

Supporting Information

Fluorescence Probe for Lysophospholipase C/NPP6 Activity and a Potent NPP6 Inhibitor

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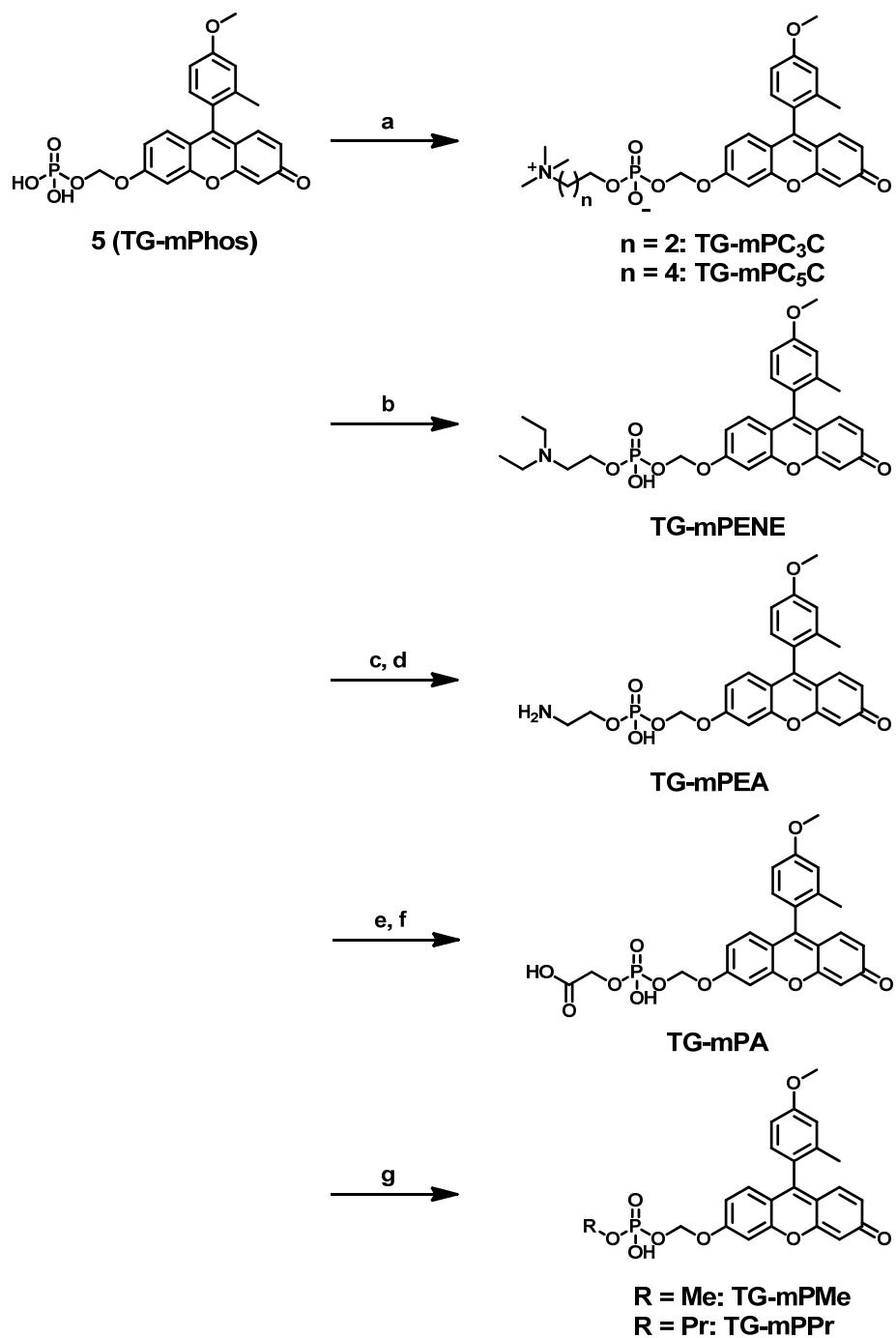
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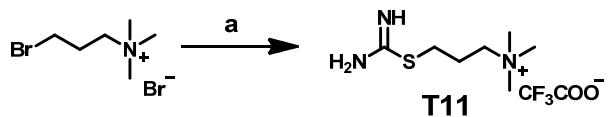
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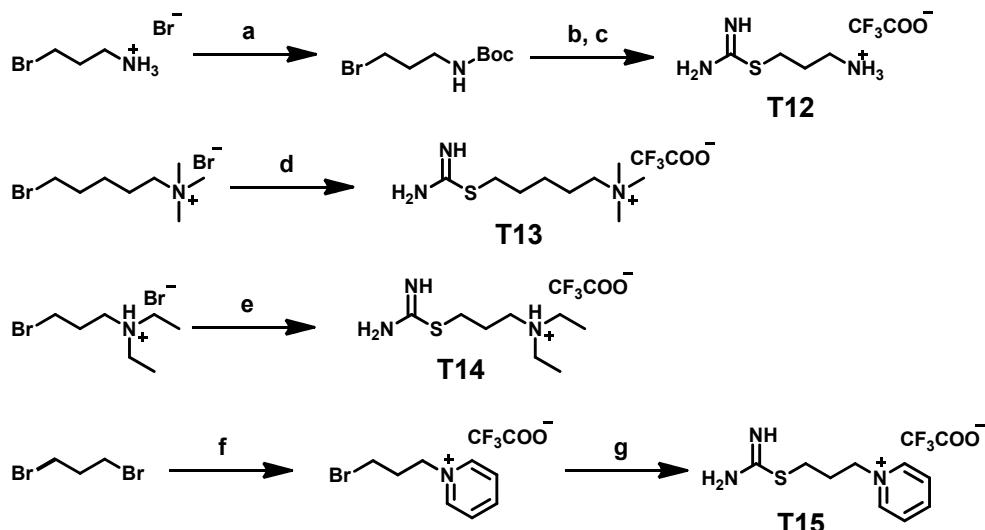
Supporting Data



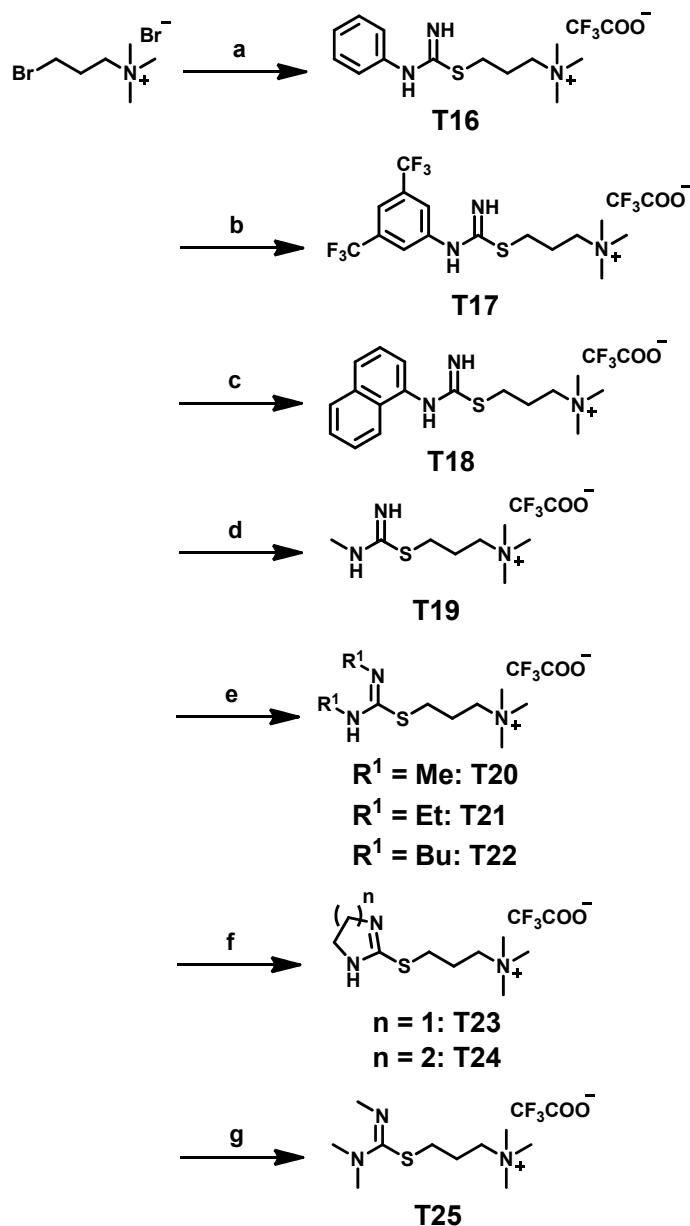
Scheme S1. Synthesis of TG-mPC analogs. Reactant and reagents: (a) (3-bromopropyl)trimethylammonium bromide or (5-bromopentyl)trimethylammonium bromide, DIEA, DMF, 50 °C, 74% and 30 %, respectively; (b) 2-diethylaminoethyl bromide hydrobromide, DIEA, DMF, 50 °C, 40%; (c) 1-*N*-Cbz-2-bromoethylamine, DIEA, DMF, 50 °C, crude; (d) Pd/C, H₂, CH₂Cl₂/MeOH, rt, 24%; (e) benzyl bromoacetate, DIEA, DMF, 50 °C, crude; (f) Pd/C, H₂, CH₂Cl₂/MeOH, rt, 55%; (g) methyl iodide or propyl iodide, Cs₂CO₃, DMF, 50 °C, 15% and 30%, respectively.



Scheme S2. Synthesis of T11. Reactant and reagents: (a) thiourea, EtOH, reflux, 85%.



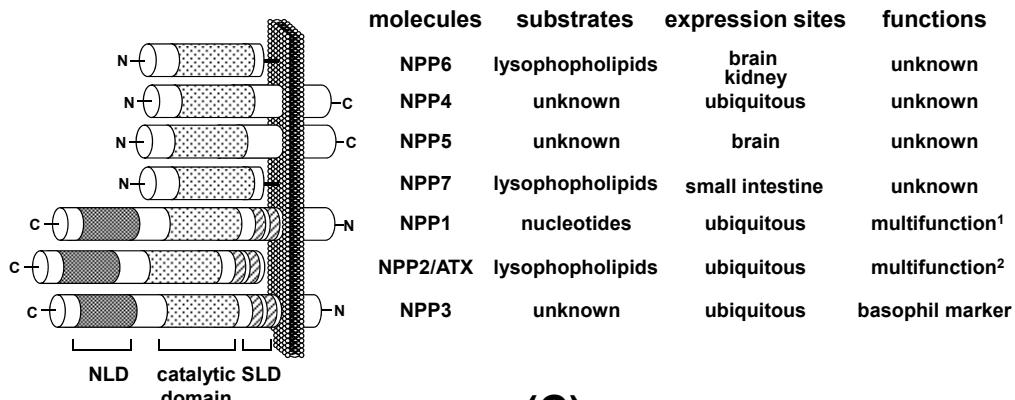
Scheme S3. Synthesis of T12-15. Reactant and reagents: (a) Boc_2O , DIEA, EtOH, 60 °C to rt, 90%; (b) thiourea, EtOH, 85 °C, crude; (c) TFA/CH₂Cl₂, 0 °C, 25% in 2 steps; (d) thiourea, EtOH, 85 °C, 72%; (e) thiourea, EtOH, 85 °C, 76%; (f) Ac₂O, pyridine, rt, 70%; (g) thiourea, EtOH, 85 °C, 37%.



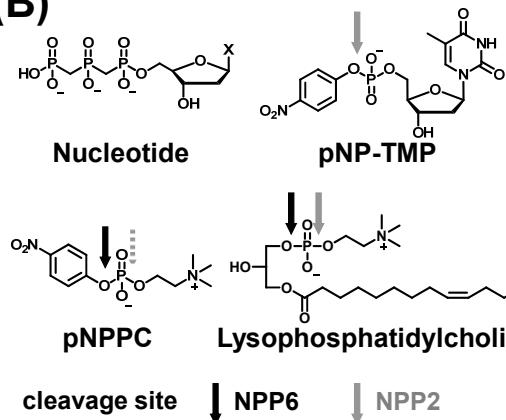
Scheme S4. Synthesis of T16-25. Reactant and reagents: (a) 1-phenylthiourea, EtOH, 85 °C, 5.7%; (b) 1-(3,5-bis(trifluoromethyl)phenyl)thiourea, EtOH, 85 °C, 10%; (c) 1-(naphthalen-1-yl)thiourea, EtOH, 85 °C, 56%; (d) 1-methylthiourea, EtOH, 85 °C, 48%; (e) 1,3-dimethylthiourea or 1,3-diethylthiourea or 1,3-dibutylthiourea, EtOH, 85 °C, 23, 48, 31%, respectively; (f) imidazolidine-2-thione or tetrahydropyrimidine-2-thione, EtOH, 85 °C, 75, 65%, respectively; (g) 1,1,3-trimethylthiourea, EtOH, 85 °C, 54%.

(A)

extracellular space intracellular space



(B)



(C)

Enzyme	Nucleotide	LPC	pNP-TMP	pNPPC	TG-mPC
NPP1	+	-	+	-	-
NPP2	-	+	+	-	-
NPP3	+	-	+	-	-
NPP4	+	-	+	-	-
NPP5	+	-	+	+	-
NPP6	-	+	-	+	+
NPP7	-	+	-	+	-

Figure S1. (A) Structures and functions of NPP6 and other NPP family members. The domain structure and membrane orientation of seven NPP members and their natural substrates, expression sites, and physiological functions are shown. NPP6 and NPP7 are anchored to plasma membrane by GPI and NPP2 is secreted extracellularly, while the others are transmembrane proteins. ¹Inhibition of bone calcification and insulin metabolism. ²Vasculature and neural tube formation in embryos, tumor progression, and development of neuropathic pain. NLD = nuclease-like domain, SLD = somatomedin-B-like domain, ATX = autotaxin. (B) Structures of native substrates, nucleotide, lysophosphatidylcholine (LPC), and synthetic absorption-based substrate, pNP-TMP and pNPPC. Black arrows indicate the cleavage site by NPP6, and gray arrows indicate the cleavage site by NPP2. (C) Reactivity of various substrates, shown in (B), and TG-mPC towards recombinant NPP1-7. For synthetic substrates, pNPPC and pNP-TMP, reactivity was evaluated in terms of absorption increase at 405 nm.

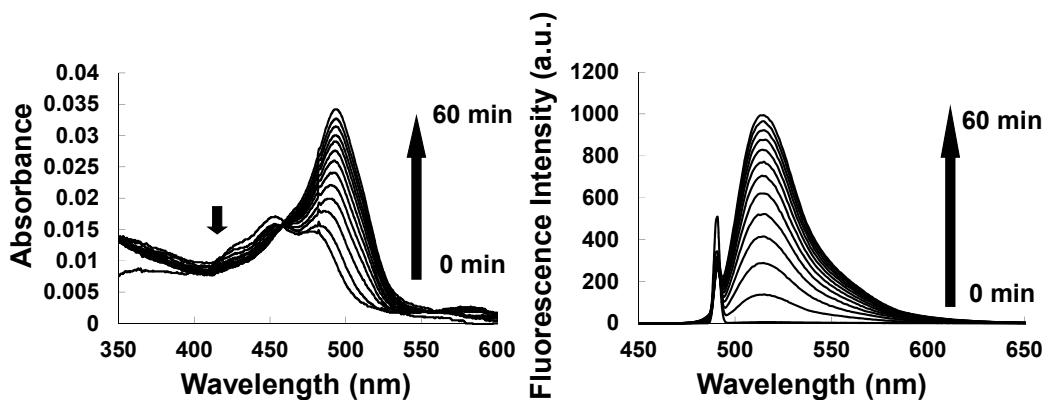


Figure S2. Changes of absorption and fluorescence spectra of TG-mPC (2 μ M) in the presence of NPP6, measured every 5 min. Reactions were performed in *NPP buffer* (100 mM Tris-HCl (pH 9.0), containing 500 mM NaCl, 5 mM MgCl₂, and 0.05% Triton-X100) at 37 °C. Excitation wavelength was 490 nm.

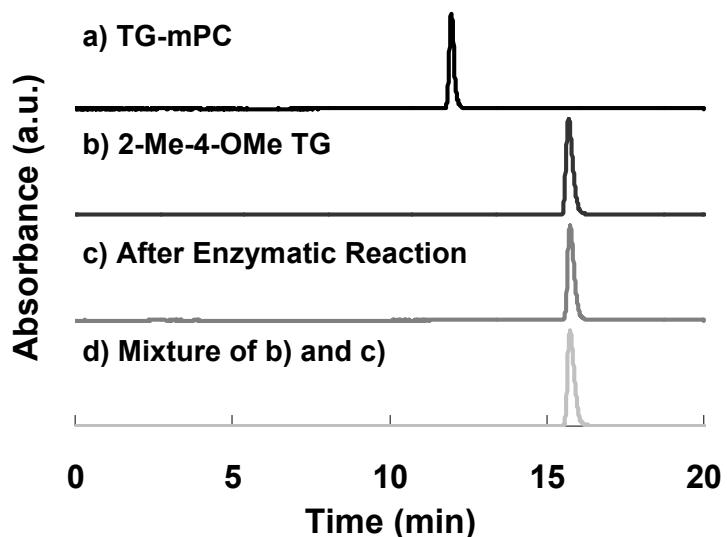


Figure S3. HPLC charts showing enzymatic reaction of TG-mPC with NPP6 to afford 2-Me-4-OMe TG: a) a solution of TG-mPC, b) a solution of 2-Me-4-OMe TG, c) reaction mixture after enzymatic reaction with NPP6, d) mixed solution of b) and c). HPLC analysis: eluent A (0.1% TFA H₂O) and eluent B (0.1% TFA, 80% acetonitrile, 20% H₂O), A/B = 80/20 \rightarrow A/B = 0/100 (30 min). For all spectra, the chromatogram was monitored at 490 nm.

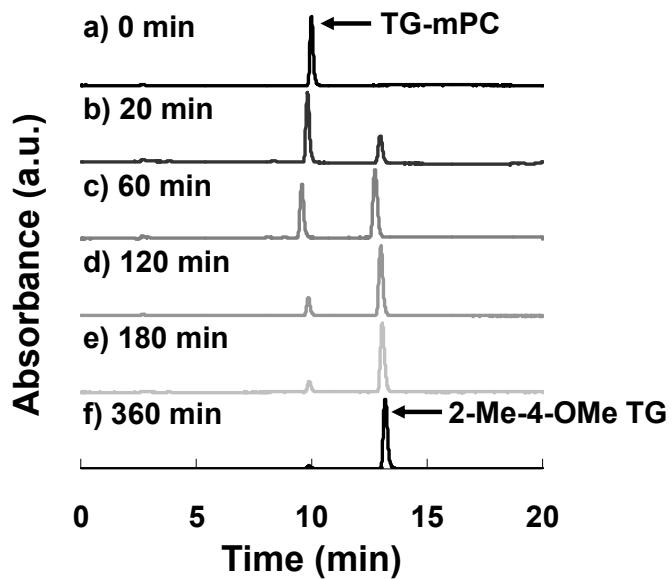


Figure S4. HPLC charts showing enzymatic reaction of TG-mPC with NPP2 to afford 2-Me-4-OMe TG: a)-f) illustrate the chromatograms at 0, 20, 60, 120, 180, 360 min after the start of enzymatic reaction with NPP2. HPLC analysis: A/B = 80/20 → A/B = 0/100 (20 min). For all spectra, the chromatogram was monitored at 490 nm.

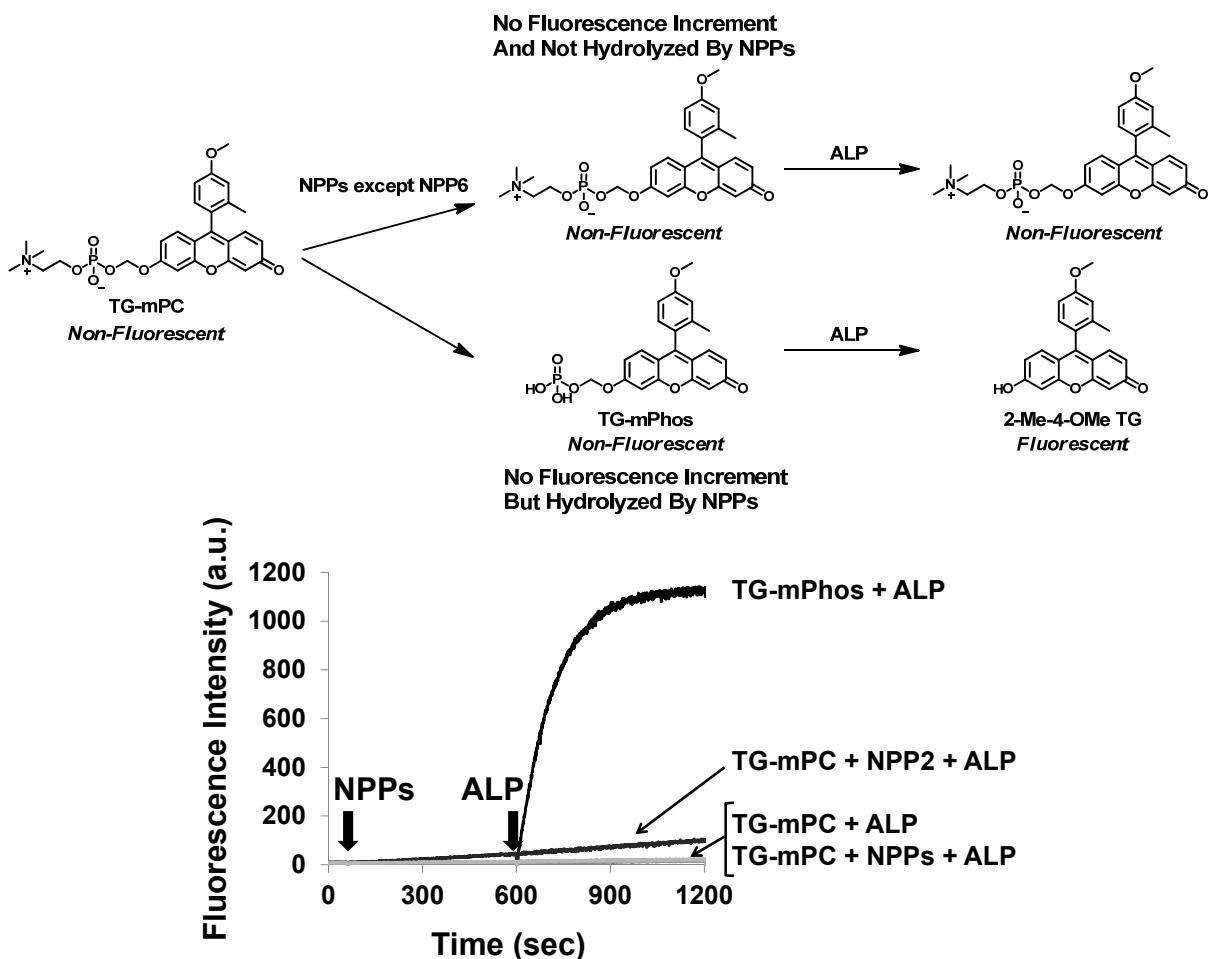


Figure S5. Reactivity of TG-mPC towards NPPs and alkaline phosphatase (ALP). Time courses of enzymatic reaction of TG-mPhos and TG-mPC with NPPs and ALP are shown. Reactions were performed in *NPP buffer* at 37 °C. Excitation and emission wavelengths were 490 nm and 510 nm, respectively. NPPs, NPP1, 2, 3, 4, 5, 7, and ALP was added at 1 min and 10 min, respectively. TG-mPhos reacted with ALP and showed a fluorescence increment. TG-mPC did not react with ALP and also showed no reactivity with NPP1, 2, 3, 4, 5, and 7, because no fluorescence increment was observed following the addition of ALP.

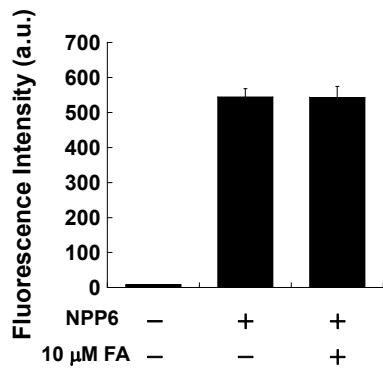


Figure S6. Fluorescence intensity of enzymatic reaction mixture of TG-mPC with NPP6 in the presence or absence of formaldehyde (FA, 10 μ M) is shown. Reactions were performed under the same conditions as in Figure S5. The results are mean \pm S.D. ($n = 3$).

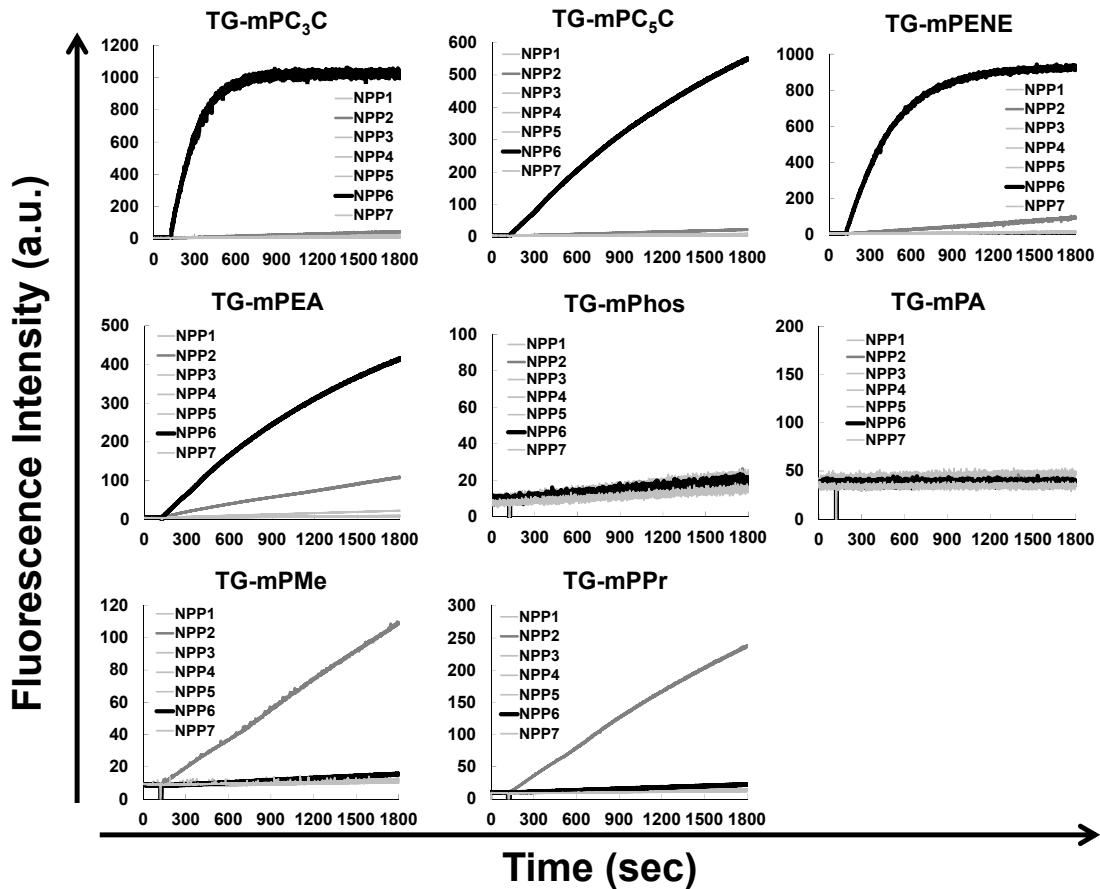


Figure S7. Reactivity of TG-mPC analogs towards NPPs. Time course of enzymatic reaction of TG-mPC₃C, TG-mPC₅C, TG-mPENE, TG-mPEA, TG-mPhos, TG-mPA, TG-mPMe, and TG-mPPr (1 μ M) with various recombinant NPPs. NPPs were added at 2 min. Bold black and brown lines indicate NPP6 and NPP2, respectively. Reactions were performed in *NPP buffer* at 37 °C. Excitation and emission wavelengths were 490 nm and 510 nm, respectively.

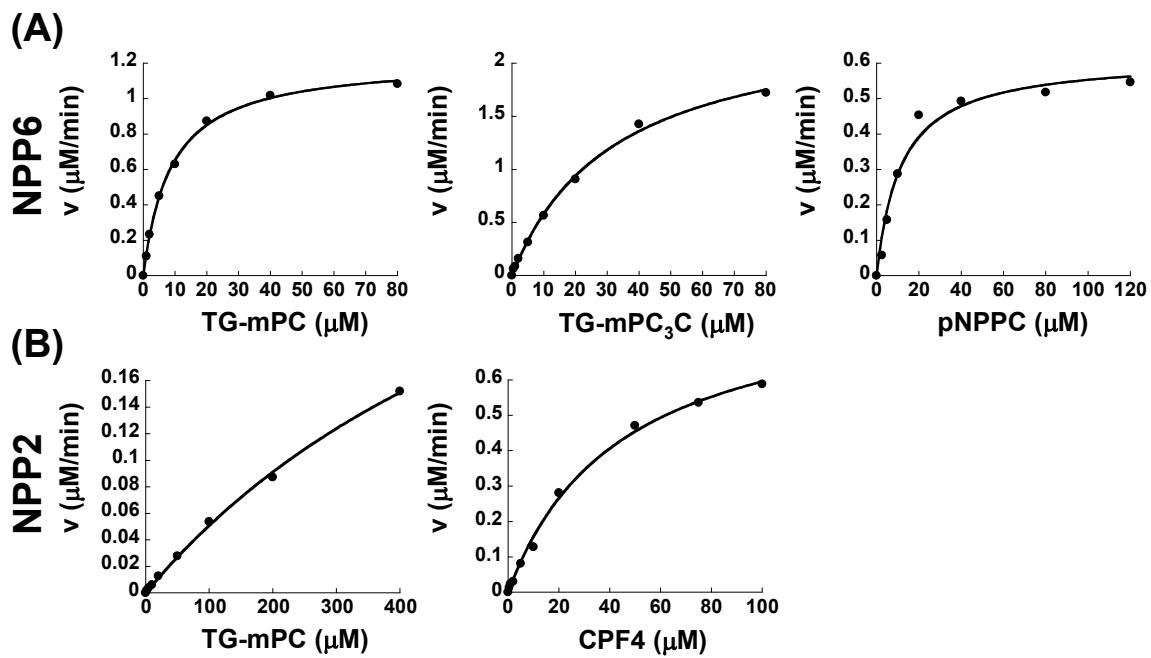


Figure S8. Kinetic study of various substrates with NPP2 and NPP6. (A) Michaelis-Menten plots and fitting curves of TG-mPC, TG-mPC₃C, and pNPPC with NPP6. (B) Michaelis-Menten plots and fitting curves of TG-mPC and CPF4 with NPP2. The initial velocity, v , was measured from the changes of fluorescence intensity (at 510 nm for TG-mPC and TG-mPC₃C, or at 380 nm for CPF4) or absorbance (at 405 nm for pNPPC).

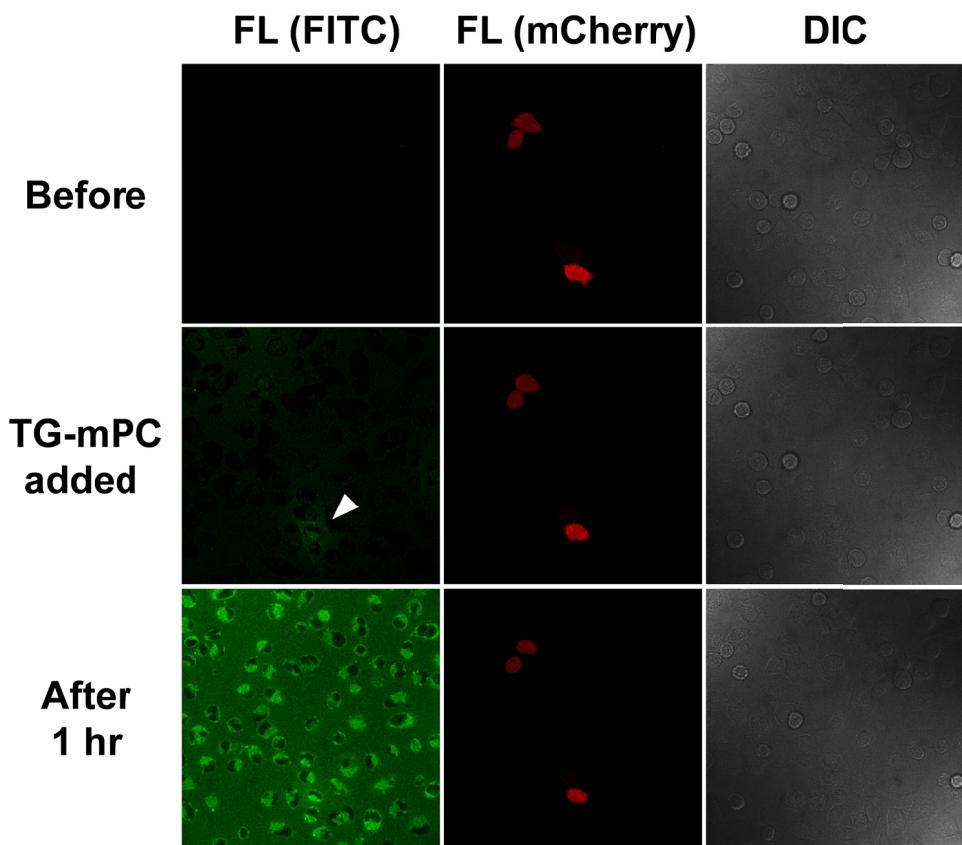


Figure S9. Fluorescence microscopic imaging of NPP6 activity in living cells with TG-mPC. HeLa cells expressing NPP6 and mCherry were incubated in HBSS at 37 °C, then TG-mPC (final 10 μ M) was added. Confocal fluorescence and DIC images were captured before and at 3 min and 1 hr after addition of TG-mPC. An arrowhead shows an NPP6 (+) cell and fluorescence increments of this cell and its surrounding media were observed immediately after addition of TG-mPC. After 1 hr, the whole fluorescence, not only that of cells but also that of the medium, was increased. This result indicates that 2-Me-4-OMe TG, which is generated by the enzymatic reaction of TG-mPC with NPP6, is not well-retained intracellularly. So, we think that further modifications of TG-mPC are still needed to obtain superior NPP6 imaging probes, e.g., with better intracellular retention of the fluorophore.^{SR1}

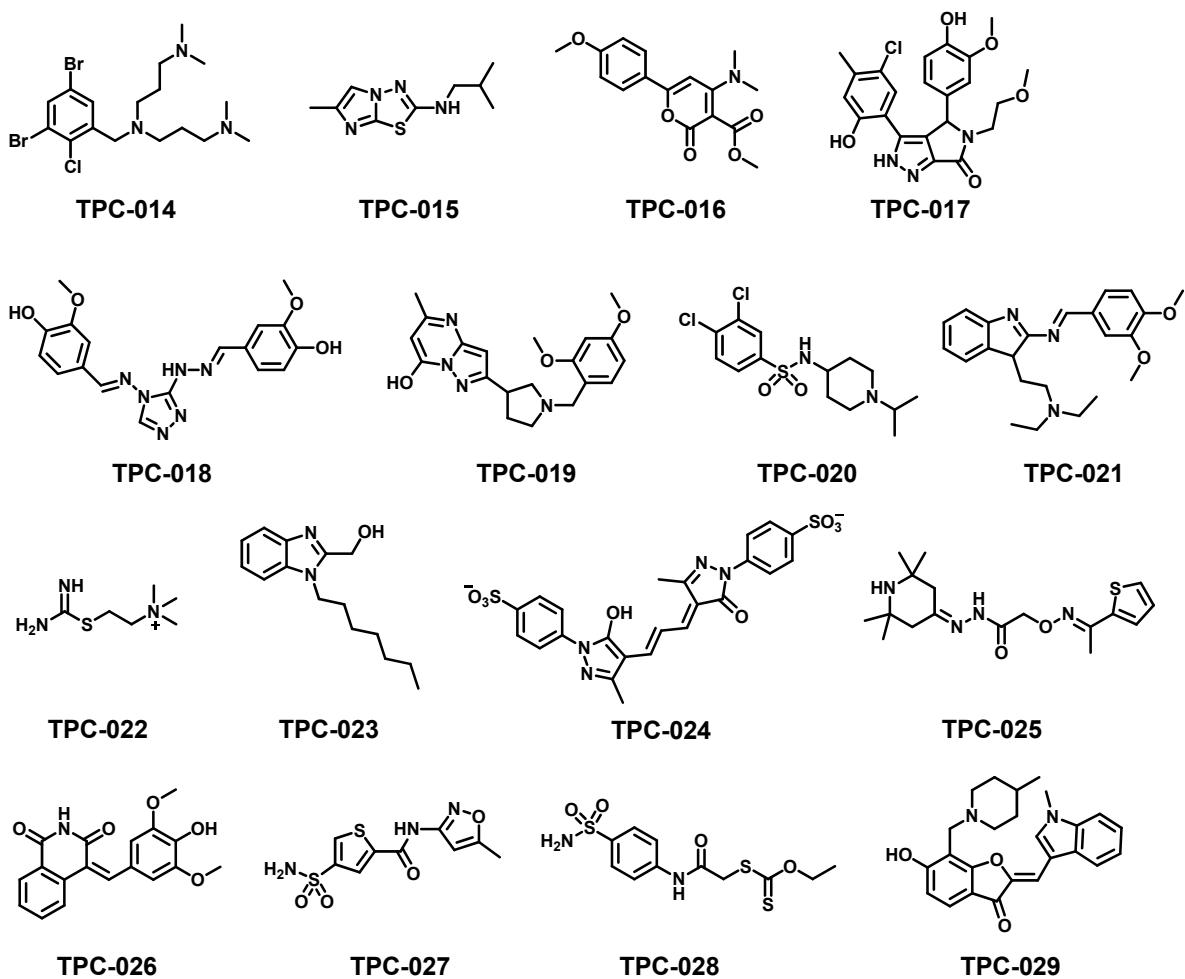


Figure S10. Structures of TPC-014 to TPC-029 shown in Table 3 (hit compounds in the NPP6 inhibitor screening).

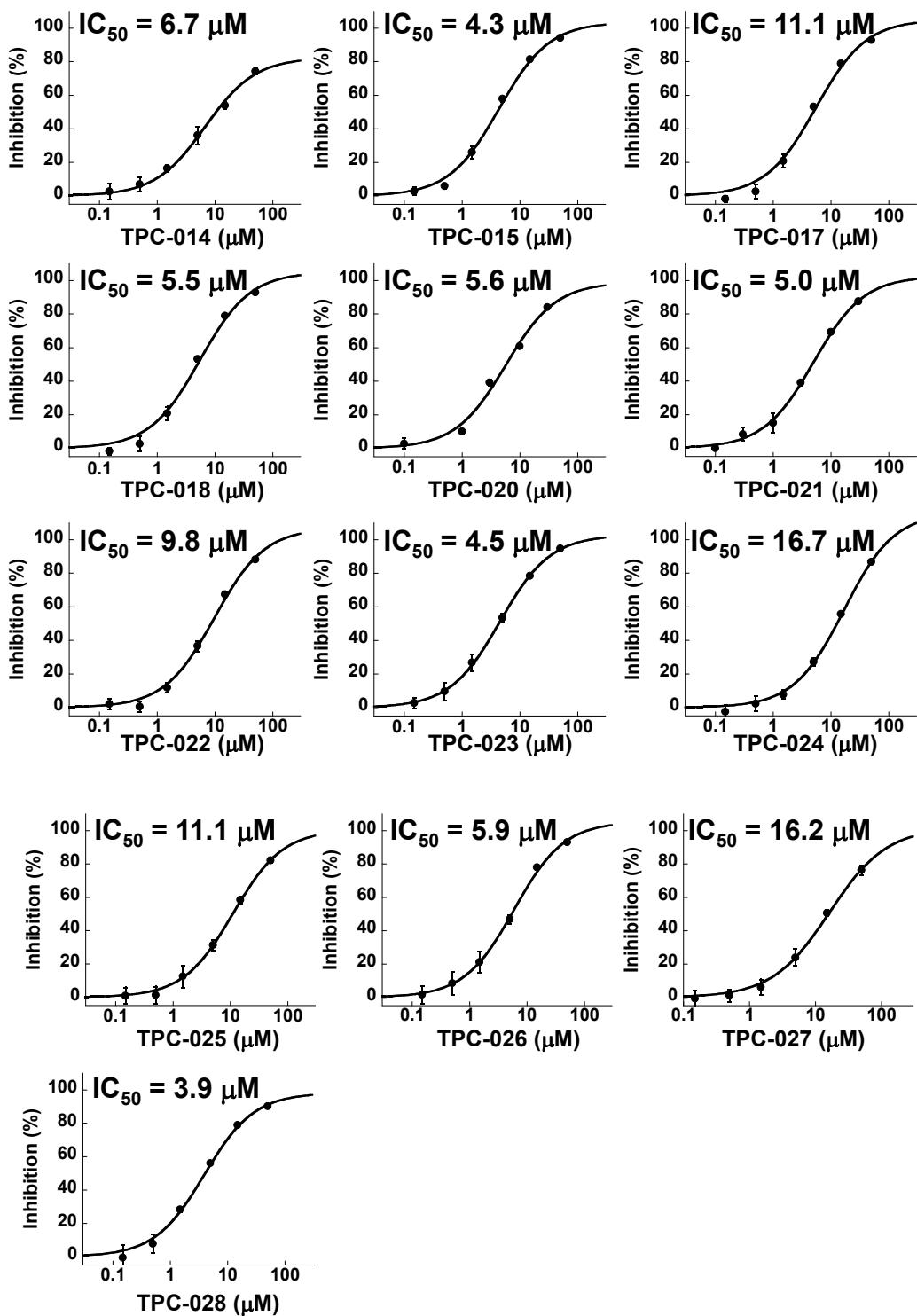


Figure S11. Inhibition plots and curves and IC_{50} values of hit compounds in the primary screening. The results are mean \pm S.D. ($n = 6$). IC_{50} values of TPC-016, 019 and 029 could not be determined.

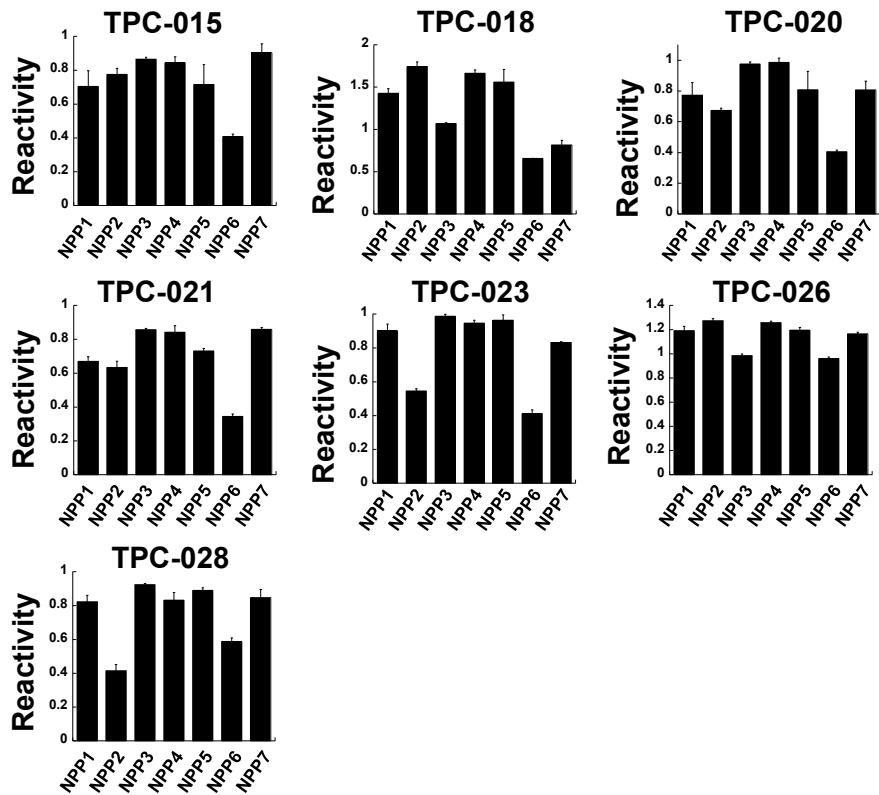


Figure S12. Selectivity test of hit compounds towards recombinant NPPs. The concentration of each compound was 50 μ M. The results are mean \pm S.D. ($n = 3$).

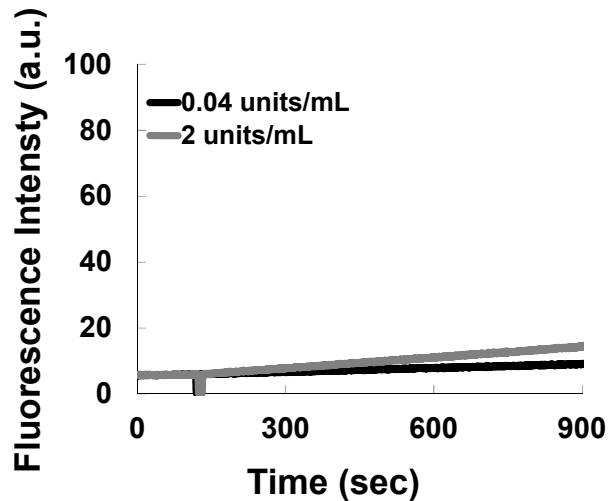


Figure S13. Cross-reactivity of TG-mPC with acetylcholinesterase (AchE). Time course of enzymatic reaction of TG-mPC (1 μ M) with AchE (0.04, 2 units/mL). AchE was added at 2 min. Reactions were performed in PBS (pH 7.4) at room temperature. Excitation and emission wavelengths were 490 nm and 510 nm, respectively.

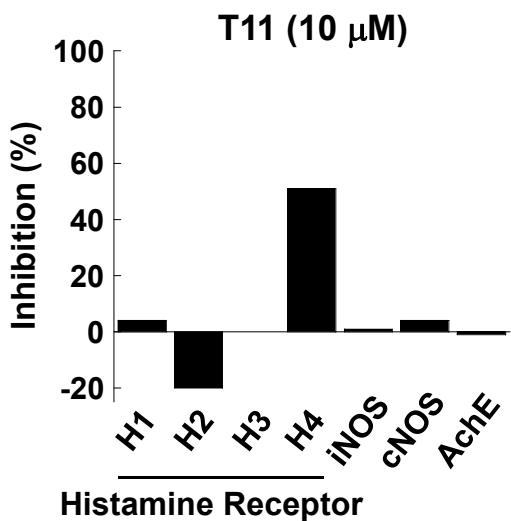


Figure S14. Off-target inhibition by T11. Inhibitory activity of T11 (10 μ M) towards histamine receptors 1-4 (H1-4), inducible NOS (iNOS), constitutive NOS (cNOS), and acetylcholinesterase (AchE). The results are means of duplicate determinations. This experiment was performed by Cerep (France).

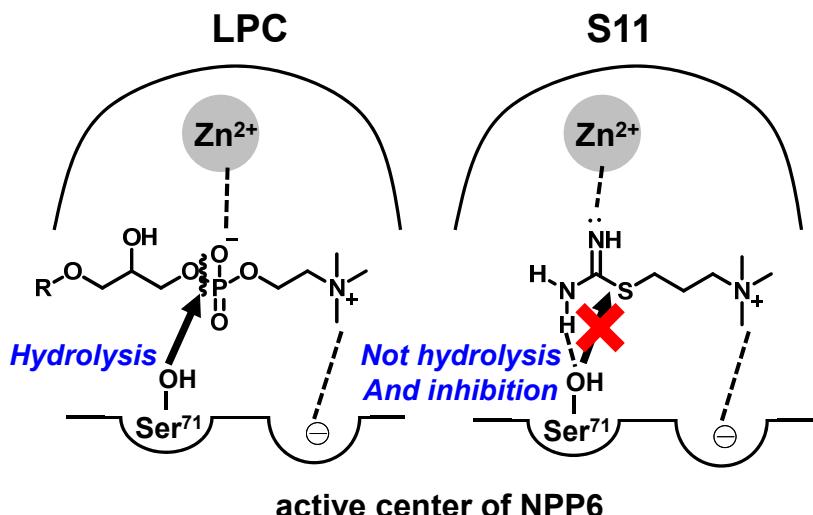


Figure S15. Proposed inhibition mechanism of T11 towards NPP6. The active center of NPP6 is shown. Ser⁷¹ is a catalytic center of NPP6 and Zn²⁺ is located close to Ser⁷¹. In the case of LPC, the choline moiety is probably trapped by negatively charged amino acid and the phosphate group chelates Zn²⁺. Then, the phosphate ester is hydrolyzed by Ser⁷¹, i.e. lysoPLC activity, and the phosphorylcholine is released. On the other hand, the choline moiety of T11 is trapped similarly and the thiourea moiety chelates Zn²⁺. However, the thiourea moiety cannot be hydrolyzed by lysoPLC activity, and as a result, T11 inhibits NPP6 activity. Because of the similarity between LPC and T11, T11 is a competitive inhibitor, as shown in Figure 8B.

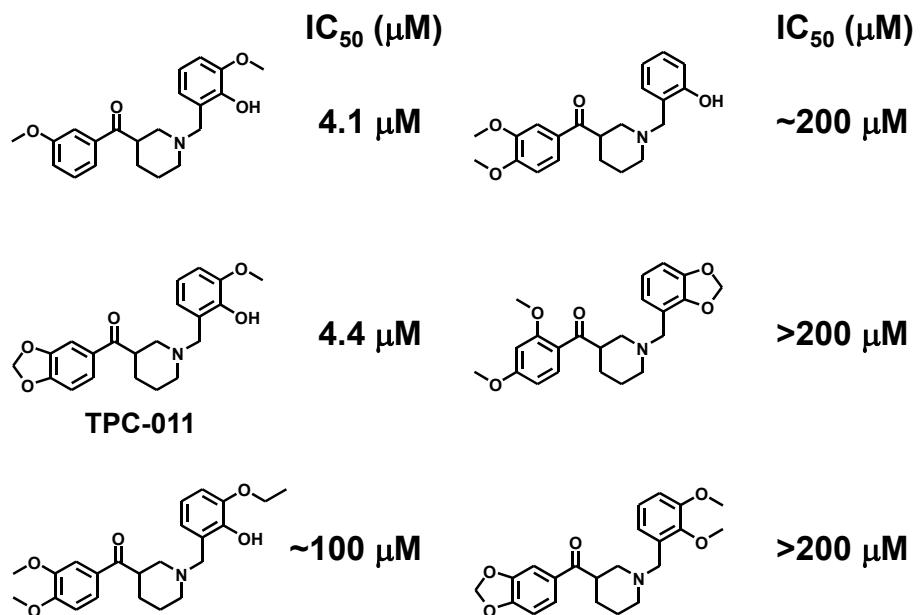


Figure S16. Structure-activity relationship (SAR) of TPC-011. Determined IC₅₀ values are shown.

Synthesis

synthesis of **2**

To a solution of dibenzyl phosphate **1** (556 mg, 2.0 mmol), NaHCO₃ (673 mg, 8.0 mmol), and *n*-Bu₄NHSO₄ (69 mg, 0.20 mmol) in distilled water (10 mL), CH₂Cl₂ was added at 0 °C. The reaction mixture was stirred for 10 min, then chloromethyl chlorosulfate (263 μL, 2.6 mmol) in CH₂Cl₂ (7 mL) was added dropwise. The whole was vigorously stirring at 0 °C for 30 min, then stirred at room temperature for 20 hr, washed with brine, dried over Na₂SO₄, and evaporated. The residue was purified by column chromatography on silica gel (eluent: CH₂Cl₂) to obtain **2** (435 mg, 1.33 mmol), yield 67%. ¹H-NMR (300 MHz, CDCl₃) δ 5.10 (d, 4H, *J* = 7.9 Hz), 5.62 (d, 2H, *J* = 15.6 Hz), 7.36 (m, 9H); ¹³C-NMR (75 MHz, CDCl₃) δ 69.8, 69.9, 73.4, 73.5, 128.0, 128.6, 128.7, 135.1, 135.2; HRMS (ESI⁺): calcd for [M+Na]⁺, 349.0372; found, 349.0345.

synthesis of **4**

To a solution of 2-Me-4-OMe TG (50 mg, 0.15 mmol) and Cs₂CO₃ (150 mg, 0.46 mmol) in DMF (5 mL), **2** (102 mg, 0.3 mmol) in DMF (2 mL) was added dropwise. The reaction mixture was stirred at room temperature for 13 hr under Ar, then evaporated. The residue was dissolved in CH₂Cl₂, washed with NaPi buffer (pH 9.2) and brine, dried over Na₂SO₄, and evaporated. The residue was purified by column chromatography on silica gel (eluent: CH₂Cl₂/MeOH = 97/3) to obtain **4** (40 mg, 0.064 mmol), yield 43%. ¹H-NMR (300 MHz, CD₃OD) δ 1.98 (s, 3H), 3.87 (s, 3H), 5.04 (d, 4H, *J* = 8.6 Hz), 5.80 (d, 2H, *J* = 14.1 Hz), 6.43 (d, 1H, *J* = 2.0 Hz), 6.58 (dd, 1H, *J* = 2.0, 9.6 Hz), 6.95-7.15 (m, 6H), 7.20 (d, 1H, *J* = 2.4 Hz), 7.22-7.28 (m, 10H); ¹³C-NMR (75 MHz, CDCl₃) δ 19.9, 55.3, 69.6, 69.7, 88.1, 88.2, 103.1, 105.9, 111.6, 113.5, 116.0, 116.3, 119.5, 124.3, 127.4, 127.8, 128.5, 128.6, 129.7, 130.3, 130.7, 135.1, 135.2, 137.8, 148.8, 153.9, 158.7, 160.2, 160.4, 185.8; MS (ESI⁺): 645 [M+Na]⁺

synthesis of **5** (TG-mPhos)

To a solution of **4** (40 mg, 0.064 mmol) in CH₂Cl₂/MeOH (3 mL/7 mL), 10% Pd/C was added. The reaction mixture was vigorously stirred at room temperature for 1 hr under H₂. Pd/C was removed by filtration, then triethylamine acetate (TEAA) buffer (5 mL) was added, and the mixture was evaporated to remove CH₂Cl₂ and MeOH. To the residual liquid, chloranil (excess) in CH₂Cl₂ (2 mL) was added. The reaction mixture was stirred at room temperature for 30 min. After removal of CH₂Cl₂, the residual liquid was purified by reversed-phase HPLC to obtain **TG-mPhos** (6.9 mg, 0.015 mmol), yield 24%. ¹H-NMR (300 MHz, CD₃OD): δ 1.30 (t, 9H, *J* = 7.3 Hz), 2.03 (s, 3H), 3.19 (q, 6H, *J* = 7.3 Hz), 3.90 (s, 3H), 5.70 (d, 2H, *J* = 10.2 Hz), 6.50 (d, 1H, *J* = 2.2 Hz), 6.63 (dd, 1H, *J* = 1.5, 9.5 Hz), 6.96-7.07 (m, 2H), 7.09-7.23 (m, 4H), 7.43 (d, 1H, *J* = 2.1 Hz); ¹³C-NMR (100 MHz, CD₃OD): δ 187.5, 164.5, 162.4, 161.6, 156.2, 155.3, 139.1, 133.0, 131.6, 131.3, 129.6, 125.4, 119.5, 117.1, 116.8, 116.5, 112.8, 105.5, 104.3, 88.9, 88.9, 55.9, 47.7, 20.0, 9.2; HRMS (ESI⁺): calcd for [M-H]⁻, 441.0739; found, 441.0716.

synthesis of **6** (TG-mPC)

To a solution of TG-mPhos (3.0 mg, 0.0068 mmol) and diisopropylethylamine (DIEA, 3.5 μL, 0.02 mmol) in DMF (1 mL), bromocholine bromide (3.3 mg, 0.014 mmol) was added. The reaction mixture was stirred at 50

°C for 24 hr, then evaporated. The residue was purified by reversed-phase HPLC to obtain **TG-mPC** (0.6 mg, 0.0011 mmol), yield 17%. ¹H-NMR (300 MHz, CD₃OD): δ 2.01 (s, 3H), 3.17 (s, 9H), 3.55-3.61 (m, 2H), 4.18-4.27 (m, 2H), 5.74 (d, 2H, *J* = 13.2 Hz), 6.49 (d, 1H, *J* = 1.5 Hz), 6.63 (dd, 1H, *J* = 1.5, 9.5 Hz), 6.97-7.08 (m, 2H), 7.09-7.25 (m, 4H), 7.43 (d, 1H, *J* = 2.2 Hz); ¹³C-NMR (100 MHz, CD₃OD): δ 187.4, 164.0, 162.4, 161.5, 156.2, 155.1, 139.1, 133.1, 131.6, 131.6, 129.8, 125.3, 119.8, 117.2, 117.1, 116.2, 112.9, 105.6, 104.2, 89.2, 89.1, 67.3, 60.6, 60.6, 55.9, 54.6, 54.6, 20.0; HRMS (ESI⁺): calcd for [M]⁺, 528.1787; found, 528.1832.

Synthesis of **TG-mPC₃C**

To a solution of **TG-mPhos** (4.0 mg, 0.0090 mmol) and DIEA (16 μL, 0.16 mmol) in DMF (1 mL), (3-bromopropyl)trimethylammonium bromide (10 mg, 0.038 mmol) was added. The reaction mixture was stirred at 40 °C for 48 hr, then evaporated. The residue was purified by reversed-phase HPLC to obtain **TG-mPC₃C** (3.6 mg, 0.0066 mmol), yield 74%. ¹H-NMR (300 MHz, CD₃OD): δ 2.03 (s, 3H), 2.05-2.10 (m, 2H), 3.10 (s, 9H), 3.39-3.47 (m, 2H), 3.87-3.95 (m, 5H), 5.70 (d, 2H, *J* = 12.5 Hz), 6.48 (d, 1H, *J* = 2.0 Hz), 6.61 (dd, 1H, *J* = 2.0, 9.5 Hz), 7.00 (dd, 1H, *J* = 2.6, 8.3 Hz), 7.05 (d, 1H, *J* = 2.6 Hz), 7.08-7.15 (m, 2H), 7.16-7.23 (m, 2H), 7.41 (d, 1H, *J* = 2.4 Hz); HRMS (ESI⁺): calcd for [M]⁺, 542.1944; found, 542.1965.

Synthesis of **TG-mPC₅C**

To a solution of **TG-mPhos** (3.1 mg, 0.0070 mmol) and DIEA (10 μL, 0.10 mmol) in DMF (1 mL), (5-bromopentyl)trimethylammonium bromide (10 mg, 0.035 mmol) was added. The reaction mixture was stirred at 40 °C for 48 hr, then evaporated. The residue was purified by reversed-phase HPLC to obtain **TG-mPC₅C** (1.2 mg, 0.0021 mmol), yield 30%. ¹H-NMR (300 MHz, CD₃OD): δ 1.40-1.52 (m, 2H), 1.58-1.69 (m, 2H), 1.75-1.87 (m, 2H), 2.03 (s, 3H), 3.10 (s, 9H), 3.80-3.90 (m, 5H), 5.69 (d, 2H, *J* = 12.3 Hz), 6.48 (d, 1H, *J* = 2.0 Hz), 6.62 (dd, 1H, *J* = 2.0, 9.7 Hz), 7.01 (dd, 1H), 7.05 (d, 1H), 7.08-7.15 (m, 2H), 7.16-7.22 (m, 2H), 7.41 (d, 1H, *J* = 2.2 Hz); HRMS (ESI⁺): calcd for [M]⁺, 570.2257; found, 570.2265.

Synthesis of **TG-mPENE**

To a solution of **TG-mPhos** (2.5 mg, 0.0056 mmol) and DIEA (10 μL, 0.10 mmol) in DMF (1 mL), 2-(diethylamino)ethyl bromide hydrobromide (10 mg, 0.038 mmol) was added. The reaction mixture was stirred at 45 °C for 48 hr, then evaporated. The residue was purified by reversed-phase HPLC to obtain **TG-mPENE** (1.2 mg, 0.0022 mmol), yield 40%. ¹H-NMR (300 MHz, CD₃OD): δ 1.28 (t, 6H, *J* = 7.1 Hz), 2.04 (s, 3H), 3.14-3.25 (m, 4H), 3.90 (s, 3H), 4.04-4.12 (m, 2H), 5.73 (d, 2H, *J* = 13.0 Hz), 6.48 (d, 1H, *J* = 2.0 Hz), 6.61 (dd, 1H, *J* = 2.0, 6.7 Hz), 7.00 (dd, 1H, *J* = 2.4, 7.9 Hz), 7.05 (d, 1H, *J* = 2.4 Hz), 7.08-7.15 (m, 2H), 7.16-7.23 (m, 2H), 7.41 (d, 1H, *J* = 2.4 Hz); HRMS (ESI⁺): calcd for [M+H]⁺, 542.1944; found, 542.1905.

Synthesis of **TG-PEA**

To a solution of **TG-mPhos** (3.0 mg, 0.0068 mmol) and DIEA (10 μL, 0.10 mmol) in DMF (1 mL), benzyl 2-bromoethylcarbamate (12 mg, 0.046 mmol) was added. The reaction mixture was stirred at 50 °C for 48 hr, then evaporated. The residue was purified by reversed-phase HPLC to obtain **TG-mPEACbz** (2.3 mg). To a

solution of TG-mPEAcbz (2.3 mg) in CH_2Cl_2 /MeOH (1 mL/1 mL), 10% Pd/C was added. The reaction mixture was stirred at room temperature for 3 hr under H_2 . Pd/C was removed by filtration, then TEAA buffer (4 mL) was added, and the mixture was evaporated to remove CH_2Cl_2 and MeOH. To the residual liquid, chloranil (excess) in CH_2Cl_2 (2 mL) was added. The reaction mixture was stirred at room temperature for 30 min. After removal of CH_2Cl_2 , the residual liquid was purified by reversed-phase HPLC to obtain **TG-mPEA** (0.8 mg, 0.0016 mmol), yield 24%. $^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 2.04 (s, 3H), 3.05-3.11 (m, 2H), 3.90 (s, 3H), 3.95-4.03 (m, 2H), 5.73 (d, 2H, J = 12.5 Hz), 6.48 (d, 1H, J = 2.2 Hz), 6.62 (dd, 1H, J = 2.0, 9.5 Hz), 6.98-7.24 (m, 6H), 7.41 (d, 1H, J = 3.0 Hz)

Synthesis of TG-mPMe

To a solution of TG-mPhos (4.6 mg, 0.010 mmol) and Cs_2CO_3 (8.1 mg, 0.025 mmol) in DMF (1 mL), methyl iodide (0.6 μL , 0.010 mmol) was added. The reaction mixture was stirred at room temperature for 48 hr, then evaporated. The residue was purified by reversed-phase HPLC to obtain **TG-mPMe** (0.7 mg, 0.0015 mmol), yield 15%. $^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 2.03 (s, 3H), 3.51 (d, 3H, J = 11.0 Hz), 3.89 (s, 3H), 5.69 (d, 2H, J = 12.1 Hz), 6.49 (d, 1H, J = 2.0 Hz), 6.61 (dd, 1H, J = 2.0, 9.7 Hz), 6.97-7.23 (m, 6H), 7.41 (d, 1H, J = 2.4 Hz); HRMS (ESI $^-$): calcd for $[\text{M}-\text{H}]^-$, 455.0896; found, 455.0914.

Synthesis of TG-mPPr· NEt_3 salt

To a solution of TG-mPhos (3.0 mg, 0.0070 mmol) and Cs_2CO_3 (8.1 mg, 0.025 mmol) in DMF (1 mL), propyl iodide (0.6 μL , 0.009 mmol) was added. The reaction mixture was stirred at room temperature for 24 hr, then evaporated. The residue was purified by reversed-phase HPLC to obtain **TG-mPPr · NEt₃ salt** (1.0 mg, 0.0021 mmol), yield 30%. $^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 0.86 (t, 3H, J = 5.4 Hz), 1.31 (t, 9H, J = 7.3 Hz), 1.49-1.62 (m, 2H), 2.04 (s, 3H), 3.20 (q, 6H, J = 7.3 Hz), 3.73-3.78 (m, 2H), 3.90 (s, 3H), 5.71 (d, 2H, J = 9.0 Hz), 6.54 (d, 1H, J = 1.6 Hz), 6.65 (dd, 1H, J = 1.6, 7.2 Hz), 7.01 (dd, 1H, J = 1.6, 6.3 Hz), 7.06 (d, 1H, J = 1.6 Hz), 7.10-7.26 (m, 4H), 7.44 (d, 1H, J = 1.8 Hz); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD): δ 186.9, 164.6, 162.5, 161.6, 156.5, 156.0, 139.1, 133.2, 131.7, 131.6, 129.4, 125.4, 119.6, 117.2, 117.1, 116.7, 112.9, 105.5, 104.2, 89.0, 88.9, 68.5, 68.5, 55.9, 47.9, 25.0, 24.9, 20.0, 10.6, 9.2; HRMS (ESI $^-$): calcd for $[\text{M}-\text{H}]^-$, 483.1209; found, 483.1194.

Synthesis of TG-mPA

To a solution of TG-mPhos (4.7 mg, 0.011 mmol) and Cs_2CO_3 (11.4 mg, 0.035 mmol) in DMF (1 mL), benzyl 2-bromoacetate (3.9 mg, 0.017 mmol) was added. The reaction mixture was stirred at room temperature for 48 hr, then evaporated. The residue was purified by reverse-phase HPLC to obtain TG-mPABz (4.0 mg). To a solution of TG-mPABz (4.0 mg) in CH_2Cl_2 /MeOH (1 mL/1 mL), 10% Pd/C was added. The reaction mixture was stirred at room temperature for 3 hr under H_2 . Pd/C was removed by filtration, then TEAA buffer (4 mL) was added, and the mixture was evaporated to remove CH_2Cl_2 and MeOH. To the residual liquid, chloranil (excess) in CH_2Cl_2 (2 mL) was added. The reaction mixture was stirred at room temperature for 30 min. After removal of CH_2Cl_2 , the residual liquid was purified by reversed-phase HPLC to obtain **TG-mPA** (3.0 mg, 0.0060 mmol), yield 55%. $^1\text{H-NMR}$ (300 MHz, CD_3OD): δ 2.03 (s, 3H), 3.89 (s, 3H), 4.25 (d, 2H, J = 8.8 Hz), 5.75 (d, 2H, J = 12.1 Hz), 6.49 (d, 1H, J = 2.0 Hz), 6.61 (dd, 1H, J = 2.0, 9.7 Hz), 6.99 (dd, 1H, J = 2.4, 8.3

Hz), 7.04 (d, 1H, *J* = 2.4 Hz), 7.10-7.15 (m, 2H), 7.16-7.22 (m, 2H), 7.41 (d, 1H, *J* = 2.2 Hz); HRMS (ESI⁺): calcd for [M]⁺, 499.0794; found, 499.0774.

Synthesis of T11

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1 mmol) in dry EtOH (10 mL), thiourea (76 mg, 1 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T11** (246 mg, 0.85 mmol), yield 85%. ¹H-NMR (300 MHz, DMSO): δ 2.01-2.16 (m, 2H), 3.11 (s, 9H), 3.22 (t, 2H, *J* = 7.3 Hz), 3.38-3.47 (m, 2H); HRMS (ESI⁺): calcd for [M]⁺, 176.1221; found, 176.1179.

Synthesis of T12

To a solution of 3-bromopropylamine hydrobromide (436 mg, 2.0 mmol), and DIEA (700 μ L, 4.0 mmol) in dry EtOH (10 mL), Boc₂O (650 mg, 3.0 mmol) was added. The reaction mixture was stirred at 60 °C for 1 hr and at room temperature for 3 hr, then evaporated. The residue was purified by column chromatography on silica gel to obtain 3-(Boc-amino)propyl bromide (430 mg, 1.8 mmol), yield 90%. To a solution of 3-(Boc-amino)propyl bromide (237 mg, 1 mmol) in dry EtOH (10 mL), thiourea (76 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was dissolved in CH₂Cl₂ (5 mL) and cooled to 0 °C, then TFA (5 mL) was added. The reaction mixture was stirred for 1 hr at 0 °C, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T12** (62 mg, 0.25 mmol), yield 25%. ¹H-NMR (300 MHz, CD₃OD): δ 2.04-2.16 (m, 2H), 3.10 (t, 2H, *J* = 7.2 Hz), 3.31 (t, 2H, *J* = 7.3 Hz)

Synthesis of T13

To a solution of (5-bromopropyl)trimethylammonium bromide (286 mg, 1.0 mmol) in dry EtOH (10 mL), thiourea (76 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T13** (240 mg, 0.72 mmol), yield 72%. ¹H-NMR (300 MHz, CD₃OD): δ 1.47-1.60 (m, 2H), 1.87-1.93 (m, 4H), 3.18 (s, 9H), 3.21 (t, 2H, *J* = 7.3 Hz), 3.34-3.43 (m, 2H); HRMS (ESI⁺): calcd for [M]⁺, 176.1221; found, 176.1179.

Synthesis of T14

To a solution of (3-bromopropyl)triethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), thiourea (76 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T14** (252 mg, 0.76 mmol), yield 76%. ¹H-NMR (300 MHz, CD₃OD): δ 1.33 (t, 9H, *J* = 7.3 Hz), 2.06-2.21 (m, 2H), 3.33-3.43 (m, 8H); HRMS (ESI⁺): calcd for [M]⁺, 218.1691; found, 218.1661.

Synthesis of T15

To a solution of 1,3-dibromopropane (402 mg, 2.0 mmol) in Ac₂O (10 mL), pyridine (155 μ L, 1.9 mmol) was added. The reaction mixture was stirred at room temperature for 20 hr, then evaporated. The residue was purified by reversed-phase HPLC to obtain (3-bromopropyl)pyrimidium trifluoroacetate (392 mg, 1.4 mmol),

yield 70%. $^1\text{H-NMR}$ (300 MHz, DMSO): δ 2.46-2.58 (m, 2H), 3.59 (t, 2H, J = 6.6 Hz), 4.73 (t, 2H, J = 7.3 Hz), 8.18 (t, 2H, J = 6.2 Hz), 8.63 (t, 1H, J = 8.1 Hz), 9.12 (d, 2H, J = 6.2 Hz); LRMS (ESI $^+$): 200 [M] $^+$. To a solution of (3-bromopropyl)pyrimidium bromide (279 mg, 1.0 mmol) in dry EtOH (10 mL), thiourea was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T15** (113 mg, 0.37 mmol), yield 37%. $^1\text{H-NMR}$ (300 MHz, CD₃OD): δ 2.41-2.53 (m, 2H), 3.32 (t, 2H, J = 7.3 Hz), 4.82 (t, 2H, J = 8.1 Hz), 8.16 (t, 2H, J = 6.2 Hz), 8.64 (t, 1H, J = 8.1 Hz), 9.09 (d, 2H, J = 6.2 Hz); HRMS (ESI $^+$): calcd for [M] $^+$, 196.0908; found, 196.0866.

Synthesis of **T16**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), 1-phenylthiourea (152 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T16** (21 mg, 0.057 mmol), yield 5.7%. $^1\text{H-NMR}$ (300 MHz, CD₃OD): δ 2.22-2.34 (m, 2H), 3.22 (s, 9H), 3.34 (t, 2H, J = 7.3 Hz), 3.44-3.52 (m, 2H), 7.34-7.39 (m, 2H), 7.46-7.58 (m, 3H); HRMS (ESI $^+$): calcd for [M] $^+$, 252.1534; found, 252.1520.

Synthesis of **T17**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), 1-(3,5-bis(trifluoromethyl)phenyl)thiourea (288 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T17** (50 mg, 0.10 mmol), yield 10%. $^1\text{H-NMR}$ (300 MHz, CD₃OD): δ 2.23-2.35 (m, 2H), 3.22 (s, 9H), 3.33 (t, 2H, J = 7.3 Hz), 3.44-3.52 (m, 2H), 7.97 (s, 2H), 8.02 (s, 1H); HRMS (ESI $^+$): calcd for [M] $^+$, 388.1282; found, 388.1286.

Synthesis of **T18**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), 1-(naphthalen-1-yl)thiourea (202 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T18** (232 mg, 0.56 mmol), yield 56%. $^1\text{H-NMR}$ (300 MHz, CD₃OD): δ 2.24-2.49 (m, 2H), 2.23 (s, 9H), 3.38-3.71 (m, 4H), 7.60-7.72 (m, 4H), 7.87-7.94 (m, 1H), 8.03-8.11 (m, 2H); HRMS (ESI $^+$): calcd for [M] $^+$, 302.1691; found, 302.1679.

Synthesis of **T19**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), 1-methylthiourea (90 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T19** (145 mg, 0.48 mmol), yield 48%. $^1\text{H-NMR}$ (300 MHz, CD₃OD): δ 2.17-2.30 (m, 2H), 3.04 (s, 3H), 3.20 (s, 9H), 3.28 (t, 2H, J = 7.3 Hz), 3.50-3.58 (m, 2H); HRMS (ESI $^+$): calcd for [M] $^+$, 190.1378; found, 190.1345.

Synthesis of **T20**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL),

1,3-dimethylthiourea (104 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T20** (73 mg, 0.23 mmol), yield 23%. ¹H-NMR (300 MHz, CD₃OD): δ 2.20-2.33 (m, 2H), 3.05 (s, 3H), 3.11 (s, 3H), 3.21 (s, 9H), 3.23 (t, 2H, *J* = 7.3 Hz), 3.54-3.63 (m, 2H); HRMS (ESI⁺): calcd for [M]⁺, 204.1534; found, 204.1500.

Synthesis of **T21**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), 1,3-diethylthiourea (132 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T21** (165 mg, 0.48 mmol), yield 48%. ¹H-NMR (300 MHz, CD₃OD): δ 1.30 (t, 6H, *J* = 7.3 Hz), 2.20-2.32 (m, 2H), 3.25 (s, 9H), 3.35 (t, 2H, *J* = 7.3 Hz), 3.44-3.64 (m, 6H); HRMS (ESI⁺): calcd for [M]⁺, 232.1847; found, 232.1821.

Synthesis of **T22**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), 1,3-dibutylthiourea (132 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T22** (124 mg, 0.31 mmol), yield 31%. ¹H-NMR (300 MHz, CD₃OD): δ 0.99 (t, 6H, *J* = 7.2 Hz), 1.34-1.50 (m, 4H), 1.60-1.75 (m, 4H), 2.18-2.33 (m, 2H), 3.19 (s, 9H), 3.34 (t, 2H, *J* = 7.4 Hz), 3.38-3.62 (m, 6H); HRMS (ESI⁺): calcd for [M]⁺, 288.2473; found, 288.2458.

Synthesis of **T23**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), imidazolidine-2-thione (102 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T23** (237 mg, 0.75 mmol), yield 75%. ¹H-NMR (300 MHz, CD₃OD): δ 2.23-2.36 (m, 2H), 3.25 (s, 9H), 3.27-3.34 (m, 2H), 3.52-3.59 (m, 2H), 4.00 (s, 4H); HRMS (ESI⁺): calcd for [M]⁺, 202.1378; found, 202.1343.

Synthesis of **T24**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), tetrahydropyrimidine-2-thione (116 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T24** (215 mg, 0.65 mmol), yield 65%. ¹H-NMR (300 MHz, CD₃OD): δ 2.00-2.12 (m, 2H), 2.17-2.32 (m, 2H), 3.21 (s, 9H), 3.24-3.29 (m, 2H), 3.50-3.61 (m, 6H); HRMS (ESI⁺): calcd for [M]⁺, 216.1534; found, 216.1520.

Synthesis of **T25**

To a solution of (3-bromopropyl)trimethylammonium bromide (261 mg, 1.0 mmol) in dry EtOH (10 mL), 1,1,3-trimethylthiourea (118 mg, 1.0 mmol) was added. The reaction mixture was stirred at 85 °C for 12 hr under Ar, then evaporated. The residue was purified by reversed-phase HPLC to obtain **T25** (178 mg, 0.54 mmol), yield 54%. ¹H-NMR (300 MHz, CD₃OD): δ 2.18-2.31 (m, 2H), 3.17 (t, 2H, *J* = 8.1 Hz), 3.19 (s, 9H), 3.28 (s, 3H), 3.38 (brs, 6H), 3.48-3.56 (m, 2H); HRMS (ESI⁺): calcd for [M]⁺, 218.1691; found, 218.1666.

Supporting Reference

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