

Electronic Supporting Information of

A NIR Dye for Development of Peripheral Nerve Targeted Probes

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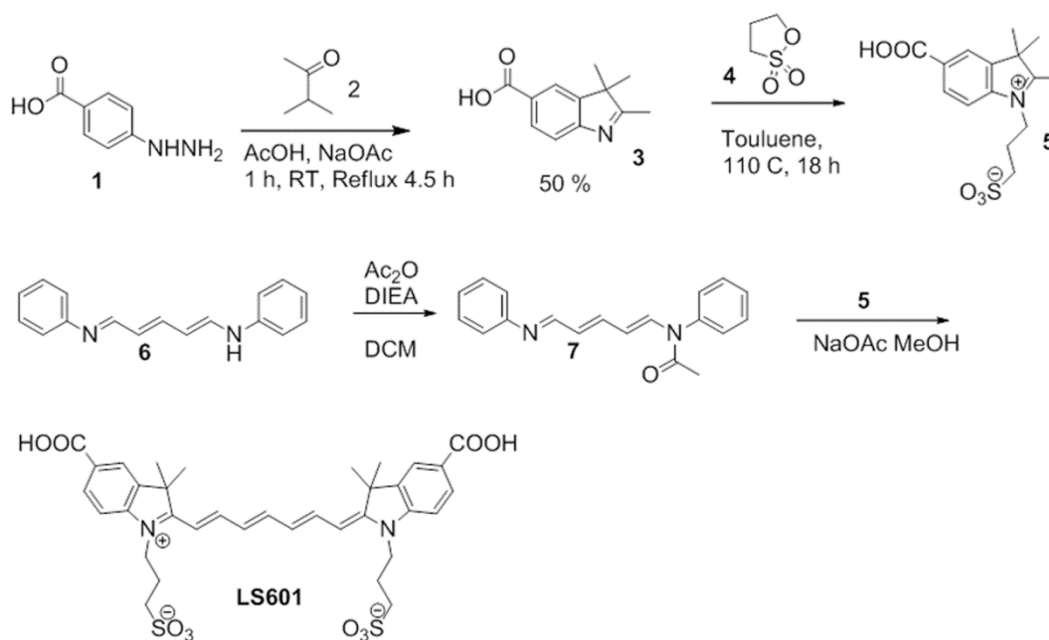
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1. Synthesis and Characterization of the NIR dye LS601

1.1. General Information

Common solvents, ICG (Cardiogreen) and reagents for synthesis were purchased from Sigma Aldrich, Alfa Aesar or TCI America and used without further purification. NMR spectra were recorded at room temperature on a Varian 400 or 600 MHz instrument, in DMSO with TMS as an internal standard (unless noted otherwise). UV/Vis spectra of samples were recorded on a Beckman Coulter DU 640 UV-visible spectrophotometer. Steady state fluorescence spectra and measurements were recorded on a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer in DMSO. Fluorescence quantum yield of LS601 was measured using ICG in DMSO as a standard ($\Phi=0.12$).¹ The compounds were analyzed using LC/MS-ESI analysis in the positive mode conducted on a Shimadzu LCMS 2010 A equipped with a UV/Vis detector at different wavelengths using a reversed-phase C-18 Vydac column (218TP, 4.6X50 mm) at a flow rate of 0.7 mL/min with a gradient 10-95% acetonitrile in water (both solvent contained 0.1 % TFA).

1.2. Synthesis and characterization



Scheme S1 Synthesis of LS601

2,3,3-trimethyl-3H-indole-5-carboxylic acid 3. To glacial acetic acid (30 mL) was added the appropriate 4-hydrazinobenzoic acid (30 mmol), sodium acetate (60 mmol), and isopropylmethyl ketone (43 mmol). The mixture was stirred at room temperature for 30 minutes and then heated at reflux for 5 hours. After cooling to room temperature the solvent was removed under vacuum and methanol (2 mL) was added to the residue. The redissolved residue was triturated with water (30 mL) to give **1a** as a yellow/brown solid. Yield 3.33 g, 55%. ¹H NMR (CDCl₃, 400 MHz): δ 8.12-8.13 (d, 1H, $J=10.44$), 8.03 (s, 1H), 7.61-7.63 (d, 1H, $J= 8.60$), 2.35 (s, 3H), 1.35 (s, 6H).

3-(5-carboxy-2,3,3-trimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate 5: The indole **3** (5.0 mmol) and 1,3-propanesultone (7.6 mmol) were suspended in 1,2-dichlorobenzene (8 mL) and heated with stirring at 110°C with a closed Teflon lined cap for 24 h. The mixture was cooled to room temperature, ether was added and the precipitate collected by filtration and washed with ether to give **5** as a brown/yellow solid which was used without further purification. Yield 0.91 g, 56 %.

Synthesis of LS601: A solution of acetic anhydride (0.057 mL) in methylene chloride (DCM, 0.5 mL) was added drop wise to a suspension of N-(5-anilino-2-4-pentadienylidene)aniline hydrochloride (0.29 mmol) and N,N-diisopropylethylamine (DIEA, 0.11 mL) in DCM (2 mL) at 0°C. The mixture was allowed to react at room temperature with stirring for 3 hours. Subsequently a solution of **5** (0.61 mmol) and sodium acetate (0.125 g) was prepared in methanol (8 mL), stirred for 30 minutes, and left at room temperature for 2.5 hours. The aniline solution was concentrated, dissolved in methanol (1.0 mL) and added slowly to the refluxing solution of indole. The reaction was refluxed for 24 h, cooled and precipitated with ether. Precipitation from methanol with ether was repeated three times to give the product **LS601** as a green powder, yield 98%. ¹H NMR (600 MHz, DMSO-*d*₆): δ 8.01 (s, 2H), 7.94 (d, $J=8.2$, 2H), 7.89 (m, 2H), 7.71 (dd, $J=17.2$, 8.9, 1H), 7.38 (d, $J=7.8$, 2H), 6.52 (m, $J=21.7$, 11.5, 4H), 4.27 – 4.19 (m, 4H), 2.58 (t, $J=6.9$, 4H), 2.03 – 1.96 (m, 4H), 1.64 (s, 12H). ESI-MS m/z : 713 [M⁺].

1.3 LCMS Reports

LS601:

Shimadzu Chromatogram(TICLC)

-----SHIMADZU LC-MS ANALYSIS REPORT-----

Sample Information

Acquired by : Mikhail Borzin
Date Acquired : 1/4/2012 5:07:20 PM
Data File : LS601-nerve_31.lcd
Sample Name : LS601-nerve
Sample ID :
Dilution Factor : 1
Vial# : 19
Injection Volume : 10
Method File : TPG ESI_pos_50mm_10min_701nm_800nm_TFA_10-95.lcm
Original Method : C:\LabSolutions\Data\Tiffany Gustafson\Gustafson Methods\Autosampler\TPG ESI_pos_50mm
Report Format : Simple4.lcr
Modified Date : 1/4/2012 5:17:22 PM

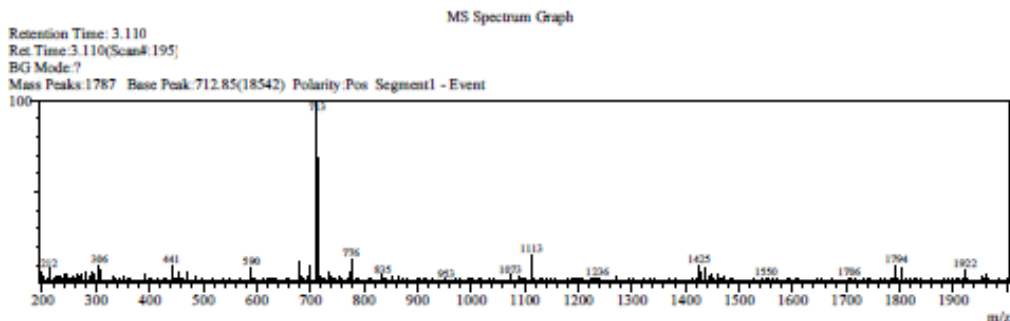
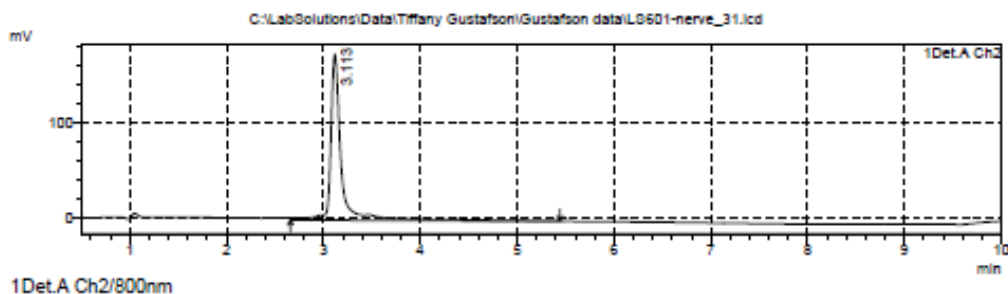
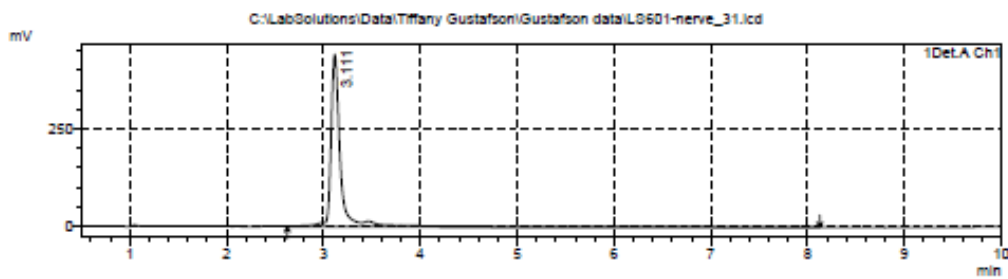
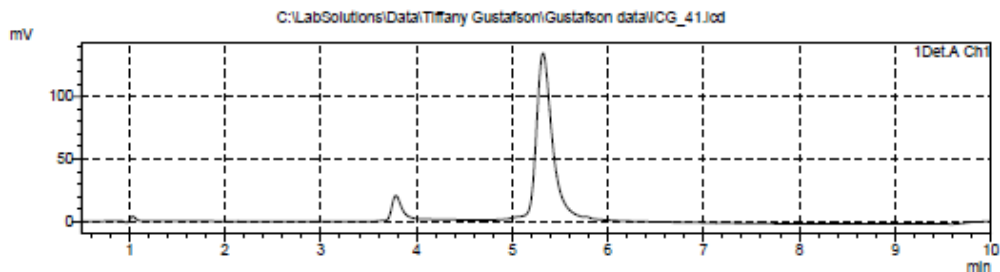


Figure S2. LCMS chromatogram of LS601. The UV/VIS detector was set to 701 nm and 800 nm. Retention time: 3.11 min

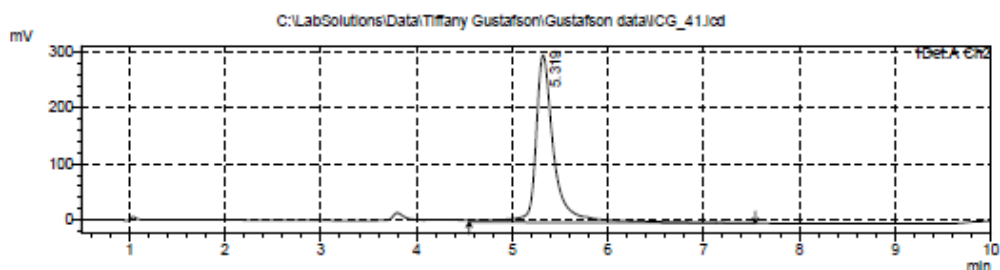
ICG (Cardiogreen, Aldrich):

Shimadzu Chromatogram(TICLC)

-----SHIMADZU LC-MS ANALYSIS REPORT-----
Sample Information
Acquired by : Mikhail Berezin
Date Acquired : 1/13/2012 4:13:56 PM
Data File : ICG_41.lcd
Sample Name : ICG
Sample ID :
Dilution Factor : 1
Vial# : 9
Injection Volume : 10
Method File : TPG ESI_pos_50nm_10min_701nm_800nm_TFA_10-95.lcm
Original Method : C:\LabSolutions\Data\Tiffany Gustafson\Gustafson Methods\Autosampler\TPG ESI_pos_50nm
Report Format : Simple4.lcr
Modified Date : 1/13/2012 4:23:58 PM



1 Det.A Ch1/701nm



1 Det.A Ch2/800nm

MS Spectrum Graph

Ret Time: 5.270(Scan#339)
BG Mode:?

Mass Peaks: 1778 Base Peak: 753.05(17489) Polarity: Pos Segment: 1 - Event: 1

MS Spectrum

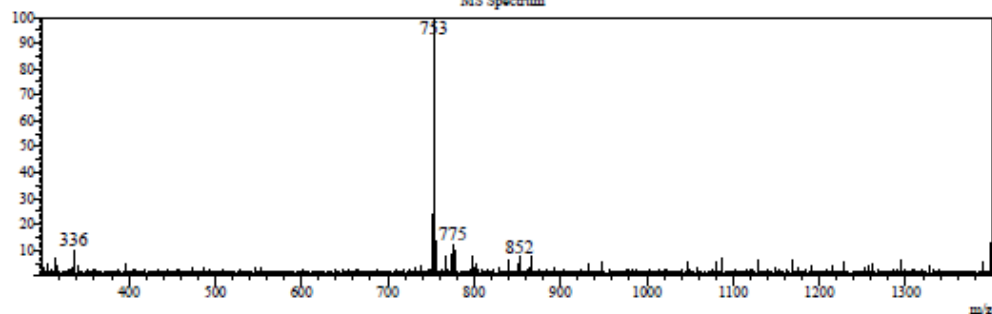


Figure S3. LCMS chromatogram of ICG. The UV/VIS detector was set to 701 nm and 800 nm. Retention time: 5.32 min

1.4 Determination of Albumin Binding Constant (K)

The binding constant (K) of LS601 and ICG to albumin was determined using our previously published protocol.²

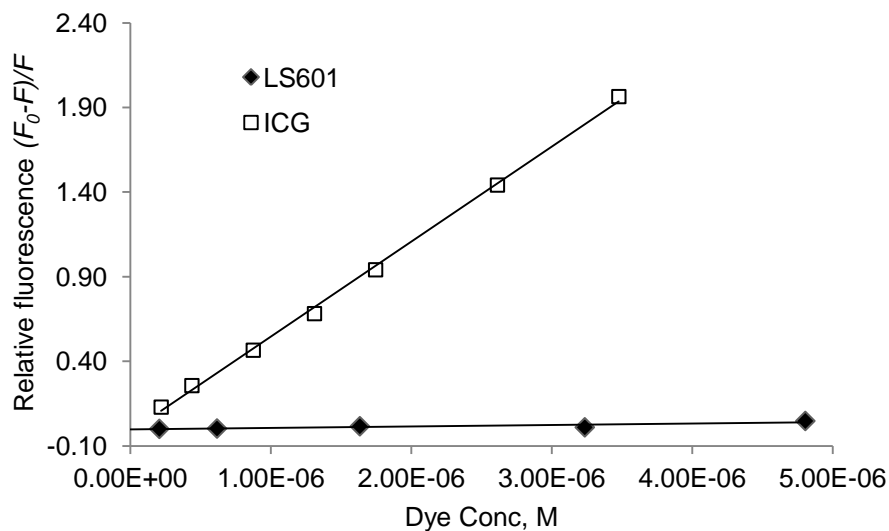


Figure S1 Relative fluorescence of tryptophan as a function of dye concentration (ex/em 275/345nm, BSA conc: 1.4×10^{-6} M in PBS). F_0 – fluorescence of albumin with no dye added. F – fluorescence of albumin upon dyes addition. Slope of the trend lines corresponds to the binding constants K . ICG possess strong binding constant to albumin with $K \sim 556,000 \text{ M}^{-1}$, LS601 shows much weaker binding with $K \sim 10,000 \text{ M}^{-1}$.

2. Small Animal Procedures

2.1. Surgical procedures

All animal procedures were approved by the Animal Studies Committee of Washington University and performed in strict accordance with their guidelines. Rats were housed in a central animal care facility and given a standard rodent diet and water ad libitum. Two to four month old female Sprague Dawley Thy1-GFP rats from our breeding facility were used.

A total of 9 rats with 18 limbs were used for the study. All limbs were randomly divided into five groups. Group I & II (3 limbs each) received ICG injections and were sacrificed at 24 and 48 h respectively. Groups III –V received LS601 injections and were sacrificed as follows: group III at 6 h (2 limbs), group IV at 24 h (3 limbs), and group V at 48 h (3 limbs). Group VI, containing 4 limbs, was sacrificed at 24 and 48 hours respectively to serve as a control receiving no injection.

Surgical procedures were performed aseptically using standard microsurgical techniques under an operating microscope. Rats were anesthetized by subcutaneous administration of 75 mg/kg ketamine hydrochloride and 0.5 mg/kg medetomidine hydrochloride. The sciatic nerve for each limb (both left and right except for the control limbs) and its three major branches (trifurcation) were exposed through a gluteal muscle-splitting incision. For injection a 20 μ M solution of dye in PBS was prepared with a DMSO concentration of 1.4% v/v. Each dye solution (8 μ L) was injected into the sciatic nerve at 5 mm proximal to the trifurcation with a Hamilton syringe (needle gauge #33) directed proximally. The site of injection was marked with a single 10-0 nylon suture. The sciatic nerve then underwent live imaging (See *in vivo* imaging section below). After imaging, the skin was re-approximated with simple interrupted 4-0 nylon sutures. Anesthesia was reversed with 0.4 mg subcutaneous injection of atipamezole HCl. At the time points indicated above for each group the animals were again anesthetized and sacrificed with 0.4 mL intracardiac injection of 200 mg/kg sodium pentobarbital. All nerves from groups I – VI were harvested and used for *ex vivo* imaging.

2.2. In vivo imaging

Live dissection imaging of each sciatic nerve receiving an injection of dye was carried out on an Olympus MVX10 fluorescent dissecting microscope, using GFP and Cy7 filter sets filter cubes, within 10 min following injection to establish initial dye location. After sacrifice each sciatic nerve was exposed and dissection imaging was repeated to determine movement of the dye.

Transdermal imaging was carried out on groups I – VI, with the exception of group III, with isoflurane anesthesia (3% v/v in 100% oxygen) administered by chamber. Time points were approximately 1.25 and 20 hours after dye injection. Planar fluorescence images were obtained on the small animal LI-COR Pearl NIR imaging system with dual channel excitation at 685 and 785 nm and emission collection at 710 and 810 nm respectively.

Fluorescence intensity analysis: Mean fluorescence intensity values were measured for 8 regions of interest (ROI) (Figure 2) along the sciatic nerve of all groups undergoing transdermal imaging. For group VI (control) the average intensity in each was subtracted from the mean intensity for that ROI in all animals from all other groups, accounting for background fluorescence within each ROI. Within each group the intensity values in each ROI were then normalized to ROI 4 and the standard deviation was obtained.

3. Ex Vivo Imaging and Histomorphometry

3.1. Confocal Microscopy

The nerves were harvested for fluorescent microscopic evaluation using a FluoView 1000 Olympus spectral confocal microscope reconfigured for NIR imaging.³ Harvested sciatic nerves were stored in optimal cutting temperature compound (OCT) at -80 °C until needed. OCT was thawed at room temperature and the nerves were removed and placed on a glass microscope slide. A coverslip was placed over the nerve and light pressure was applied to flatten the tissue. Microscopy was carried out imaging for both GFP and Cy7 fluorescence.

3.2. Histomorphometry

Thy1-GFP rats underwent the above surgical procedure on both limbs receiving either LS601 or saline injections. The rat was allowed to recover for two weeks, at which time the animal was anesthetized and sacrificed with 0.4 mL intracardiac injection of 200 mg/kg sodium pentobarbital and the sciatic nerves were harvested and used for histomorphometry.

Detailed histomorphometric analysis was performed as previously described.⁴ Briefly, harvested sciatic nerves were placed in 3% glutaraldehyde solution overnight. After washing with phosphate buffer, nerves were post-fixed with 1% osmium tetroxide overnight at 4°C. Specimens were then dehydrated in graded alcohols and embedded in 100% epoxy. Blocked nerves were cut into 1 µm cross-sections and stained with toluidine blue for light-microscopy imaging and qualitative analysis. All nerves underwent qualitative neural architecture assessment for total number of nerve fibers, nerve fiber width (microns), percent neural tissue (100x neural area/intrafascicular area), and nerve fiber density (fiber number/mm²) in cross-sections acquired 3–5 mm proximal, distal, and at the site of injection.

References:

1. R. C. Benson and H. A. Kues, *Phys. Med. Biol.*, 1978, **23**, 159-163.
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4. D. A. Hunter, A. Moradzadeh, E. L. Whitlock, M. J. Brenner, T. M. Myckatyn, C. H. Wei, T. H. Tung and S. E. Mackinnon, *J Neurosci Methods*, 2007, **166**, 116-124.