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Myelin-targeted, texaphyrin-based multimodal imaging agent for magnetic resonance and optical imaging

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Abstract

Reliable methods of imaging myelin are essential to investigate the causes of demyelination and to study drugs that promote remyelination. Myelin-specific compounds can be developed into imaging probes to detect myelin with various imaging techniques. The development of multimodal myelin-specific imaging probes enables the use of orthogonal imaging techniques to accurately visualize myelin content and validate experimental results. Here, we describe the synthesis and application of multimodal myelin-specific imaging agents for light microscopy and magnetic resonance imaging. The imaging agents were synthesized by incorporating the structural features of luxol fast blue MBS, a myelin-specific histological stain, into texaphyrins coordinated to Gd^{III}. These new complexes demonstrated absorption of visible light, emission of near-IR light, and relaxivity values greater than clinically approved contrast agents for magnetic resonance imaging. These properties enable the use of optical imaging and magnetic resonance imaging for visualization of myelin. We performed section- and *en block*-staining of *ex vivo* mouse brains to investigate the specificity for myelin of the new compounds. Images obtained from light microscopy and magnetic resonance imaging demonstrate that our complexes are retained in white matter structures and enable detection of myelin.

Keywords

contrast agents; gadolinium; MRI; multimodal probes; myelin; optical imaging; paramagnetic agents; texaphyrin

INTRODUCTION

The ability to view changes in the brain and peripheral nervous system is of great importance to the study of neurodegenerative diseases and aging (1–5). Among the components of the nervous system, myelin is a critical target for understanding neuronal anatomy and function (6–8). Myelin is a characteristic lipid-protein membrane in the nervous system that aids with the proper conduction of nerve impulses. Changes in

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myelinated nerve structure often result from nerve cell deterioration caused by inherited or acquired neuropathologies and brain injuries (9–13). Hence, effective monitoring of the morphology of myelinated regions of the brain is important in the study of neurodegenerative diseases and therapies for these diseases.

Histology using light microscopy with chromophoric or fluorophoric stains was the primary method of imaging myelin until recently (7,8). Advancements in imaging technologies have permitted the use of other modalities for imaging myelin including positron emission tomography (PET) and magnetic resonance imaging (MRI). Although several PET tracers that target myelin have been reported (1,14,15), the use of PET for imaging myelinated structures in brains is limited because of its low (1–2 mm) resolution (1,16). Currently, the most promising non-optical imaging method for myelin is MRI (1–3,6,17,18). The ability to acquire whole brain images using MRI with high resolution is important for *in vivo* imaging and *ex vivo* pre-clinical research. Typically with *in vivo* MRI scans, a spatial resolution of 0.5–1 mm can be obtained (16), whereas with *ex vivo* imaging the resolution can be down to 25–100 μm (17). Various MRI techniques have been investigated for imaging myelin including ultrashort echo time MRI, diffusion tensor imaging, magnetization transfer, and T_2 relaxometry (3,12,13,20–22). The specificity of these methods for myelin is not ideal because these methods struggle to distinguish changes in myelination from hemorrhages, iron deposits, and changes in the water content of tissue (6).

Myelin-specific contrast agents provide a way to augment the lack of specificity when imaging myelin. Myelin-specific Gd^{III} -containing small molecules were reported that are similar in efficiency to nonspecific clinically approved contrast agents (2, 23, 24). These contrast agents that have longitudinal relaxivity (r_1) of $\sim 5.1 \text{ mM}^{-1} \text{ s}^{-1}$ at 1.41 T were able to highlight myelination in MRI via T_1 maps; however, the images acquired with these agents demonstrate the need for contrast agents with higher relaxivity values to produce images of myelinated structures with increased signal-to-noise ratios (2,23,24). Further, the Cu^{II} -containing myelin-specific histology stain Luxol fast blue MBS (LFB MBS, Figure 1) was reported for imaging of myelinated brains with magnetic resonance spectroscopy (25). However, LFB MBS did not produce appreciable contrast because of the extremely low relaxivity, r_1 ($0.09 \text{ mM}^{-1} \text{ s}^{-1}$ at 4.7 T) of the complex as a contrast agent for MRI (25). Investigation of efficient myelin-specific agents for contrast-enhanced MRI is crucial for effective visualization of myelination.

Sensitive measurements of differences in tissue structure require high precision and accuracy, and one way to ensure the reliability of findings is with verification using orthogonal imaging techniques. Here, we report new multimodal myelin-specific contrast agents for MRI and that can be used with light and near-IR fluorescence microscopies. We also report the imaging of myelinated structures in *ex vivo* samples using our new agents.

RESULTS AND DISCUSSION

Design, Synthesis, and Characterization

A well-known myelin-specific histological stain LFB MBS was used as our structural inspiration because it contains a metal-ligand motif that is amenable to adaptation into a

contrast agent for MRI. However, the ligand framework of LFB MBS cannot be directly used for the synthesis of stable Gd^{III} -containing complexes because the cavity size of the phthalocyanine in LFB MBS does not match well with the ionic radius of Gd^{III} (26). Sessler and co-workers reported the use of expanded porphyrins, known as texaphyrins, to form stable complexes with lanthanides (27,28). Their studies with Gd^{III} -containing texaphyrins have shown that complexes of these macrocycles have higher relaxivity values ($\sim 18 \text{ mM}^{-1} \text{ s}^{-1}$ at 1.2 T) than clinically approved contrast agents for MRI (29). With that knowledge, we reasoned that texaphyrins would have the potential to mimic the structural features of phthalocyanine—including a planar aromatic macrocycle, peripheral functional groups, and charge—while forming complexes with Gd^{III} . Although LFB MBS interacts with myelin, the important structural features for interaction are not well established (7). Therefore, five different texaphyrin complexes that vary in the number and identity of functional groups were synthesized (**1–5**, Figure 1). The functional groups introduced to the texaphyrin periphery include sulfonates, carboxylates, and methyl esters. Sulfonates and carboxylates were used to test the importance of negatively charged functional groups for interactions with myelin, and methyl esters were used as a control to study the need for negatively charged functional groups.

The functional group selection enabled exploration of the influence of a variety of properties on staining. Complex **1** has an overall charge of +2, and it does not have negatively charged functional groups. This molecule was designed to test if negatively charged functional groups are crucial for myelin-specific interactions. Complex **2** has carboxylate groups and is neutral. This complex is a carboxylate analogue for the sulfonate containing texaphyrins and was synthesized to study the importance of sulfonates as the negatively charged functional groups. Complexes **3–5** have sulfonate groups and charges of 0, -1 , -2 , respectively, and these complexes were designed to study the effect of the number of sulfonate groups on interaction with myelin.

Although many texaphyrin derivatives have been reported, texaphyrins **1–5** are new derivatives, and the synthetic routes to complexes **1–5** are shown in Scheme 1 with detailed procedures provided in the experimental section. Incorporation of different types and numbers of functional groups into the periphery of texaphyrins resulted in different physicochemical properties including solubility.

Complexes **1–3** are sparingly soluble in water but soluble in methanol, ethanol, and acetonitrile, while complexes **4** and **5** are soluble in water. Texaphyrins **1** and **3–5** are soluble in mixtures of water with methanol, ethanol or acetonitrile, and solutions of **1** and **3–5** did not yield precipitate after 3 months; however, texaphyrin **2** produced an insoluble precipitate in the presence of 95% ethanol after 15 min of dissolution. The use of absolute ethanol avoided the rapid precipitation of **2**, but upon storage, precipitation was observed after 2 days. Avoidance of precipitation is important to enable staining with a known concentration of complex; therefore, freshly prepared stain **2** was used for each experiment.

In addition to solubility, optical imaging-relevant properties of complexes **1–5** were studied. The extended conjugation of texaphyrins produces a characteristic sharp absorption in the visible region (Figure 2) that causes the metal complexes to appear dark green in color. All

five texaphyrin complexes demonstrated similar absorptions regardless of the number or type of the functional groups on the side chains. The deep green color of the texaphyrin complexes enabled the visualization with light microscopy similar to the visualization of the blue histology stain LFB MBS. The fluorescence emissions of the five complexes were also similar to each other regardless of functional group identity (Figure 3), exhibiting near-IR emission when excited by 468–492 nm light.

The efficiencies of complexes **1–5** as contrast agents for MRI were determined by measuring their relaxivities at 1.4 T and 37 °C using solvent systems that demonstrated the ability to dissolve the complexes. The solvent system that was used for dissolution was measured as the blank in each case. All five complexes demonstrated 3.5–4-fold higher relaxivity values than a clinically used contrast agent for MRI (Gd^{III}1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acetate, DOTA, Table 1). These high relaxivities likely result from a combination of slow tumbling due to the size of the molecule and the availability of more than one site for water coordination. Texaphyrin **1** had the highest relaxivity (19.5 mM⁻¹ s⁻¹) and was similar to other reported texaphyrins with side chains containing neutral functional groups (29). The relaxivity of methyl ester-containing texaphyrin **1** is 1.8–1.5-fold greater than the sulfonate and carboxylate-containing texaphyrins **2–5**, possibly because interaction of negatively charged groups with Gd^{III} causes a reduction of the available sites for water coordination. High relaxivity is desirable for contrast agents because it enables high levels of contrast enhancement, which is especially important for detection of fine structures (30), including white matter structures.

In the preparation of staining solutions, the ditolylguanidinium ion was selected as the counter cation for texaphyrin complexes containing anionic functional groups because the counter cation used in LFB MBS is ditolylguanidinium. The use of this organic cation improves the lipophilic nature of the overall molecule, facilitating penetration through brain tissues (7). Ditolylguanidinium was not used in the preparation of stain using texaphyrin complex **1** because that complex does not contain anionic functional groups.

Tissue staining

Initial staining was performed with coronal sections of mouse brain tissue to visualize the interactions of texaphyrins **1–5** with myelinated structures. Because of the optical absorptions of texaphyrins, we initially used light microscopy to gauge the interaction of the texaphyrins with myelin. Texaphyrin complexes have a characteristic dark green color due to the absorption of visible light in the 700–750 nm range. Thus, texaphyrins are chromophoric probes similar to LFB MBS. Additionally, the fluorescence emission studies demonstrated a near-IR emission and, consequently, the potential for use as fluorescence probes. The staining of mouse brain tissue was performed by incubating tissue samples (200 µm thick) in stain solutions at 54 °C for 5 h. At the end of the incubations, color transfer was observed from the stain solutions to the mouse brain sections. When comparing texaphyrin stains **1–5** to each other, the staining solution of complex **1** demonstrated the lowest reduction of color after incubation and resulted in only lightly colored tissue. Tissue sections stained with texaphyrins **2–5** were dark green. Brain sections stained with texaphyrins were differentiated with Li₂CO₃ to remove excess and loosely bound texaphyrins, under the assumption that

mild alkali solutions interrupt the interaction between sulfonates and myelin (7). Because hydrophilic alkali solutions do not enter the hydrophobic domains of myelin to interrupt the interactions, the stain is retained in myelinated structures (7). However, the use of aqueous Li_2CO_3 , which is used with LFB MBS (7), led to under-differentiation for tissue samples stained with texaphyrins **2** and **3** and over-differentiation for texaphyrins **4** and **5**. An ethanolic Li_2CO_3 solution was selected as the optimal differentiation solution because it demonstrated appreciable differentiation for texaphyrins **2–5**.

Methyl ester-containing texaphyrin **1** did not differentiate with aqueous or ethanolic Li_2CO_3 as indicated by the dark coloration of the entire slice (Figure 4, row 1). Because of the inability to differentiate myelinated and non-myelinated regions with texaphyrin **1**, it was not used for further studies. Texaphyrins **2** and **3** provided a visible differentiation of well-established myelinated regions (31) from non-myelinated regions (Figure 4, rows 2 and 3), and texaphyrins **4** and **5** were removed partially or completely from tissue upon exposure to water during the hydration step prior to microscopy (Figure 4, rows 4 and 5). The removal of stain with exposure to water is likely due to the hydrophilicity from the presence of three or more negatively charged sulfonate groups and is an undesirable feature for *ex vivo* staining of myelin. In contrast, texaphyrins **2** and **3** that were differentiated with ethanolic Li_2CO_3 were stable upon exposure to water; thus, texaphyrins **2** and **3** were found to be viable candidates for staining myelin. Myelin-rich regions were green compared to myelin-poor surrounding tissues in coronal brain sections stained with texaphyrins **2** or **3**. Specifically, the myelin-rich corpus callosum, striations in the caudate putamen, anterior commissure, and mammillothalamic tract appeared green in the optical images (Figure 4).

The observations with staining studies of texaphyrins **1–5** reveal that the presence of a negatively charged functional group is vital for interaction with myelin. Furthermore, light microscopy images of mouse brain sections stained with texaphyrins showed that complexes **2** and **3**, which are less hydrophilic than complexes **4** and **5**, differentiate better than the other complexes. Consequently, complexes **2** and **3** highlight myelinated regions in the brain tissue more efficiently than the other complexes. Texaphyrins **4** and **5** that have more than two negatively charged functional groups did stain myelin, but the high solubility of these texaphyrins in water interferes with the retention of the stain in myelinated regions. Texaphyrin **1** did not highlight myelin and demonstrated nonspecific staining of brain tissue. Unlike texaphyrins **2–5**, Li_2CO_3 was not able to remove texaphyrin **1** from tissue samples. It is assumed that the interaction of LFB MBS and myelin occurs through an acid–base type interaction between the negatively charged sulfonate groups of the stain and positively charged amine residues of the basic myelin proteins (7). Our observations support the presence of this type of interaction because texaphyrin **1** with neutral methyl ester groups did not demonstrate myelin-specific staining of brain tissues.

The nonspecific labeling observed with texaphyrin **1** likely arises because the positively charged molecule interacts with negatively charged lipids in the tissue. Furthermore, maintaining some hydrophobicity seems to be critical to attain desirable staining and differentiation. A possible explanation for our observations with texaphyrins **4** and **5** is that the hydrophilic stain is unable to enter the hydrophobic domains of the myelin sheath.

Instead, these molecules might interact with the surface proteins, making them easily removed upon exposure to water.

From light microscopy, texaphyrins **2** and **3** that contain two carboxylates and two sulfonates, respectively, demonstrated better staining of myelinated regions in brain tissues than texaphyrins **1**, **4**, or **5**. This observation indicates that the negatively charged functional groups do not need to be sulfonates. However, handling of texaphyrin **2** is difficult because of the formation of precipitate upon storage that likely results because of oligomerization into coordination polymers. Consequently, texaphyrin **3** was selected as the best myelin-specific texaphyrin of the five complexes for staining of intact brains and observation with MRI.

When staining intact brains with texaphyrin **3**, the optimal incubation time for the diffusion of the texaphyrin was found to be 24–26 h likely because of the thickness of the tissue relative to the 200 μm sections (stained for 5 h). The differentiation time for intact brain was 65 h, and an incision along the midline of the brain was made prior to differentiation to facilitate the diffusion of Li_2CO_3 into the tissue. The long differentiation times and mechanical opening of tissue were necessary for penetration of ionic Li_2CO_3 through hydrophobic tissue. Stained and differentiated whole mouse brain was used for MRI to investigate the ability of texaphyrin **3** to act as a myelin-specific contrast agent.

MRI of a mouse brain stained with texaphyrin **3**

Relaxation rate (R_1)-mapping of intact mouse brain stained with texaphyrin **3** was performed to investigate the contrast enhancing properties of the complex during MRI. An intact mouse brain stained with texaphyrin **3** and an unstained control brain were embedded in agarose prior to MRI. The R_1 -maps of the mouse brains stained with texaphyrin **3** and unstained control were obtained using longitudinal relaxation time (T_1) measurements and converting to R_1 . The R_1 -maps of the mouse brain stained with texaphyrin **3** demonstrated a clear difference between myelin-rich (bright) and myelin-poor (dark) regions in comparison to the unstained control (Figure 5). Using the average of five regions of interest from R_1 -maps of stained brain and unstained control brain (Figure S4), the enhancement of R_1 in myelin-rich regions with respect to myelin-poor regions was calculated. Myelinated regions of the R_1 -map of the brain samples stained with texaphyrin **3** demonstrated a faster (2.6-fold) longitudinal relaxation rate (R_1) than myelin-poor regions of the stained tissue. The enhancement of R_1 of the myelin-rich regions from the surroundings was only 1.6-fold for the unstained control. This increase of R_1 in stained brain tissue was statistically significant with respect to unstained brain tissue (Student t test, 95% confidence interval). Thus, the myelin-rich regions in the R_1 -map of the stained brain appear bright and are distinguishable due to the additional contrast enhancement afforded by texaphyrin **3**. Water protons tend to relax faster in the presence of paramagnetic Gd^{III} ; thus, the increase of the R_1 relaxation rate indicates the presence of Gd^{III} and regions with more Gd^{III} appear brighter in the R_1 -map. Hence, Gd^{III} -containing complex **3** demonstrates a potential to remain in myelin-rich regions.

Dual-modality contrast agents are ideally suited for confirmation of imaging results because the conditions that might confound the interpretation of one imaging modality rarely affect the other. Thus, enabling the collection of more reliable and detailed information about samples. Furthermore, a dual-modality contrast agent can be designed to exploit the strengths of two complimentary imaging modalities that can increase the usefulness of the agent for biomedical imaging (33,34). For example, optical imaging is a sensitive technique, but it suffers from low penetration depths, whereas MRI has no practical depth limits but is less sensitive. A contrast agent that can facilitate both optical and MR imaging will complement the weaknesses of each imaging technique and improve overall usefulness (35, 36).

Because the optical and magnetic properties of our synthesized texaphyrin complexes, they are expected to function as dual-modality imaging agents. Hence, after performing MRI, the brain sample was sectioned and observed with light microscopy to verify the MRI findings (Figure 5). In the slices from the brain that was imaged with MRI, white matter structures appeared green under the light microscope and confirmed that texaphyrin **3** was distributed in regions that showed a positive enhancement in R_1 -map. The imaging studies from these orthogonal imaging modalities confirmed the ability of texaphyrin **3** to diffuse into intact mouse brains, differentiate to be retained specifically in myelinated regions, and to act as a multimodal imaging agent facilitating the visualization of myelin-rich structures.

CONCLUSIONS

In conclusion, we synthesized small molecules for myelin-specific imaging using structure-mimicking strategy (30). *Ex vivo* studies demonstrated that texaphyrins **2** and **3** interact with myelin. The optical and magnetic properties of these texaphyrin complexes allowed the imaging of myelin in intact brains using light microscopy and MRI. These findings are expected to facilitate pre-clinical research of neuronal disease by enabling effective visualization of myelination and demyelination of mouse models.

EXPERIMENTAL

Commercially available chemicals were of reagent-grade purity or better and were used without purification unless otherwise noted. Water was purified using a PURELAB Ultra Mk2 water purification system (ELGA). Compounds **3a** (37), **3b** (38), and **6a** (39) were synthesized using previously published procedures.

Analytical thin-layer chromatography (TLC) was carried out on ASTM TLC plates precoated with silica gel 60 F₂₅₄ (250 μ m layer thickness). Visualization of TLC was accomplished using a UV lamp followed by charring with potassium permanganate stain (3 g KMnO₄, 20 g K₂CO₃, 5 mL 5% w/v aqueous NaOH, 300 mL H₂O). Flash chromatography was performed using silica gel 60, 230–400 mesh (EMD Chemicals) or aluminum oxide, activated, acidic, Brockmann I, standard grade, ~150 mesh, 58 Å (Sigma Aldrich). Preparative reverse-phase chromatography was performed using RP-tC18 SPE Sep-Pak columns (Waters) and analytical HPLC analyses were performed with a C18 column (Restek International, Viva C18, 5 μ m, 250 $\times$ 4.6 mm²). Analytical HPLC used a

binary gradient method (pump A: water, pump B: acetonitrile; 5% B for 5 min, 5–95% B over 7 min, 95% B for 1 min, 95–5% B over 1 min for complexes **4** and **5**, and isocratic 94.8% CH₃OH, 5% water, and 0.2% formic acid for 45 min for complex **2**). The flow rates used for analytical column was 1 mL min⁻¹ and detection was carried out with a photodiode array detector.

¹H NMR spectra were obtained using a Varian Mercury 400 (400 MHz), an Agilent 400 (400 MHz), a Varian 500 (500 MHz), or an Agilent 600 (600 MHz) spectrometer, and ¹³C NMR spectra were obtained using a Varian Mercury 400 (101 MHz), an Agilent 400 (101 MHz), a Varian 500 (126 MHz), or an Agilent 600 (151 MHz) spectrometer. Chemical shifts are reported relative to residual solvent signals unless otherwise noted (CDCl₃, ¹H: δ 7.27, ¹³C: δ 77.16; dimethylsulfoxide-*d*₆, ¹H: 2.50, ¹³C: 39.52; D₂O, ¹H: 4.79, ¹³C: 39.52 for an internal standard of dimethyl sulfoxide-*d*₆; CD₃OD/D₂O mixture, ¹H: 4.79, ¹³C: 49.00). NMR data are assumed to be first order, and the apparent multiplicity is reported as “s” = singlet, “dd” = doublet of doublets, “t” = triplet, “q” = quartet, “m” = multiplet, or “brs” = broad singlet. Italicized elements are those that are responsible for the chemical shifts. High-resolution mass spectra (HRMS) were obtained on an electrospray time-of-flight high-resolution Waters Micromass LCT Premier XE mass spectrometer or a Waters Synapt G2S mass spectrometer equipped with an intermediate pressure ionization source. The matrix 2-nitrophloroglucinol (40) was used at 5 mg/200 μL in 1:1 acetonitrile/H₂O prior to combination with analyte solution 1:1 (v/v) and used with the latter mass spectrometer for compounds **2**, **4**, and **5**. UV–vis spectra were obtained on Shimadzu UVmini-1240 UV–vis spectrophotometer. Data are reported as UV–vis (solvent) λ_{max}, nm (ε), where λ_{max} is the wavelength of maximum absorption in nanometers; ε is the molar absorption coefficient; and “sh” is the wavelength of shoulder. Inductively coupled plasma optical emission spectroscopy (ICP–OES) analyses were performed using a HORIBA Jobin Yvon ULTIMA spectrometer.

Sonication was performed using a FS60H sonicator (Fisher Scientific). Freeze drying was performed using a Freezone 2.5 freeze dryer (LABCONCO). Elemental analyses were performed by Midwest Microlab LLC, Indianapolis, Indiana, USA.

Benzyl 4-(3-hydroxypropyl)-3,5-dimethyl-1*H*-pyrrole-2-carboxylate (**1b**)

Compound **1a** (1.5 g, 4.7 mmol, 1 equiv) was cooled to 0 °C, and to it was added BH₃·THF (1 M, 24 mL, 24 mmol, 5 equiv) dropwise while stirring. The reaction mixture was allowed to warm to ambient temperature and stirred for 5 h. Excess BH₃·THF was quenched with CH₃OH (20 mL), and the solvent was removed under reduced pressure. The resulting residue was dissolved in CH₂Cl₂ and washed with HCl (0.5 M, 2 × 20 mL). The organic layer was dried with anhydrous K₂CO₃, and the solvent was removed under reduced pressure to yield 1.34 g (98%) of **1b** as a white powder. ¹H NMR (400 MHz, CDCl₃, δ): 1.49 (brs, OH, 1H), 1.64–1.86 (m, CH₂, 2H), 2.20 (s, CH₃, 3H), 2.30 (s, CH₃, 3H), 2.47 (t, *J* = 7.4 Hz, CH₂, 2H), 3.64 (t, *J* = 5.6 Hz, CH₂, 2H), 5.30 (s, CH₂, 2H), 7.29–7.56 (m, CH, 5H), 8.79 (brs, NH, 1H); ¹³C NMR (101 MHz, CDCl₃, δ): 10.8 (CH₃), 11.6 (CH₃), 20.3 (CH₂), 33.6 (CH₂), 62.5 (CH₂), 65.6 (CH₂), 116.6, 121.5, 127.8, 128.1 (CH), 128.2 (CH),

128.6 (CH), 130.2, 136.7, 161.5; HRMS (m/z): $[M + H]^+$ calcd for $C_{17}H_{22}NO_3$, 288.1600; found, 288.1641.

Benzyl 4-(3-bromopropyl)-3,5-dimethyl-1H-pyrrole-2-carboxylate (1c)

To a solution of **1b** (0.90 g, 3.2 mmol, 1 equiv) in anhydrous CH_2Cl_2 was added CBr_4 (1.6 g, 4.8 mmol, 1.5 equiv). The resulting mixture was cooled to 0 °C and stirred for 10 min under Ar in the dark. To this mixture was added PPh_3 (1.3 g, 4.8 mmol, 1.5 equiv), and the mixture was stirred for 1.5 h at ambient temperature under Ar in the dark. Solvent was removed under reduced pressure, and the resulting viscous oil was purified by silica gel chromatography (1:5 ethyl acetate/hexanes) to obtain 0.83 g (74%) of **1c** as an off white powder. 1H NMR (400 MHz, $CDCl_3$, δ): 1.92–2.12 (m, CH_2 , 2H), 2.23 (s, CH_3 , 3H), 2.32 (s, CH_3 , 3H), 2.55 (t, J = 8.0 Hz, CH_2 , 2H), 3.40 (t, J = 8.0 Hz, CH_2 , 2H), 5.32 (s, CH_2 , 2H), 7.31–7.51 (m, CH , 5H), 8.99 (brs, NH , 1H); ^{13}C NMR (101 MHz, $CDCl_3$, δ): 10.9 (CH_3), 11.6 (CH_3), 22.4 (CH_2), 33.5 (CH_2), 33.6 (CH_2), 65.6 (CH_2), 116.7, 120.4, 127.7, 128.1 (CH), 128.6 (CH), 130.6, 136.7; HRMS (m/z): $[M + H]^+$ calcd for $C_{17}H_{21}NO_2Br$, 350.0756; found, 350.0692; TLC: R_f = 0.44 (1:5 ethyl acetate/hexanes).

Benzyl 5-(acetoxymethyl)-4-(3-bromopropyl)-3-methyl-1H-pyrrole-2-carboxylate (2c)

To a stirring mixture of **1c** (0.44 g, 1.2 mmol, 1 equiv) in glacial acetic acid (17 mL) was added acetic anhydride (5 mL) dropwise under Ar at ambient temperature. The reaction mixture was stirred for 15 min; $Pb(OAc)_4$ (1.1 g, 2.5 mmol, 2 equiv) was added; and stirring was continued for 1 h. The reaction mixture was poured on to ice, and the resulting precipitate was collected and washed with water (10 $\times$ 50 mL). The precipitate was dried under reduced pressure, dissolved in CH_2Cl_2 (2 mL), and filtered. The filtrate was precipitated using petroleum ether (100 mL) to yield 0.45 g (93%) of **2c** as a white fluffy powder. 1H NMR (400 MHz, $CDCl_3$, δ): 1.98–2.06 (m, CH_2 , 2H), 2.06 (s, CH_3 , 3H), 2.30 (s, CH_3 , 3H), 2.62 (t, J = 7.4 Hz, CH_2 , 2H), 3.39 (t, J = 6.4 Hz, CH_2 , 2H), 5.05 (s, CH_2 , 2H), 5.31 (s, CH_2 , 2H), 7.31–7.50 (m, CH , 5H), 9.23 (brs, NH , 1H); ^{13}C NMR (101 MHz, $CDCl_3$, δ): 10.7 (CH_3), 21.1 (CH_3), 22.2 (CH_2), 33.3 (CH_2), 33.8 (CH_2), 57.0 (CH_2), 65.9 (CH_2), 119.3, 123.3, 126.8, 127.8 (CH), 128.3 (CH), 128.7 (CH), 136.4, 161.2, 171.7; HRMS (m/z): $[M + H]^+$ calcd for $C_{19}H_{23}NO_4Br$, 408.0811 found, 408.0588.

Benzyl-5,5'-(3,4-diethyl-1H-pyrrole-2,5-diyl)bis(methylene)bis(4-3-bromopropyl)-3-methyl-1H-pyrrole-2-carboxylate (3c)

Compound **2c** (0.39 g, 0.96 mmol, 2 equiv) and 3,4-diethylpyrrole (65 μ L, 0.48 mmol, 1 equiv) were dissolved in CH_3OH (27 mL) under Ar in dark and heated at 60 °C for 1 h until a clear solution was obtained. To this clear solution was added *p*-toluene sulfonic acid (7.9 mg, 0.041 mmol, 0.085 equiv) in CH_3OH (1 mL). The resulting reaction mixture was stirred for 1.5 h at 60 °C, at which point the volume was reduced by sparging N_2 through the reaction mixture (5–10 min) until a precipitate started to form. Once precipitation started, the N_2 sparging was stopped, and the reaction was stirred for 4 h at 60 °C. The reaction mixture was concentrated to 10 mL under reduced pressure and cooled to –20 °C for 2 h to promote precipitation. The precipitate was separated, washed with cold CH_3OH (0 °C, 2 mL) and dried under reduced pressure to yield 0.24 g (60%) of **3c** as a pink powder. 1H

NMR (400 MHz, CDCl₃, δ): 1.19 (t, J = 7.4 Hz, CH₃, 6H), 1.84–2.03 (m, CH₂, 4H), 2.26 (s, CH₃, 6H), 2.42–2.66 (m, CH₂, 8H), 3.36 (t, J = 6.2 Hz, CH₂, 4H), 3.62 (brs, CH₂, 4H), 4.35 (brs, CH₂, 4H), 6.96–7.10 (m, CH, 4H), 7.24–7.39 (m, CH, 6H), 8.85 (brs, NH, 1H), 11.33 (brs, NH, 2H); ¹³C NMR (101 MHz, CDCl₃, δ): 11.3 (CH₃), 17.0 (CH₃), 17.8 (CH₂), 21.9 (CH₂), 22.1 (CH₂), 33.3 (CH₂), 33.6 (CH₂), 65.2 (CH₂), 117.3, 118.8, 119.3, 122.0, 126.2 (CH), 126.6, 127.1 (CH), 127.9 (CH), 133.7, 136.5, 162.5; HRMS (m/z): [M + H]⁺ calcd for C₄₂H₅₀N₃O₄Br₂, 818.2168; found, 818.2140.

Dimethyl-3,3'-(2,2-(3,4-diethyl-1H-pyrrole-2,5-diyl)bis(methylene)bis(5-formyl-4-methyl-1H-pyrrole-3,2-diyl))dipropionate (4a)

A mixture of tripyrrane **3a** (0.38 g, 0.51 mmol, 1 equiv) and Pd/C (10%, 0.070 g, 0.067 mmol) in anhydrous THF (12 mL) was degassed under reduced pressure and then saturated with hydrogen (1 atm), and the reaction mixture was stirred for 6 h at ambient temperature. The reaction mixture was filtered through celite, and the solvents were removed under reduced pressure to obtain deprotected **3a** as a pink powder. To deprotected **3a** (0.29 g, 0.51 mmol, 1 equiv) was added trifluoroacetic acid (0.90 mL, 13 mmol, 25 equiv) dropwise under Ar at ambient temperature while stirring. Upon complete addition of trifluoroacetic acid, the resulting solution was stirred for 10 min. The reaction mixture was cooled to –20 °C, and triethylorthoformate (0.96 mL, 5.7 mmol, 11 equiv) was added dropwise. The resulting reaction mixture was stirred at –20 °C for 10 min before allowing the reaction mixture to warm to ambient temperature. Water (3.0 mL) was added dropwise to the stirring mixture immediately after the temperature warmed above –20 °C, and stirring at ambient temperature was continued for 15 min. A dark red precipitate was collected and washed with water (5 × 5 mL). The precipitate was suspended in a mixture of water/ethanol/ammonium hydroxide (1:2:1 v/v/v, 5.0 mL) and stirred at ambient temperature for 1 h. The resulting yellow precipitate was filtered and washed with water (3 × 3 mL) followed by cold ethanol (0 °C, 2.0 mL). The precipitate was dissolved in a methanol/water mixture (5:1, 3.6 mL), heated to 60 °C for 5 min, cooled to ambient temperature, and concentrated under reduced pressure. The resulting suspension was cooled at –20 °C for 4 h then filtered, and the precipitate was washed with water (4.0 mL). Solvents were removed under reduced pressure to yield 171 mg (63%) of **4a** as a light brown solid. ¹H NMR (400 MHz, CDCl₃, δ): 1.11 (t, J = 7.4 Hz, CH₃, 6H), 2.21 (s, CH₃, 6H), 2.31 (t, J = 7.8 Hz, CH₂, 4H), 2.45 (q, J = 7.4 Hz, CH₂, 4H), 2.71 (t, J = 7.8 Hz, CH₂, 4H), 3.64 (s, CH₃, 6H), 3.87 (s, CH₂, 4H), 9.09 (s, CH, 2H), 9.30 (s, NH, 1H), 10.52 (s, NH, 2H); ¹³C NMR (101 MHz, CDCl₃, δ): 8.9 (CH₃), 16.7 (CH₃), 17.8 (CH₂), 19.3 (CH₂), 22.8 (CH₂), 34.5 (CH₂), 51.7 (CH₃), 120.7, 120.9, 121.8, 128.2, 133.0, 138.7, 173.6 (CH), 175.6; HRMS (m/z): [M + H]⁺ calcd for C₃₀H₄₀N₃O₆, 538.2917; found, 538.2917.

5,5'-(3,4-Diethyl-1H-pyrrole-2,5-diyl)bis(methylene)bis(4-ethyl-3-methyl-1H-pyrrole-2-carbaldehyde) (4b)

Compound **4b** was synthesized according to the procedure in reference 38 with modifications. The modifications included addition and stirring of trifluoroacetic acid at ambient temperature, addition and stirring of triethylorthoformate at –20 °C, and purification by precipitation with methanol at –20 °C. This precipitation method yielded 305 mg (73%) of a light brown solid. ¹H NMR (400 MHz, CDCl₃, δ): 0.80–1.29 (m, CH₃, 12H),

2.20 (s, CH_3 , 6H), 2.32–2.56 (m, CH_2 , 8H), 3.84 (s, CH_2 , 4H), 9.14 (s, CH , 2H), 9.39 (s, NH , 1H), 10.11 (s, NH , 2H); ^{13}C NMR (101 MHz, CDCl_3 , δ): 8.8 (CH_3), 15.1 (CH_3), 16.5 (CH_3), 16.9 (CH_2), 17.7 (CH_2), 22.7 (CH_2), 120.8, 121.5, 124.8, 128.2, 132.9, 138.0, 175.6 (CH); HRMS (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{26}\text{H}_{36}\text{N}_3\text{O}_2$, 422.2808; found, 422.2808.

5,5'-(3,4-Diethyl-1H-pyrrole-2,5-diyl)bis(methylene)bis(4-3-bromopropyl)-3-methyl-1H-pyrrole-2-carbaldehyde) (4c)

A mixture of tripyrrane **3c** (0.60 g, 0.73 mmol, 1 equiv) and Pd/C (10%, 0.10 g, 0.095 mmol) in anhydrous THF (18 mL) was degassed under reduced pressure and saturated with hydrogen (1 atm), and the reaction mixture was stirred for 6 h at ambient temperature. The reaction mixture was filtered through celite, and solvent was removed under reduced pressure to obtain deprotected **3c** as a pink powder. To deprotected **3c** (0.46 g, 0.73 mmol, 1 equiv) was added trifluoroacetic acid (0.67 mL, 8.9 mmol, 12 equiv) dropwise under Ar at ambient temperature while stirring. Upon complete addition of trifluoroacetic acid, the resulting solution was stirred for 10 min. The reaction mixture was cooled at -20°C , and triethylorthoformate (0.79 mL, 4.8 mmol, 6.5 equiv) was added dropwise over 15 min. The resulting reaction mixture was stirred at -20°C for 15 min; the cold bath was removed; and water (22 mL) was added dropwise without stirring immediately after the temperature warmed above -20°C . The reaction mixture was left to stand for 30 min at ambient temperature, and the dark red precipitate was collected and washed with water (5×10 mL). The precipitate was suspended in ethanol (8.6 mL), and a mixture of ammonium hydroxide/water (1:3 v/v, 8.5 mL) was added. The reaction mixture was stirred at ambient temperature for 20 min. The resulting precipitate was separated and washed with water (5×20 mL) until the washings had a pH of 7 as measured by pH paper. The resulting solid was dissolved in CH_2Cl_2 (5 mL) and precipitated using CH_3OH /water (8:1, 45 mL). The resulting suspension was cooled to -20°C for 24 h, filtered, and washed with water (5.0 mL). Solvents were removed under reduced pressure to yield 371 mg (84%) of **4c** as a light brown solid. ^1H NMR (400 MHz, CDCl_3 , δ): 1.12 (t, $J = 7.4$ Hz, CH_3 , 6H), 1.82–1.96 (m, CH_2 , 4H), 2.20 (s, CH_3 , 6H), 2.46 (q, $J = 7.4$ Hz, CH_2 , 4H), 2.54 (t, $J = 7.2$ Hz, CH_2 , 4H), 3.31 (t, $J = 6.4$ Hz, CH_2 , 4H), 3.87 (brs, CH_2 , 4H), 9.07 (s, CH , 2H), 9.26 (s, NH , 1H), 10.74 (brs, NH , 2H); ^{13}C NMR (101 MHz, CDCl_3 , δ): 9.2 (CH_3), 16.8 (CH_3), 17.9 (CH_2), 22.3 (CH_2), 22.9 (CH_2), 33.3 (CH_2), 33.4 (CH_2), 120.6, 121.2, 121.8, 128.2, 139.0, 175.5 (CH); HRMS (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{28}\text{H}_{38}\text{N}_3\text{O}_2\text{Br}_2$, 606.1331; found, 606.1338.

Sodium 3,3'-(2,2'-(3,4-diethyl-1H-pyrrole-2,5-diyl)bis(methylene)bis(5-formyl-4-methyl-1H-pyrrole-3,2-diyl))dipropene-1-sulfonate (4d)

To a solution of **4c** (0.20 g, 0.33 mmol, 1 equiv) in *p*-dioxane (12 mL) was added an aqueous solution of KI (0.14 g, 0.80 mmol, 2.5 equiv, 2.0 mL), and the resulting mixture was stirred for 30 min at 80°C under Ar. To the resulting mixture was added an aqueous solution of Na_2SO_3 (0.24 g, 1.9 mmol, 6.0 equiv, 2.0 mL), and the mixture was stirred for 24 h under Ar at 80°C . Solvents were removed under reduced pressure, and the resulting residue was purified by reverse-phase chromatography using RP-tC18 SPE Sep-Pak (stepwise gradient of 1:0 $\rightarrow$ 7:3 water/ CH_3OH). Volatiles were removed under reduced pressure, and the resulting residue was dissolved in water (2 mL) and freeze dried to yield 0.16 g (76%) of **4d** as a brown fluffy powder. ^1H NMR (400 MHz, D_2O , δ): 0.91 (t, $J = 6.6$ Hz, CH_3 , 6H), 1.61–

1.74 (m, CH_2 , 4H), 2.14 (s, CH_3 , 6H), 2.21–2.44 (m, CH_2 , 8H), 2.70 (t, $J = 7.4$ Hz, CH_2 , 4H), 3.80 (s, CH_2 , 4H), 9.06 (s, CH , 2H); ^{13}C NMR (101 MHz, D_2O with dimethylsulfoxide- d_6 internal standard δ): 9.8 (CH_3), 17.4 (CH_3), 18.6 (CH_2), 23.3 (CH_2), 24.3 (CH_2), 26.4 (CH_2), 52.1 (CH_2), 122.7, 123.0, 123.9, 129.0, 137.6, 141.8, 178.7 (CH); HRMS (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{28}\text{H}_{38}\text{N}_3\text{O}_8\text{S}_2$, 608.2100; found, 608.2116.

3,3'-(1,2-Phenylenebis(oxy))dipropene-1-sulfonic acid (6b)

Catechol **5a** (0.72 g, 6.5 mmol, 1 equiv) was dissolved in a mixture of NaOH (0.65 g, 16 mmol, 2.7 equiv) and ethanol (35 mL) under Ar, and the resulting mixture was heated at reflux until a homogeneous solution was obtained. This mixture was added to propane sultone (1.8 g, 15 mmol, 2.5 equiv) dropwise over 30 min under Ar. The reaction mixture was heated at reflux under Ar for 48 h. The reaction mixture was centrifuged while hot; liquid was decanted; and the resulting residue was washed with hot ethanol (60 °C, 3×25 mL). The residue was dissolved in water (10 mL) and sparged with gaseous HCl for 3 min. The acid-saturated solution was centrifuged, and the liquid was decanted and concentrated under reduced pressure. The concentrated liquid (3 mL) was precipitated using CH_3OH (47 mL) and cooled to -20 °C. The solid residue was isolated, dissolved in water (5 mL), and purified by re-precipitation using CH_3OH /diethyl ether (8:1 v/v, 45 mL) at -20 °C. The precipitate was separated and washed with diethyl ether (2×25 mL). The volatiles were removed under reduced pressure to obtain 0.82 g (37%) of **6b** as an off white solid. ^1H NMR (400 MHz, D_2O , δ): 2.15–2.30 (m, CH_2 , 4H), 3.05–3.17 (m, CH_2 , 4H), 4.12 (t, $J = 6.4$ Hz, CH_2 , 4H), 7.03 (s, CH , 4H); ^{13}C NMR (101 MHz, D_2O with internal standard of dimethylsulfoxide- d_6 , δ): 25.8 (CH_2), 49.4 (CH_2), 69.2 (CH_2), 116.1 (CH), 123.7 (CH), 149.3; HRMS (m/z): $[\text{M} - 2\text{H} + \text{Na}]^-$ calcd for $\text{C}_{12}\text{H}_{16}\text{O}_8\text{S}_2\text{Na}$, 375.0184; found, 375.0247.

Sodium 3-(2-methoxyphenoxy)propane-1-sulfonate (6c)

2-Methoxyphenol **5b** (0.6 mL, 4 mmol, 1 equiv) was added to a mixture of NaOH (0.24 g, 6.0 mmol, 1.5 equiv) and ethanol (15 mL) under Ar in the dark, and the resulting reaction mixture was heated at reflux until a homogeneous solution was obtained. This mixture was added to propane sultone (0.74 g, 6.0 mmol, 1.5 equiv) dropwise under Ar. The reaction mixture was heated at reflux under Ar for 20 h in the dark. The reaction mixture was centrifuged while hot; liquid was decanted; and the resulting residue was washed with hot ethanol (60 °C, 3×20 mL). The residue was dissolved in water (5 mL), precipitated using ethanol (40 mL), and cooled to -20 °C. The solid residue was isolated, and the volatiles were removed under reduced pressure to obtain 0.83 g (84%) of **6c** as an off white solid. ^1H NMR (400 MHz, D_2O , δ): 2.14–2.27 (m, CH_2 , 2H), 3.04–3.15 (m, CH_2 , 2H), 3.84 (s, CH_3 , 3H), 4.14 (t, $J = 6.4$ Hz, CH_2 , 2H), 6.95–7.14 (m, CH , 4H); ^{13}C NMR (101 MHz, D_2O with internal standard of dimethylsulfoxide- d_6 , δ): 25.6 (CH_2), 49.2 (CH_2), 56.9 (CH_3), 68.6 (CH_2), 113.6 (CH), 114.9 (CH), 122.9 (CH), 123.2 (CH), 148.5, 149.8; HRMS (m/z): $[\text{M} - \text{H}]^-$ calcd for $\text{C}_{10}\text{H}_{13}\text{O}_5\text{S}$, 245.0484; found, 245.0491.

3,3'-(4,5-Diamino-1,2-phenylene)bis(oxy)dipropenoic acid hydrochloride salt (7a)

A mixture of dinitro compound **6a** (0.40 g, 1.2 mmol, 1 equiv), Pd/C (10%, 0.11 g, 0.10 mmol), and ethanol (18 mL) was sonicated until **6a** was dissolved. The resulting reaction

mixture was degassed under reduced pressure, saturated with hydrogen (1 atm), and heated at reflux for 3 h under an atmosphere of hydrogen. Solvent was removed under reduced pressure; HCl (1 M, 10 mL) was added; and the resulting mixture was filtered through a 0.2 μ m filter. The filtrate was concentrated by removing solvent under reduced pressure to yield 367.3 mg (88%) of **7a** as a purple hygroscopic solid. ^1H NMR (400 MHz, D_2O , δ): 2.70 (t, J = 6.0 Hz, CH_2 , 4H), 4.15 (t, J = 5.6 Hz, CH_2 , 4H), 6.96 (s, CH , 2H); ^{13}C NMR (101 MHz, D_2O with internal standard of dimethylsulfoxide- d_6 , δ): 35.4 (CH_2), 67.3 (CH_2), 111.9 (CH), 120.8, 149.1, 176.8; HRMS (m/z): $[\text{M} - \text{H}]^-$ calcd for $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_6$, 283.0930; found, 283.0936.

3,3'-(4,5-Dinitro-1,2-phenylene)bis(oxy)dipropene-1-sulfonic acid (**7b**)

Sulfonic acid **6b** (0.24 g, 0.68 mmol, 1 equiv) was dissolved in glacial acetic acid (3.9 mL) and stirred for 15 min at 15 $^\circ\text{C}$. To this mixture was added aqueous HNO_3 (70%, 2.5 mL) dropwise over a period of 15 min while maintaining the temperature of the reaction mixture at 15 $^\circ\text{C}$. After the addition, the reaction mixture was warmed to ambient temperature and stirred for 15 min. The reaction mixture was cooled to 15 $^\circ\text{C}$, and fuming HNO_3 (6.6 mL) was added dropwise over a period of 30 min while the temperature of the reaction mixture was held at 15 $^\circ\text{C}$. After the addition, the reaction mixture was warmed to ambient temperature and stirred for 64 h. The reaction mixture was concentrated under reduced pressure, and cold methanol (0 $^\circ\text{C}$, 2.0 mL) was added to form a precipitate. The precipitate was collected and dried under reduced pressure. Purification was performed using an RP- C_{18} SPE Sep-Pak column (stepwise gradient of 1:9 $\rightarrow$ 1:4 CH_3OH /water) to yield 151.6 mg (50%) of **7b** as a yellow solid. ^1H NMR (400 MHz, D_2O , δ): 2.22–2.37 (m, CH_2 , 4H), 3.11 (t, J = 7.4 Hz, CH_2 , 4H), 4.32 (t, J = 6.4 Hz, CH_2 , 4H), 7.60 (s, CH , 2H); ^{13}C NMR (101 MHz, D_2O with internal standard of dimethylsulfoxide- d_6 , δ): 25.5 (CH_2), 49.1 (CH_2), 70.2 (CH_2), 110.5 (CH), 137.6, 152.7; HRMS (m/z): $[\text{M} - \text{H}]^-$ calcd for $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_{12}\text{S}_2$, 443.0066; found, 443.0062.

3-(2-Methoxy-4,5-dinitrophenoxy)propane-1-sulfonic acid (**7c**)

Sulfonic acid **6c** (0.80 g, 3.3 mmol, 1 equiv) was dissolved in glacial acetic acid (19 mL) and stirred for 10 min at 15 $^\circ\text{C}$. To this mixture was added aqueous HNO_3 (70%, 12 mL) dropwise over a period of 15 min while maintaining the temperature of the reaction mixture at 15 $^\circ\text{C}$. After the addition, the reaction mixture was warmed to ambient temperature and stirred for 15 min. The reaction mixture was cooled to 15 $^\circ\text{C}$, and fuming HNO_3 (30 mL) was added dropwise over a period of 30 min while the temperature of the reaction mixture was held at 15 $^\circ\text{C}$. After the addition, the reaction mixture was warmed to ambient temperature and stirred for 46 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was dissolved in water (0.5 mL), and precipitated using CH_3OH /diethyl ether (1:1, 50 mL). The precipitate was collected and dried under reduced pressure to yield 0.80 g (72%) of **7c** as a yellow solid. ^1H NMR (400 MHz, D_2O , δ): 2.22–2.33 (m, CH_2 , 2H), 3.09 (t, J = 7.6 Hz, CH_2 , 2H), 3.96 (s, CH_3 , 3H), 4.24–4.32 (m, CH_2 , 2H), 7.51–7.56 (m, CH , 2H); ^{13}C NMR (101 MHz, D_2O with internal standard of dimethylsulfoxide- d_6 , δ): 25.3 (CH_2), 48.8 (CH_2), 58.3 (CH_3), 69.9 (CH_2), 109.0 (CH), 109.7 (CH), 137.3, 152.2, 153.1; HRMS (m/z): $[\text{M} - \text{H}]^-$ calcd for $\text{C}_{10}\text{H}_{11}\text{N}_2\text{O}_9\text{S}$, 335.0185; found, 335.0189.

4,5-Diethyl-10,23-dimethyl-9,24-bis(methylpropanoate)-16,17-bis((methylpropanoate)oxy)-13,20,25,26,27-pentaazapentacyclo-[20.2.1.1^{3,6}.1^{8,11},0^{14,19}]heptacos-3,5,8,10,12,14,16,18,20,22,24-undecaene (1L)

To a mixture of tripyrrole **4a** (0.12 g, 0.22 mmol, 1 equiv) in toluene/CH₃OH (6:1, 140 mL) under Ar was added a solution of diamine salt **7a** (0.080 g, 0.22 mmol, 1 equiv) in CH₃OH (4.0 mL). To the resulting reaction mixture was added HCl (11.6 M, 0.05 mL, 0.6 mmol, 3 equiv), and the mixture was heated at 80 °C for 6 h under Ar. The reaction mixture was cooled to ambient temperature; K₂CO₃ (83 mg, 0.60 mmol, 3 equiv) was added; and stirring was continued for 30 min. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. Solids were dissolved in methanol (4.0 mL) and cooled at –20 °C for 16 h to promote precipitation. The precipitate was collected, washed with diethyl ether (2.0 mL), and solvents were removed under reduced pressure to yield 76.4 mg (58%) of **1L** as a bright red solid. ¹H NMR (500 MHz, CDCl₃, δ): 1.06 (t, *J* = 7.5 Hz, CH₃, 6H), 2.27 (s, CH₃, 6H), 2.38 (q, *J* = 7.5 Hz, CH₂, 4H), 2.46 (t, *J* = 7.8 Hz, CH₂, 4H), 2.70–2.91 (m, CH₂, 8H), 3.71 (s, CH₃, 6H), 3.73 (s, CH₃, 6H), 3.96 (dd, *J* = 15.5, 123.4 Hz, CH₂, 4H), 4.25 (brs, CH₂, 4H), 7.16 (s, CH, 2H), 8.30 (s, CH, 2H), 11.07 (s, NH, 1H), 12.25 (s, NH, 2H); ¹³C NMR (126 MHz, CDCl₃, δ): 9.8 (CH₃), 16.7 (CH₃), 17.8 (CH₂), 19.5 (CH₂), 22.2 (CH₂), 34.4 (CH₂), 35.2 (CH₂), 51.81 (CH₃), 51.82 (CH₃), 65.2 (CH₂), 102.6 (CH), 120.3, 120.4, 121.2, 125.3, 131.0, 133.5, 140.2, 141.6 (CH), 148.0, 171.5, 173.2; HRMS (*m/z*): [M + H]⁺ calcd for C₄₄H₅₆N₅O₁₀, 814.4027; found, 814.4015; UV–vis (30% CH₃OH in water) λ_{max}, nm (ε): 364 (48400).

4,5-Diethyl-10,23-dimethyl-9,24-bis(ethyl)-16,17-bis((propanesulfonic acid)oxy)-13,20,25,26,27-pentaazapentacyclo-[20.2.1.1^{3,6}.1^{8,11},0^{14,19}]heptacos-3,5,8,10,12,14,16,18,20,22,24-undecaene (3L)

To a mixture of dinitrosulfonic acid **7b** (52.8 mg, 0.118 mmol, 1 equiv) in water/CH₃OH (1:2, 3.6 mL) was added Pd/C (10%, 26.8 mg, 0.0252 mmol). The resulting reaction mixture was degassed under reduced pressure and saturated with H₂ (1 atm). The reaction mixture was heated at reflux for 40 min under an atmosphere of hydrogen until the suspension became colorless. The resulting suspension was filtered through a 0.2 μm filter into a mixture of **4b** (50.4 mg, 0.118 mmol, 1 equiv) in CH₃OH/toluene/water (25:5:1, 62 mL) and HCl (11.6 M, 0.030 mL, 0.35 mmol, 3 equiv). The reaction mixture was stirred under Ar for 6.5 h. Solvents were removed under reduced pressure, and the resulting residue was precipitated with CH₃OH (5 mL) at –20 °C. The precipitate was isolated and purified by reverse-phase chromatography using an RP-tC18 SPE Sep-Pak (stepwise gradient of 1:1→1:4 water/CH₃OH). Volatiles were removed under reduced pressure to yield 37 mg (41%) of **3L** as a red powder. ¹H NMR (600 MHz, dimethylsulfoxide-*d*₆, δ): 1.01 (t, *J* = 7.5 Hz, CH₃, 6H), 1.11 (t, *J* = 7.5 Hz, CH₃, 6H), 2.01–2.07 (m, CH₂, 4H), 2.30–2.37 (m, CH₂, CH₃, 10H), 2.50–2.53 (m, CH₂, 4H), 2.58 (t, *J* = 6.9 Hz, CH₂, 4H), 3.95 (s, CH₂, 4H), 4.26 (t, *J* = 6.9 Hz, CH₂, 4H), 7.57 (s, CH, 2H), 8.72 (s, CH, 2H), 10.43 (s, NH, 1H), 11.17 (s, NH, 2H); ¹³C NMR (151 MHz, dimethylsulfoxide-*d*₆, δ): 8.5 (CH₃), 14.4 (CH₃), 15.1 (CH₃), 15.9 (CH₂), 16.1 (CH₂), 22.0 (CH₂), 23.6 (CH₂), 46.7 (CH₂), 66.6 (CH₂), 101.3 (CH), 119.4, 120.0, 123.5, 125.1, 140.5 (CH), 147.1; HRMS (*m/z*): [M – H][–] calcd for

$C_{38}H_{50}N_5O_8S_2$, 768.3101; found, 768.3099; UV-vis (30% CH_3OH in water) λ_{max} , nm (ϵ): 368 (38417).

Diethyl-10,23-dimethyl-9,24-bis(propanesufonicacid)-16-((propanesufonicacid)oxy)-17-methoxy-13,20,25,26,27-pentaazapentacyclo-[20.2.1.1^{3,6}.1^{8,11},0^{14,19}]heptacosa-3,5,8,10,12,14,16,18,20,22,24-undecaene (4L)

To a mixture of dinitrosulfonic acid **7c** (17 mg, 0.050 mmol, 1 equiv) in water/ CH_3OH (1:2, 1.8 mL) was added Pd/C (10%, 11 mg, 0.0010 mmol). The resulting reaction mixture was degassed under reduced pressure and saturated with H_2 (1 atm). The reaction mixture was heated at reflux for 75 min under an atmosphere of hydrogen until the suspension became colorless. The resulting suspension was filtered through a 0.2 μm filter into a mixture of **4d** in CH_3OH /water (15:1, 32 mL) and HCl (11.6 M, 0.020 mL, 0.23 mmol, 4.6 equiv). The reaction mixture was stirred under Ar for 6 h. Solvents were removed under reduced pressure, and the resulting residue was purified by reverse-phase chromatography using an RP-tC18 SPE Sep-Pak (stepwise gradient of 1:0 $\rightarrow$ 7:3 water/ CH_3OH). Volatiles were removed under reduced pressure, and the resulting residue was dissolved in water (2 mL) and freeze dried to yield 24 mg (57%) of **4L** as a red fluffy powder. 1H NMR (600 MHz, CD_3OD/D_2O (5:2), δ): 0.87 (brs, CH_3 , 6H), 1.90 (brs, CH_2 , 4H), 2.09 (brs, CH_2 , 2H), 2.20–2.36 (m, CH_2 , CH_3 , 10H), 2.65 (brs, CH_2 , 4H), 2.81 (brs, CH_2 , 6H), 3.88 (s, CH_3 , 3H), 4.02 (brs, CH_2 , 4H), 4.18 (brs, CH_2 , 2H), 7.27–7.40 (m, CH , 2H), 8.43 (s, CH , 2H); ^{13}C NMR (151 MHz, CD_3OD/D_2O (5:2), δ): 10.0 (CH_3), 16.8 (CH_3), 18.2 (CH_2), 23.3 (CH_2), 24.2 (CH_2), 25.1 (CH_2), 26.2 (CH_2), 49.8 (CH_2), 51.7 (CH_2), 57.4 (CH_3), 68.9 (CH_2), 103.8 (CH), 104.6 (CH), 121.9, 123.5, 123.7, 124.5, 124.6, 126.1, 126.5, 127.0, 127.2, 142.6 (CH), 142.9 (CH), 143.6, 149.3, 150.7, 152.5; HRMS (m/z): $[M - H]^-$ calcd for $C_{38}H_{50}N_5O_{11}S_3$, 848.2669; found, 848.2674; UV-vis (30% CH_3OH in water) λ_{max} , nm (ϵ): 365 (49529), 459 (sh, 15588).

4,5-Diethyl-10,23-dimethyl-9,24-bis(propanesufonicacid)-16,17-bis((propanesufonicacid)oxy)-13,20,25,26,27-pentaazapentacyclo-[20.2.1.1^{3,6}.1^{8,11},0^{14,19}]heptacosa-3,5,8,10,12,14,16,18,20,22,24-undecaene (5L)

To a mixture of dinitrosulfonic acid **7b** (23 mg, 0.050 mmol, 1 equiv) in water/ CH_3OH (1:2, 1.8 mL) was added Pd/C (10%, 11 mg, 0.0010 mmol). The resulting reaction mixture was degassed under reduced pressure and saturated with H_2 (1 atm). The reaction mixture was heated at reflux for 35 min under an atmosphere of hydrogen until the suspension became colorless. The resulting suspension was filtered through a 0.2 μm filter into a mixture of **4d** in CH_3OH /water (15:1, 32 mL) and HCl (11.6 M, 0.020 mL, 0.23 mmol, 4.6 equiv). The reaction mixture was stirred under Ar for 6 h. Solvents were removed under reduced pressure, and the resulting residue was purified by reverse-phase chromatography using an RP-tC18 SPE Sep-Pak (stepwise gradient of 1:0 $\rightarrow$ 4:1 water/ CH_3OH). Volatiles were removed under reduced pressure, and the resulting residue was dissolved in water (2 mL) and freeze dried to yield 25 mg (53%) of **5L** as a red fluffy powder. 1H NMR (600 MHz, CD_3OD/D_2O (5:2), δ): 0.86 (t, $J = 7.5$ Hz, CH_3 , 6H), 1.86–1.94 (m, CH_2 , 4H), 2.05–2.11 (m, CH_2 , 4H), 2.34 (brs, CH_2 , CH_3 , 10H), 2.61–2.68 (m, CH_2 , 4H), 2.78–2.84 (m, CH_2 , 8H), 4.04 (s, CH_2 , 4H), 4.15–4.20 (m, CH_2 , 4H), 7.35 (s, CH , 2H), 8.45 (s, CH , 2H); ^{13}C NMR (151 MHz, CD_3OD/D_2O (5:2), δ): 10.2 (CH_3), 16.9 (CH_3), 18.2 (CH_2), 23.3 (CH_2),

24.4 (CH₂), 25.1 (CH₂), 26.3 (CH₂), 49.8 (CH₂), 51.8 (CH₂), 69.0 (CH₂), 105.6 (CH), 121.7, 123.8, 124.4, 125.3, 127.7, 142.9 (CH), 144.7, 149.8, 153.9; HRMS (*m/z*): [M – H][–] calcd for C₄₀H₅₄N₅O₁₄S₄, 956.2550; found, 956.2527; UV–vis (30% CH₃OH in water) λ_{max}, nm (ε): 363 (57217), 467 (sh, 16283).

Texaphyrin complex 1

To a stirring solution of texaphyrin **1L** (0.060 g, 0.074 mmol, 1 equiv) in a mixture of CH₂Cl₂/CH₃OH (1:4, 5 mL) was added triethylamine (99 μL, 0.74 mmol, 10 equiv). To the resulting solution was added Gd(OAc)₃·4H₂O (60 mg, 0.15 mmol, 2 equiv), and the reaction mixture was heated at 60 °C while sparging with air until the peak intensity ratio (742 nm/364 nm) become constant (4.5 h). Solvents were removed under reduced pressure, and the resulting residue was purified using silica gel chromatography (stepwise gradient of 1:9→1:4 CH₃OH/CH₂Cl₂). Purification by precipitation from CH₃OH/CH₂Cl₂ (1:2, 0.5 mL) layered with CH₃OH/diethyl ether (1:1, 3 mL) yielded 36.4 mg (45%) of **1** as a green solid. HRMS (*m/z*): [M + AcO[–]]⁺ calcd for C₄₆H₅₃N₅O₁₂Gd, 1022.2917; found, 1022.2921; Anal. Calcd for C₄₄H₅₀N₅O₁₀GdCl₃·CH₃CO₂H·3H₂O: C, 46.60; H, 5.02; N, 5.91. Found: C, 46.78; H, 4.64; N, 6.10; UV–vis (30% CH₃OH in water) λ_{max}, nm (ε): 742 (32291), 470 (90902), 416 (sh, 52916); TLC: *R*_f = 0.24 (1:9 CH₃OH/CH₂Cl₂).

Texaphyrin complex 2

To a mixture of tripyrrole **4b** (72 mg, 0.16 mmol, 1 equiv) in THF/toluene (5:1 v/v, 90 mL) and HCl (11.6 M, 0.1 mL, 0.8 mmol, 5 equiv) was added a solution of diamine salt **7a** (0.060 g, 0.16 mmol, 1 equiv) in water (1.0 mL), and the mixture was heated at 80 °C for 8.5 h under Ar. Solvents were removed under reduced pressure. To the resulting residue (0.10 g, 0.15 mmol, 1 equiv) in CH₃OH (8 mL) was added triethylamine (0.21 mL, 1.5 mmol, 10 equiv). To the resulting solution was added Gd(OAc)₃·4H₂O (0.30 g, 0.75 mmol, 5 equiv), and the reaction mixture was heated at 60 °C while sparging with air until the peak intensity ratio (740 nm/370 nm) became constant (20 h). Solvents were removed under reduced pressure. The resulting residue was washed with CH₃OH, dissolved in CH₂Cl₂, and purified by chromatography with acidic alumina (stepwise gradient of 1:1:0→0:1:0.01 CH₂Cl₂/CH₃OH/AcOH). Volatiles were removed under reduced pressure to yield 27 mg (22%) of **2** as a green powder (purity 98.4% based on HPLC). HRMS (*m/z*): [M – H + 2-nitrophenylroglucinol]⁺ calcd for C₄₄H₄₆N₆O₁₁Gd, 992.2476; found, 992.2478; UV–vis (30% CH₃OH in water) λ_{max}, nm (ε): 740 (737), 474 (1995), 416 (1045).

Texaphyrin complex 3

To a stirring solution of texaphyrin **3L** (22 mg, 0.028 mmol, 1 equiv) in a mixture of CH₂Cl₂/CH₃OH (1:2.5 v/v, 2.8 mL) was added triethylamine (40 μL, 0.28 mmol, 10 equiv). To the resulting solution was added Gd(OAc)₃·4H₂O (46 mg, 0.11 mmol, 4 equiv), and the reaction mixture was heated at 60 °C while sparging with air until the peak intensity ratio (738 nm/368 nm) became constant (4 h). Solvents were removed under reduced pressure, and purified using silica gel chromatography (stepwise gradient of 1:9→1:4 CH₃OH/CH₂Cl₂). Purification by precipitation from CH₃OH (5 mL) layered with diethyl ether (25 mL) yielded 9.7 mg (37%) of **3** as a green solid. HRMS (*m/z*): [M + Na]⁺ calcd for

$C_{38}H_{44}N_5O_8S_2GdNa$, 940.1755; found, 940.1725; Anal. Calcd for $C_{38}H_{45}N_5O_8GdS_2Na_2 \cdot 3H_2O$: C, 44.69; H, 5.03; N, 6.86. Found: C, 44.86; H, 5.03; N, 6.49; UV-vis (30% CH_3OH in water) λ_{max} , nm (ϵ): 738 (27878), 473 (88060), 416 (sh, 44727); TLC: R_f = 0.26 (1:4 CH_3OH/CH_2Cl_2).

Texaphyrin complex 4

To a stirring solution of texaphyrin **4L** (50 mg, 0.059 mmol, 1 equiv) in CH_3OH (6 mL) was added triethylamine (0.080 mL, 0.59 mmol, 10 equiv). To the resulting solution was added $Gd(OAc)_3 \cdot 4H_2O$ (120 mg, 0.29 mmol, 5 equiv), and the reaction mixture was heated at 60 °C while sparging with air until the peak intensity ratio (742 nm/365 nm) became constant (22 h). Solvents were removed under reduced pressure, and the resulting residue was purified by reverse-phase chromatography using an RP-tC18 SPE Sep-Pak (stepwise gradient of 1:0 $\rightarrow$ 7:3 water/ CH_3OH). Volatiles were removed under reduced pressure. The resulting residue was dissolved in water (2 mL) and freeze dried to yield 30.2 mg (51%) of **4** as a green fluffy powder. HRMS (m/z): $[M - H]^+$ calcd for $C_{38}H_{45}N_5O_{11}S_3Gd$, 1001.1522; found, 1001.1520; Anal. Calcd for $C_{38}H_{43}N_5O_{11}GdS_3Na_5 \cdot 5H_2O$: C, 37.90; H, 4.44; N, 5.82. Found: C, 37.77; H, 3.87; N, 5.82; UV-vis (30% CH_3OH in water) λ_{max} , nm (ϵ): 742 (34571), 470 (93523), 416 (sh, 46904). HPLC chromatogram in Figure S2.

Texaphyrin complex 5

To a stirring solution of texaphyrin **5L** (0.048 g, 0.049 mmol, 1 equiv) in CH_3OH (8 mL) was added triethylamine (0.070 mL, 0.49 mmol, 10 equiv). To the resulting solution was added $Gd(OAc)_3 \cdot 4H_2O$ (0.10 g, 0.25 mmol, 5 equiv), and the reaction mixture was heated at 60 °C while sparging with air until the peak intensity ratio (743 nm/363 nm) became constant (6 h). Solvents were removed under reduced pressure, and the resulting residue was purified by reverse-phase chromatography using an RP-tC18 SPE Sep-Pak (stepwise gradient of 1:0 $\rightarrow$ 7:3 water/ CH_3OH). Volatiles were removed under reduced pressure. The resulting residue was dissolved in water (2 mL) and freeze dried to yield 30.7 mg (55%) of **5** as a green fluffy powder. HRMS (m/z): $[M - H]^+$ calcd for $C_{40}H_{49}N_5O_{14}S_4Gd$, 1109.1403; found, 1109.1405; Anal. Calcd for $C_{40}H_{46}N_5O_{14}GdS_4Na_8 \cdot 8H_2O$: C, 33.50; H, 4.36; N, 4.88. Found: C, 33.55; H, 3.84; N, 4.85; UV-vis (30% CH_3OH) λ_{max} , nm (ϵ): 743 (30486), 472(82734), 416 (sh, 43146). HPLC chromatogram in Figure S3.

Texaphyrin stain 1

To a mixture of ethanol (95%, 2 mL) and acetic acid (10%, 100 μ L) was added texaphyrin **1** (4.4 mg, 0.0045 mmol, 1 equiv). The reaction mixture was heated at 60 °C for 18 h, cooled to ambient temperature, and filtered through a 0.2 μ m filter to obtain stain **1** as a dark green solution. The stain solution was stored at 4 °C. The concentration of the stain solution was determined by ICP-OES.

Texaphyrin stain 2

To a mixture of ethanol (100%, 2 mL) and acetic acid (10%, 100 μ L) was added texaphyrin **2** (4.0 mg, 0.0048 mmol, 1 equiv) followed by ditolylguanidine (4.7 mg, 0.019 mmol, 4 equiv) dissolved in acetic acid (1 M, 19 μ L, 4 equiv). The reaction mixture was filtered

through a 0.2 μm filter and heated at 60 °C for 18 h. The solution was cooled to ambient temperature, and filtered through a 0.2 μm filter to obtain stain **2** as a dark green solution. The stain solution was stored at 4 °C. The concentration of the stain solution was determined by ICP–OES.

Texaphyrin stain 3

To a mixture of CH_3OH /water (3:2 v/v, 1 mL) was added texaphyrin **3** (4.2 mg, 0.0045 mmol, 1 equiv) followed by ditolylguanidine (4.4 mg, 0.018 mmol, 4 equiv) dissolved in acetic acid (1 M, 18 μL , 4 equiv). The reaction mixture was heated at 60 °C for 6 h. The solution was cooled to ambient temperature, and the solvent was removed under reduced pressure. The residue was dissolved in a mixture of ethanol (95%, 2 mL) and acetic acid (10%, 100 μL), and the solution was heated at 60 °C for 14 h. The solution was cooled to ambient temperature and filtered through a 0.2 μm filter to obtain stain **3** as a dark green solution. The stain solution was stored at 4 °C. The concentration of the stain solution was determined by ICP–OES.

Texaphyrin stain 4

To a mixture of CH_3OH /water (1:1 v/v, 1 mL) was added texaphyrin **4** (4.1 mg, 0.0041 mmol, 1 equiv) followed by ditolylguanidine (6.0 mg, 0.025 mmol, 6 equiv) dissolved in acetic acid (1 M, 25 μL , 6 equiv). The reaction mixture was heated at 60 °C for 6 h. The solution was cooled to ambient temperature, and the solvent was removed under reduced pressure. The residue was dissolved in a mixture of ethanol (95%, 2 mL) and acetic acid (10%, 100 μL), and the solution was heated at 60 °C for 14 h. The solution was cooled to ambient temperature and filtered through a 0.2 μm filter to obtain stain **4** as a dark green solution. The stain solution was stored at 4 °C. The concentration of the stain solution was determined by ICP–OES.

Texaphyrin stain 5

To a mixture of CH_3OH /water (1:2 v/v, 1 mL) was added texaphyrin **5** (4.2 mg, 0.0038 mmol, 1 equiv) followed by ditolylguanidine (8.0 mg, 0.033 mmol, 8 equiv) dissolved in acetic acid (1 M, 33 μL , 8 equiv). The reaction mixture was heated at 60 °C for 6 h. The solution was cooled to ambient temperature, and the solvent was removed under reduced pressure. The residue was dissolved in a mixture of ethanol (95%, 2 mL) and acetic acid (10%, 100 μL), and the solution was heated at 60 °C for 14 h. The solution was cooled to ambient temperature and filtered through a 0.2 μm filter to obtain stain **5** as a dark green solution. The stain solution was stored at 4 °C. The concentration of the stain solution was determined by ICP–OES.

Relaxivity measurements of texaphyrins 1–5

Longitudinal relaxation times (T_1) were measured using a standard inversion recovery method with a Bruker Minispec mq 60 at 1.4 T and 37 °C in water or 30% or 50% methanol in water. The slope of a plot of $1/T_1$ vs concentration of Gd^{III} was used to obtain relaxivity. Five different concentrations of Gd^{III} (0–1 mM) were used. Measurements were repeated 3

times with independently prepared samples, and Gd concentrations were determined by ICP-OES.

UV-vis and fluorescence emission measurements of texaphyrins 1–5

UV-vis spectra were obtained on Shimadzu UVmini-1240 UV-vis spectrophotometer. Texaphyrins **1–5** were dissolved in 30% methanol in water (0.05–0.25 mM) and absorbance was measured using a 3 mm quartz cuvette. Fluorescence spectra were obtained using a HORIBA Jobin Yvon Fluoromax-4 spectrofluorometer. Texaphyrins **1–5** were dissolved in 30% methanol in water and a 3 mm quartz cuvette was used. Fluorescence emissions were measured using 5 nm slit width and 468–492 nm excitation wavelengths.

Animals

Animal care and use were in accordance with the National Institutes of Health Animal Care Guidelines and approved by the Wayne State University Institutional Animal Care and Use Committee. Intact brain tissue used for the section- and *en bloc*-staining techniques were obtained from euthanized C57Bl/6 mice that were bred in-house (41). Prior to euthanizing, all efforts were made to minimize suffering.

Tissue Staining

Tissue Preparation—Formaldehyde (37% wt/v), lithium carbonate, ethanol (200 proof), and agarose were purchased from Sigma Aldrich, Acros Organics, Decon Laboratories Inc., and OmniPur, respectively. Tissues were acquired within 5 min of animal sacrifice and placed in a beaker of 3.7% (wt/v) aqueous formalin solution. Fixed tissue was stored at 4 °C in formalin. Prior to staining, fixed tissues were removed from formalin storage and rinsed with water (~10 mL) to remove formalin.

Section-Staining Procedure—Tissue sections (200 µm thick) were obtained from fixed intact tissues using a vibrotome 3000 (MyNeuroLab). For each stain, three tissue sections were stained that provided observation of a diverse assortment of brain regions: 0.50 to 1.54, 0.14 to –0.58, and –1.58 to –2.18 mm from Bregma (31,32). Each tissue section was incubated in staining solution (0.8–2.5 mM, 0.2 mL) for 5 h at 54 °C. Tissue sections were removed from the staining solution and rinsed by gentle swirling with ethanol (95%, 1 mL, 1 min) followed by water (1 mL, 1 min). Tissues stained with texaphyrin **1** were differentiated with a solution of Li₂CO₃ (4.5 mM) in ethanol (65%, 4 mL) for 30 min, sequentially with three concentrations of ethanol (100, 95, and 70%, 4 mL each) for 45 min each, and water (4 mL) for 5 min. All differentiations and rinsings were performed on a rotator (Thermo Labquake Tube Shaker/Rotator). Tissues stained with texaphyrins **2–5** were differentiated with a solution of Li₂CO₃ (4.5 mM) in ethanol (65%, 4 mL) for 1–2 h, rinsed with ethanol (95%, 4 mL, 1 min), and hydrated in water (4 mL, 5 min for complexes **2** and **3**, 1 min for complexes **4** and **5**). After differentiation and hydration, tissue sections were mounted on microscope slides for imaging.

En bloc-Staining Procedure—Intact mouse brains were incubated in solutions of texaphyrin **3** (0.27 mM, 1.8 mL) for 24 h at 54 °C. At the end of the incubation, the brains were rinsed with ethanol (95%, 25 mL, 1 min) then water (25 mL, 1 min). The brain tissue

was pat dried with tissue paper, and an incision was made using a scalpel along the midline on the dorsal face of the intact brain, such that the incision reached the dorsal third ventricle. Brains were placed in a solution of Li_2CO_3 (11 mM) in ethanol (40%, 25 mL) and the vial containing the brains in the Li_2CO_3 solution was rotated. The solution of Li_2CO_3 was changed after 6 and 24 h, and the differentiation was continued for total of 65 h. At the end of the differentiation, the mouse brain was rinsed with water (25 mL, 1 min) and hydrated in water (25 mL) for 10 min. The hydrated brain was pat dried and suspended in agarose gel (2%) for MRI.

Optical Imaging

Optical images were acquired using an Olympus SZX7 (model number SZ2-ILST) microscope (1.25 $\times$ or 5.6 $\times$ magnification) with an attached SPOT idea microscope camera (model number 27.2–3.1 MP). Differentiated section-stained mouse brain tissues were mounted on microscope slides to obtain images. *En bloc*-stained mouse brains were sliced (200 μm) using a vibrotome after obtaining MR images, and the slices were mounted on microscope slides for optical imaging. To compare with the MR images, brain slices were selected using a rough calculation of distance from the Bregma accounting for slice thicknesses of 500 and 200 μm for MRI and optical microscopy, respectively.

MRI

MRI scans were performed with a 7 T Varian small animal MRI scanner (299.44 MHz, 7.0 T) equipped with a 12 cm bore magnet and a 38 mm diameter homemade transmit/receive quadrature birdcage coil. Samples included a whole mouse brain stained with texaphyrin **3** and an unstained control whole mouse brain suspended in agarose. T_1 -maps were acquired at ambient temperature using a T-One by Multiple Read-Out Pulses (TOMROP) (41), which is a Look–Locker sequence (43). At least one dummy cycle (N pulses followed by T_{relax}) was applied before the start of data acquisition. Inversion of the longitudinal magnetization was accomplished using a non-selective hyperbolic secant adiabatic pulse of duration 12 ms. One phase-encode line of 40 small-tip-angle (approximately 10° 3-lobe sinc shaped pulses) gradient-echo images (4 ms) was acquired after each such adiabatic inversion (50 ms intervals) for a total recovery time of 3000 ms with a 3 s relaxation interval between each adiabatic inversion. Matrix size = 256 $\times$ 192; 32 mm² field-of-view; three image slices at 0.5 mm thickness; and eight averages. Analysis proceeded as described previously (44), with a modification that is now described. Because the 3 s relaxation time was not sufficient to allow the longitudinal magnetization to re-equilibrate after inversion, the equilibrated magnetization of the sequence was iteratively estimated along with the other model parameters (proton density, T_1 , and tip-angle). ImageJ was used to process the R_1 -maps. Brightness and contrast were adjusted to 50 and color balance was adjusted to 244. Five regions of interest were used to calculate the percent increase of R_1 in myelin-rich regions with respect to myelin-poor regions in the stained and unstained brains (Figure S4). The difference between the average R_1 values of the regions of interest from myelin-rich and myelin-poor regions were used to calculate percent increase of R_1 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Chemical structures of **a** LFB MBS and **b** texaphyrins **1–5**. Counter ions are not shown, but counter cations are ditolylguanidinium ions and counter anions are acetate ions.

Figure 2.
UV-vis absorption spectra of texaphyrins **1–5**: **1** (...); **2** (---); **3** (=); **4** (· —); and **5** (—).

Figure 3.

Emission spectra of texaphyrins **1–5**: **1** (...), $\lambda_{\text{ex}} = 485$ nm; **2** (---), $\lambda_{\text{ex}} = 470$ nm; **3** (=), $\lambda_{\text{ex}} = 468$ nm; **4** (· —), $\lambda_{\text{ex}} = 490$ nm; and **5** (—), $\lambda_{\text{ex}} = 492$ nm.

Figure 4.

Light microscopy images of mouse brain slices stained with texaphyrins. Columns A–C contain representative images from three different regions of the brain (A, 1.54 to 0.5; B, 0.14 to –0.58; and C, –1.58 to –2.18 mm from Bregma) that were treated with texaphyrins 1–5. Row 6 shows mouse brain atlas corresponding to matching coronal section treated with texaphyrin 3 (32). The images from the mouse brain atlas are modified to color well-established myelin-rich regions green, and the following parts are labeled: anterior commissure (aco), corpus callosum (cc), caudate putamen (CPu), fornix (f), fimbria of the

hippocampus (fi), internal capsule (ic), mammillothalamic tract (mmt), and optic track (ot).
The slice thickness is 200 μm for all slices, and the scale bar represents 1 mm for all images.

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Figure 5.

Representative coronal images (−1.58 mm from Bregma) of intact brain tissue from two different imaging modalities: **a** MR image (R_1 -map) of unstained control brain; **b** Light microscopy image of unstained control brain from the same brain used for MRI; **c** Image from the mouse brain atlas corresponding to a matching coronal section modified to color myelin-rich regions green (32). The corpus callosum (cc), caudate putamen (CPu), fornix (f), fimbria of the hippocampus (fi), internal capsule (ic), mammillothalamic tract (mmt), and optic tract (ot) are labeled in the image; **d** R_1 -map of a mouse brain stained with texaphyrin **3**; **e** Light microscopy image of a mouse brain stained with texaphyrin **3** from the same brain used for MRI; and **f–h** high magnification images of myelinated regions denoted in **e**. Myelinated regions of brains stained with texaphyrin **3** produce similar staining patterns in both imaging modalities. The slice thickness was 500 μm for the R_1 -map and 200 μm for optical imaging. Scale bars = 1 mm; Panels **a–e** use the same scale bar, and panels **f–h** use the same scale bar.

Scheme 1.

Synthesis route to complexes **1–5**. Detailed procedures are provided in the experimental section.

Relativity (r_1) values of texaphyrins **1–5** and Gd^{III}DOTA measured at 1.4 T and 37 °C in water or *30% or †50% methanol in water.

Table 1

Texaphyrin	1	2	3	4	5	Gd ^{III} DOTA
Relativity (mM ⁻¹ s ⁻¹)	19.5 ± 0.1 ^a	13.3 ± 0.2 [*]	11.6 ± 0.1 [†]	11.3 ± 0.2	11.7 ± 0.1	3.11 ± 0.01

Results are reported as mean ± standard error of three independently prepared samples.