_2$**C**H$_2$SO$_3$), 115.9, 120.9, 126.9, 142.6, 142.8, 144.1 (Ar-**C**), 199.3 (**C**=N). MS (ES$^+$) *m/z* 376.2 [M+H]$^+$; $C_{15}H_{22}NO_6S_2$ requires 376.0889; found 376.0884. M.Pt > 250 °C.

(*E*)-2-Chloro-3-(hydroxymethylene)cyclohex-1-enecarbaldehyde, 3[3]



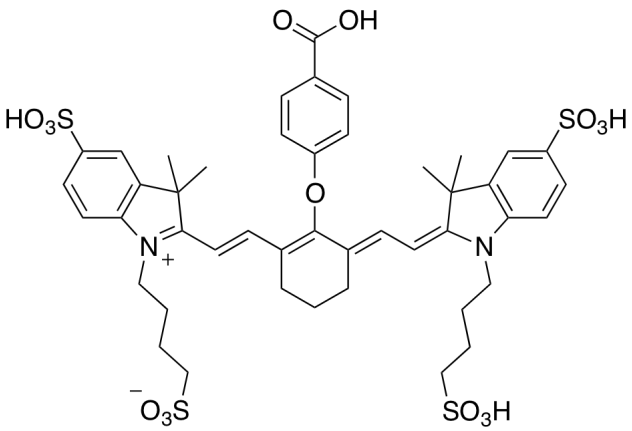
Anhydrous DMF (8 mL, 103 mmol) in anhydrous DCM (8 mL) were cooled in an ice bath to 0 °C and a solution of $POCl_3$ (7.4 mL, 79.1 mmol) in anhydrous DCM (7 mL) was added dropwise with stirring. Cyclohexanone (2.0 g, 21.5 mmol) was then added and the resulting solution heated 80 °C for 3 hours. After this time, the reaction mixture was cooled to room temperature before being poured onto ice and left overnight. The solution was decanted and the resulting yellow crystals dried under vacuum for several hours (1.58 g, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.71 (2H, quin, J = 6, CH$_2$C**H**$_2$CH$_2$), 2.46 (4H, t, J = 6, C**H**$_2$CH$_2$C**H**$_2$), 4.30-4.88 (1H, br. s, C**H**OH), 8.89 (2H, br. s, O**H** and CO**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 18.7(CH$_2$**C**H$_2$CH$_2$), 20.4, 23.8 (**C**H$_2$CH$_2$**C**H$_2$), 24.0 (**C**), 56.9 (**C**), 136.6 (**C**Cl), 190.6 (**C**OH), 197.4 (**C**O). MS (ES$^+$) *m/z* 173.3 [M+H]$^+$; $C_8H_{10}ClO_2$ requires 173.0369; found 173.0374. M. Pt. 129-131 °C.

4-(2-((*E*)-2-((*E*)-2-Chloro-3-((*E*)-2-(3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-5-sulfo-3*H*-indol-1-ium-1-yl)butane-1-sulfonate, 4[4]



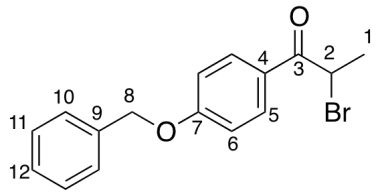
Potassium 2,3,3-trimethyl-1-(4-sulfonatobutyl)-3*H*-indol-1-ium-5-sulfonate (**2)** (40 mg, 0.11 mmol) and (*E*)-2-chloro-3-(hydroxymethylene)cyclohex-1-enecarbaldehyde (**3**) (9.2 mg, 0.05 mmol) were dissolved in a mixture of anhydrous butan-1-ol (2.8 mL) and anhydrous benzene (1.2 mL) and heated to 110 °C overnight. After this period, the solvents were removed under reduced pressure and the residue washed with diethyl ether (3 x 5 mL). This gave **4** as green powder, which was dried for several hours and used directly in the next step without further purification (47 mg, 100%). ^{1}H NMR (400 MHz, D_2O) δ 1.61 (12H, s, C**H**$_3$), 1.76-1.97 (10H, m, **H**^{1}, **H**^{6}, **H**^{7}), 2.05-2.18 (4H, m, **H**^{2}), 2.98 (4H, t, J = 7, **H**^{5}), 4.54 (4H, t, J = 7, **H**^{8}), 6.83 (2H, d, **H**^{4}), 7.51-7.61 (2H, m, **H**^{3}), 7.92 (2H, d, J = 8, **H**^{9}), 8.03 (2H, d, J = 8, **H**^{10}), 8.12 (2H, s, **H**^{11}). MS (ES^-) *m/z* 885.2 $[M-H]^-$; $C_{38}H_{46}N_2ClO_{12}S_4$ requires 885.1622; found 885.1634. (HPLC) t_R = 13.05 min; λ_{max} (H_2O) 784 nm.

4-(2-((*E*)-2-((*E*)-2-(4-carboxyphenoxy)-3-((*E*)-2-(3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-5-sulfo-3*H*-indol-1-ium-1-yl)butane-1-sulfonate, 5



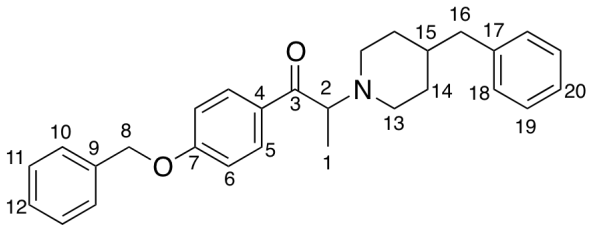
4-hydroxybenzoic acid (15 mg, 0.106 mmol) and NaH (5.1 mg, 0.212 mmol) were stirred as a solution in anhydrous DMF (700 μL) at 0 °C for 15 mins. 4-(2-((*E*)-2-((*E*)-2-chloro-3-((*E*)-2-(3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-5-sulfo-3*H*-indol-1-ium-1-yl)butane-1-sulfonate (47 mg, 0.053 mmol) was added dropwise as a solution in anhydrous DMF (1 mL) and the resulting mixture heated to 70 °C. The progress of the reaction was monitored by analytical RP-HPLC until complete conversion of the starting material was obtained [note: may take several additions of 4-hydroxybenzoic acid and NaH]. Upon completion of the reaction, the solvent was removed under reduced pressure and the crude product purified by preparative RP-HPLC to give **5** as green powder (15 mg, 29%). MS (ES^-) *m/z* 987.1 $[M-H]^-$; $C_{45}H_{51}N_2O_{15}S_4$ requires 987.2172; found 987.2167. λ_{max} (H_2O) 772 nm. RP-HPLC: t_R = 12.6 mins [2-40% MeCN in Triethylammonium Acetate Buffer (25 mM) over 20 mins].

1-(4-(Benzyloxy)phenyl)-2-bromopropan-1-one, 6[7]



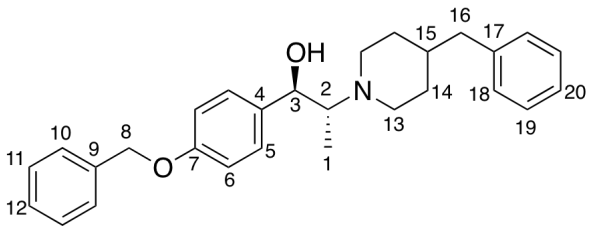
Bromine (512 μL, 10.00 mmol) was added to a solution of 1-(4-(benzyloxy)phenyl)propan-1-one (2.40 g, 10.00 mmol) in diethyl ether (25 mL). The mixture was stirred at room temperature for 30 minutes when a colour change from red to yellow was observed. At this point, the reaction was quenched by the addition of H_2O (10 mL) and the mixture diluted with Et_2O (25 mL). The organic layer was separated and washed with $NaHCO_3$ (30 mL), $Na_2S_2O_3$ (30 mL) and brine (30 mL), before drying over $MgSO_4$. Filtration and evaporation of the solvent gave the crude product, which was purified by column chromatography (DCM/Hexane, 50:50; R_f = 0.33) to give **6** an orange solid (2.61 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.89 (3H, d, J = 7, C**H**$_3$), 5.15 (2H, s, OC**H**$_2$Ph), 5.26 (2H, q, J = 7, C**H**Br), 7.03 (2H, d, J = 9, H^5), 7.33-7.46 (5H, m, Ar-**H**), 8.02 (2H, d, J = 9, H^6). ^{13}C NMR (101 MHz, $CDCl_3$) δ 20.4, (**C**H$_3$) 41.6 (**C**HBr), 70.4 (O**C**H$_2$Ph), 114.9 (**C**^{6}), 127.2 (**C**^{10}), 127.6 (**C**^{12}), 128.4 (**C**^{11}), 128.8 (**C**^{5}), 131.5 (**C**^{4}), 136.1 (**C**^{9}), 163.2 (**C**^{7}), 192.1 (**C**O). MS (ES^+) *m/z* 319.1 $[M+H]^+$; $C_{16}H_{16}{}^{79}BrO_2$ requires 319.0337; found 319.0336. M. Pt. 76-78 °C.

1-(4-(Benzyloxy)phenyl)-2-(4-benzylpiperidin-1-yl)propan-1-one, 7[8]



4'- (benzyloxy)-2-bromopropiophenone (**6**) (2.52 g, 7.91 mmol) and 4-benzyl piperidine (2.78 mL, 15.82 mmol) were stirred as a solution in anhydrous ethanol (11 mL) at 78 °C overnight. Upon no further reaction as observed by TLC, the reaction was concentrated and the white solid that remained triturated with Et_2O. The solid was filtered and the filtrate concentrated and purified by column chromatography (Hexane/EtOAc, 50:50; R_f = 0.53) to give **7** as yellow liquid (2.78 g, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (3H, d, J = 7, C**H**$_3$), 1.28-1.31 (2H, m, **H**^{14}), 1.45-1.55 (1H, m, **H**^{15}), 1.56-1.68 (2H, m, **H**$^{14'}$), 2.07-2.16 (1H, m, **H**^{13}), 2.34-2.43 (1H, m, **H**^{13}), 2.52 (2H, d, J = 7, C**H**$_2$Ph), 2.78-2.86 (1H, m, **H**^{13}), 2.91-2.98 (1H, m, **H**^{13}), 4.07 (1H, q, J = 7, C**H**CH$_3$), 5.15 (2H, s, OC**H**$_2$Ph), 7.01 (2H, d, J = 9, H^5), 7.11-7.48 (10H, m, Ar-**H**), 8.13 (2H, d, J = 9, H^6). ^{13}C NMR (101 MHz, $CDCl_3$) δ 11.9 (**C**H$_3$), 32.5, 32.9 (**C**^{14}), 38.1 (**C**^{15}), 43.3 (C^{16}), 48.8, 51.8 (**C**^{13}), 64.9 (O**C**H$_2$Ph), 70.2 (**C**HCH$_3$), 114.4 (**C**^{6}), 125.8 (**C**^{20}), 127.6 (**C**^{10}), 128.2 (**C**^{12}), 128.3 (**C**^{18}), 128.8 (**C**^{19}), 129.2 (**C**^{11}), 129.7 (**C**^{5}), 131.4 (**C**^{4}), 136.4 (**C**^{9}), 140.8 (**C**^{17}), 162.5 (**C**^{7}), 199.6 (**C**O). MS (ES$^+$) *m/z* 414.5 [M+H]$^+$; $C_{28}H_{32}NO_2$ requires 414.2433; found 414.2440.

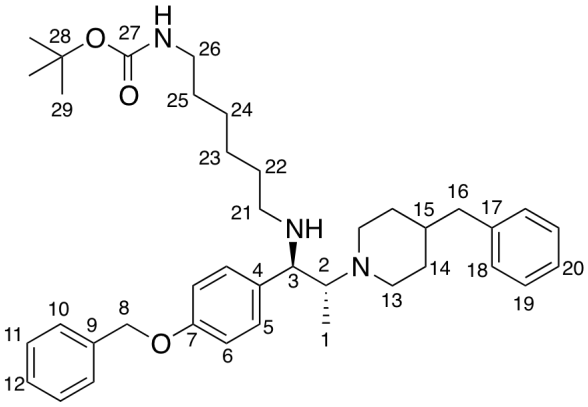
(1*R*,2*R*)-1-(4-(Benzyloxy)phenyl)-2-(4-benzylpiperidin-1-yl)propan-1-ol, 8[8]



A suspension of $LiAlH_4$ (241 mg, 6.34 mmol) in anhydrous THF (10 mL) was treated with a solution of 1-(4-(Benzyloxy)phenyl)-2-(4-benzylpiperidin-1-yl)propan-1-one (**7**) (1.31 g, 3.17 mmol) in anhydrous THF (10 mL) at 0 °C over a 10 minute period. The resulting mixture was stirred for a further 2 hours and the reaction progress monitored by ESI-MS. Upon complete reduction, the reaction was quenched by the addition of H_2O (300 µL), NaOH (300 µL, 1M) and finally H_2O (300 µL). The lithium salts were filtered and the solvent reduced to give **8** as white solid (1.00 g, 76%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.77 (3H, d, J = 7, C**H**$_3$), 1.26-1.53 (2H, m, **H**^{14}), 1.54-1.64 (1H, m, **H**^{15}), 1.70-1.80 (2H, m, **H**^{14}), 2.09-2.19 (1H, m, **H**^{13}), 2.50-

2.65 (2H, m, $\mathbf{H}^{2}$ + $\mathbf{H}^{13}$), 2.61 (2H, d, J = 7, C$\mathbf{H}_2$Ph), 2.68-2.77 (1H, m, $\mathbf{H}^{13}$), 2.84-2.92 (1H, m, $\mathbf{H}^{13}$), 4.28 (1H, d, J = 10, $\mathbf{H}^{3}$), 5.09 (2H, s, OC$\mathbf{H}_2$Ph), 5.20-5.47 (1H, br. s, O**H**), 6.98 (2H, d, J = 9, H^{5}), 7.17-7.49 (12H, m, Ar-**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 8.1 (**C**H_3), 32.6, 33.1 (**C**^{14}), 38.4 (**C**^{15}), 43.3 (**C**H_2Ph), 44.5, 53.0 (**C**^{13}), 66.8 (O**C**H_2Ph), 70.2 (**C**HCH$_3$), 73.9 (**C**HOH), 114.8 (**C**^{6}), 126.0 (**C**^{20}), 127.6 (**C**^{10}), 128.0 (**C**^{12}), 128.3 (**C**^{5}), 128.6 (**C**^{18}), 128.7 (**C**^{19}), 129.2 (**C**^{11}), 134.6 (**C**^{4}), 137.2 (**C**^{9}), 140.7 (**C**^{17}), 158.5 (**C**^{7}). MS (ES^+) *m/z* 416.2 $[M+H]^+$; $C_{28}H_{34}NO_2$ requires 416.2590; found 416.2589. M. Pt. 117-119 °C.

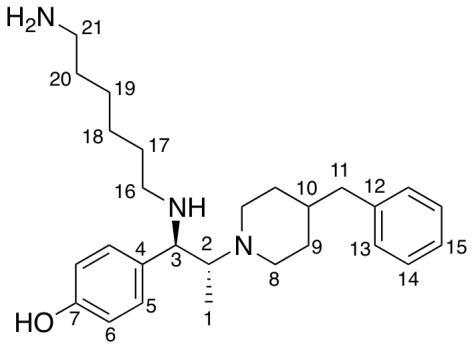
tert-Butyl (6-(((1*R*,2*R*)-1-(4-(benzyloxy)phenyl)-2-(4-benzylpiperidin-1-yl)propyl)amino)hexyl)carbamate, 9[9]



To a stirred solution of *O*-benzylated (*threo*)-ifenprodil (247 mg, 0.595 mmol) in anhydrous THF (7 mL) at 0 °C, was added triethylamine (249 μL, 1.79 mmol) and methansulfonyl chloride (116 μL, 1.50 mmol). The resulting mixture was stirred for 30 mins until complete reaction was confirmed by TLC. To the mesylate was added triethylamine (166 μL, 1.19 mmol) and *N*-Boc-1,6-hexyldiamine (322 mg, 1.49 mmol) and the resulting mixture stirred at room temperature for 24 hours. Upon completion of the reaction as verified by ESI-MS, the reaction mixture was partitioned between DCM (30 mL) and H_2O (30 mL). The layers were separated and the aqueous extracted with DCM (3 x 30 mL). The combined organic portions were washed with sat. $NaHCO_3$ (50 mL) and brine (50 mL), before being dried over $MgSO_4$ and filtered. The solvent was concentrated under reduced pressure and the crude residue purified by column chromatography (DCM/MeOH, 100 to 95:5; R_f = 0.31) to give **9** as white solid (231 mg, 63%). ^{1}H NMR (700 MHz, $CDCl_3$) δ 0.61 (3H, d, J = 7, C$\mathbf{H}_3$), 1.13-1.33 (4H, m, $\mathbf{H}^{14}$ + $\mathbf{H}^{24}$), 1.37-1.56 (6H, m, $\mathbf{H}^{14}$ + $\mathbf{H}^{25}$ + $\mathbf{H}^{23}$), 1.44 (9H, s, C(C$\mathbf{H}_3$)$_3$), 1.52 (1H, m, $\mathbf{H}^{15}$), 1.63-1.73 (2H, m, $\mathbf{H}^{22}$), 2.04-2.35 (2H, m, $\mathbf{H}^{21}$), 2.39-2.47 (2H, m, $\mathbf{H}^{13}$), 2.55 (2H, d, J = 7, C$\mathbf{H}_2$Ph), 2.66-2.78 (2H, m, $\mathbf{H}^{13}$), 3.07-3.12 (2H, m, $\mathbf{H}^{26}$), 3.32 (1H, d, J = 9, $\mathbf{H}^{3}$), 4.50 (1H, br.m, $\mathbf{H}^{2}$), 5.04 (2H, s, OC$\mathbf{H}_2$Ph), 6.93 (2H, d, J = 9, $\mathbf{H}^{6}$), 7.15 (2H, d, J = 9, $\mathbf{H}^{5}$), 7.19 (1H,

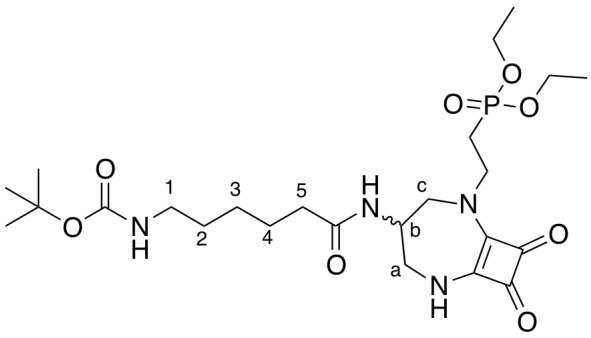
dd, J = 8, 5, **H**^{20}), 7.23-7.30 (4H, m, **H**^{18} +**H**^{19}), 7.32 (1H, dd, J = 8, 5, **H**^{12}), 7.38 (2H, t, J = 8, **H**^{11}), 7.44 (2H, d, J = 8, **H**^{10}). ^{13}C NMR (176 MHz, $CDCl_3$) δ 9.0 (**C**H_3), 26.8 (**C**^{24}), 27.1 (**C**^{25}), 27.2 (**C**^{23}), 28.6 (C(**C**$H_3)_3$), 30.2 (**C**^{22}), 33.1, 33.2 (**C**^{14}), 38.6 (**C**^{15}), 40.7(**C**^{26}), 43.5 (**C**H_2Ph), 44.5, 47.1 (**C**^{13}), 52.9(**C**^{21}), 63.8 (**C**HCH_3), 65.7 (**C**HNH), 70.2 (O**C**H_2Ph), 79.2 (**C**$(CH_3)_3$), 114.7 (**C**^{6}), 125.9 (**C**^{20}), 127.7 (**C**^{10}), 128.1 (**C**^{12}), 128.3 (**C**^{5}), 128.7 (**C**^{18}), 129.2 (**C**^{19}), 129.6 (**C**^{11}), 133.9 (**C**^{4}), 137.3 (**C**^{9}), 140.9 (**C**^{17}), 156.1 (**C**O), 158.2 (**C**^{7}). MS (ES^+) *m/z* 614.0 $[M+H]^+$; $C_{39}H_{56}N_3O_3$ requires 614.4322; found 614.4327. M. Pt. 97-99 °C.

N^1-((1*R*,2*R*)-1-(4-(Benzyloxy)phenyl)-2-(4-benzylpiperidin-1-yl)propyl)hexane-1,6-diamine, 10[9]



To a solution of *tert*-butyl (6-(((1*R*,2*R*)-1-(4-(benzyloxy)phenyl)-2-(4-benzylpiperidin-1-yl)propyl)amino)hexyl)carbamate (**9**) (109 mg, 0.180 mmol) in acetic acid (3.3 mL), was added HBr (47% in H_2O, 0.3 mL) and the solution heated to 100 °C overnight. After cooling to room temperature, aqueous NaOH (3M) was added until pH 12. The aqueous layer was extracted with $CHCl_3$ (3 x 20 mL) and the organic dried over $MgSO_4$, filtered and concentrated. The crude residue was then purified by column chromatography (DCM/MeOH, 100 to 85:15; R_f = 0.12) to give **10** as pale yellow solid (24 mg, 32%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.59 (3H, d, J = 7, C**H**$_3$), 1.22-1.70 (13H, m, **H**^{9} + **H**^{10} + **H**^{17} + **H**^{18} + **H**^{19} + **H**^{20}), 2.05 (1H, m, **H**^{8}), 2.29-2.48 (3H, m, **H**^{8}), 2.56 (2H, d, J = 7, C**H**$_2$Ph), 2.63-2.75 (5H, m, **H**^{2} + **H**^{16} + **H**^{21}), 3.28 (1H, d, J = 10, **H**^{3}), 3.52-3.89 (4H, br. m, N**H**$_2$ + N**H** + O**H**), 6.71 (2H, d, J = 9, **H**^{6}), 7.09-7.13 (2H, m, **H**^{5}), 7.14-7.22 (3H, m, **H**^{13} + **H**^{15}), 7.25-7.29 (2H, m, **H**^{14}). ^{13}C NMR (101 MHz, $CDCl_3$) δ 8.9 (**C**H_3), 26.7 (**C**^{18}), 27.2 (**C**^{19}), 29.5 (**C**^{17}), 33.1 (**C**^{20}), 33.3, 33.4 (**C**^{9}), 38.6 (**C**^{10}), 42.0 (**C**^{21}), 43.5 (**C**H_2Ph), 44.4, 47.3 (**C**^{8}), 52.9 (**C**^{16}), 64.1 (**C**HCH_3), 65.7 (**C**HNH), 115.7 (**C**^{6}), 125.9 (**C**^{15}), 128.3 (**C**^{13}), 129.2 (**C**^{5}), 129.6 (**C**^{14}), 132.4 (**C**^{4}), 141.0 (**C**^{12}), 156.6 (**C**^{7}). MS (ES^+) *m/z* 424.1 $[M+H]^+$; $C_{27}H_{42}N_3O$ requires 424.3328; found 424.3330. M. Pt. 56-58 °C.

tert-Butyl (6-((2-(2-(diethoxyphosphoryl)ethyl)-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-4-yl)amino)-6-oxohexyl)carbamate, 11



N-Boc-6-aminohexanoic acid (52 mg, 0.22 mmol), EDC (51 mg, 0.264 mmol) and HOBt (36 mg, 0.264 mmol) were dissolved in anhydrous DMF (2 mL) and stirred at room temperature under an atmosphere of argon for 20 minutes. After this period, a pre-stirred solution of diethyl (2-(4-amino-8,9-dioxo-2,6-diazabicyclo[5.2.0]non-1(7)-en-2-yl)ethyl)phosphonate[10] (74 mg, 0.22 mmol) and NMM (48 μL, 0.44 mmol) in anhydrous DMF (1.5 mL) was added drop wise and the resulting solution stirred at room temperature until complete consumption of the starting materials by ESI-MS. After this period, the solvent was removed under reduced pressure and the crude oil taken up into EtOAc (15 mL). $NaHCO_3$ (15 mL) was added, the layers separated and the aqueous fraction washed with EtOAc (3 x 20 mL). The combined organic portions were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH, 100% to 90:10 in 1% increments; R_f =0.25) to give **11** as yellow oil (52 mg, 43%). ^{1}H NMR (700 MHz, $CDCl_3$) δ 1.23-1.29 (2H, m, **H**^{3}), 1.31 (6H, 2 x t, J = 7, P(OCH$_2$C**H**$_3$)$_2$), 1.40 (9H, s, C(C**H**$_3$)$_3$), 1.42-1.44 (2H, m, **H**^{4}), 1.56-1.63 (2H, m, **H**^{2}), 2.09-2.24 (2H, m, PC**H**$_2$CH$_2$), 2.25-2.34 (2H, m, **H**^{5}), 3.00-3.14 (2H, m, **H**^{1}), 3.20-3.38 (1H, m, **H**^{c}(axial)), 3.51 (1H, d, J = 14, **H**$^{c/a}$(eq)), 3-60-3.73 (2H, m, PCH$_2$C**H**$_2$), 3.87-3.95 (1H, br. m, **H**$^{c/a}$(eq)), 4.02-4.13 (4H, qd, $^3J_{H\text{-}H}$ = 7, $^3J_{H\text{-}P}$ = 3, P(OC**H**$_2$CH$_3$)$_2$), 4.23-4.39 (2H, m, **H**^{a}(axial) + N**H**), 4.82-4.87 (1H, br. m, **H**^{b}), 7.58 (1H, br. N**H**), 8.01 (1H, br. N**H**). ^{13}C NMR (176 MHz, $CDCl_3$) δ 16.5 (2 x d, ^{3}J = 6, P(OCH$_2$**C**H$_3$)$_2$), 24.7 (d, 1J = 140, P**C**H$_2$CH$_2$), 25.4 (**C**^{3}), 26.4 (**C**^{4}), 28.6 (C(**C**H$_3$)$_3$), 29.8 (**C**^{2}), 35.7 (**C**^{5}), 40.5 (**C**^{1}), 48.6 (**C**^{c}), 49.4, (d, ^{2}J = 16, PCH$_2$**C**H$_2$), 55.3 (**C**^{a}), 62.1 (d, ^{2}J = 7, P(O**C**H$_2$CH$_3$)$_2$), 62.6 (**C**H), 79.1 (**C**(CH$_3$)$_3$), 156.2 (**C**O), 167.6, 168.6 (**C**=**C**), 174.6, 180.6, 182.2 (**C**O). ^{31}P NMR (162 MHZ, $CDCl_3$) δ 27.24. MS (ES$^+$) *m/z* 545.0 $[M+H]^+$; $C_{24}H_{42}N_4O_8P$ requires 545.2740; found 545.2745.

The Boc-group was subsequently hydrolysed in a solution of DCM/TFA (1:1, 2 mL) to give the TFA-salt of the amine (***12***), which was used directly in the coupling step without further purification. MS (ES^+) *m/z* 446.0 $[M+H]^+$.

Synthesis of L^1: 4-(2-((*E*)-2-((*E*)-2-(4-carboxyphenoxy)-3-((*E*)-2-(3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-5-sulfo-3*H*-indol-1-ium-1-yl)butane-1-sulfonate (6.5 mg, 0.0066 mmol) and HATU (3.3 mg, 0.0085 mmol) were dissolved in anhydrous DMF (500 µL) and stirred under argon at room temperature for 10 mins. N^1-((1*R*,2*R*)-1-(4-(Benzyloxy)phenyl)-2-(4-benzylpiperidin-1-yl)propyl)hexane-1,6-diamine (2.78 mg, 0.0066 mmol) and diisopropylethylamine (2.3 µL, 0.013 mmol) were then added as a solution in anhydrous DMF (300 µL) and the resulting mixture stirred at room temperature overnight. After this period a second addition of the amine and coupling reagent were added and stirring continued for a further 5 hours. After no further reaction was observed by analytical RP-HPLC, the solvent was removed under reduced pressure and the crude residue purified by preparative RP-HPLC to give $\mathbf{L^1}$ as light green solid (1.5 mg, 16%). MS (ES^-) *m/z* 1392.5 $[M-H]^-$; $C_{72}H_{90}N_5O_{15}S_4$ requires 1392.532; found 1392.529. λ_{max} (H_2O) 776 nm. RP-HPLC: t_R = 18.3 mins [2-40% MeCN in Triethylammonium Acetate Buffer (25 mM) over 20 mins].

Synthesis of L^2: 4-(2-((*E*)-2-((*E*)-2-(4-carboxyphenoxy)-3-((*E*)-2-(3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-5-sulfo-3*H*-indol-1-ium-1-yl)butane-1-sulfonate (8.2 mg, 0.0083 mmol) and HATU (4.1 mg, 0.011 mmol) were dissolved in anhydrous DMF (600 µL) and stirred under argon at room temperature for 10 mins. The TFA salt of the amine, ***12*** (4.6 mg, 0.0083 mmol) and diisopropylethylamine (2.9 µL, 0.017 mmol) were then added as a solution in anhydrous DMF (400 µL) and the resulting mixture stirred at room temperature overnight. After this period a second addition of the amine and coupling reagent were added and stirring continued for a further 5 hours. After no further reaction was observed by analytical RP-HPLC, the solvent was removed under reduced pressure and the crude residue purified by preparative RP-HPLC to give the phosphonate ethyl ester as a light green solid. The phosphonate ethyl ester (1.7 mg, 0.0012 mmol) was dissolved in anhydrous DMF (300 µL) to which bromotrimethylsilane (1.2 µL, 0.009 mmol) was added. The resulting solution was stirred under argon at 60 °C and the reaction monitored by analytical RP-HPLC. Upon completion after 5 hours, the solvent was removed under reduced pressure and the crude residue purified

by preparative RP-HPLC to give **L²** as green powder (0.65 mg, 6% over two steps). MS (ES^-) *m/z* 1357.3 $[M-H]^-$; $C_{60}H_{74}N_6O_{20}S_4P$ requires 1357.359; found 1357.358. λ_{max} (H_2O) 776 nm. RP-HPLC: t_R = 17.0 mins [2-40% MeCN in Triethylammonium Acetate Buffer (25 mM) over 20 mins].

2.3 Cell culture

In short, the NSC-34 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's (DMEM) and F12 medium supplemented with fetal bovine serum (FBS, 10% v/v), non essential amino acids (1%), sodium glutamate (1%) and Penicillin/Streptomycin (0.1%). At approximately 90% confluence after 3 healthy passages, cells were sub-cultured into a different growth medium, which contained a mixture of DMEM/Ham's F12 (1:1), altering the FBS content to 1% . Cells were allowed to proliferate over a period of several days to allow for the growth of functional NMDA receptors.

NIH-3T3 cells were maintained in growth phase as monolayers in an appropriate medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS), sodium pyruvate and glucose (5%) and 0.5% penicillin and streptomycin. All cell lines were incubated at 37 °C, 90% average humidity and 5% (v/v) CO_2.

2.4 Cytotoxicity

IC_{50} values were determined using the MTT assay, which makes use of the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product by the mitochondrial dehydrogenase of viable cells. This insoluble formazan was quantified spectrophotometrically upon dissolution in DMSO. Approximately 5×10^3 differentiated NSC-34 cells in 100 μL culture medium were seeded into each well of flat-bottomed 96-well plates and allowed to attach overnight. Complex solutions were added to triplicate wells to give final concentrations over a 2-log range. Following 24 h incubation, MTT (1.0 mM) was added to each well, and the plates incubated for a further 4 h. The culture medium was removed, and DMSO (150 μL) was added. The plates were shaken for 20 seconds and the absorbance measured immediately at 540 nm in a microplate reader.

2.5 Epi-fluorescence microscopy and image analysis.

In first microscopic method, fluorescence images were recorded using a Leica DMI3000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC360 FX camera. To record live cell fluorescence of treated cells a filter set with

excitation 750 nm and emission 800 nm was used. Images were analyzed using the integrated software from Leica.

Second cell microscopy imaging of **L^1** and **L^2** in cells was achieved using a custom built epi-florescence microscope (modified Zeiss Axiovert 200M)[10], using a Zeiss APOCHROMAT 63x/1.40 NA objective combined with a low voltage 365 nm pulsed UV LED focused, collimated excitation source (1.2W). For rapid spectral acquisition the microscope was equipped at the X1 port with a Peltier cooled 2D-CCD detector (Ocean Optics) used in an inverse 100 Hz time gated sequence. The spectrum was recorded from 400-800 nm with a resolution of 0.24 nm and the final spectrum was acquired using an averaged 10,000 scan duty cycle. Probe lifetimes were measured on the same microscope platform using a novel cooled PMT detector (Hamamatsu H7155) interchangeable on the X1 port. Both the control and detection algorithm were written in LabView2011. Time gated images were recorded using a high resolution cooled EOS CCD camera (Thor labs). All duty cycle and gating sequence were established and controlled by dedicated custom developed LabView software.

2.6 Multi-spectral optoacoustic imaging. In short, a tunable optical parametric oscillator pumped by an Nd:YAG laser allows excitation from 700 nm to 900 nm. Laser pulse duration is below 10 ns and a pulse repetition frequency of 10 Hz was used. The beam was coupled into a custom fiber bundle that is divided into 10 output arms, allowing even illumination around the circumference of the mouse with a ring-shape pattern. A custom-made piezo composite ultrasonic cylindrically focused transducer array with 256 elements and a central frequency of 5 MHz was used for detection. Transducer array, fiber bundle outputs and sample holder were submerged in a water bath maintained at 34°C. Samples were placed in a horizontal position inside a thin polyethylene membrane without direct contact with water. This arrangement allowed a convenient acoustic coupling between the sample and the transducer array.

Differentiated NSC-34 cells were grown to confluency and trypsinised with Trypsin/EDTA 0.05/0.02% (w/v), centrifuged and re-suspended to 2.5 x 10^5 cells/ 250 µL in incubation buffer (HBSS supplemented with 20mM HEPES, pH 7.2) in 750 µL Eppendorf tubes. A maximum of 20 µL of highly concentrated stock solutions of either L^1 or L^2 were added to give solutions with the desired concentrations. Cells were incubated for 20 minutes at 37 °C and 5% CO_2. Afterwards, cells were centrifuged (300×g, 5 min., RT) and the supernatant was kept for MSOT-measurements. Cells were washed once with HBSS, centrifuged again

(300×g, 5 min., RT) and re-suspended carefully in 250 µL of HBSS. The cell-solution was then embedded in an agar phantom for MSOT-imaging. In short, the labeled cells were enclosed within a 3 mm diameter plastic tube embedded into an 2 cm-diameter cylindrical phantom made of 1.3% agar and 1.2% by volume of Intralipid emulsion (Sigma-Aldrich). This mix leads to an optically diffusive medium with acoustic properties similar those of tissue. MSOT imaging was then done in one cross-sectional imaging plane (~200 µm in plane resolution). The phantom images were reconstructed using the interpolated model-matrix inversion.[18] Afterwards, linear spectral unmixing was applied to each set of multiwavelengths images to resolve absorber specific signals. For each pixel the total measured optoacoustic spectrum was fitted to the known spectra of the contrast agent.

2.7 *In vivo* intracranial injection and cryoslicer-imaging.

To verify the visualization capacity of MSOT in the brain, a mouse was injectecd intracranially under isoflurane-anesthesia with 1µL of a 3.6 mM solution of **L[1]** and **5** into the left and right side of the brain, respectively. The injection coordinates were, given from Bregma according to Paxinos and Franklin[11] anterior 1.10 mm and 1.75 mm left or right. The depth of the injection was ~2.00 mm, placing the injection inside the motor cortex, which is known for a high expression of the NMDA receptor[12]. For the overall injection procedure a motorized stereotaxic drill and injection robot equipped with a software interface that utilizes a stereotactic mouse brain atlas (based on reference 11) was used (Neurostar, Germany). Directly after the injection, the mice were transferred to the same pre-clinical small animal MSOT scanner and multispectral optoacoustic data was recorded and analyzed as described above. Furthermore, ex vivo cryoslicer-imaging[13] was used to validate the presence of **L[1]** in the brain.

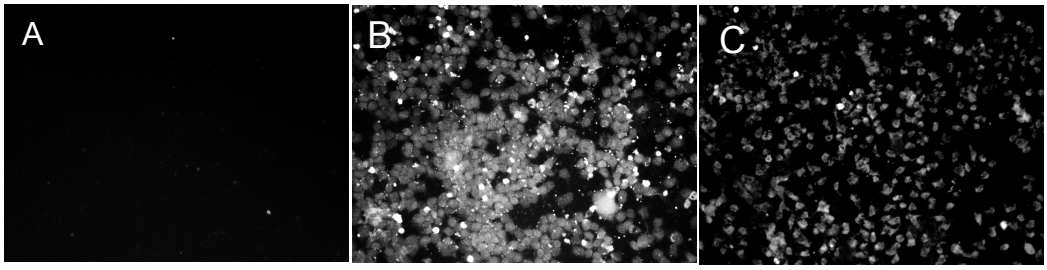


Figure S3. Live cell fluorescence microscopy images of differentiated NSC-34 cells ($\lambda_{ex}/\lambda_{em}$ = 780/800 nm). (A) Treatment with compound ***5*** (10 μM, 30 minutes). (B) Treatment with $\mathbf{L^1}$ (10 μM, 30 minutes); (C) Treatment with $\mathbf{L^2}$ (10 μM, 30 minutes). ($\lambda_{ex}/\lambda_{em}$= 750/800 nm).

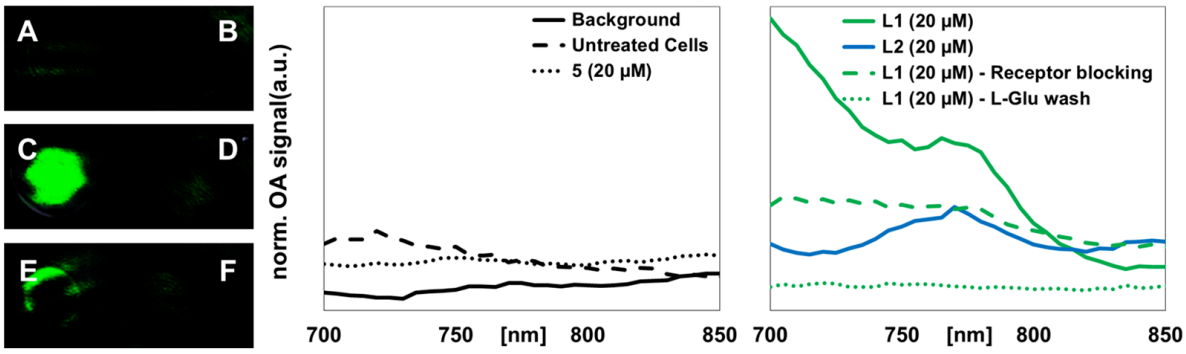


Figure S4 (Detailed figure of Figure 3). Live cell OA signal of differentiated NSC-34 cells following treatment with **5**, $\mathbf{L^1}$ or $\mathbf{L^2}$ (each 20 µM, 30 min, 37°C and 5% CO_2). **(Left)** ***A)*** Untreated cells; ***B)*** Cells treated with control dye, **5**, no OA signal; ***C)*** Cells treated with $\mathbf{L^1}$, strong OA signal; ***D)*** Cells treated with $\mathbf{L^2}$, faint OA signal; ***E)*** Cells treated with the $\mathbf{L^1}$ corresponding antagonist, Ifenprodil (40 µM, 10 min), followed by treatment with $\mathbf{L^1}$, OA signal decreased vs. ***C***. ***F)*** Cells treated with L^1 and washed with Glu (2X, 1 mM), strong reduction in OA signal. **(Right)** Absorbance spectrum of **5**, $\mathbf{L^1}$ and $\mathbf{L^2}$ that are fed into the unmixing algorithm. Wavelength-dependent analysis of NSC-34 labelled with **5**, $\mathbf{L^1}$ or $\mathbf{L^2}$.

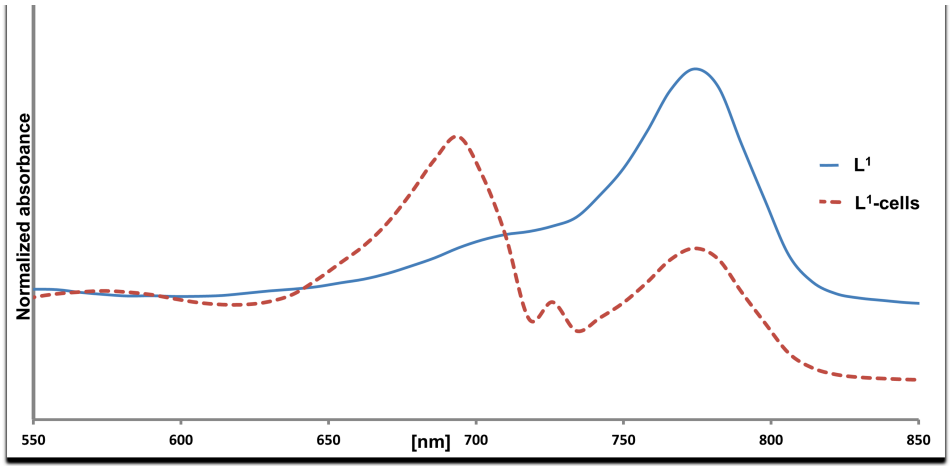


Figure S5. Normalized absorbance spectra of **L**[1] (1 μM) and **L**[1] (1 μM) labelled on cells for 30 min (2X HBSS wash).

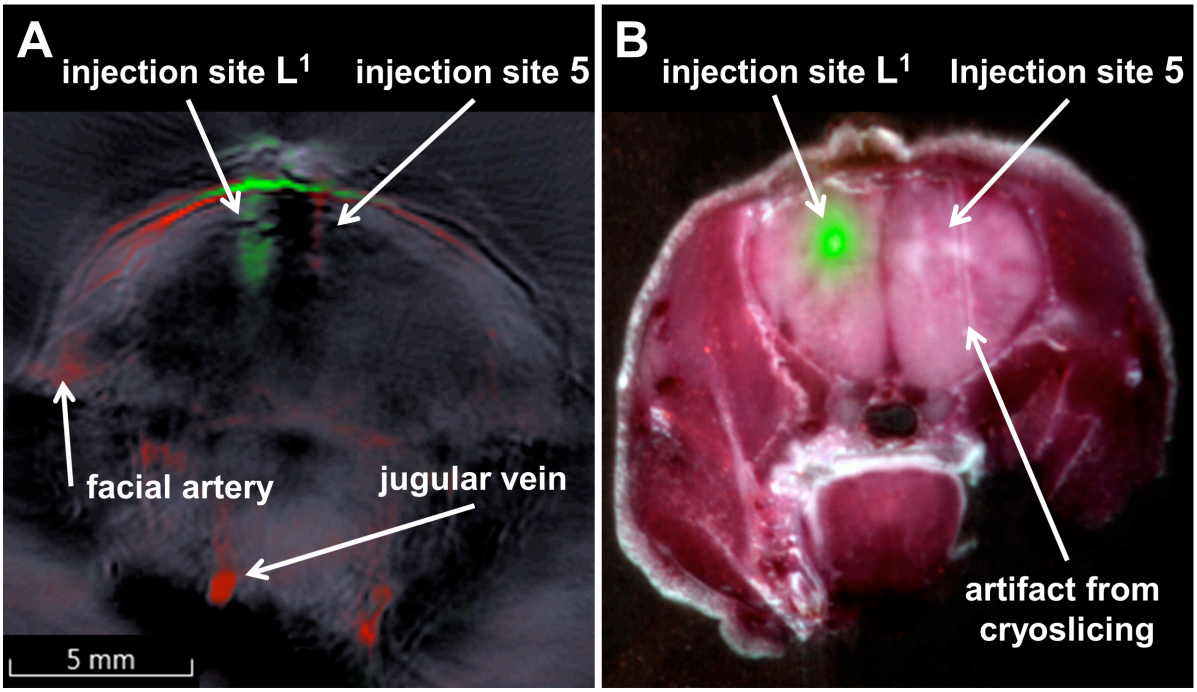


Figure S6. In vivo MSOT and ex vivo cryoslicer-imaging after intracranial injection of **L**[1] and **5**. (A) In vivo MSOT cross-sectional image of a mouse head ~30 min. after intracranial injection of 3.6 nmol of **L**[1] (left side, green) and 3.6 nmol of **5** (right side, not visualized). The unmixed data (green: probe signal and red: signal from oxygenized hemoglobin) was overlayed onto an anatomical image showing the signal from measurements at the wavelength 800 nm. The probe signal on top of the injection site stems from leakage of the probe after the injection. (B) Ex vivo cryoslicer-imaging clearly validates the presence of the fluorescent probe **L**[1] inside the brain (green). Absence of the likewise fluorescent molecule **5** can be explained by flushing away of the non-binding **5** via blood flow. (Note: the two streaks that look like an injection site are in fact an artifact from cryoslicing, the actual injection site of **5** is indicated).

References

1. Lopalco M., Koini E. N., Cho J. K., Bradley M. Org. & Biomolecular Chem. **2009**, *7*, 856-859.
2. Mujumdar R. B., Ernst L. A., Mujumdar S. R., Lewis C. J., Waggoner A. S., Bioconjug. Chem. **1993**, *4*, 105-111.
3. Reynolds G. A., Drexhage K. H. The J. of Org. Chem. **1977**, *42*, 885-888.
4. Hilderbrand S. A., Kelly K. A., Weissleder R., Tung C.-H., Bioconjug. Chem. **2005**, *16*, 1275-1281.
5. Strekowski L., Lipowska M., Patonay G. The J.of Org. Chem. **1992**, *57*, 4578-4580.
6. Li Q., Tan J., Peng B.-X. Molecules, **1997**, *2*, 91-98.
7. Lou S., Fu G. C. J. Am. Chem. Soc. **2010**, *132*, 1264-1266.
8. Chenard B. L., Shalaby I. A., Koe B. K., Ronau R. T., Butler T. W., Prochniak M. A., Schmidt A. W., Fox C. B. J. Med. Chem. **1991**, *34*, 3085-3090.
9. Marchand P., Becerril-Ortega J., Mony L., Bouteiller C., Paoletti P., Nicole O., Barré L., Buisson A., Perrio C. Bioconjug. Chem. **2011**, *23*, 21-26.
10. Pal R., Beeby A. Methods Appl. Fluoresc. **2014,** *2*, 037001.
11. Paxinos G, Franklin KBJ. The Mouse Brain in Stereotaxic Coordinates. 2. San Diego, CA: Academic; 2001.
12. Newell KA, Karl T, Huang XF Neuroscience, 2013, *248*, 670.
13. Barapatre N, Symvoulidis P, Möller W, Prade F, Deliolanis NC, Hertel S, Winter G, Yildirim AÖ, Stoeger T, Eickelberg O, Ntziachristos V, Schmid O J. Pharm. Biomed. Anal., **2015**, *102*, 129.