Improved Synthesis of 2-Trifluoromethyl-10-aminopropylphenothiazine: Making 2-Trifluoromethyl-10-aminopropylphenothiazine Readily Available for Calmodulin Purification

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Supporting Information

ABSTRACT: An improved and high yielding three-step synthesis for the production of 2-trifluoromethyl-10-aminopropylphenothiazine (TAPP) using less hazardous and more inexpensive reagents, its coupling to Sepharose-4B resin, and its ability to purify calmodulin are described. The overall yield of TAPP, starting with 3-aminopropyl bromide hydrobromide and 2-(trifluoromethyl)phenothiazine, was 96%.

INTRODUCTION

Calmodulin (CaM) is a calcium-ion sensing protein that is a major translator of calcium signaling in cells. In general, CaM binds up to four calcium ions when cellular calcium levels rise and can then bind to and alter the function of many other proteins depending on calcium concentration, cell type, and availability of its binding target.1,2 CaM is composed of two lobes connected by a central linker helix, often referred to as a dumbbell shape (Figure 1a). Each lobe can bind two calcium ions, for a total of four calcium binding sites. Upon calcium binding, the CaM central linker helix becomes flexible, and the lobes wrap around the target to form a compact ellipsoid (Figure 1b).3,4 CaM binds to and regulates ∼300 cellular targets, involving the protein in many critical processes, such as smooth muscle contraction, intracellular movement, memory, and metabolism.5 Alterations in CaM regulation contribute to multiple disease states, such as Alzheimer’s disease, Parkinson’s disease, and Down’s syndrome.5,6 Because CaM binds to so many proteins, it must be able to differentiate between targets depending on its affinity for each, the cell type, and the spatial and temporal nature of the calcium signal. Thus, the intricacies of CaM’s complex regulation network are still being unraveled.

To structurally and functionally investigate CaM, it must first be fully purified, generally from Escherichia coli that have been engineered to make CaM. The most common method for CaM purification is hydrophobic interaction chromatography via a phenyl Sepharose resin. For CaM, this is actually pseudoaffinity chromatography as its hydrophobicity can be modulated by calcium ions. When CaM binds the calcium ion, its exposed hydrophobicity is greatly increased. Unfortunately, phenyl Sepharose purification has drawbacks including the necessity of two consecutive columns and frequent additional purification methods such as ion exchange chromatography or heat treatment as phenyl Sepharose alone does not fully purify CaM as determined by gel electrophoresis.7−12 Another method for CaM purification is the use of a 2-trifluoromethyl-10-aminopropylphenothiazine (TAPP) Sepharose affinity
In our experience, TAPP Sepharose requires only one column and fully purifies CaM from bacterial lysate without any additional steps. Despite its superiority, TAPP Sepharose is rarely used for CaM purification as it is not commercially available. Instead, the TAPP molecule must be synthesized and coupled to epoxide-activated Sepharose. TAPP (1), along with APP (2), CAPP (3), and TAPP-O (4) (Figure 2), is a structural class of 2-substituted-10-aminophenothiazines that act as CaM antagonists.

Figure 2. 2-Substituted-10-aminophenothiazines CaM antagonists.

RESULTS AND DISCUSSION

Currently, only one synthesis method is reported for TAPP in 1983 with moderate yields. The synthetic scheme (Scheme 1) calls for hazardous reagents (acrylonitrile, LAH, and HCl gas), complicated reaction setup (reflux, Soxhlet extractor), long work-up times (36 h), and difficult precipitation.

These reaction conditions make TAPP very inaccessible to those who need it for CaM purification, primarily biochemists with limited organic synthesis training. Herein, we describe improved synthesis for TAPP (Scheme 2) using low-cost and safer reagents, as well as simple organic chemistry reaction procedures and purification resulting in a 96% overall yield. It is the hope that these changes will allow TAPP to be more accessible for those who need it.

In lieu of the highly reactive, volatile, and toxic acrylonitrile, the aminopropyl arm was added via a substitution reaction with 3-aminopropyl bromide hydrobromide (7). The terminal amine first had to be protected, so 7 was treated with di-tert-butyl dicarbonate in the presence of trimethylamine to yield 8 in 98% yield. Phenothiazine 5 was then substituted with 8 in the presence of KOH to give boc-protected TAPP (9) in 90% yield. After purification by flash chromatography (5% EtOAc/95% hexanes) 9 was deprotected using a 4:1 ratio of CH2Cl2 and trifluoroacetic acid (TFA) to yield TAPP (1) in 96% overall yield in three synthetic steps.

Scheme 1. Literature-Reported Synthesis of TAPP

Scheme 2. Improved Synthesis of TAPP from 2-(Trifluoromethyl)phenothiazine and 3-Aminopropyl Bromide Hydrobromide
Testing the binding capacity of TAPP Sepharose with pure CaM resulted in a 5.5 ± 1.3 mg CaM bound and eluted per mL settled resin, a comparable binding capacity to the previously reported 5.7 mg CaM/mL settled resin.\(^\text{14,15}\) Testing the purification ability of 10 mL settled TAPP Sepharose resin with CaM-expressing bacterial lysate resulted in 95 mg of CaM at ≥95% purity as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Supporting Information). CaM purified by TAPP Sepharose resin was compared to that purified by the more common phenyl Sepharose resin. The purity of each CaM was assessed by SDS-PAGE, and the activity of the two CaM types was compared by its ability to activate the phosphatase calcineurin. The two purification methods yielded CaM with similar ability to activate calcineurin while TAPP Sepharose purification resulted in CaM of higher purity (Supporting Information).

## CONCLUSIONS

Purifying CaM for structural and functional studies is most effective when using TAPP Sepharose. However, this method is infrequently used as the previously reported TAPP synthesis was involved and had moderate yields. With the efficient and high-yield method of TAPP synthesis and coupling to Sepharose, we present here CaM purification by TAPP Sepharose which will be much more accessible to those studying CaM.

## EXPERIMENTAL SECTION

Unless stated otherwise, all reactions were carried out in oven-dried glassware. NMR spectra were obtained using a JEOL ECX-300 spectrometer (Peabody, MA) at 300 MHz (\(^1\)H NMR) and 75 MHz (\(^13\)C NMR). Chemical shifts are in ppm relative the CDCl\(_3 \) resonance. Spin–spin coupling constants (\(J\)) are given in hertz. Mass spectra were obtained using a Waters MALDI micro MX TOF spectrometer (Milford, MA) matrix-free. UV/vis spectra were obtained using an Agilent Cary 60-02 spectrophotometer (Santa Clara, CA) at 280 nm. Analytical TLC was performed on Sorbtech (Norrscot, GA) polyester-backed TLC plates (TLC silica gel 60 UV254), and compounds were detected with a UV lamp. Flash chromatography was performed on Silicycle (Quebec, Canada) SiliaFlash P60 silica gel (40–63 μm, 230–400 mesh). All gel electrophoresis supplies were purchased from Bio-Rad. All reagents and solvents were used as is, without further purification. All reagents were purchased from Fisher Scientific, with the exception of Sepharose 4B (Millipore-Sigma) and Terrific Broth (IBI Scientific).

### Synthesis of TAPP. tert-Butyl (3-Bromopropyl)carbamate (8).

Triethylamine (1.1 mL, 8.0 mmol) was added to a 0 °C suspension of 3-aminopropyl bromide hydrobromide (1.8 g, 8.0 mmol) and dichloromethane (DCM; 30 mL). Di-tert-butyl dicarbonate (1.8 g, 8.0 mmol), dissolved in DCM (80 mL), was then added dropwise to the 0 °C suspension, and the reaction mixture was allowed to gradually warm to room temperature and was stirred overnight. The reaction was diluted with EtOAc (50 mL), and the organic layer was washed with 5% (w/w) NaOH (3 × 50 mL), water (3 × 50 mL), and brine (3 × 50 mL). The organic layer was dried over MgSO\(_4\) and concentrated under reduced pressure to afford 8, a colorless oil, in 98% yield (1.86 g). All spectroscopic data agrees with previously reported literature values.\(^\text{19}\)

**tert-Butyl (3-(3-(Trifluoromethyl)-10H-phenothiazin-10-yl)propyl)carbamate (9).** To a suspension of potassium hydroxide (0.63 g, 11.2 mmol) and 8 in dimethylformamide (2.5 mL), under inert atmosphere, was added 2-(trifluoromethyl)phenothiazine (0.50 g, 1.87 mmol) in one portion. After stirring for 16 h at room temperature, water (10 mL) was added to the reaction mixture, and the subsequent solution was extracted with DCM (3 × 10 mL). The combined organic extracts were then washed with water (3 × 10 mL) and brine (10 mL), dried over MgSO\(_4\) and concentrated under reduced pressure. The crude product was then purified by flash chromatography (5% EtOAc/95% hexanes → 20% EtOAc/80% hexanes) to afford 9, a pale yellow oil, in 90% yield (0.72 g).\(^\text{1}\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 1.38 (s, 9H), 1.98 (pentet, 2H), 3.23 (q, 2H), 3.95 (t, 2H), 4.77 (br s, 1H), 6.90 (dd, 1H, \(J = 1.05 \text{ Hz}, J = 8.25 \text{ Hz})\), 6.97 (td, 1H, \(J = 1.02 \text{ Hz}, J = 7.53 \text{ Hz})\), 7.05 (s, 1H), 7.14–7.18 (m, 2H), 7.22–7.26 (m, 2H). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 27.26, 28.39, 38.52, 45.19, 79.31, 112.09 (q, \(J\)\(_{\text{CF}} = 3.59 \text{ Hz})\), 116.12 (2C), 119.40 (q, \(J\)\(_{\text{CF}} = 3.59 \text{ Hz})\), 123.43, 124.08 (q, \(J\)\(_{\text{CF}} = 96.80 \text{ Hz})\), 127.77, 127.82, 127.85, 129.79 (q, \(J\)\(_{\text{CF}} = 32.27 \text{ Hz})\), 130.60, 144.42, 145.88, 156.09. HRMS (MALDI) \(m/z\): calcd for C\(_{21}\)H\(_{23}\)F\(_3\)N\(_2\)O\(_2\)S, 424.1432; found, 424.1140.

### 3-(3-(Trifluoromethyl)-10H-phenothiazin-10-yl)propan-1-amine, TAPP (1).

A 4:1 mixture of DCM/TFA (8 mL:2 mL) was added to 9 (0.50 g, 1.18 mmol), and the reaction mixture was stirred at room temperature. After 2 h, the solvent was removed under reduced pressure, and the crude product was dissolved in 5 mL of H\(_2\)O and 5 mL of MeOH. The solution’s pH was adjusted to 10 with 1 M NaOH and was extracted with DCM (3 × 5 mL). The combined organic extracts were dried over MgSO\(_4\) and concentrated under reduced pressure to afford 1, a pale yellow oil, in 99% yield (0.38 g). All spectroscopic data agrees with previously reported literature values.\(^\text{13}\)

### Coupling TAPP to Sepharose 4B Resin. Activation of Sepharose 4B.

A 100 mL suspension of Sepharose 4B resin (20% EtOH/80% H\(_2\)O) was filtered, washed with deionized water (3 L), and dried until water no longer dripped from the
funnel (Warning: Do not let Sepharose 4B dry out, make sure it stays damp). The resin (~50 g) was then collected and transferred to an Erlenmeyer flask and diluted in 1 M NaOH (30 mL) followed by the addition of sodium borohydride (0.21 g). The activation of the resin was initiated by the addition of 60% 1,4-butanediol diglycidyl ether (90 mL). The suspension was then stirred gently for 8 h at room temperature. After 8 h, the activated resin was filtered and washed with deionized water (6 L) and dried until the resin was still slightly damp (~50 g).

**Coupling of TAPP to Resin.** A solution of 1 (0.50 g, 1.18 mmol), dioxane (25 mL), and water (25 mL) was added to the activated resin (~50 g), and the pH of the suspension was adjusted to 12 with 1 M NaOH. The suspension was then placed on a shaker at 50 rpm and room temperature for 40 h at which time the TAPP-coupled resin was filtered and washed with deionized water (500 mL), dioxane (300 mL), deionized water (500 mL), 95% EtOH (500 mL), and deionized water (2.5 L). The resin was dried until water no longer dripped from the funnel (Warning: Do not let Sepharose 4B dry out, make sure it stays damp).

**Deactivation of Remaining Active Epoxide Groups.** A suspension of TAPP-coupled resin (~50 g) and 2 M glycine (100 mL, pH = 9.6) was placed on a shaker (50 rpm, room temperature). After 24 h, the TAPP-activated resin was filtered, washed with deionized water (1 L), 1 M NaCl (1 L), and deionized water (1.5 L). The resin was then stored in 20% (v/v) EtOH in a light-limiting bottle.

**Testing TAPP Sepharose for CaM Binding and Purity.**

**Testing CaM Binding Capacity of TAPP Sepharose.** Prepurified CaM (40 mg) in 20 mM Tris pH 7.5, 200 mM NaCl, 10 mM CaCl₂ (TAPP loading buffer) was added to 5 mL settled TAPP Sepharose, equilibrated with TAPP loading buffer, in a gravity column and was allowed to flow through. The resin was washed with TAPP loading buffer until the absorbance at 280 nm returned to the buffer baseline. CaM was eluted with 20 mM Tris pH 7.5, 200 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) (TAPP elution buffer) in 5 mL fractions until the absorbance at 280 nm returned to the buffer baseline. Absorbance at 280 nm was measured for the elutions to determine total CaM bound using an extinction coefficient of 2980 cm⁻¹ M⁻¹. Binding capacity testing was repeated twice more for a total of three tests. CaM binding capacity was 5.5 mg CaM per 1 mL settled TAPP Sepharose ± 1.3 mg.

**Testing CaM Purification by TAPP Sepharose.** CaM was expressed from the pETCaMI vector in _E. coli_ BL21 (DE3). The pETCaMI vector was a kind gift of Dr. Anthony Persechini at the University of Missouri at Kansas City. Cells were added to Terrific Broth (1 L) with 0.5% glycerol, 0.05% glucose, 0.2% lactose, and 50 mg kanamycin and incubated at 37 °C and 300 rpm for 22 h. The cells were then collected by centrifugation at 6000g and 4 °C for 10 min, and the cell pellets were stored at −20 °C until purification could be performed. For every 1 g of the cell pellet, 1 mL of 20 mM Tris pH 7.4 and 200 mM NaCl were added, and cells were resuspended. 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/mL lysozyme, and one EDTA-free protease inhibitor tablet was added. Cell resuspension was sonicated, on ice, for 20 s at 90% amplitude three times with 30 s on ice between sonications. Lysed cells were centrifuged at 4 °C and 20 000g for 30 min, and then the lysate was filtered with a 0.45 μm syringe filter and CaCl₂ concentration was adjusted to 20 mM. Lysate was added to a 10 mL TAPP Sepharose gravity column equilibrated with TAPP loading buffer and allowed to flow through. The resin was washed with 40 mL of TAPP loading buffer three times. CaM was eluted with TAPP elution buffer in 10 × 6 mL fractions, and the absorbance at 280 nm was measured to determine total CaM bound. CaM purity was assessed SDS-PAGE (Supporting Information).

**Comparison of TAPP Sepharose-Purified CaM to Phenyl Sepharose-Purified CaM.** _CaM Expression and Purification by Phenyl Sepharose._ CaM was purified by phenyl Sepharose resin similarly to previously described. CaM was expressed, lysed, and clarified as described above. After 0.45 μm syringe filtration, lystate was adjusted to 2 mM EDTA, loaded onto a 10 mL phenyl Sepharose gravity column equilibrated with 20 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, and allowed to flow through. The column was washed with 15 mL of the same buffer. The combined flow-through and wash was adjusted to 10 mM CaCl₂ and loaded onto a 10 mL phenyl Sepharose gravity column equilibrated with 20 mM Tris pH 7.5, 100 mM NaCl, and 10 mM CaCl₂ and allowed to flow through (FT2). The column was washed with 30 mL of 20 mM Tris pH 7.5, 10 mM CaCl₂ (W2); 30 mL 20 mM Tris pH 7.5, 100 mM NaCl, 10 mM CaCl₂ (W3); and 30 mL 20 mM Tris pH 7.5, 10 mM CaCl₂ (W4). CaM was eluted from the column with 20 mM Tris pH 7.5, 10 mM EDTA in 10 × 5 mL fractions. CaM purity was assessed by SDS-PAGE (Supporting Information).

**Activity of CaM Purified by TAPP Sepharose Compared to Phenyl Sepharose.** Calcineurin phosphatase activity against the small molecule substrate p-nitrophenyl phosphate (pNPP) was determined as a function of CaM concentration. pNPP was obtained from MP Biomedicals, and calcineurin was a kind gift of Dr. Trevor Creamer at the University of Kentucky. Reaction mixtures contained 30 nM calcineurin, 100 mM pNPP, and 0–120 nM CaM purified with TAPP Sepharose or phenyl Sepharose in assay buffer: 100 mM NaCl, 50 mM Tris, 6 mM MgCl₂, 0.5 mM CaCl₂, 0.5 mM dithiothreitol, 0.025% NP-50, pH 7.5. Reactions were incubated at 37 °C for 90 min, and pNP release was monitored by its absorbance at 405 nm. Each reaction was performed four times for each CaM concentration and type (Supporting Information).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02146.

Spectroscopic data for all new compounds, SDS-PAGE for CaM purity determination, and activity of CaM-activated calcineurin (PDF)

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The manuscript was written through contributions of all authors.
Notes
The authors declare no competing financial interest.

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