Synthesis of a Fluorescent Gabapentin-Choline Conjugate for Single Molecule Detection

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Background

Voltage-gated Ca^{2+} channels are important molecular devices allowing changes in membrane potential to control various Ca^{2+}-dependent cell functions such as muscle contraction, hormone and neurotransmitter release, neuronal plasticity and sensory cell signaling. There are three subfamilies (Ca_v1, Ca_v2 and Ca_v3), ten different pore-forming α_1 subunit isoforms that control the Ca^{2+} influx to the needs of individual cells, and also accessory subunits (α_2-δ, β and γ) involving alternative splicing and regulatory proteins.[1] The nicotinic acetylcholine receptor (nAChR), for example, is a well characterized ligand-gated ion channel,[2] plays important roles in neurotransmission and in Alzheimer's disease,[3] as well as in anti-inflammatory effects.[4]

Because of the lack of tools for the simultaneous measurement of ligand binding and channel gating, their relationship has been inferred only indirectly, and an apparatus was developed recently to investigate signal transduction processes of channel molecules at the single molecule level.[5] Most recently, a fluorescent GABA-choline conjugate ligand for single molecule observation of ligand–channel interactions was developed and binding of this ligand to nAChR was visualized by total internal reflection fluorescence (TIRF) microscopy.[6] Although shown to be a powerful tool for investigation of single channel–ligand interactions, the synthesis of this promising ligand was only performed in an extremely low yield (0.06%) (**Scheme 1**).

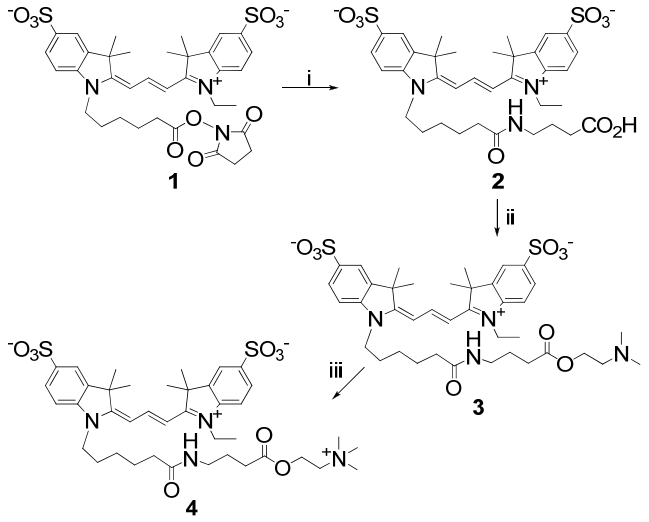
The FDA-approved anticonvulsant drug gabapentin, an analogue of GABA, down-regulates activity of voltage-activated calcium channels by specifically interacting with the extracellular auxiliary $\alpha_2\delta$ subunit, and its mechanisms of action have been reviewed.[7] We were asked to synthesize a fluorescent derivative of gabapentin for voltage-gated calcium channel studies and

developed the alternative synthetic route shown (**Schemes 2 and 3**). The synthesis used the N-hydroxysuccinimide-activated monofunctional ester of Cy3, **1** and the Fmoc-protected gabapentin-choline conjugate, **7** to give the final conjugate product, **8** in one step in an isolated yield of 90%.

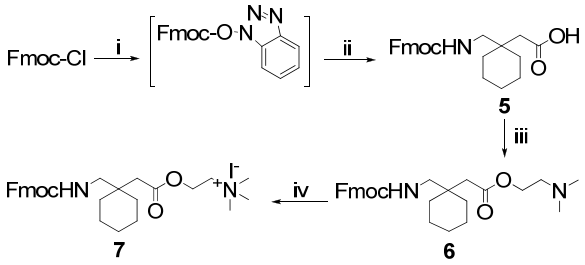
Chemistry

This route is particularly attractive since it not only gives a high yield of the final product but also mitigates the need for time-consuming chromatography of intermediates, as the Fmoc-protected intermediates are synthesized in organic solvents and are isolated and purified by simple extractive work-ups. This route proved successful with high yields for each step. One challenge was purification of Fmoc-protected gabapentin-*N*, *N*-dimethylaminoethyl ester[8] as purity is vital for quantitative conversion to Fmoc protected gabapentin-choline conjugate, but a quick wash of its ethyl acetate solution with cold saturated aqueous sodium bicarbonate solution during work-up proved safe for the base sensitive Fmoc protecting group. Then, methylation with methyl iodide proceeded quantitatively to yield the Fmoc-protected gabapentin-choline conjugate which was used without further purification.[9, 10]

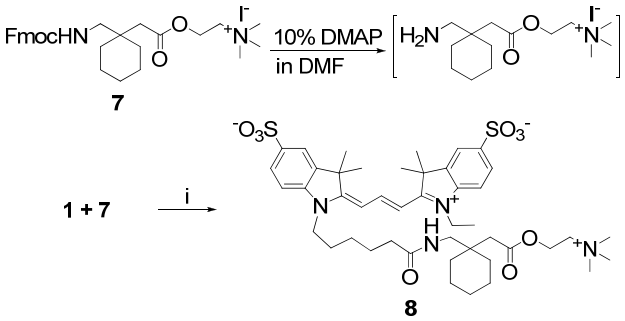
Scheme 1. Reagents and conditions: (i) 4-aminobutanoic acid (GABA), 0.1 M sodium borate buffer, pH 8.5, room temperature, 2h, 41%; (ii) oxalyl chloride, *N, N*-dimethylethanolamine, DMF, room temperature, 4h, 0.1%; (iii) CH_3I, DMF, room temperature, 2 days, 14%.



Scheme 2. Reagents and conditions: (i) HOBt, Pyridine, DCM, room temperature, 1 h; (ii) gabapentin, DIPEA, DMF, room temperature, 5h, 94%; (iii) N, N-dimethylaminoethanol, 2-dipyrodinyl carbonate, DMAP, toluene, room temperature, 24h, 72%; (iv) CH_3I, $CHCl_3$, room temperature, 24h, quantitative.



Scheme 3. Reagents and conditions: (i) 10% DMAP in DMF, room temperature, 12h, 90%.



Experimental

General. Cy3-NHS was purchased from GE Healthcare UK, Ltd (Buckinghamshire, England). All other chemicals were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI). HPLC was performed using an Agilent 1200 series instrument equipped with a multi-wavelength detector. The detector was set to 280 nm and 540 nm. The ^{1}H NMR and the ^{13}C NMR spectra were recorded on a Varian 400 MHz spectrometer operating at 400 MHz and 100 MHz, respectively. Chemical shifts are reported in parts per million (δ) and referenced internally to deuterated solvents. Mass spectroscopy data were measured on an Agilent 1200 series LC equipped with an Agilent G2440A LC/MSD-Trap-XCT ion-trap mass spectrometer. ESI-TOF High Accuracy MS was measured by The Scripps Research Institute (La Jolla, CA).

Synthesis of Fmoc-Gabapentin (5). Fmoc-Cl (2 g, 7.8 mmol, 1 equiv) was added to a suspension of *N*-hydroxybenzotriazole (1.05 g, 7.8 mmol, 1 equiv) in dichloromethane (10 mL),

followed by the addition of pyridine (0.87 g, 11 mmol, 1.4 equiv). The resultant clear solution was stirred at r.t. for 1 h and a white precipitate formed. The solvent was removed under reduced pressure and the residue was suspended in DMF (50 mL) followed by the addition of gabapentin (1.37 g, 8 mmol, 1 equiv). To the suspension was added *N, N*-diisopropylethylamine (2mL) and a clear solution resulted after the mixture was stirred at r.t. for 10 min. The solution was stirred at r.t. for a further 2 h and the solvents were removed under reduced pressure. The residue was dissolved in ethyl acetate (50 mL) and the solution was washed with 5 M HCl (10 mL × 2), and dried over sodium sulfate. Solvents were evaporated and the residue was dried *in vacuo* to afford 3.44 g of a white solid. The crude product was further purified by recrystallization in ethanol to afford product, **5** (2.9 g, 94 %). TLC in hexane : ethyl acetate 3 : 1 R_f = 0.32. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.76 (d, 2H, *J* = 7.4 Hz, Ar-H), 7.59 (d, 2H, *J* = 7.2 Hz, Ar-H), 7.42 (t, 2H, *J* = 7.3 Hz, Ar-H), 7.31(t-d, 2H, *J* = 7.5, 1.0 Hz, Ar-H), 5.20 (t, 1H, *J* = 6.8 Hz, NH), 4.48 (d, 2H, *J* = 6.4 Hz, Fmoc-CH_2), 4.21 (t, 1H, *J* = 6.4 Hz, Fmoc-CH), 3.21 (t, 2H, *J* = 6.8 Hz, CH_2-NH), 2.26 (s, 2H, CH_2-CO_2H), 1.50-1.28 (m, 10H, cyclohexane-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 176.7, 157.7, 144.0, 141.5, 127.9, 127.3, 125.2, 120.2, 67.0, 47.9, 47.6, 41.1, 38.0, 34.1, 26.0, 21.5. APCI-MS (positive): calculated for $C_{24}H_{27}NO_4$ 393.2; found 394.2 (MH^+).

Synthesis of Fmoc-Gabapentin *N, N*-Dimethylaminoethyl Ester (6). To a suspension of Fmoc-gabapentin (787 mg, 2 mmol) in toluene (30 mL) was added 2-dipyridinyl carbonate (475 mg, 2.2 mmol) along with 4-dimethylaminopyridine (DMAP) (24 mg, 0.2 mmol). After addition of *N*, *N*-dimethylaminoethanol (890 mg, 10 mmol), the reaction mixture became clear. The solution was stirred at r. t. for 24 h. Solvents were evaporated under reduced pressure. The residue was purified by preparative HPLC on a Zorbax XDB C18 (21.2 × 50 mm, 5 µm) column at a 20 mL/min flow rate. The solvent system consisted of solvents A (0.1 % TFA in water) and B (0.1 % TFA in acetonitrile) with a gradient of solvent B from 40% to 60 % over 4 minutes. The title compound was eluted with R_t = 2 min. Solvents were evaporated under reduced pressure to afford a colorless oil. This residue was dissolved in ethyl acetate (10 mL) and washed with cold saturated sodium bicarbonate solution (10 mL) and brine (5 mL), then dried over sodium sulfate. Removal of the solvent *in vacuo* afforded an amorphous white solid product, **6** (0.67 g, 72 %). TLC in hexane:ethyl acetate, 3:1 R_f = 0.46. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.74 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.60 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.37 (t-m, 2H, *J* = 7.5 Hz, Ar-H), 7.29

(t-d, 2H, J = 7.5, 1.2 Hz, Ar-H), 5.97 (t, 1H, J = 6.6 Hz, NH), 5.25 (s, CH_2Cl_2), 4.43 (d, 2H, J = 6.4 Hz, 2H, Fmoc-CH_2), 4.22 (t, 1H, J = 6.4 Hz, Fmoc-CH), 4.15 (t, 2H, J = 5.6 Hz, O-$\underline{CH_2}$-CH_2-N), 3.17 (d, 2H, J = 6.8 Hz, CH_2-NH), 2.49 (t, 9H, J = 5.6 Hz, O-CH_2-$\underline{CH_2}$-N), 2.30 (s, 2H, CH_2-CO_2H), 2.16 (s, 6H, NMe_2), 1.55 -1.30 (m, 10H, cyclohexane-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.3, 157.2, 144.1, 141.4, 127.6, 127.0, 125.1, 120.0, 66.3, 61.2, 57.7, 54.5, 47.5, 45.2, 41.1, 38.2, 34.3, 26.0, 21.5. ESI MS(positive): calculated for $C_{28}H_{36}N_2O_4$ 464.6; found 465.4 (MH^+).

Synthesis of Fmoc-Gabapentin-Choline (7). A solution of Fmoc-gabapentin *N*, *N*-dimethylaminoethyl ester, **6** (0.16 g, 0.34 mmol) and iodomethane (1 mL) in chloroform (10 mL) was stirred at r.t. for 24 h. The solvent was evaporated to afford, quantitatively, a yellowish solid product, **7** (0.16 g) which was stored in a freezer and used without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.65 (d, 2H, J = 8.0 Hz, Ar-H), 7.54 (d, 2H, J = 8.0 Hz, Ar-H), 7.29 (t, 2H, J = 7.5 Hz, Ar-H), 7.22 (t, 2H, J = 7.5 Hz, Ar-H), 5.39 (t, 1H, J = 5.6 Hz, NH), 4.42 (m, 2H, O-$\underline{CH_2}$-CH_2-N^+), 4.25 (d, 2H, J = 6.7 Hz, Fmoc-CH_2), 4.11 (t, 1H, J = 6.7 Hz, Fmoc-CH), 3.93 (m, 2H, O-CH_2-$\underline{CH_2}$-N^+), 3.34 (s, 9H, NMe_3^+), 3.14 (d, 2H, J = 5.6 Hz, hexane-CH_2-NH), 2.26 (s, 2H, CH_2-CO_2H), 1.40-1.27 (m, 10H, cyclohexane-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.0, 156.8, 143.6, 141.0, 127.6, 127.0, 125.0, 119.8, 66.4, 64.9, 57.5, 54.4, 47.0, 39.6, 37.3, 33.4, 25.6, 21.2. ESI-MS(positive): calculated for $C_{29}H_{38}N_2O_4$ 478.3; found 479.1(MH^+).

Synthesis of Cy3-gabapentin-choline (8). The monofunctionalyzed Cy3-NHS ester, **1** (8.8 mg, 0.012 mmol) and Fmoc-gabapentin-choline, **7** (28 mg, 0.046 mmol) were dissolved in a freshly prepared solution of 10 % DMAP in DMF (0.10 mL). The mixture was stirred at r.t. overnight and monitored by LC/MS (ESI). The product was isolated by preparative HPLC on a Zorbax XDB C18 (21.2 × 100 mm, 5 µm) column run at a 20 mL/min flow rate. The solvent system consisted of solvents A (0.05 % TFA in water) and B (0.05 % TFA in acetonitrile) with a gradient of solvent B from 20 % to 55 % over 5 minutes. The title compound was eluted with an R_t = 2.6 min and was collected and lyophilized to afford a red solid product, **8** (9.0 mg, 90 %). Analytical HPLC was performed on a Zorbax XDB C18 (4.6 × 50 mm, 5 µm) column at a 1 mL/min flow rate. The solvent system consisted of solvents A (0.05 % TFA in water) and B (0.05 % TFA in acetonitrile) with a gradient of solvent B from 20 % to 95 % over 6 minutes. The

title compound was eluted with an R_t = 5.1 min. TLC in water:acetonitrile 3:1 gave an R_f = 0.41. ^{1}H NMR (DMSO-d_6, 400 Hz): δ 8.36 (t, 1H, J = 13.2 Hz, beta-H), 7.82 (m, 2H, Ar-H), 7.68 (d-t, 2H, J = 8.4, 1.4 Hz, Ar-H), 7.59 (t, 1H, J = 6.4 Hz, NH), 7.41 (d, 1H, J = 8.4 Hz, Ar-H), 7.35 (d, 1H, J = 8.4 Hz, Ar-H), 6.53 (d, 2H, J = 13.2 Hz, alpha-H), 4.39 (m, 2H, O-$\underline{CH_2}$-CH_2-N^+), 4.16 (m, 4H, 2 NCH_2-), 3.64 (m, 2H, O-CH_2-$\underline{CH_2}$-N^+), 3.14 (d, 2H, J = 6.4 Hz, hexane-CH_2-NH), 3.10 (s, 9H, NMe_3^+), 2.25 (s, 2H, CH_2-CO_2H), 2.11 (t, 2H, J = 7.0 Hz, -CH_2CONH-), 1.70 (s, 12H, 4CH_3), 1.53 (t, 3H, J = 7.2 Hz, $NCH_2\underline{CH_3}$), 1.50-1.30 (m, 16H, 8CH_2). ^{13}C NMR (DMSO-d_6,100 Hz): δ 174.0, 173.8, 172.3, 170.6, 149.8, 145.9, 145.7, 141.8, 141.2, 140.1, 140.0, 126.2, 119.8, 110.5, 110.4, 106.9, 102.9, 102.7, 63.6, 57.3, 52.8, 48.9, 48.8, 48.5, 43.9, 43.8, 36.6, 34.8, 32.5, 27.4, 27.2, 26.7, 25.6, 25.4, 25.0, 20.9, 12.2. ESI-TOF High Accuracy MS (positive): calculated for $C_{45}H_{64}N_4O_9S_2$ (MH^+) 869.4187; found 869.4163.

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