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An improved synthesis of a fluorescent gabapentin–choline conjugate for single molecule detection

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ABSTRACT

Voltage-gated calcium ion channels comprise pore-forming α_1 and auxiliary $\alpha_2\delta$, β, and γ subunits. They are important molecular devices involved in a variety of cell functions. Fluorescently labeled acylcholine analogues are important in studies such as ion channel regulation. Cy3-3-acetylcholine has recently been synthesized for single molecule detection studies; albeit in an extremely low overall yield (0.06%). In this work, an alternative route to that used in the previous Cy3-3-acetylcholine synthesis was developed with a 90% yield at a significantly lower material cost.

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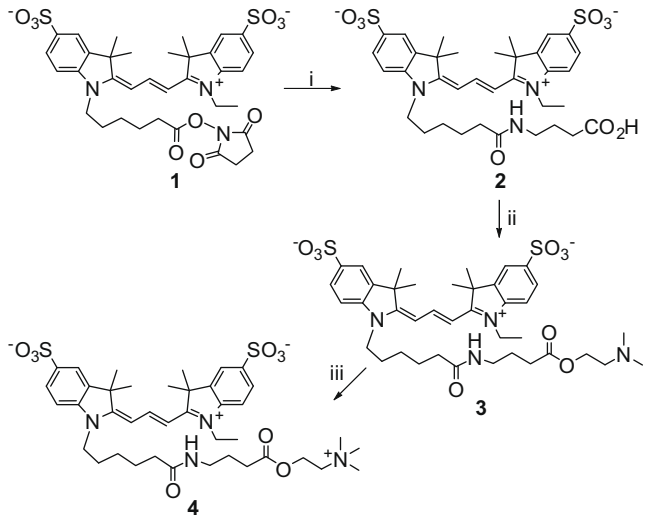
Voltage-gated Ca^{2+} channels are important molecular devices allowing changes in membrane potential to control various Ca^{2+}-dependent cell functions such as muscle contraction, hormone and neurotransmitter release, neuronal plasticity, and sensory cell signaling. There are three subfamilies (Ca_v1, Ca_v2, and Ca_v3), 10 different pore-forming α_1 subunit isoforms that control the Ca^{2+} influx to the needs of individual cells, and also accessory subunits ($\alpha_2\delta$, β, and γ) involving alternative splicing and regulatory proteins.[1] The nicotinic acetylcholine receptor (nAChR), for example, which 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 synthesis of the Cy3-3-acetylcholine fluorescent analogue of acetylcholine, as described by Fujimoto et al., was performed in three steps (Scheme 1). The commercially available monofunctionalized Cy3 dye moiety (~US$800/5 mg) was first attached to 4-aminobutyric acid (GABA), and the conjugated acid was esterified with *N*,*N*-dimethylethanolamine via reaction with oxalyl chloride. Quaternization of the amine with methyl iodide yielded the desired fluorescent conjugate. The three reactions proceed in yields



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

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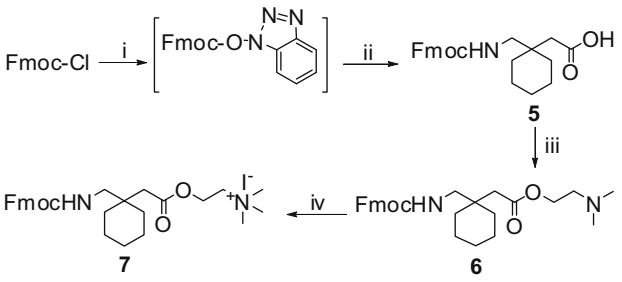
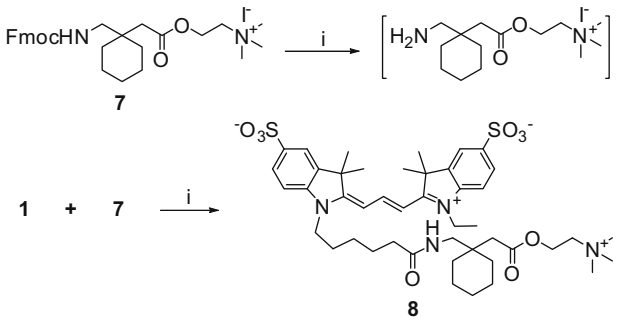


Scheme 2. Reagents and conditions: (i) HOBt, pyridine, DCM, room temperature, 1 h; (ii) gabapentin, DIPEA, DMF, room temperature, 5 h, 94%; (iii) *N*,*N*-dimethylaminoethanol, 2-dipyrodinyl carbonate, DMAP, toluene, room temperature, 24 h, 72%; (iv) CH_3I, $CHCl_3$, room temperature, 24 h, quantitative.



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

of 41%, 0.1%, and 14%, respectively, with the 0.1% yield obtained during the esterification reaction making the reaction sequence impracticable for scale-up and prohibitively expensive in light of the cost of the starting Cy3 dye. As such agents are likely to be important in future single molecule studies, we designed an alternative 'one-step' synthesis strategy [from the expensive Cy3 monofunctional ester] for a similar acylcholine agent based on a gabapentin analogue. The calcium ion channel regulation study using this agent is in progress.

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 succinimido-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%. 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.

Attaching the expensive Cy3 dye during the last step of the synthesis is very attractive in order to mitigate costs, even without the extremely poor esterification yield obtained in the Fujimoto route.[6] In an alternative strategy we decided to first make a gabapentin–choline conjugate. Direct coupling of GABA and choline was successful using hydrogen chloride-saturated anhydrous dioxane,[8] and although this method might have proved applicable to a gabapentin–choline analogue in our hands attempts to isolate a pure gabapentin–choline conjugate were not successful due to the relative instability of the conjugate during work-up. A Boc-protected gabapentin–choline conjugate precursor was then tried but also proved useless due to instability of intermediates during work-ups as well as having the inconvenience of the lack of a suitable UV absorption for reaction monitoring and analyses. An Fmoc-protected gabapentin–choline conjugate and its intermediates would have proper UV absorption for detection and can be deprotected under mild conditions. Furthermore, we intended to perform the final coupling reaction in an anhydrous organic phase using an in situ generation of gabapentin–choline from a stable lipophilic precursor in order to diminish competing hydrolysis of the activated Cy3 dye and reduce effects due to the observed instability of free gabapentin–choline. This route proved successful with high yields for each step. One challenge was purification of the Fmoc-protected gabapentin–*N*,*N*-dimethylaminoethyl ester[9] as purity is vital for quantitative conversion to the 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.[10,11]

In the final Cy3 coupling step, choice of an appropriate base which would remove the Fmoc protecting group, while not interfering with the concomitant coupling reaction, was crucial since the initially tried piperidine, even at low concentration (5%) in DMF, seriously competes with in situ-generated gabapentin–choline for the Cy3 monofunctional ester. A 10% dimethylaminopyridine (DMAP) solution in DMF, which served as both base and solvent, proved to be safe. The DMAP slowly cleaves off the Fmoc group and the slowly formed and unstable gabapentin–choline conjugate immediately reacts with the Cy3 monofunctional ester that is present to form the stable final product. Eventually, the final product was isolated in ~90% yield by injecting the reaction mixture directly onto a preparative HPLC and then lyophilizing the product fraction. Finally, attempted coupling reactions with a cyanine dye monoacid to an Fmoc-protected gabapentin–choline conjugate catalyzed by HBTU, HATU, or EDC coupling agents in situ with Fmoc deprotecting agents such as piperidine, DIPEA, or TBAF were all tried but proved less successful due to higher by-product levels and lower yields.

In conclusion, we have developed an efficient synthesis of the Cy3-gabapentin–choline conjugate, using an approach which should also prove useful for the generation of other similar fluorescent dye conjugates of biologically important acylcholine analogues.

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Supplementary data

Experimental procedures and complete spectral data and the copies of ^1H and ^{13}C NMR spectra of all intermediates and final product are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.02.125.

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