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Structural Requirements for a Lipoamino Acid in Modulating the Anticonvulsant Activities of the Systemically-Active Galanin Analogs

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Abstract

Introduction of lipoamino acid (LAA), Lys-palmitoyl, and cationization into a series of galanin analogs yielded systemically-active anticonvulsant compounds. To study the relationship between the LAA structure and anticonvulsant activity, orthogonally protected LAAs were synthesized in which the Lys side chain was coupled to fatty acids varying in length from C_8 to C_{18}, or to a monodispersed polyethylene glycol, PEG_4. Galanin receptor affinity, serum stability, lipophilicity (logD) and activity in the 6 Hz mouse model of epilepsy of each of the newly synthesized analogs was determined following systemic administration. The presence of various LAAs or Lys($MPEG_4$) did not affect the receptor binding properties of the modified peptides, but their anticonvulsant activities varied substantially, and were generally correlated with their lipophilicity. Our results suggest that varying the length or polarity of the LAA residue adjacent to positively-charged amino acid residues may effectively modulate the antiepileptic activity of the galanin analogs.

Introduction

When delivered directly into the brain, many neuropeptides, including galanin, neuropeptide Y, somatostatin, neurotensin or opioid peptides can modulate excitatory or inhibitory circuits and suppress seizures and/or pain sensation.[1-4] Despite a considerable interest in generating blood-brain barrier (BBB) permeable analogs of neuropeptides, only a few successful examples have been reported to date.[4-12] To improve central nervous system (CNS) bioavailability of neuroactive peptides, various strategies have been explored; including lipidization, cationization or glycosylation.[13-15] Banks and Kastin showed that the lipophilicity of peptides improved their permeability through the BBB.[16] Despite the finding that only small amounts of the peptides entered the brain, the authors noted a direct correlation between the logD values and blood-to-brain ratio. Reversible lipidization of opioid peptides also improved centrally-mediated analgesic effects.[17] It is also important to note that oral bioavailability of the somatostatin analog TT-232 was achieved by coupling various LAAs to either N- or C-terminal

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Supporting Information Available: Two supplemental tables and one supplemental figure are available as supporting information. Supplemental Tables S1 and S2 summarize the purity and logD calculations; whereas Supplemental Figure S1 shows the HPLC chromatograms obtained with the galanin analogs. This material is available free of charge via the Internet at http://pubs.acs.org.

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components of the analog.[18] Although lipophilicity has been acknowledged to play an important role in structure-bioavailability relationships,[16, 19] very few examples are available where lipidization of neuropeptides has been found to improved their activity in the brain.[17, 20, 21] As pointed out in a review by Witt *et al*,[13] "lipidization of peptides may increase plasma protein binding, systemic elimination and intracellular sequestration; thus hampering their efficient penetration into the CNS." Indeed, when just the lipidization strategy was applied to galanin, the truncated galanin analog containing a Lys-palmitoyl residue was inactive as an anticonvulsant, despite displaying high affinity toward galanin receptors and a high logD.[22]

In contrast, lipidization appeared very effective in improving the CNS bioavailability of the truncated galanin analogs when the Lys(palmitoyl) residue was introduced in concert with cationization.[22] The most active analog, Gal-B2, contained the C-terminal "-Lys-Lys-Lys (palmitoyl)-Lys-NH_2" motif (Figure 1). The combined lipidization and cationization resulted in very potent antiepileptic compounds; *e.g*., the anticonvulsant ED_{50} of Gal-B2 was found to be 0.8 mg/kg when tested intraperitoneally (*i.p*.) in the 6 Hz pharmacoresistant model of epilepsy. Since galanin suppresses seizures by activating GalR1 and GalR2 located in the hippocampus and other limbic structures, our data suggest that the combination of cationization and lipidization is effective in improving the BBB penetration of Gal-B2 without negatively affecting receptor affinity. Furthermore, our results suggest that the sequence position of LAAs is important for the systemic activity of Gal-B2, suggesting that the lipidization/cationization motif was not simply producing the additive effect by combining hydrophobicity and positive charges.

In order to define the structural requirements of the LAA residue for improving the anticonvulsant activity of Gal-B2, we synthesized a series of LAAs in which the Lys side chain was coupled to either fatty acids, varying in length from C_8 to C_{18}, or to a monodispersed PEG_4 ($MPEG_4$). These LAAs were used to replace the Lys-palmitoyl residue in position 16 of Gal-B2 (Figure 1). The binding affinity, octanol/water partitioning coefficient, and ability to suppress seizures in the *in vivo* 6 Hz model of epilepsy was determined following *i.p.* administration. Our findings suggest that the length and polarity of the LAAs are important for maintaining antiepileptic activity of the galanin analogs.

Results

Design and chemical synthesis

To study the role of Lys-palmitoyl, residue 16, in the activity of Gal-B2, we designed a series of analogs where we systematically changed the length of the fatty acid or replaced the palmitoyl motif with a $MPEG_4$ (Figure 1). The length of the LAAs varied from C_8 to C_{18}, by two carbon atom increments (Table 1). The $MPEG_4$-containing Gal-B2 analog was designed to dissect the lipophilic contribution of the Lys-palmitoyl residue, since $MPEG_4$ is significantly more polar than C_{16} despite having an identical number of atoms. To synthesize these new structure activity relationship (SAR) analogs of Gal-B2, we applied a direct coupling of the orthrogonally protected N^{ε}-lipolysine intermediates (N^{ε}-palmitoyl-N^{α}-Fmoc-lysine is commercially available). For the synthesis of N^{ε}-octanoyl **1** (C_8), -decanoyl **2** (C_{10}), -lauryl **3** (C_{12}), -myristoyl **4** (C_{14}) and -stearoyl **5** (C_{18}) substituted N^{α}-Fmoc-lysine, a convenient method was applied using Na_2CO_3 as a coupling reagent with a corresponding acid halide in dioxane/water solvent system (Scheme 1). Synthesis of Fmoc-Lys-($MPEG_4$)-OH **6**, was achieved by coupling Fmoc-Lys-OH with perfluorophenol activated $MPEG_4$-acetic acid **7**. Compound **7** was synthesized from the substitution reaction of $MPEG_4$-OH with ethyl bromoacetate followed by ester hydrolysis (Scheme 2). The modified amino acids were purified by flash chromatography and used for solid-phase peptide synthesis (SPPS).

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The SPPS was carried out using an automated peptide synthesizer at 25 μmol scale on preloaded Fmoc-Lys(Boc)-Rink amide AM resin. Five-fold Fmoc-amino acids/PyBop/DIPEA (1:1:2) were used in peptide synthesis. Two-fold of the Fmoc protected LAAs or $MPEG_4$-Lysine were used in manual coupling synthesis. The synthesized peptides were cleaved from the resin using reagent K (trifluoroacetic acid (TFA)/water/thioanisole/phenol/ethanedithiol; 90/5/5/7.5/2.5 v/v), followed by precipitation and washing with ice cold methyl *tert*-butyl ether (MTBE). Crude peptides were purified by preparative reversed-phase HPLC using diphenyl column with a linear gradient of water/acetonitrile (both buffered with 0.1% TFA). The purities of peptides were greater than 95% by analytical HPLC analyses (Supplemental Figure S1, Supplemental Table S1). Purified analogs were quantified by UV absorbance (λ = 279.8 nm, ε = 7000 cm^{-1} M^{-1}), and their monoisotopic mass was confirmed by MALDI-TOF mass spectrometry (Table 2).

Physicochemical and structural properties

To determine the lipophilicity of the analogs, logD values were calculated using the combination of the HPLC retention times and the logD determined from the classical shake-flask method, as previously described.[22] Calculations of logD values are provided in Supplemental Table S2. For the LAA containing analogs, the logD values ranged from 0.78 for Gal-B2-C_8 to 1.35 for Gal-B2-C_{18}. As shown in Figure 2, the logD values linearly correlated with the carbon chain length of the LAA (linear fit yielded R^2 = 0.98). Since the logD values increased four-fold by replacing one Lys residue with Lys-stearoyl (compare logD of Gal-(K) 4 (0.34)[22] with Gal-B2-C_{18}, 1.35), and almost doubled by changing the LAA from C_8 to C_{18}, these results emphasize the effectiveness of the LAAs to increase the lipophilicity of short peptides. The analog with the $MPEG_4$-amino acid isostere had approximately two-fold lower logD (0.57), as compared to the Lys-palmitoyl residue (1.24).

Gal-B2 is largely unstructured in water, but contains 23% α-helical content in the presence of 50% trifluoroethanol (TFE).[22] In our previous work,[22] we hypothesized that the presence of LAA could stabilize the helical conformation of the systemically-active galanin analogs by hydrophobic interactions with Tyr9 and/or Trp2. To test this hypothesis, the conformational properties of the Gal-B2 analogs were investigated by circular dichroism (CD). In the presence of 5 mM potassium phosphate containing 150 mM NaF (pH 7.5), the helical content varied among the analogs from 1% to 7% (only Gal-B2-C_8 had 15%). The helical content in 50% TFE increased for most analogs as compared to that determined in “buffer only” conditions (Table 2). No apparent correlations were observed between the length of the LAA and percentage of helical content in the analogs.

Metabolic stability

To investigate how the length of the LAA residue might affect resistance of the galanin analogs to proteolytic degradation, we employed the *in vitro* metabolic stability assay. The half-life of the analogs was determined in buffered 25% rat blood serum incubated at 37 °C. The amount of each analog remaining after an appropriate time interval was quantified by HPLC and the time-course of disappearance of the analogs is shown in Figure 3A. Calculated half-live values for the analogs are summarized in Figure 3B. All analogs exhibited pronounced resistance to proteolytic degradation (for comparison, the unmodified Gal(1-16) analog had 7.8 min half-time under these conditions). [22] Interestingly, the galanin analogs containing the shortest or the longest aliphatic chain were the least stable, whereas the analog containing C_{14} had the longest half-life of 12 h (Figure 3B). This correlation suggested a rather complex mechanism by which the LAAs might increase the stability of galanin analogs in plasma. We hypothesized that the metabolic stability of the galanin analogs might be correlated with their serum albumin binding properties (e.g., binding properties of long-chain carboxylic acids to albumin are dependent on aliphatic chain length).[23] However, our efforts in determining the protein binding

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properties of Gal-B2 by microdialysis failed due to possible micelle formation.[22] The Gal-B2-$MPEG_4$ analog exhibited a very long half-life (12.4 ± 0.65 h), suggesting that the extended amino acid residue might provide steric hindrance for access of proteinases.

In vitro and *in vivo* pharmacology

Since two galanin receptors, GalR1 and GalR2, are known to be involved in controlling seizures in the brain,[24, 25] we investigated the interactions of the galanin analogs with both receptor subtypes using a competitive binding assay. As described in our previous work, K_i values were determined using a time-resolved fluorescence binding assay with a europium-labeled galanin.[22] The GPCR membrane preparations used in the assay were commercially available (Perkin-Elmer or Millipore) and were derived from recombinant human GalR1 or GalR2 gene sequences. As shown in Table 3, when compared to each other, none of the analogs studied here exhibited significantly different affinities toward either receptor subtype. This suggested that the relatively large LAA moiety did not affect the affinity of the analogs towards the galanin receptors. All analogs maintained several-fold preference in binding to GalR1 subtype, as compared to GalR2, similar to that of unmodified Gal(1-16).[22]

The anticonvulsant activity of the analogs was studied in the 6 Hz (32 mA) model of pharmacoresistant epilepsy following *i.p.* administration of a bolus dose of 4 mg/kg. The analogs were evaluated for their ability to suppress seizures at various times (0.25 to 4 h) after peptide administration. The time-response curves were then integrated to provide a qualitative measurement of efficacy; i.e., area under the curve (AUC) values (Table 3, Figure 4). The analogs differed significantly in their ability to protect mice from seizures. Based on the AUC values, the most active galanin analogs contained Gal-B2-C_{16} and Gal-B2-C_{18}, whereas the least active analogs included C_8 and C_{10}. Interestingly, the Lys-$MPEG_4$-containing analog was also active as an anticonvulsant, albeit significantly less when compared to Gal-B2.

Discussion

To study the structural requirements for the LAA residue 16 in mediating the antiepileptic activity of the systemically-active galanin analogs,[22] we synthesized and characterized a new series of analogs that differed in their length/polarity of the fatty acid moiety coupled to the Lys16 residue. The major finding of this work was that, the six galanin analogs (despite having comparable affinities towards the galanin receptors and differing in the length of the fatty acid from C_8 to C_{18}), exhibited pronounced differences in their anticonvulsant activities following *i.p.* administration. The analogs containing shorter fatty acids were less active as compared to those with longer chains. These results suggest that the increased lipophilicity provided by the longer fatty acids is an important factor in improving the anticonvulsant activity of galanin analogs following systemic administration. Further work will be required to determine whether the resulting compounds are agonists, partial agonists, or antagonists. Interestingly, both Gal-B2 and Gal-B2-C_8 showed comparable analgesic activities in a mouse pain assay following *i.p.* administration (E. Adkins-Scholl, H.S. White, G. Bulaj, unpublished results), suggesting that systemically-active galanin analogs varying in the length of a LAA may control seizures or pain via galanin receptors in the CNS or peripheral nerves, respectively.[26-28] Our previous data indicated that the position of the LAA residue appeared to be important for the anticonvulsant activity of Gal-B2,[22] thus implying that lipophilicity alone (which would be expected to increase passive diffusion) is not the sole important factor for improving seizure suppression by this analog. Although the "-Lys-Lys-Lys(palmitoyl)-Lys-NH_2" motif appeared to be the most effective in increasing the anticonvulsant activity of the galanin analogs following *i.p.* administration. Clearly, more SAR studies, or perhaps even a combinatorial approach, will be needed to further improve the potency of the Gal-B2 related analogs.[22]

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Introduction of the LAA to the galanin analogs significantly improved their *in vitro* metabolic stability; this effect may be, at least in part, accounted for by steric effects of the long side chain in position 16 of the analogs. This finding is not surprising in light of previous reports with lipopeptides, in which prolonged *in vitro* and/or *in vivo* half-lives were observed,[29] including: gonadotropin-releasing hormone analogs,[30] glucagon-like peptide 1 analogs,[31] insulin,[32] and octreotide.[20] The metabolic stability of lipopeptides may also be affected by their interactions with fatty acid binding sites in serum albumin.[32] As shown in Figure 3B, the longest serum half-lives were observed for Gal-B2-C_{14} and Gal-B2-C_{16}, whereas the shortest were observed for Gal-B2-C_8. Interestingly, the C_8 (octanoic acid) moiety binds with 11-fold higher affinity to human serum albumin compared to the C_{14} (myristic acid).[23]

Another aspect of this work is our less conventional approach of using orthogonally protected LAAs and $MPEG_4$-amino acids as building blocks for SPPS. Introduction of LAA into peptides have been reported using three distinct strategies: 1) on-resin fatty acid acylation of N^{α}-amino acids,[33, 34] or N^{ε}-lysine,[35] 2) esterification of fatty acids,[36] and 3) conjugation at the C-terminus of peptide.[18, 37] Our strategy of using the Fmoc protected LAAs directly coupled during the solid-phase peptide synthesis offers apparent advantages in applications such as routine synthesis of lipopeptide analogs for SAR studies or synthesis of peptide-based combinatorial libraries directed toward optimization of the chain length of the LAAs in the cationic/lipidic motif.

To what extent the systemically-active galanin analogs may have improved the BBB permeability remains unanswered. However, since some of the modified galanin analogs possess pronounced antiepileptic activity, believed to be mediated by the galanin receptors located in the limbic system and thus, behind the blood-brain-barrier, these data suggest that sufficient quantities of the analogs must penetrate into the brain. As we have suggested previously,[22] lipidization may improve CNS bioavailability of Gal-B2 via a combination of adsorptive-mediated endocytosis and passive diffusion.[38, 39] More mechanistic studies assessing the mechanism of transport of galanin analogs across the cell-based models of BBB are underway. Regardless of the mechanism by which these analogs penetrate into the brain, our data suggest that changing the length and/or polarity of the LAA residue in the context of cationization may offer a useful strategy to modulate CNS bioavailability of galanin analogs. In summary, we believe that the variations in lipidization and cationization appear to provide a useful strategy for modulating CNS bioavailability of not only the galanin analogs described here, but perhaps even other neuropeptides.

Experimental section

General synthetic procedures

Reagent chemicals were obtained from Aldrich Chemical Corporation and were used without prior purification. Reactions were performed under N_2 atmosphere, unless otherwise indicated. Chromatography refers to flash chromatography on silica gel (Whatman 230-400 mesh ASTM silica gel). Preparative HPLC was performed on a Waters 600 pump system equipped with a Waters 2487 dual wavelength detector (λ_1 = 220 nm, λ_2 = 280 nm) and a preparative Vydac diphenyl column (219TP101522); analytical HPLC used an analytical Vydac diphenyl column (219TP54). The HPLC mobile phases are Buffer A, 100% water (0.1% TFA) and Buffer B, 90% acetonitrile (0.1% TFA). Metabolic stability used a Waters Alliance 2695 system equipped with an autosampler, dual wavelength detector, and a Waters YMC ODS-A diphenyl column (AA125052503WT). NMR spectra were recorded at 400 MHz (^{1}H), 101 MHz (^{13}C) at 25 °C. Proton and carbon chemical shifts are given in ppm relative to TMS internal standard. MALDI-TOF MS were determined at the University of Utah Core Facility. CD spectra were obtained on an Aviv 62DS CD spectropolarimeter at room temperature. Optical rotations were

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measured on a Perkin–Elmer polarimeter (model 343) using a 1 mL capacity quartz cell with a 10 cm path length.

Fmoc-Lys(octanoyl)-OH (1)

Fmoc-L-lysine (0.737 g, 2.0 mmol) was added to dioxane (10 mL), and then a solution of Na_2CO_3 (0.636 g, 6.0 mmol) in H_2O (12.6 mL) was added drop wise at 0 °C. After 5 min, a solution of octanoyl chloride (0.325 g, 2.0 mmol) in dioxane (10 mL) was added to the mixture. The solution was allowed to warm to room temperature and stirred overnight. The solvents were extracted with CH_2Cl_2 (250 ml), washed with saturated $NaHCO_3$ (20 mL), 1M HCl (2 ml) and brine (20 mL), dried over Na_2SO_4 and concentrated. Flash chromatography (CH_2Cl_2/CH_3OH, 6:1) gave a white amorphous solid **1** (0.86 g, 87%). TLC R_f = 0.29 (CH_2Cl_2/CH_3OH, 10:1); $[\alpha]^{20}_D$ +10.2° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ (ppm) 7.68 (d, *J* = 7.6 Hz, 2H), 7.53 (m, 2H), 7.32 (m, 2H), 7.23 (m, 2H), 5.67 (m, 1H), 4.29 (m, 2H), 4.13 (m, 1H), 3.19 (m, 2H), 2.10 (tm, 2H), 1.84-1.17 (m, 16H), 0.78 (m, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 174.40, 143.95, 141.40, 127.90, 127.29, 125.39, 120.12, 67.15, 47.30, 39.42, 36.84, 31.94, 29.52, 29.27, 26.07, 22.84, 14.31; HRMS (MALDI) (*m/z*) (MNa^+): found 517.2673; Calcd for $C_{29}H_{38}N_2O_5Na$ 517.2678.

Fmoc-Lys(decanoyl)-OH (2)

Compound **2** was submitted to the same procedure described above for the preparation of **1** and gave a white amorphous solid (yield, 78%); TLC R_f = 0.40 (CH_2Cl_2/CH_3OH, 8:1); $[\alpha]^{20}_D$ +12.0° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ (ppm) 7.67 (d, *J* = 7.6 Hz, 2H), 7.52 (m, 2H), 7.30 (m, 2H), 7.21 (m, 2H), 5.78 (d, *J* = 7.6 Hz,1H), 4.27 (m, 2H), 4.12 (m, 1H), 3.17 (m, 1H), 2.10 (tm, 2H), 1.83-1.14 (m, 20H), 0.78 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 174.69, 156.64, 144.14, 143.91, 141.45, 127.94, 127.31, 125.38, 120.17, 98.99, 67.27, 66.36, 61.17, 54.04, 47.31, 39.41, 36.87, 32.26, 32.09, 29.72, 29.60, 29.53, 29.04, 26.08, 22.90, 22.54, 14.36; HRMS (MALDI) (*m/z*) (MNa^+): found 545.2978; Calcd for $C_{31}H_{42}N_2O_5Na$ 545.2991.

Fmoc-Lys(lauroyl)-OH (3)

Compound **3** was submitted to the same procedure described above for the preparation of **1** and gave a white solid (yield, 64%); mp 126.0-128.0 °C; TLC R_f = 0.76 (CH_2Cl_2/CH_3OH, 4:1); $[\alpha]^{20}_D$ +14.3° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ (ppm) 7.67 (d, *J* = 7.6 Hz, 2H), 7.52 (m, 2H), 7.30 (m, 2H), 7.21 (m, 2H), 5.82-5.77 (m, 2H), 4.26-4.39 (m, 2H), 4.12 (t, *J* = 7.2 Hz, 1H), 3.17 (m, 2H), 2.09 (m, 2H), 1.83-1.35 (m, 6H), 1.48 (m, 17H), 0.79 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 175.11, 174.81, 156.56, 143.93, 141.49, 127.93, 127.31, 125.38, 120.18, 67.32, 53.76, 47.34, 39.36, 36.94, 32.13, 29.84, 29.72, 29.56, 29.14, 26.03, 22.91, 22.34, 14.35; HRMS (MALDI) (*m/z*) (MNa^+): found 573.3299, Calcd for $C_{33}H_{46}N_2O_5Na$ 573.3304.

Fmoc-Lys(myristoyl)-OH (4)

Compound **4** was submitted to the same procedure described above for the preparation of **1** and gave a white solid (yield, 64%); mp 132.0-134.0 °C; TLC R_f = 0.81 (CH_2Cl_2/CH_3OH, 3:1); $[\alpha]^{20}_D$ +13.4° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ (ppm) 7.73 (d, *J* = 7.6 Hz, 2H), 7.59 (m, 2H), 7.37 (m, 2H), 7.27 (m, 2H), 5.97 (br, 1H), 5.79 (d, *J* = 7.6 Hz, 1H), 4.41-4.33 (m, 2H), 4.18 (t, *J* = 7.6 Hz, 1H), 3.23 (m, 2H), 2.15 (m, 2H), 1.89-1.41 (m, 6H), 1.21 (m, 22H), 0.87 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 175.27, 174.86, 156.58, 144.11, 143.92, 141.48, 127.94, 127.32, 125.39, 120.18, 67.37, 53.78, 47.33, 39.39, 36.91, 32.16, 29.93, 29.89, 29.75, 29.59, 29.53, 29.08, 26.06, 22.93, 22.40, 14.36; HRMS (MALDI) (*m/z*) (MNa^+): found 601.3623, Calcd for $C_{35}H_{50}N_2O_5Na$ 601.3617.

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Fmoc-Lys(stearoyl)-OH (5)

Compound **5** was submitted to the same procedure described above for the preparation of **1** and gave a white solid (yield, 68%); mp 133.0-135.0 °C; TLC R_f = 0.62 (CH_2Cl_2/CH_3OH, 5:1); $[\alpha]^{20}_D$ +11.8° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ (ppm) 7.68 (d, *J* = 7.6 Hz, 2H), 7.52 (m, 2H), 7.31 (m, 2H), 7.22 (m, 2H), 5.73 (d, *J* = 7.2, Hz, 1H), 4.28 (m, 2H), 4.12 (m, 1H), 3.19 (m, 2H), 2.09 (m, 2H), 1.84-1.18 (m, 37H), 0.80 (m, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 175.27, 174.69, 156.54, 144.13, 143.94, 141.41, 127.94, 127.31, 125.40, 120.19, 67.32, 53.75, 47.35, 39.29, 36.97, 32.15, 29.93, 29.58, 29.10, 29.19, 26.03, 22.92, 22.33, 14.35; HRMS (MALDI) (*m/z*) (MNa^+): found 657.4240; Calcd for $C_{39}H_{58}N_2O_5$ Na 657.4243.

$MPEG_4$-acetic acid (7)

To a solution of $MPEG_4$-OH (2.20 g, 10.6 mmol) in THF (20 mL) was added NaH (60%, 0.635 g, 15.9 mmol) at 0 °C. The solution was stirred for 1 h at room temperature, and then ethyl bromoacetate (1.4 mL, 12.6 mmol) was added dropwise. After 16 h at 50 °C, the solution was concentrated *in vacuo*. The residue was hydrolyzed with 1M LiOH (8 mL) and CH_3OH (8 mL) for 6 h at room temperature. The solution was acidified with 1M HCl to pH 1, and then the solvents were removed under reduced pressure. The residue was purified by chromatography (CH_2Cl_2/CH_3OH 10:1) to afford **7** (1.66 g, 59%) as a colorless oil. TLC R_f = 0.15 (CH_2Cl_2/CH_3OH, 4:1); ^{1}H NMR ($CDCl_3$): δ (ppm) 9.10 (br, 1H), 4.03 (s, 2H), 73.57-3.44 (m, 16H), 3.25 (s, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 172.94, 71.93, 71.09, 70.64, 70.58, 70.55, 70.51, 70.45, 68.61, 61.61, 59.02; HRMS (MALDI) (*m/z*) (MNa^+): found 289.1248, Calcd for $C_{11}H_{22}O_7$Na 289.1263.

Fmoc-Lys($MPEG_4$)-OH (6)

To a solution of $MPEG_4$-acetic acid **7** (0.266 g, 1.0 mmol) and perfluorophenol (0.202 g, 1.1 mmol) in CH_2Cl_2 (5 mL) was added DCC (0.247 g, 1.2 mmol) at room temperature. After stirring overnight, the mixture was diluted with acetone (50 mL) and filtered; the residue was dried *in vacuo* to give the activated ester, which was used directly in the next step. The crude oil in DMF (5 mL) was added to a solution of Fmoc-L-lysine (0.442 g, 1.2 mmol) and DIPEA (0.7 mL, 4 mmol) in DMF (10 mL) at 0 °C. The solution was stirred overnight at room temperature, and then poured into brine (50 mL), extraction with CH_2Cl_2 (150 mL), washed with 1M HCl, brine, dried and purified by flash chromatography (CH_2Cl_2/CH_3OH, 5:1) to afford an amorphous solid **6** (0.37 g, 60%). TLC R_f = 0.21 (CH_2Cl_2/CH_3OH, 5:1); $[\alpha]^{20}_D$ +19.5 ° (*c* 3, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ (ppm) 7.67 (d, *J* = 7.2 Hz, 2H), 7.52 (m, 2H), 7.29 (m, 2H), 7.21 (m, 2H) ,5.78 (d, *J* = 6.8 Hz, 1H), 4.29 (d, *J* = 6.8 Hz, 2H), 4.11 (m, 1H), 3.91 (s, 2H), 3.54-3.52 (m, 14H), 3.45-3.43 (m, 2H), 3.27 (s, 3H), 1.81-1.18 (m, 6H); ^{13}C NMR ($CDCl_3$): δ (ppm) 171.04, 156.36, 144.18, 144.03, 141.48, 127.90, 127.30, 125.39, 120.15, 72.07, 71.09, 70.69, 70.61, 70.27, 67.05, 59.15, 53.92, 47.38, 38.54, 31.78, 29.91, 29.00, 22.27; HRMS (MALDI) (*m/z*) (MNa^+): found 639.2917, Calcd for $C_{32}H_{44}N_2O_{10}$Na 639.2893.

Peptide synthesis

All galanin analogs were synthesized on a Symphony peptide synthesizer (Protein Technologies Inc.) using Fmoc-based coupling protocols as previously described.[22] Cleaved peptides were purified by reversed-phase HPLC using preparative HPLC column (Vydac diphenyl, 219TP1011522) and eluted with a linear gradient of acetonitrile (0.1% TFA). The flow rate was 10 mL/min, and the elution was monitored by UV detection at 220 nm and 280 nm. Buffer A (0.1% TFA in water) and buffer B (0.1% TFA, v/v, in 90% aqueous acetonitrile) were used to produce a linear gradient from 20 to 100% of buffer B over 40 min. Purified analogs were quantified by measuring UV absorbance at 279.8 nm (molar absorbance coefficient ε = 7,000 cm^{-1} M^{-1}).

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Partitioning coefficient, logD

LogD values were determined based on calculated capacity factors derived from HPLC retention times as previously described.[22] The logD for five galanin analogs were determined using conventional shake flask methods, using 400 μg of peptide reconstituted in 1 mL of phosphate buffered saline. The aqueous peptide solution was shaken with an equal volume of water saturated octanol for 24 h. Concentration of the aqueous layer was determined by HPLC. Retention times for the analogs was determine on a linear gradient of 20% to 100% buffer B in 40 min, with immediate return to initial conditions and a 20 min re-equilibration. A linear correlation between logD and HPLC retention times gave calculated logD values.

Circular dichroism

α-Helical conformation of each analog was studied using CD as previously described.[22] Peptides were reconstituted in NaF buffer to a concentration of 0.1 mg/mL. The peptides were scanned from 250 nm to 200 nm, with a 1 nm step size and 1.0 s dwell time. Data were averaged from 5 scans.

Metabolic stability assay

Peptide stability was assessed in a buffered 25% rat blood serum assay as previously described.[22] To 200 μL of buffered serum, 2.5 μg of peptide was added and the solution was incubated at 37 °C. Reactions were quenched at select time points upon the addition of "quenching solution" (15% trichloroacetic acid in 40% isopropanol), and the remaining amount of intact peptide was determined by HPLC.

Pharmacological characterization

Galanin receptor binding assay and the anticonvulsant screening were carried out as previously described in detail.[22] Briefly, the competitive binding assay was performed using human GalR1 and GalR2 receptor membrane preparations and europium-labeled galanin (Perkin-Elmer). Samples were incubated at room temperature for 90 min, followed by 4 washings with wash buffer (50 mM Tris-HCl pH 7.5 and 5 mM $MgCl_2$) using a vacuum manifold. Enhancement solution (200 μL) was added, and the plates were incubated at room temperature for 30 min. The plates were read on a VICTOR^3 spectrofluorometer. Competition binding curves were analyzed using GraphPad Prism software, using a nonlinear regression, sigmoidal dose-response (variable slope) curve with no constraints or weights, seen below. Top and Bottom are the plateaus on the y-axis. With this model, we hold the K_d and concentration of europium-labeled galanin constant (4.3 nM and 2 nM, respectively) and assume one binding site with reversible binding at equilibrium.

$$Y=\text{Bottom}+\frac{\text{Top}\ \ \text{Bottom}}{1+10^{(X\ \log EC_{50})}} \qquad EC_{50}=10^{\left[\log K_i*\left(1+\frac{[\text{Eugalanin}]}{K_d}\right)\right]}$$

Anticonvulsant efficacy was assessed following i.p. administration to five groups of CF-1 mice (n = 4 mice) at a dose of 4 mg/kg. At various times (i.e., 0.25, 0.5, 1, 2, and 4 h) after administration, mice were challenged with a 6 Hz corneal stimulation (32 mA, 3 sec, 6 Hz). Mice not displaying a characteristic limbic seizure were considered protected. The percent of animals protected at each time-point was plotted against time, and the AUC values were calculated with GraphPad Prism.

Supplementary Material

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Acknowledgments

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Abbreviations

AUC
area under the curve

BBB
blood-brain barrier

CD
circular dichroism

CNS
central nervous system

DCC
N,*N*'-dicyclohexylcarbodiimide

DIPEA
N,*N*-diisopropylethylamine

Fmoc
N-(9-fluorenyl)methoxycarbonyl

GalR1
galanin receptors subtype 1

GalR2
galanin receptors subtype 2

GPCRs
G-protein coupled receptors

i.p
intraperitoneally

LAA
Lipoamino acid

$MPEG_4$
monodispersed polyethylene glycol PEG_4

PyBop
(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate

SAR
structure-activity relationship

SPPS
solid phase peptide synthesis

TFA
trifluoroacetic acid

TFE
2,2,2-trifluoroethanol

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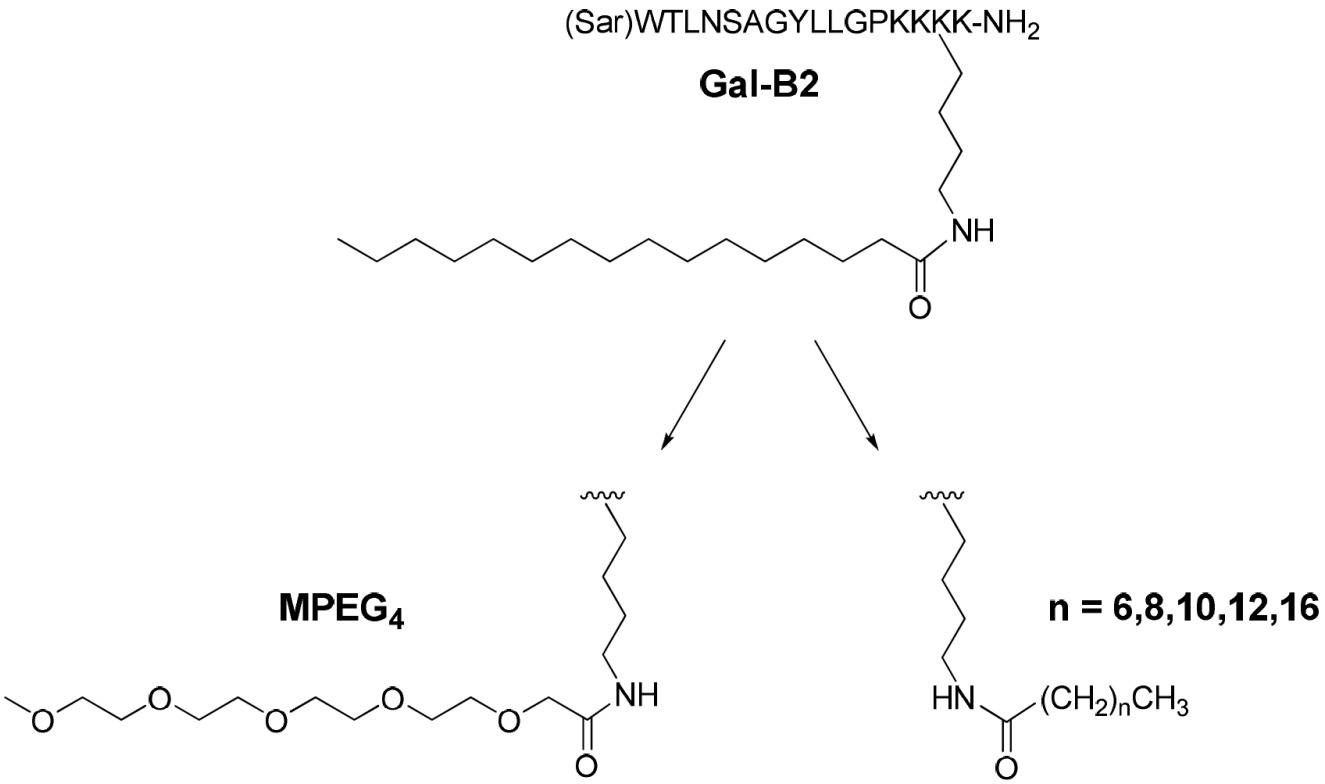

Figure 1.
Structures of Gal-B2 and LAAs used in this SAR study. Note that $MPEG_4$ has an identical number of atoms as the C_{16} (palmitoyl), but is significantly less lipophilic.

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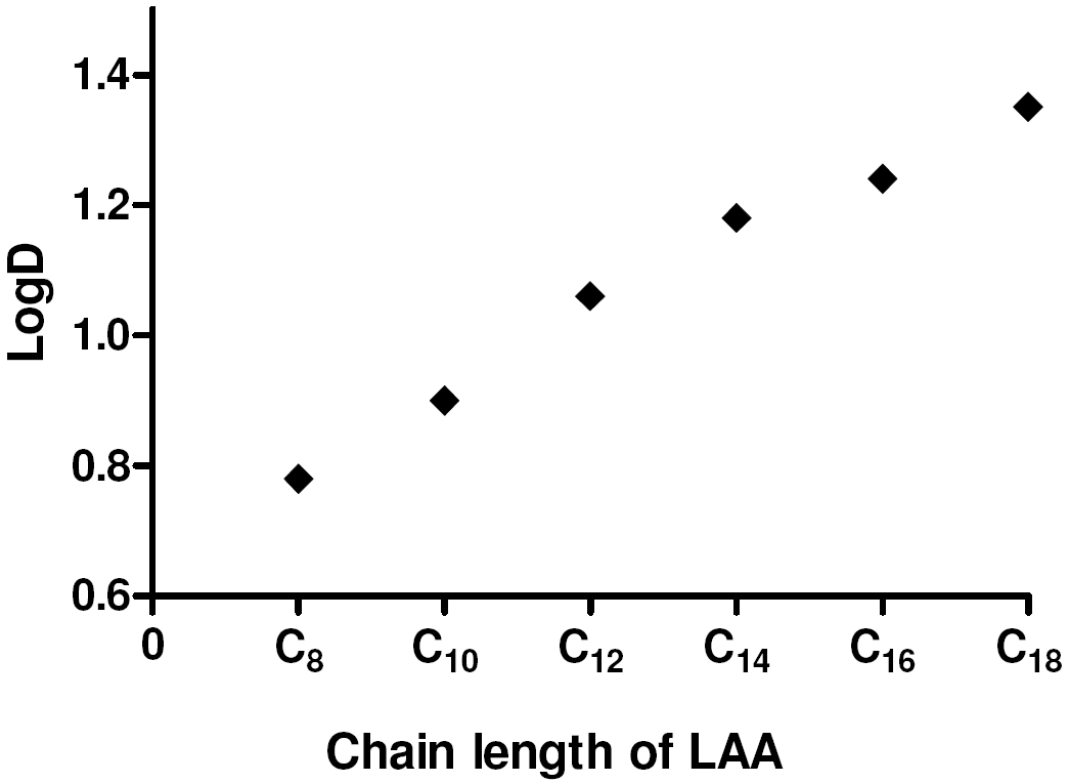

Figure 2.
Relationships between the aliphatic chain length of the LAAs in Gal-B2 analogs and the octanol/water partition coefficient (logD). Linear fit yielded R^2=0.9839, P<0.0001.

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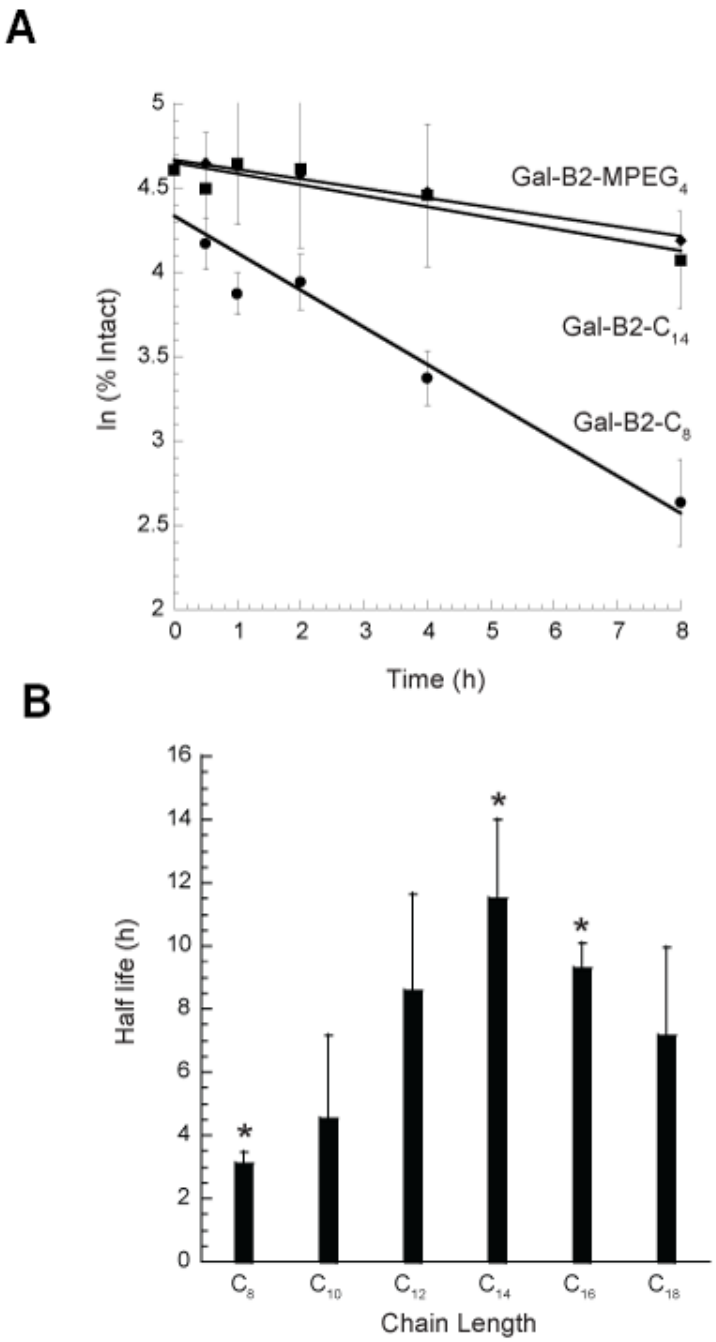

Figure 3.

(A) *In vitro* serum stability assay for the galanin analogs. Representative plots showing a time-course of degradation for C_8 (●), C_{14} (■), and $MPEG_4$ (◆)-containing galanin analogs in the presence of 25% rat serum at 37 °C. Degradation of analogs was monitored by analytical HPLC. Data points were obtained at 0, 0.5, 1, 2, 4 and 8 h from the average of at least three independent experiments. (B) Relationships between the length of the LAAs and the *in vitro* metabolic stability of the galanin analogs. The half-lives were determined by incubating the peptides in 25% diluted rat serum at 37 °C. The concentrations of the remaining analogs were determined

by HPLC. Data were obtained from at least three independent experiments. ANOVA single factor analysis yielded P-value of 0.004 for C_8, C_{14}, and C_{16}-containing species (*).

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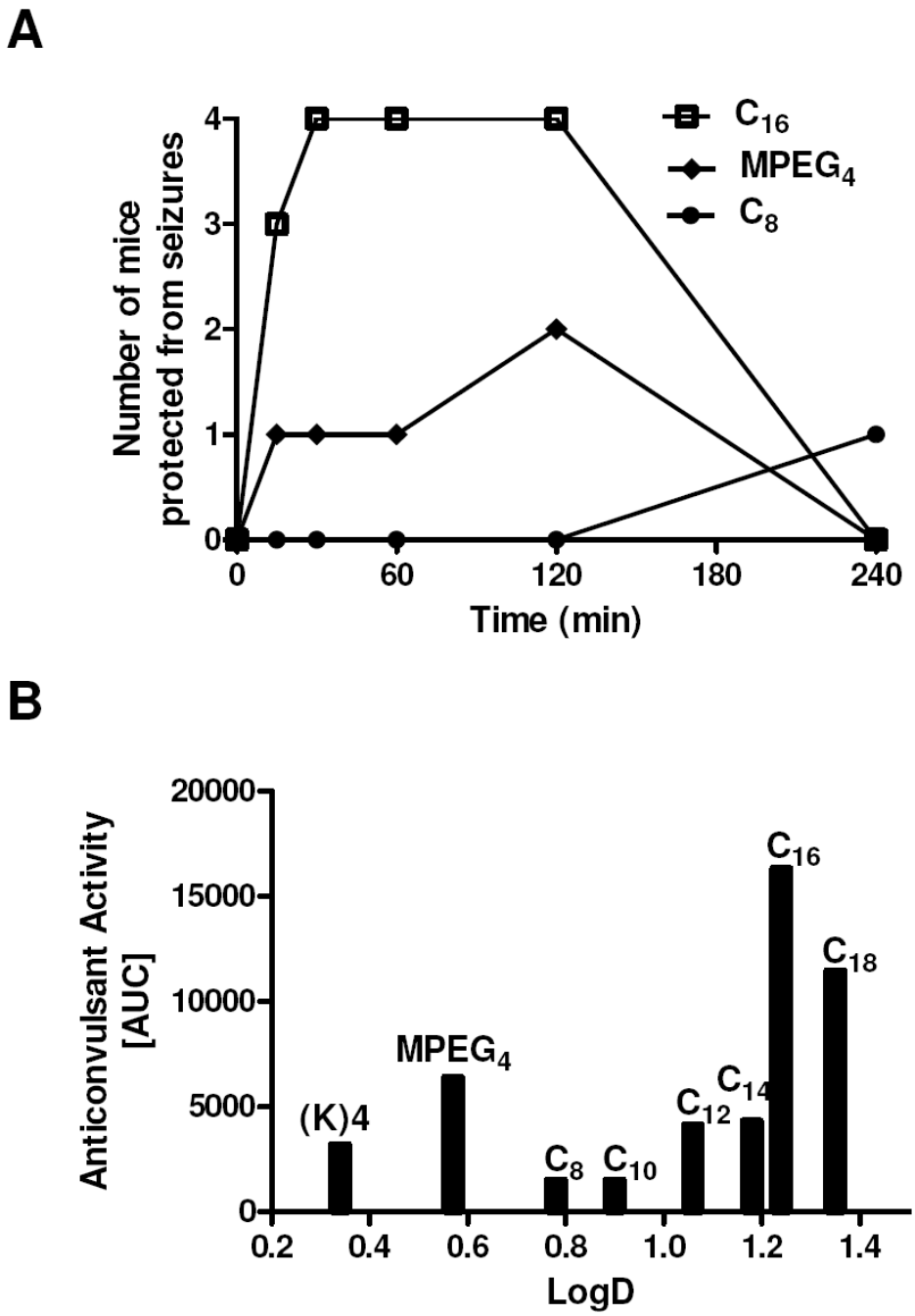
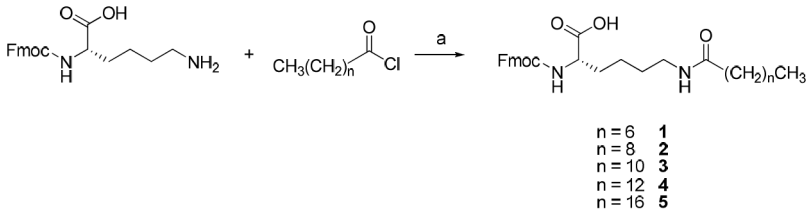


Figure 4.

Anticonvulsant activity of the galanin analogs. The analogs were administered *i.p.* as a bolus dose of 4 mg/kg. (A) time-response studies ranging from 15 min to 4 h were used to calculate area under the curve values (AUC). (B) Relationships between logD and the anticonvulsant activity (AUC values) of the galanin analogs in the 6 Hz model of epilepsy.



Scheme 1. Synthesis of the Fmoc-protected LAAs[a]

[a]Key: (a) Na_2CO_3, H_2O/dioxane, rt, overnight, 64~87%.

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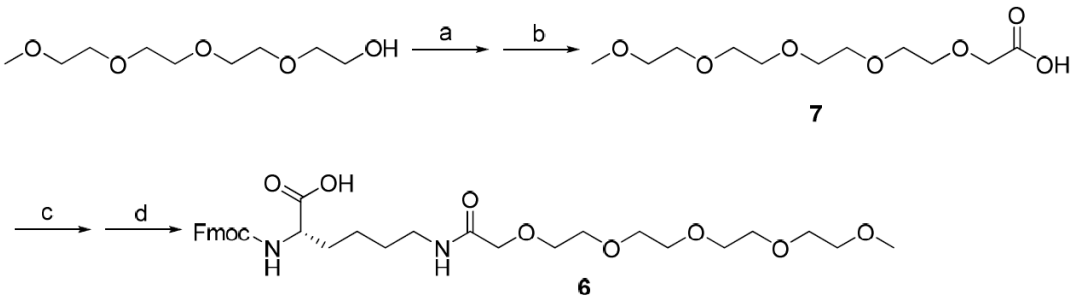

Scheme 2. Synthesis of Fmoc-Lys(MPEG$_4$)-OH[a]

[a] Key: (a)NaH 0 °C, 1 h; Ethyl bromoacetate, 50 °C, 16 h; (b) 1M LiOH/CH_3OH 6h, 59% (from two steps); (c) Perfluorophenol, DCC, overnight; (d) Fmoc-L-lysine, DIPEA, 0 °C, 60% (from two steps).

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Table 1

Structures of LAAs used for SPPS of the systemically-active galanin analogs.

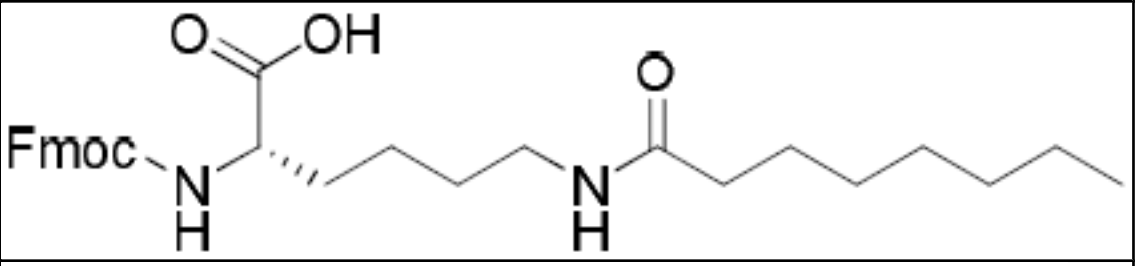
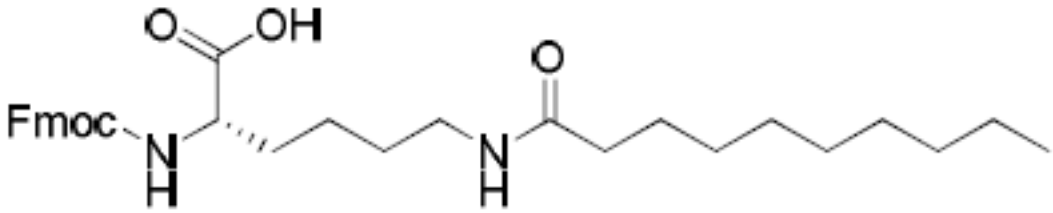
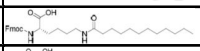
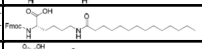
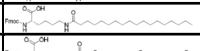
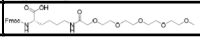
LAA	Name	Structure
1	Fmoc-Lys(octanoyl)-OH	
2	Fmoc-Lys(decanoyl)-OH	
3	Fmoc-Lys(lauroyl)-OH	
4	Fmoc-Lys(myristoyl)-OH	
5	Fmoc-Lys(stearoyl)-OH	
6	Fmoc-Lys($MPEG_4$)-OH	

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Table 2

Structure and properties of the galanin analogs. All analogs are amidated at the C-terminus.

Analog	Structure	HPLC retention time[a]	α-helix[b]	Mass spec. (MALDI, MH^+) Calcd/found
Gal-(K)4[c]	(Sar)WTLNSAGYLLGP**KKKK**	15.13 ± 0.42	14%	1873.09/1873.14
Gal-B2-C_8	(Sar)WTLNSAGYLLGP**KK(Lys-octanoyl)K**	20.13 ± 0.17	9%	2001.21/2001.17
Gal-B2-C_{10}	(Sar)WTLNSAGYLLGP**KK(Lys-decanoyl)K**	21.49 ± 0.05	6%	2029.24/2029.45
Gal-B2-C_{12}	(Sar)WTLNSAGYLLGP**KK(Lys-lauroyl)K**	22.51 ± 0.03	16%	2057.28/2057.26
Gal-B2-C_{14}	(Sar)WTLNSAGYLLGP**KK(Lys-myristoyl)K**	23.76 ± 0.18	16%	2085.31/2085.33
Gal-B2[c]	(Sar)WTLNSAGYLLGP**KK(Lys-palmitoyl)K**	25.84 ± 0.10	23%	2112.31/2112.33
Gal-B2-C_{18}	(Sar)WTLNSAGYLLGP**KK(Lys-stearoyl)K**	26.73 ± 0.18	5%	2141.33/2141.48
Gal-B2-$MPEG_4$	(Sar)WTLNSAGYLLGP**KK(Lys-$MPEG_4$)K**	17.52 ± 0.03	18%	2123.23/2123.29

[a] A linear gradient of water/90% acetonitrile (buffered with 0.1% TFA) on a Vydac diphenyl column, starting from 20% acetonitrile to 100% acetonitrile in 40 min.

[b] Determined in the presence of 50% v/v 2,2,2-trifluoroethanol.

[c] Data from [22].

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Table 3

In vitro and *in vivo* pharmacological properties of the galanin analogs

	***In vitro* assay (receptor binding)**		***In vivo* assay (anticonvulsant activity)**				
Analog	**GalR1 K_i (nM)**	**GalR2 K_i (nM)**	**(6 Hz, 32 mA, 4 mg/kg)**				
			15'	**30'**	**1h**	**2h**	**4h**
Gal-(K)4 [a]	0.4 ± 0.1	24.0 ± 9.9	0/4	3/4	1/3	0/3	0/4
Gal-B2-C_8	0.7 ± 0.1	14.9 ± 0.6	0/4	0/4	0/4	0/4	1/4
Gal-B2-C_{10}	1.3 ± 0.7	14.4 ± 0.6	2/4	2/4	0/4	0/4	0/4
Gal-B2-C_{12}	1.4 ± 0.4	16.1 ± 8.5	1/4	3/4	2/4	0/4	0/4
Gal-B2-C_{14}	2.6 ± 0.1	18.2 ± 1.6	2/4	3/4	2/4	0/4	0/4
Gal-B2 [a]	3.5 ± 1.0	51.5 ± 34.4	3/4	4/4	4/4	4/4	0/4
Gal-B2-C_{18}	4.0 ± 2.0	15.0 ± 1.0	4/4	3/4	4/4	2/4	0/4
Gal-B2-$MPEG_4$	0.5 ± 0.1	20.5 ± 6.5	1/4	1/4	1/4	2/4	0/4

[a] Data from [22].