

# A Use-dependent, Subtype-selective Inhibitor of Native AMPA Receptors

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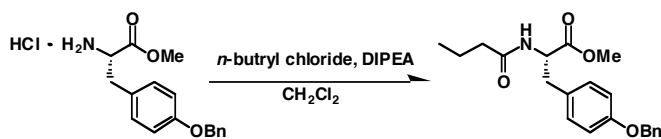
## Supporting Information

### Synthesis

An examination of previously reported philanthotoxin syntheses revealed no single method for preparing PhTx-74 with the desired level of efficiency, cost per gram, and scalability. Solid phase synthesis (SPS), while ideal for rapidly preparing small quantities of structurally related compound libraries, is expensive and cannot be reasonably scaled to provide gram quantities of material. Thus, we developed an efficient, comparatively cost-efficient, solution phase synthesis of PhTx-74.

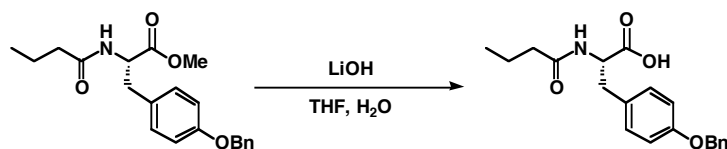
One of the most challenging aspects of synthesizing polyamine toxins is the formation of the secondary amine in the polyamine side chain. While many reactions result in the desired amine (e.g. Fukuyama-Mistunobu coupling between an activated amine and an alcohol, coupling of an acid to an amine followed by reduction of the amide), isolating the polyamine product from the reaction byproducts is typically extremely challenging. Therefore, we selected a secondary amine forming reaction that required no additional reagents and formed no difficult to remove byproducts. In particular, we converted the alcohol into the mesylate, which upon reaction with the primary amine yields the desired product and a water-soluble byproduct (methanesulfonic acid). Another key aspect of the synthesis involves selecting the appropriate protecting groups for the phenolic oxygen (e.g. benzyl ether) and the primary amine (e.g. *t*-butyl carbamate, Boc). We note that protecting the phenolic oxygen with an acid-labile protecting group (e.g. *t*-butyl ether) would eliminate one step in the procedure, as this group may be removed under acidic conditions along with the Boc protecting group. However, we found that the orthogonal two-step deprotection strategy provides the best method for obtaining pure PhTx-74.

All commercially available reagents were used without further purification unless otherwise indicated. <sup>1</sup>H NMR spectra were recorded using a 400 MHz Varian Inova NMR spectrometer. Chemical shifts (δ) are reported relative to the internal standard tetramethylsilane (TMS). Thin-layer chromatography (TLC) was performed using silica gel 60 F<sub>254</sub>-coated plates (EM Science, Gibbstown, NJ). Flash column chromatography was carried out using silica gel 60 (EMD Chemical, Inc., Cincinnati, OH). All reactions were carried out under anhydrous conditions (i.e. dry solvents/reagents and an inert atmosphere of nitrogen) unless otherwise indicated.

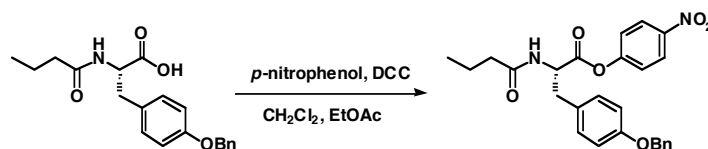


**3-(4-Benzyloxy-phenyl)-2-butyrylamino-propionic acid methyl ester:** To a 0 °C solution of O-benzyl-L-tyrosine methyl ester hydrochloride (11.8 g, 36.5 mmol, Bachem) in dichloromethane (300 mL) was added diisopropylethylamine (15.9 mL, 91.3 mmol), followed by *n*-butyryl chloride (4.2 mL, 40.2 mmol) dropwise. Upon complete addition, the mixture was allowed to warm to ambient temperature. After 16 h, the mixture was washed three times with 50 mL saturated sodium chloride

(adjusted to pH 3 with hydrochloric acid) and two times with 50 mL saturated sodium bicarbonate. The organic extract was then dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The resulting residue was taken up in dichloromethane and passed through a plug of silica gel (5% methanol/dichloromethane) to afford a light brown solid that was used without further purification (13.0 g, 100%).  $R_f = 0.41$  (1:1 ethyl acetate/hexanes).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.43–7.30 (m, 5H, ArH), 7.00 (d, 2H,  $J = 8.6$  Hz, ArH), 6.89 (d, 2H,  $J = 8.6$  Hz, ArH), 5.84 (d,  $J = 7.5$ , NH), 5.03 (s, 2H,  $\text{OCH}_2\text{Ph}$ ), 4.86 (m, 1H,  $J = 7.5$ ,  $J = 5.7$ , CH- $\text{CH}_2\text{Ph}$ ), 3.72 (s, 3H,  $\text{OCH}_3$ ), 3.05 (m, 2H,  $J = 5.7$ , CH- $\text{CH}_2\text{-Ph}$ ), 2.15 (t, 2H,  $J = 14.8$ , CO- $\text{CH}_2\text{-CH}_2\text{CH}_3$ ), 1.62 (m, 2H,  $J = 7.3$  Hz,  $J = 14.8$  Hz,  $\text{COCH}_2\text{-CH}_2\text{-CH}_3$ ), 0.91 (t, 3H,  $J = 7.3$  Hz,  $\text{COCH}_2\text{CH}_2\text{-CH}_3$ ).



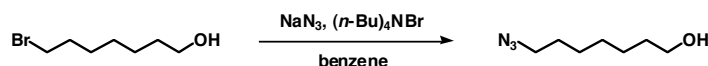
**3-(4-Benzyloxy-phenyl)-2-butyrylamino-propionic acid** To a solution of 3-(4-Benzyloxy-phenyl)-2-butyrylamino-propionic acid methyl ester (13.0 g, 36.5 mmol) in tetrahydrofuran (100 mL) was added 1N LiOH (150 mL). The mixture was vigorously stirred at ambient temperature for 16 h and then acidified to pH 3 with concentrated hydrochloric acid. The aqueous and organic phases were separated and the aqueous phase was saturated with solid sodium chloride and then washed five times with 200 mL ethyl acetate. The combined organic extracts were dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to afford a white solid (12.5 g, 100%).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.03 (d, 1H,  $J = 8.4$ , NH), 7.44–7.30 (m, 5H, ArH), 7.13 (d, 2H,  $J = 8.8$  Hz, ArH), 6.90 (d, 2H,  $J = 8.4$  Hz, ArH), 5.05 (s, 2H, -O- $\text{CH}_2\text{-Ph}$ ), 4.86 (ddd, 1H,  $J = 4.8$ ,  $J = 8.4$ ,  $J = 9.2$ , CH- $\text{CH}_2\text{Ph}$ ), 2.97 (dd, 1H,  $J = 4.8$  Hz, 14.0, CH- $\text{CH}_2\text{-Ph}$ ), 2.76 (dd, 1H,  $J = 9.6$  Hz, 13.6 Hz, CH- $\text{CH}_2\text{-Ph}$ ), 2.02 (t, 2H,  $J = 7.2$ , CO- $\text{CH}_2\text{-CH}_2\text{CH}_3$ ), 1.42 (quint, 2H,  $J = 7.2$  Hz,  $\text{COCH}_2\text{-CH}_2\text{-CH}_3$ ), 0.75 (t, 3H,  $J = 7.2$  Hz,  $\text{COCH}_2\text{CH}_2\text{-CH}_3$ ).



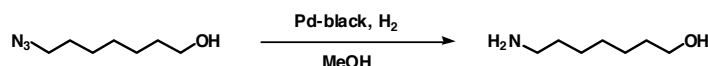
### 3-(4-Benzyloxy-phenyl)-2-butyrylamino-propionic acid 4-nitro-phenyl ester

This reaction was carried out essentially as previously reported (Choi et al., 1992; Nakanishi et al., 1997). To a solution of 3-(4-benzyloxy-phenyl)-2-butyrylamino-propionic acid (3.50 g, 10.3 mmol) and *p*-nitrophenol (1.43 g, 10.3 mmol) in dichloromethane and ethyl acetate (1:1, 200 mL) was added 1,3-dicyclohexylcarbodiimide (2.56 g, 12.4 mmol). A white precipitate began to form after five minutes. The mixture was stirred for an additional 4 h at room temperature and then filtered to remove dicyclohexylurea (DCU). The filter cake was washed with 200 mL dichloromethane, and the combined filtrates concentrated *in vacuo*. The resulting residue was purified by flash silica gel chromatography (1:49 methanol/ dichloromethane) to yield a light yellow solid (4.73 g, 99%).  $R_f = 0.66$  (1:19 methanol/ dichloromethane). Note: Because methanol reacts exothermically with silica gel, column cracking will occur when a methanol solution is added to a column. To avoid this cracking one should fill the column with the appropriate amount of dry silica gel, add 1% methanol in dichloromethane and shake the column lengthwise to create a slurry. The methanol / silica gel reaction occurs in the slurry; subsequent packing under pressure will provide an uncracked column. *p*-Nitrophenol runs quickly in methanol in dichloromethane (and inconsistently in ethyl acetate in hexanes) because of the acidity of the phenolic proton. Unreacted *p*-nitrophenol can be removed from the column by patient elution with 1% methanol in dichloromethane. The desired product can then be removed with 2% to 5% methanol in

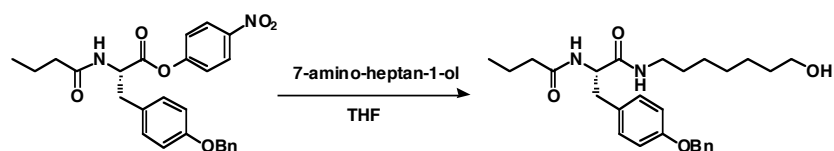
dichloromethane. Methanol content greater than 5% will co-elute dicyclohexylurea with the product. A methanol content greater than 10% should not be used as silica gel will be dissolved by the mobile phase. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 8.11 (d, 2H, *J* = 9.2 Hz, *ArH*), 7.51-7.42 (m, 5H, *ArH*), 6.91-6.68 (m, 4H, *ArH*), 7.00 (d, 2H, *J* = 8.4 Hz, *ArH*), 6.00 (d, 1H, *J* = 7.6 Hz, CO-NH-CH-), 5.03 (s, 2H, O-CH<sub>2</sub>-Ph), 4.85 (m, 1H, *J* = 6.0 Hz, *J* = 13.6 Hz, α-CH-CH<sub>2</sub>-Ph), 3.10 (dd, 1H, *J* = 6.0 Hz, *J* = 14.0 Hz, -CH-CH<sub>2</sub>-Ph), 3.04 (dd, 1H, *J* = 6.0 Hz, *J* = 14.0 Hz, -CH-CH<sub>2</sub>-Ph), 2.19 (t, 2H, *J* = 7.6 Hz, -CO-CH<sub>2</sub>-CH<sub>2</sub>), 1.63 (sextet, 2H, *J* = 7.2 Hz, -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>), 0.92 (t, 3H, *J* = 7.2 Hz, -CH<sub>2</sub>-CH<sub>3</sub>).



**7-Azido-heptan-1-ol** To a solution of 7-bromo-heptan-1-ol (10.0 g, 51.2 mmol, Aldrich) in benzene (200 mL) at ambient temperature was added tetra-*n*-butylammonium bromide (1.65 g, 5.12 mmol), followed by sodium azide (6.65 g, 102 mmol). The mixture was warmed to reflux and stirred for 16 h. After cooling to ambient temperature, the mixture was filtered through celite and the cake was washed with 100 mL dichloromethane. The combined filtrates were concentrated *in vacuo* and the resulting residue passed through a plug of silica gel (1:1 ethyl acetate/dichloromethane) to yield a gold oil (8.02 g, 99%). *R<sub>f</sub>* = 0.61 (1:1 ethyl acetate/hexanes). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 3.65 (t, 2H, *J* = 6.0, CH<sub>2</sub>-CH<sub>2</sub>-N<sub>3</sub>), 3.40 (q, 2H, *J* = 6.8 Hz, CH<sub>2</sub>-CH<sub>2</sub>-OH), 3.26 (t, 1H, *J* = 6.8 Hz, OH), 1.87 (quint, 2H, *J* = 6.8 Hz), 1.50-1.30 (m, 6H), 1.73-1.52 (m, 2H).

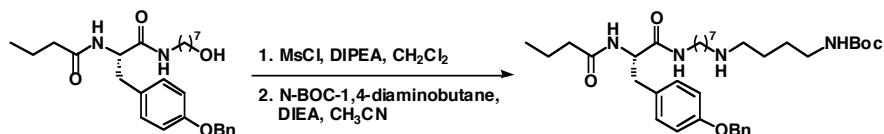


**7-Amino-heptan-1-ol** To a solution of 7-azido-heptan-1-ol (8.00 g, 50.9 mmol) in methanol (200 mL) was added palladium black (500 mg). The mixture was stirred for 16 h at ambient temperature under hydrogen gas (1 atm) and then filtered through celite. The filter cake was washed with 200 mL methanol, the combined filtrates were concentrated *in vacuo* and the resulting residue purified by flash silica gel chromatography (1:9 methanol/ dichloromethane to 1:1:8 isopropylamine/ methanol/ dichloromethane) to yield a light gold oil (5.33 g, 80%). *R<sub>f</sub>* = 0.67 (1:1:8 isopropylamine/ methanol/ dichloromethane). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 3.63 (t, 2H, *J* = 6.4, CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>), 3.39 (t, 2H, *J* = 7.2 Hz, CH<sub>2</sub>-CH<sub>2</sub>-OH), 3.26 (t, 1H, *J* = 6.8 Hz, OH), 1.69 (m, 2H), 1.61-1.50 (m, 4H), 1.40-1.30 (m, 4H).



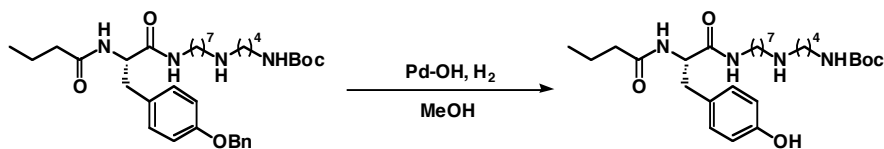
***N*-[2-(4-Benzyloxy-phenyl)-1-(7-hydroxy-heptylcarbonyl)-ethyl]-butyramide** To a solution of 3-(4-benzyloxy-phenyl)-2-butrylamino-propionic acid 4-nitro-phenyl ester (4.73 g, 10.2 mmol) in tetrahydrofuran (100 mL) was added a solution of 7-amino-heptan-1-ol (2.68 g, 20.4 mmol in 10 mL tetrahydrofuran). The mixture was stirred for 16 h at ambient temperature and then concentrated *in vacuo*. The resulting residue was purified by flash silica gel chromatography (1:49 methanol/ dichloromethane to 1:9 methanol/ dichloromethane) to yield a light yellow solid (4.65 g, 99%). *R<sub>f</sub>* = 0.45 (1:9 methanol/ dichloromethane). *Note:* This reaction can be driven to completion by the addition of excess amino alcohol. No ester formation was detected in this reaction. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.40-7.27 (m, 5H, *ArH*), 7.13 (d, 2H, *J* = 8.4 Hz, *ArH*), 6.91 (d, 2H, *J* = 8.8 Hz, *ArH*), 6.21 (d, 1H, *J* = 7.6 Hz, NH), 5.79 (bs, 1H, NH), 5.04 (s, 2H, O-CH<sub>2</sub>-Ph), 4.54 (m, 1H, *J* = 8.0 Hz, *J* = 14.4 Hz, CH-CH<sub>2</sub>Ph), 3.61 (m, 2H, *J* = 6.4 Hz, *J* = 11.2 Hz, CH<sub>2</sub>-CH<sub>2</sub>-OH), 3.14 (m, 2H, *J* = 6.4 Hz, CONH-CH<sub>2</sub>-

CH<sub>2</sub>), 3.02 (dd, 1H,  $J = 6.4$  Hz,  $J = 14.0$  Hz, CH-CH<sub>2</sub>-Ph), 2.92 (dd, 1H,  $J = 8.8$  Hz,  $J = 14.0$  Hz, CH-CH<sub>2</sub>-Ph), 2.16 (t, 2H,  $J = 7.2$  Hz, CO-CH<sub>2</sub>-CH<sub>2</sub>CH<sub>3</sub>), 1.63-1.46 (m, 4H), 1.35-1.13 (m, 8H), 0.91 (t, 3H,  $J = 7.2$  Hz, COCH<sub>2</sub>CH<sub>2</sub>-CH<sub>3</sub>).

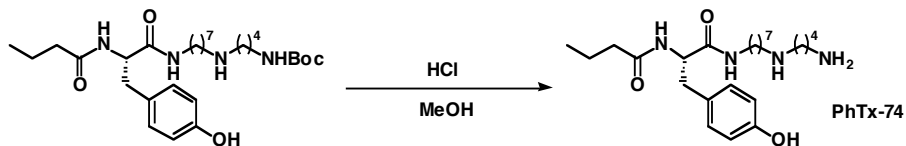


**(4-{7-[3-(4-Benzyloxy-phenyl)-2-butyrylamino-propionylamino]-heptylamino}-butyl)-carbamic**

**acid *t*-butyl ester** This sequence of reactions is based on a previous report (Gardner et al., 2004). To a solution of *N*-[2-(4-benzyloxy-phenyl)-1-(7-hydroxy-heptylcarbamoyl)-ethyl]-butyramide (4.65 g, 10.2 mmol) in dichloromethane (100 mL) was added diisopropylethylamine (3.60 mL, 20.4 mmol), followed by methanesulfonyl chloride (1.60 mL, 20.4 mmol) dropwise. After stirring for 4 h, the mixture was diluted with 1M sodium hydroxide (50 mL), followed by water (50 mL). The organic and aqueous phases were separated and the aqueous layer was washed with 50 mL dichloromethane. The combined organic layers were washed with 50 mL saturated sodium chloride, dried over anhydrous sodium sulfate and then concentrated *in vacuo*. To a solution of the resulting light brown solid (5.40 g,  $R_f = 0.63$ , 1:9 methanol/dichloromethane) in acetonitrile (80 mL), was added diisopropylethylamine (1.80 mL, 10.2 mmol) followed by *N*-BOC-1,4-diaminobutane (4.00 mL, 20.4 mmol, Sigma-Aldrich). The mixture was stirred for 48 h at 35 °C and then concentrated *in vacuo*. The resulting residue was dissolved in 150 mL dichloromethane and washed four times with 50 mL saturated sodium carbonate. The organic layer was dried over anhydrous sodium sulfate and then concentrated *in vacuo*. The resulting residue was purified by flash chromatography (1:19 methanol/ dichloromethane to 1:1:8 isopropylamine/ methanol/ dichloromethane) to yield a light brown solid (5.82 g, 91% over two steps).  $R_f = 0.65$  (1:1:8 isopropylamine/ methanol/ dichloromethane). *Note:* This S<sub>N</sub>2 reaction is extremely slow; *stoichiometric* reaction of the protected diamine and mesylate can take as long as 7 days at ambient temperature. The reaction rate can be increased by heat or the addition of excess protected diamine. The secondary amine product runs slowly ( $R_f \sim 0.1$ ) and with streaking in 10% methanol in dichloromethane. Thus, the product was purified by flash chromatography with the addition of isopropylamine (IPA) to avoid streaking. Residual starting materials were removed with 5-10% methanol in dichloromethane, and the desired product was eluted with 1-2% IPA in 10% methanol in dichloromethane. Because IPA boils as 32.4 °C, it can easily be removed *in vacuo*. The reaction can be monitored by TLC using a cobalt stain (1 g cobalt chloride heptahydrate and 3 g ammonium thiocyanate in 20 mL H<sub>2</sub>O), which colors amines (bright blue) and amides (light blue) without heat. Because methanesulfonic acid is produced in the reaction, a base, diisopropylethylamine (DIPEA), is required to avoid formation of the protected di-ammonium sulfonate salt. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  7.43-7.32 (m, 5H, *ArH*), 7.14 (d, 2H,  $J = 8.4$  Hz, *ArH*), 6.90 (d, 2H,  $J = 8.8$  Hz, *ArH*), 6.18 (bs, 1H, *NH*), 5.70 (bs, 1H, *NH*), 5.04 (s, 2H, O-CH<sub>2</sub>-Ph), 4.86 (bs, 1H, *NH*), 4.52 (m, 1H, -CH-CH<sub>2</sub>Ph), 3.16-3.08 (m, 4H), 3.02 (dd, 1H,  $J = 6.0$  Hz, 13.6 Hz, CH-CH<sub>2</sub>-Ph), 2.92 (dd, 1H,  $J = 8.0$  Hz, 13.6 Hz, CH-CH<sub>2</sub>-Ph), 2.64-2.56 (m, 4H,  $J = 7.2$ ), 2.15 (t, 2H,  $J = 7.2$  Hz, CO-CH<sub>2</sub>-CH<sub>2</sub>CH<sub>3</sub>), 1.61 (quint, 2H,  $J = 7.2$  Hz, COCH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>), 1.52-1.44 (m, 4H), 1.44 (s, 9H, *t*-butyl), 1.37-1.19 (m, 10H), 0.90 (t, 3H,  $J = 7.2$  Hz, COCH<sub>2</sub>CH<sub>2</sub>-CH<sub>3</sub>).

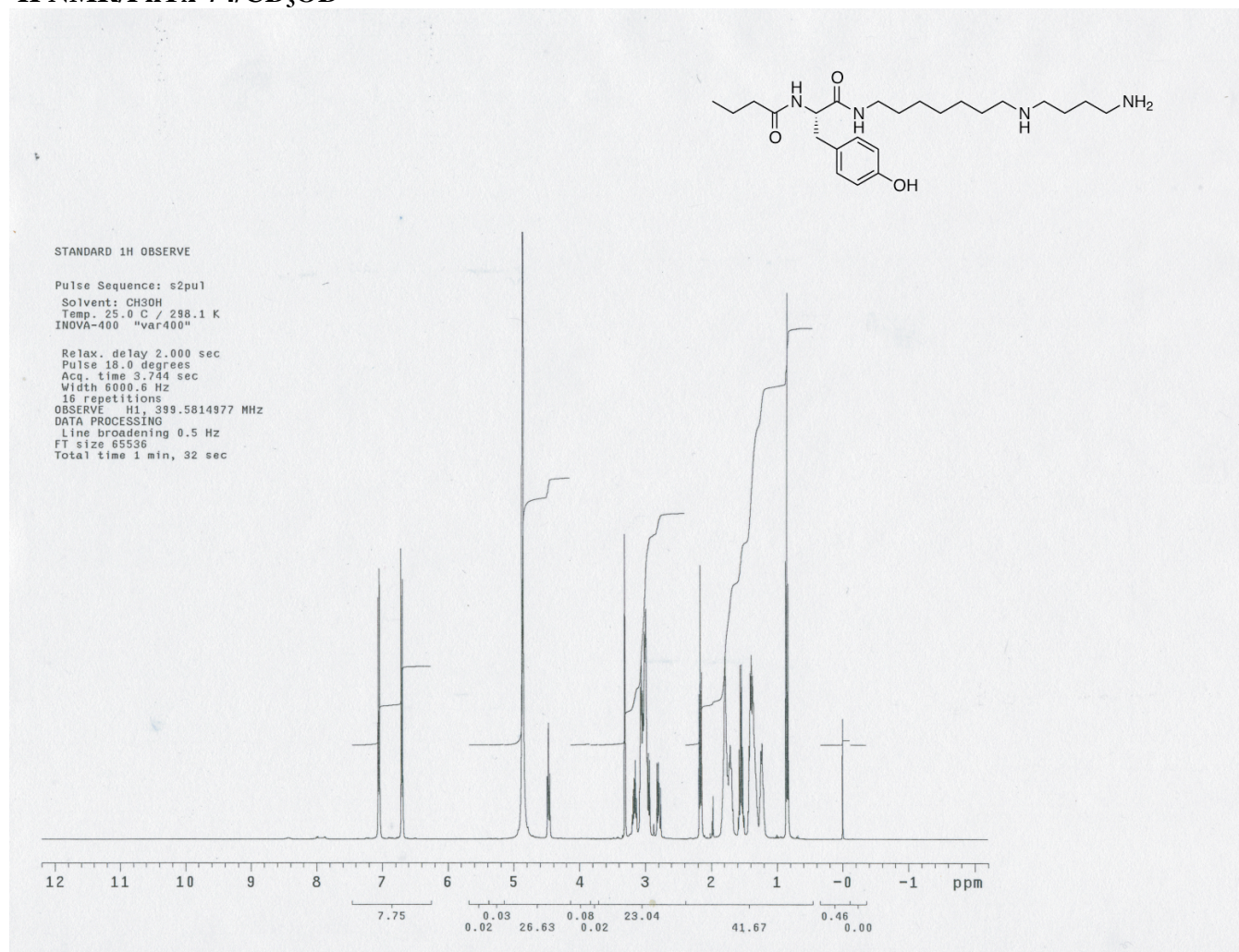


**(4-{7-[2-Butyrylamino-3-(4-hydroxy-phenyl)-propionylamino]-heptylamino}-butyl)-carbamic acid *t*-butyl ester** To a solution of (4-{7-[3-(4-Benzyloxy-phenyl)-2-butyrylamino-propionylamino]-heptylamino}-butyl)-carbamic acid *t*-butyl ester (5.86 g, 9.38 mmol) in methanol (100 mL) was added palladium hydroxide (1.16 g). The mixture was stirred for 16 h at ambient temperature under hydrogen gas (1 atm) and then filtered through celite. The filter cake was washed with 20 mL methanol and the filtrate was concentrated *in vacuo* to yield a light brown solid that was used without further purification (5.00 g, 99%).  $R_f$  = 0.63 (1:1:8 isopropylamine/ methanol/ dichloromethane). *Note:* Hydrogenation ( $H_2$ , 1 atm) over palladium on carbon in ethanol gave partial reduction of the benzyl group (by  $^1H$ -NMR). Similar results were obtained with a slightly more acidic solvent (methanol) and a more reactive palladium source (palladium black). Pearlman's catalyst (20% palladium hydroxide on carbon) in methanol gave complete deprotection.  $^1H$ -NMR (400 MHz,  $CDCl_3$ ):  $\delta$  7.08 (d, 2H,  $J$  = 8.4 Hz, ArH), 6.71 (d, 2H,  $J$  = 8.4 Hz, ArH), 6.36 (d, 1H,  $J$  = 7.6 Hz, NH), 5.26 (bs, 1H, NH), 4.49 (m, 1H, -CH- $CH_2$ Ph), 3.14-3.08 (m, 2H), 2.76-2.66 (m, 6H,  $J$  = 7.2), 2.20 (t, 2H,  $J$  = 7.2 Hz, -CO- $CH_2$ - $CH_2$ CH $_3$ ), 1.72-1.38 (m, 6H), 1.43 (s, 9H, *t*-butyl), 1.36-1.13 (m, 6H), 0.95 (t, 3H,  $J$  = 7.6 Hz, COCH $_2$ CH $_2$ -CH $_3$ ).



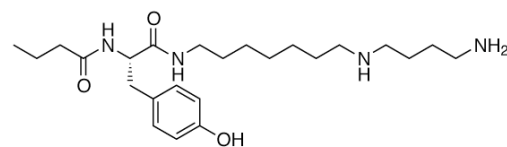
**N-[1-[7-(4-Amino-butylamino)-heptylcarbonyl]-2-(4-hydroxy-phenyl)-ethyl]-butyramide hydrochloride** A solution of (4-{7-[2-Butyrylamino-3-(4-hydroxy-phenyl)-propionylamino]-heptylamino}-butyl)-carbamic acid *t*-butyl ester (5.00 g, 9.38 mmol) in 80 mL 3 M anhydrous hydrochloric acid in MeOH was stirred for 3 h at ambient temperature. The mixture was concentrated *in vacuo* to yield a white solid (4.70 g, 99%).  $R_f$  = 0.15 (1:1:8 isopropylamine/ methanol/ dichloromethane). *Note:* Anhydrous HCl was obtained by slow addition of acetyl chloride to anhydrous methanol at 0 °C. TLC samples were neutralized with a drop of IPA. Complete deprotection occurs within 2-3 hours with longer reactions times resulting in methanolysis of the amide. The crude product (PhTx-74  $\cdot$  2HCl), may be further purified by recrystallization from ethanol, ethyl acetate and hexanes. The salt is dissolved in a minimum volume of hot ethanol, and the solution is allowed to cool to ambient temperature. Ethyl acetate is added dropwise just until the solution first became cloudy, and then hexane is added to induce crystallization. Cooling to 0 °C followed by collection by filtration provides pure recrystallized PhTx-74 salt.  $^1H$ -NMR (400 MHz,  $CD_3OD$ ):  $\delta$  7.03 (d, 2H,  $J$  = 8.0 Hz, ArH), 6.68 (d, 2H,  $J$  = 7.6, ArH), 4.46 (t, 1H,  $J$  = 8.0 Hz,  $J$  = 8.0 Hz, -CH- $CH_2$ Ar), 3.15 (m, 1 H), 3.08-2.92 (m, 8H), 2.78 (dd, 1H,  $J$  = 8.0 Hz, 13.2 Hz, -CH- $CH_2$ -Ar), 2.15 (t, 2H,  $J$  = 7.2 Hz), 1.82-1.64 (m, 6H), 1.54 (sextet, 2H,  $J$  = 7.6 Hz, -CH $_2$ -CH $_2$ -CH $_3$ ), 1.43-1.29 (m, 6H), 1.22 (m, 2H), 0.84 (t, 3H,  $J$  = 7.2 Hz, COCH $_2$ CH $_2$ -CH $_3$ ).

**<sup>1</sup>H NMR/PhTx-74/CD<sub>3</sub>OD**



# <sup>1</sup>H NMR/PhTx-74/DMSO-d<sup>6</sup> + D<sub>2</sub>O

		<b>Nucleus</b>	1H	<b>Number of Transients</b>	20	<b>Frequency (MHz)</b>	399.57
<b>Pulse Sequence</b>	s2pul	<b>Solvent</b>	DMSO-D6 + D2O	<b>Temperature (degree C)</b>	25.000	<b>Sweep Width (Hz)</b>	6000.60

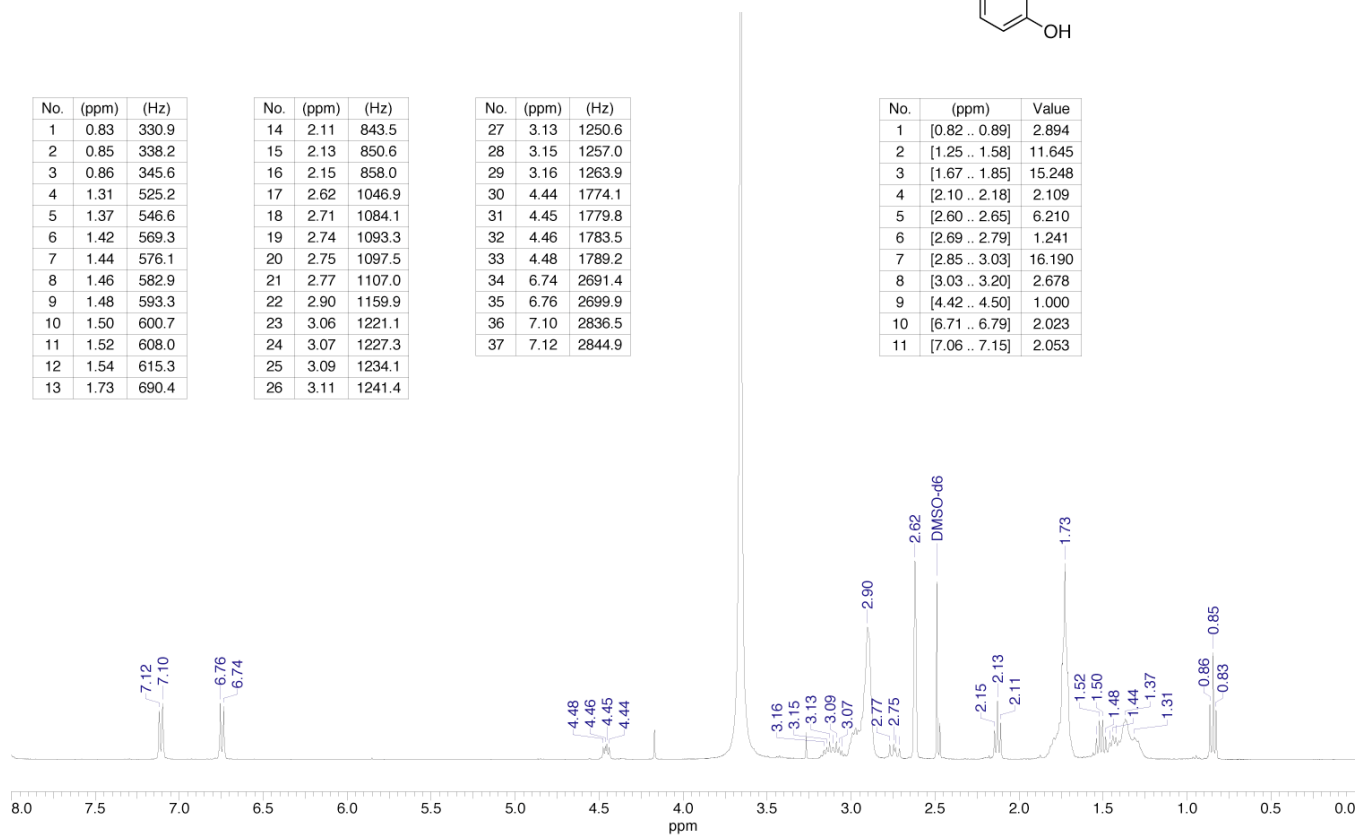


No.	(ppm)	(Hz)
1	0.83	330.9
2	0.85	338.2
3	0.86	345.6
4	1.31	525.2
5	1.37	546.6
6	1.42	569.3
7	1.44	576.1
8	1.46	582.9
9	1.48	593.3
10	1.50	600.7
11	1.52	608.0
12	1.54	615.3
13	1.73	690.4

No.	(ppm)	(Hz)
14	2.11	843.5
15	2.13	850.6
16	2.15	858.0
17	2.62	1046.9
18	2.71	1084.1
19	2.74	1093.3
20	2.75	1097.5
21	2.77	1107.0
22	2.90	1159.9
23	3.06	1221.1
24	3.07	1227.3
25	3.09	1234.1
26	3.11	1241.4

No.	(ppm)	(Hz)
27	3.13	1250.6
28	3.15	1257.0
29	3.16	1263.9
30	4.44	1774.1
31	4.45	1779.8
32	4.46	1783.5
33	4.48	1789.2
34	6.74	2691.4
35	6.76	2699.9
36	7.10	2836.5
37	7.12	2844.9

No.	(ppm)	Value
1	[0.82 .. 0.89]	2.894
2	[1.25 .. 1.58]	11.645
3	[1.67 .. 1.85]	15.248
4	[2.10 .. 2.18]	2.109
5	[2.60 .. 2.65]	6.210
6	[2.69 .. 2.79]	1.241
7	[2.85 .. 3.03]	16.190
8	[3.03 .. 3.20]	2.678
9	[4.42 .. 4.50]	1.000
10	[6.71 .. 6.79]	2.023
11	[7.06 .. 7.15]	2.053





## Electrophysiology

The AMPAR subunit (rat GluR1-3, flip isoforms) and  $\gamma$ -TARP plasmids, gifts from Dr. David Brecht, were subcloned into pGEMHE for expression in oocytes. The GluR3<sub>CT1</sub> chimeric subunit was constructed by replacing the GluR3 C-terminal domain (coding amino acids 831-888) with the GluR1 C-terminal domain (coding amino acids 819-907) using standard techniques in molecular biology. Plasmid DNAs were linearized with *Nhe I* and capped mRNA transcripts were synthesized *in vitro* using T7 polymerase (mMessage Machine Kit; Ambion, Austin, TX). All purified mRNA transcripts were rigorously quantitated to ensure the desired ratios of subunit mRNAs were co-injected in oocytes. In particular, aliquots of each transcript reaction were electrophoresed (to separate full length transcripts from residual nucleotides) and the relative concentrations of full-length transcripts determined using fluorescence detection (of ethidium bromide stained mRNA) with an Alpha-Innotech 8900 image analyzer. The concentrations of the mRNA stocks were then normalized to one another. All subunit mRNAs were prepared and quantitated side-by-side on the same day, using the same transcription kit. When one subunit mRNA stock is consumed (~ 6 months), all of the subunits are prepared again.

Stage V-VI oocytes were surgically obtained from adult *Xenopus laevis* frogs (Nasco Intl., Fort Atkinson, WI) according to the methods approved by the UCSF Institutional Animal Care and Use Committee, as previously described (Quick *et al.* 1994), and stored at 18 °C in ND96-storage solution (96 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, and 5.0 mM HEPES, 1% horse serum, 2.5 mM Na pyruvate, 0.5 mM theophylline, 50 mg/mL gentamycin, pH 7.5). Oocytes were injected in the cytoplasm (50 nL/oocyte) with AMPA receptor subunit mRNAs and the  $\gamma$ -2 TARP subunit using a positive-displacement injection apparatus (Drummond Nanoject). Homomeric receptors were prepared by co-injecting the individual AMPA receptor subunit (GluR1, GluR2, or GluR2) with a  $\gamma$ -2 TARP in 1:1 ratio at a total mRNA concentration ranging from 0.002-0.02 ng/nL, depending on the AMPA receptor subunit, to provide peak glutamate-evoked (100  $\mu$ M) responses ~ 0.2-1.0  $\mu$ A. Heteromeric receptors were prepared by co-injecting AMPA receptor subunits with  $\gamma$ -2 TARP in a 1:1:2 ratio at a total mRNA concentration of 0.002-0.02 ng/nL, depending on the AMPA receptor subunits, to provide peak glutamate-evoked (100  $\mu$ M) responses ~ 0.2-1.0  $\mu$ A. Injected oocytes were deemed appropriate for experiments only if the standing currents were < 10% of the peak current at a holding potential of -80 mV.

Glutamate-evoked currents (100  $\mu$ M) were recorded ~24-36 h post-injection in the two-electrode voltage clamp recording configuration (GeneClamp 500B, Axon Instruments, Union City, CA). Electrodes (0.5 to 2 M $\Omega$  resistance) were filled with 3 M KCl. Steady-state currents were recorded at a holding potential of -80 mV. Current-voltage (I/V) relationships were measured by ramping from positive (+20 mV) to negative (-80 mV) potentials in the presence and absence of 100  $\mu$ M glutamate and then subtracted. All drug solutions were prepared from aqueous stocks, diluted in the oocyte recording solution ND96: (96 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, and 5.0 mM HEPES, pH 7.5). Solution changes were controlled using an electronic valve control system with pinch-type valves (Valvelink 16, Automate Scientific, Inc., San Francisco, CA). Current responses were acquired using pClamp 9.0 software (Axon Instruments, Union City, CA) and a Digidata 1322A interface (Axon Instruments). Bar graph data (mean  $\pm$  SEM) were analyzed and graphed using KaleidaGraph 3.5.

Following our observation that PhTx-74 blocks GluR1/2 receptors, we expected that the toxin would also inhibit GluR2/3 receptors. This assumption was based on the knowledge that the receptor transmembrane sequences, including those lining the channel pore, are identical in GluR1/2 and GluR2/3 receptors (Figure S1). However, such sequence identity does not ensure that the quaternary structures of the GluR1/2 and GluR2/3 channel pores are identical. Since C-terminal domain



modifications have been previously shown to modulate the structure of an AMPA receptor pore (i.e. rate of ion permeation, see Banke et al. 2000, Derkach et al. 1999), we explored the possibility that the observed subtype selectivity is due in part to differences between the C-terminal domains of the GluR1 and GluR3 subunits, which then translate into structural differences between the GluR1/2 and GluR2/3 receptor pores. Our experiments indeed suggest that the GluR3 C-terminal domain affects the affinity of GluR2/3 receptors for PhTx-74 (see Figure 1).

### Figure S1

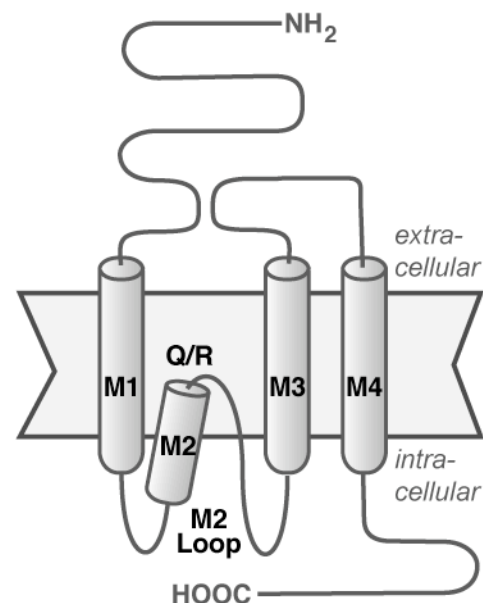
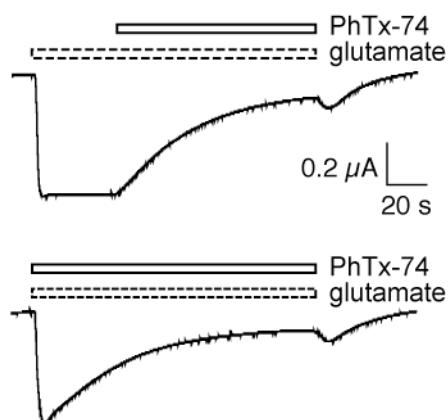


Figure S1. Amino acid sequence alignment of the GluR1 and GluR3 AMPAR subunits. Amino acid differences are indicated by bolded text (•), phosphorylation sites by red italicized text and approximate sites of interaction with intracellular proteins indicated by blue italicized text. Cartoon (right) depicts the proposed membrane topology of an AMPAR subunit. The putative pore-lining Q/R site residue, which is a glutamine in GluR1 and GluR3 subunits and an arginine residue in mRNA-edited GluR2 subunits, is a key determinant of Ca<sup>+2</sup> permeability. LBD = ligand-binding domain.

## Figure S2

Figure S2. Representative traces from the same cell showing the effect of PhTx-74 on Glu1/2 receptors. Top trace shows the effect of PhTx-74 (100  $\mu$ M, solid drug bar) on steady-state glutamate-evoked (100  $\mu$ M, dashed drug bar) currents. Bottom trace shows the effect of co-applying glutamate (100  $\mu$ M) and PhTx-74 (100  $\mu$ M). The peak current in the two traces is comparable, consistent with open channel (use-dependent) block by PhTx-74.



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