Electronic Supplementary Material (ESI) for Molecular BioSystems

Supplementary Information

CDC25A-inhibitory RE Derivatives Bind at Pocket Adjacent to the Catalytic Site

Ayako Tsuchiya,[a] Miwako Asanuma,[b] Go Hirai,[a] Kana Oonuma,[a] Muhammad Muddassar,[a] Eri Nishizawa,[a] Yusuke Koyama,[a] Yuko Otani,[a] Kam Y. J. Zhang,[a] and Mikiko Sodeoka[*a,b]

[a]RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan,
[b]Sodeoka Live Cell Chemistry Project, ERATO, Japan Science and Technology Agency

Organic Synthesis

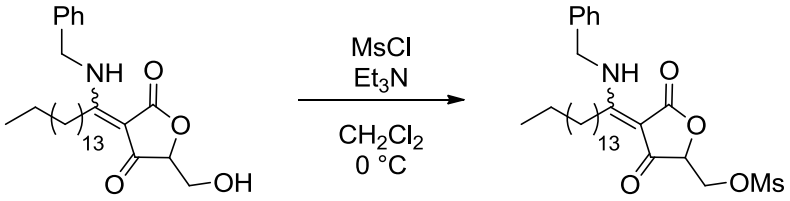
Experimental procedure & spectral data

***General*:** NMR spectra were recorded on a JEOL-AL300, JEOL JNM-AL400, JEOL-ECS400 or JEOL JNM-ECP500 spectrometer, operating at 300 MHz, 400 MHz or 500 MHz for ^{1}H-NMR, and 100 MHz or 125 MHz for ^{13}C-NMR. Chemical shifts were reported in the scale relative to $CHCl_3$ as an internal reference. MALDI-TOF/MS was taken on a BrukerDaltonics Microflex with matrix (α-cyano-4-hydroxycinnamic acid) dimer and angiotensin I as internal standards. HRMS was taken on a BrukerDaltonics micrOTOF II. Reactions were carried out under a nitrogen atmosphere, unless noted otherwise. Tetrahydrofuran (THF), diethyl ether, dichloromethane (CH_2Cl_2), *N,N*-dimethylformamide (DMF), and methanol were purified with a GlassContour Solvent Dispensing System.

Synthesis of RE Derivatives

Synthesis of compounds **1**~**3** has been already reported.[1]

Synthesis of compound **4**



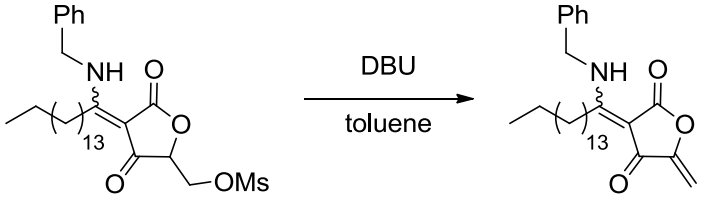
1 **4**

Triethylamine (30.5 μL, 219 μmol) and methanesulfonyl chloride (17.0 mg, 219 μmol) were added to a solution of **1** (50.0 mg, 109 μmol) in tetrahydrofuran (500 μL) at 0 °C. The reaction mixture was stirred for 10 min at 0 °C, then diluted with ethyl acetate, and phosphate buffer (pH = 7.0) was added at the same temperature. The organic layer was separated and washed with water. The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was purified by GPC (eluent: chloroform) to give **4** (tautomeric mixture: 54:46 in $CDCl_3$, 57.7 mg, 99%) as a white amorphous solid.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.88 (brt, J = 6.4 Hz, 3H), 1.17-1.64 (m, 26H), 2.86-3.08 (m, 2H), 3.03 (s, 1.38H), 3.04 (s, 1.62H), 4.46-4.52 (m, 1H), 4.59-4.69 (m, 4H), 7.26-7.28 (m, 2H), 7.37-7.43 (m, 3H), 10.4 (br, 0.46H), 11.2 (br, 0.54H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 14.17, 22.73, 27.63, 27.66, 27.73, 28.05, 29.21, 29.38, 29.44, 29.46, 29.60, 29.66, 29.69, 29.71, 29.72, 29.83, 31.94, 37.59, 37.77, 47.06, 47.34, 67.69, 67.89, 77.61, 79.57, 89.96, 91.48, 127.11, 128.62, 128.64, 129.28, 129.29, 134.42, 134.50, 170.10, 174.53, 175.00, 175.17, 189.73, 194.39; MALDI-TOF/MS (*m*/*z*): $[M+Na]^+$ calcd for $C_{29}H_{45}NNaO_6S$, 558.29; found, 558.40; HRMS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{29}H_{46}NO_6S$, 536.3046; found, 536.3028.

[1] G. Hirai, A. Tsuchiya, Y. Koyama, Y. Otani, K. Oonuma, K. Dodo, S. Simizu, H. Osada and M. Sodeoka, *ChemMedChem*, 2011, **6**, 617-622.

Synthesis of compound **5**

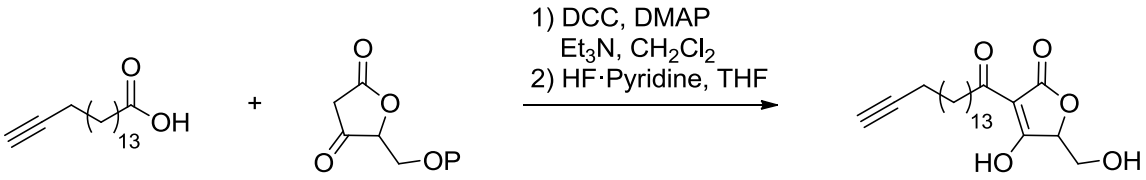


4 **5**

To a solution of **4** (15.0 mg, 28.0 μmol) in toluene (1.0 mL) was added DBU (20.9 μL, 140 μmol) at 0 °C. The reaction mixture was heated at 110 °C for 10 seconds, then diluted with ethyl acetate. Water was added, and the resulting mixture was extracted five times with ethyl acetate. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give a crude product, which was purified by preparative thin-layer chromatography (eluent: hexane/ethyl acetate = 3/2) to give **5** (tautomeric mixture: 74:26 in $CDCl_3$, 7.0 mg, 57%) as a colorless syrup.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.88 (brt, *J* = 6.4 Hz, 3H), 1.13-1.59 (m, 26H), 2.97 (m, 1.48H), 3.06 (m, 0.52H), 4.65-4.68 (m, 2H), 4.93 (brd, *J* = 2.0 Hz, 1H), 5.22 (brd, *J* = 2.0 Hz, 0.74H), 5.30 (br, 0.26H), 7.26-7.29 (m, 2H), 7.34-7.43 (m, 3H), 10.1 (br, 0.26H), 11.5 (br, 0.74H); ^{13}C-NMR (77.5 MHz, $CDCl_3$) 14.08, 22.65, 22.66, 27.58, 27.62, 27.81, 27.93, 29.14, 29.31, 29.35, 29.40, 29.51, 29.58, 29.61, 29.64, 29.65, 29.72, 29.75, 29.80, 31.88, 31.91, 46.98, 47.25, 90.34, 91.50, 91.65, 92.01, 127.10, 128.61, 129.31, 134.67, 134.74, 151.64, 152.86, 167.10, 171.96, 174.79, 175.12, 180.07, 184.46; HRMS-ESI (*m*/*z*): $[M+Na]^+$ calcd for $C_{28}H_{41}NNaO_3$, 462.2984; found, 462.2982.

Synthesis of compound **12**

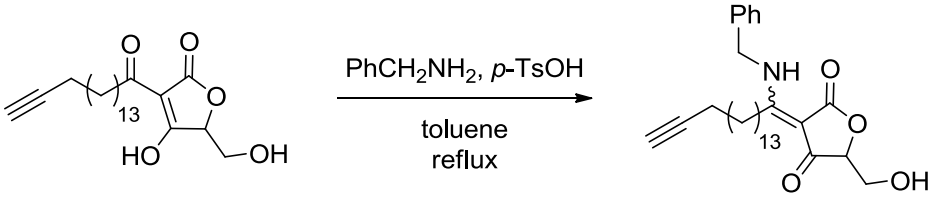


10 **11** **12**

Compounds **10** (119 mg, 448 μmol) and **11**[1] (150 mg, 407 μmol) were dissolved in dichloromethane (2.0 mL), and then Et_3N (62.4 μL, 448 μmol), DCC (109 mg, 529 μmol), and DMAP (14.9 mg, 122 μmol) were added to the solution at 0 °C. The reaction mixture was stirred for 9 h at room temperature, then diluted with chloroform. A 1 N aqueous solution of HCl was added at 0 °C, and the resulting mixture was extracted with chloroform twice. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give a yellow oil, which was used in the next step without further purification. To a solution of the crude product in THF (2.0 mL) was added HF·pyridine complex (0.5 mL). The reaction mixture was stirred at 50 °C for 5 h, and further HF·pyridine complex (0.5 mL) was added. Stirring was continued for 5 h at 50 °C, and then the reaction mixture was diluted with chloroform. A 1 N aqueous solution of HCl was added at 0 °C. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give a crude product, which was purified by MPLC on a diol packed column (eluent: $CHCl_3$ / MeOH = 39 / 1) to give **12** (98.6 mg, 64%) as a white amorphous solid containing some impurities.

HRMS-ESI (*m*/*z*): $[M+Na]^+$ calcd for $C_{22}H_{34}NaO_5$, 401.2304; found, 401.2295.

Synthesis of compound **6**

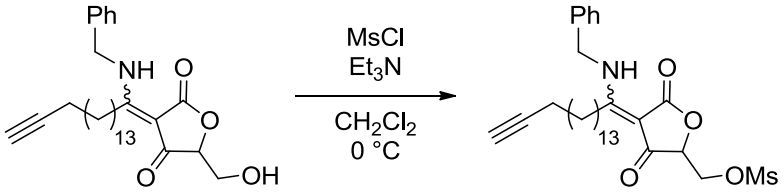


12 **6**

p-Toluenesulfonic acid (10 mg) and benzylamine (28.1 mg, 262 μmol) were added to a solution of **12** (66.1 mg, 52.8 μmol) in toluene (1.0 mL) at room temperature. The reaction mixture was heated at reflux for 30 min, then further benzylamine (28.1 mg, 262 μmol) was added, and the resulting mixture was stirred for 3.0 h at reflux temperature. After cooling to room temperature, the mixture was purified by preparative thin-layer chromatography on silica gel (eluent: chloroform/methanol = 10/1) to give **6** (tautomeric mixture: 58:42 in $CDCl_3$, 57.4 mg, 70%) as a white amorphous solid.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.24-1.62 (m, 24H), 1.93 (t, *J* = 2.7 Hz, 1H), 2.16 (dt, *J* = 2.7 Hz, 7.1 Hz, 2H), 2.31 (t, *J* = 7.1 Hz, 0.42H), 2.87-3.09 (m, 2H), 3.90-3.99 (m, 2H), 4.51 (t, *J* = 3.9 Hz, 0.42H), 4.52 (t, *J* = 3.9 Hz, 0.58H), 4.63 (brs, 0.84H), 4.65 (brs, 1.16H), 7.24-7.28 (m, 2H), 7.33-7.42 (m, 3H), 10.4 (br, 0.42H), 11.3 (br, 0.58H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 18.49, 24.84, 27.64, 27.69, 27.77, 28.00, 28.55, 28.83, 29.17, 29.25, 29.27, 29.33, 29.48, 29.51, 29.56, 29.63, 29.65, 29.67, 29.88, 33.94, 46.90, 47.21, 61.50, 61.64, 68.03, 80.86, 82.67, 84.75, 90.49, 91.94, 126.95, 127.02, 128.38, 128.41, 129.10, 129.11, 134.56, 134.69, 171.08, 174.10, 174.61, 175.69, 192.94, 197.28; HRMS-ESI (*m*/*z*): $[M+Na]^+$ calcd for $C_{29}H_{41}NO_4Na$, 490.2933; found, 490.2930.

Synthesis of compound **7**

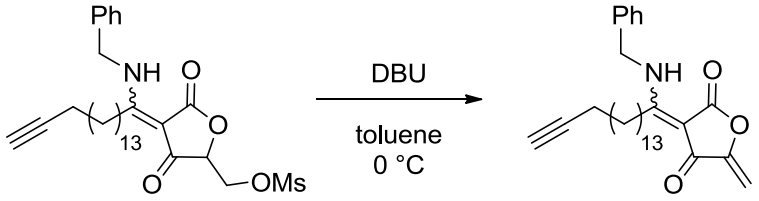


6 **7**

Triethylamine (14.1 μL, 101 μmol) and methanesulfonyl chloride (7.8 μL, 101 μmol) were added to a solution of **6** (23.7 mg, 50.7 μmol) in dichloromethane (1.0 mL) at 0 °C. The reaction mixture was stirred for 15 min at 0 °C, then diluted with dichloromethane, and phosphate buffer (pH = 7.0). Water were added, and the resulting mixture was extracted three times with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to give a crude product, which was purified by preparative thin-layer chromatography (eluent: hexane/ethyl acetate = 2/3) to give **7** (tautomeric mixture: 55:45 in $CDCl_3$, 25.9 mg, 93%) as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.19-1.62 (m, 24H), 1.93 (t, *J* = 2.7 Hz, 1H), 2.17 (brdt, *J* = 2.7 Hz, 7.1 Hz, 2H), 2.89-3.08 (m, 2H), 3.035 (s, 1.35H), 3.037 (s, 1.65H), 4.46-4.50 (m, 1H), 4.61 (brdd, *J* = 2.4 Hz, 11.4 Hz, 1H), 4.63-4.68 (m, 3H), 7.25-7.28 (m, 2H), 7.36-7.43 (m, 3H), 10.4 (br, 0.45H), 11.2 (br, 0.55H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 18.42, 27.60, 27.64, 27.70, 28.03, 28.50, 28.77, 29.12, 29.19, 29.42, 29.43, 29.51, 29.57, 29.60, 29.80, 37.57, 37.74, 47.05, 47.33, 67.68, 67.87, 68.02, 77.60, 79.54, 84.77, 89.94, 91.45, 127.10, 128.60, 128.63, 129.26, 129.28, 134.41, 134.48, 170.11, 174.52, 175.00, 175.15, 189.73. 194.37; HRMS-ESI (*m*/*z*): $[M+Na]^+$ calcd for $C_{30}H_{43}NNaO_6S$, 568.2709; found, 568.2713.

Synthesis of compound **8**

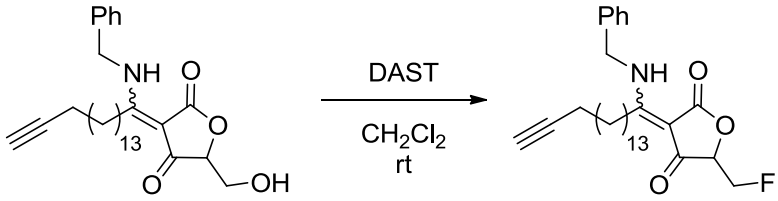


7 **8**

To a solution of **7** (25.9 mg, 47.5 μmol) in toluene (200 μL) was added DBU (10.6 μL, 71.1 μmol) at 0 °C. The reaction mixture was stirred for 30 min at 0 °C, then diluted with ethyl acetate. Phosphate buffer (pH = 7.0) and water were added, and the resulting mixture was extracted three times with ethyl acetate. The organic layers were combined, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give a crude product, which was purified by preparative thin-layer chromatography (eluent: hexane/ethyl acetate = 2/3) to give **8** (tautomeric mixture: 70:30 in $CDCl_3$, 12.8 mg, 61%) as a colorless syrup.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.24-1.61 (m, 24H), 1.93 (t, *J* = 2.8 Hz, 1H), 2.17 (dt, *J* = 2.8 Hz, 7.4 Hz, 2H), 2.97 (m, 1.40H), 3.05 (m, 0.60H), 4.65-4.68 (m, 2H), 4.92 (m, 1H), 5.21 (d, *J* = 2.3 Hz, 0.70H), 5.29 (d, *J* = 2.3 Hz, 0.30H), 7.26-7.28 (m, 2H), 7.34-7.42 (m, 3H), 10.1 (br, 0.30H), 11.5 (br, 0.70H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 18.36, 27.60, 27.63, 27.82, 27.93, 28.46, 28.72, 29.07, 29.14, 29.16, 29.35, 29.41, 29.46, 29.51, 29.55, 29.72, 29.80, 47.00, 47.26, 68.00, 84.78, 90.36, 91.53, 91.67, 92.04, 127.12, 128.63, 128.64, 129.33, 134.66, 134.74, 151.65, 152.86, 167.11, 171.98, 174.79, 175.13, 180.09, 184.48; HRMS-ESI (*m*/*z*): $[M+Na]^+$ calcd for $C_{29}H_{39}NNaO_3$, 472.2828; found, 472.2813.

Synthesis of compound **9**

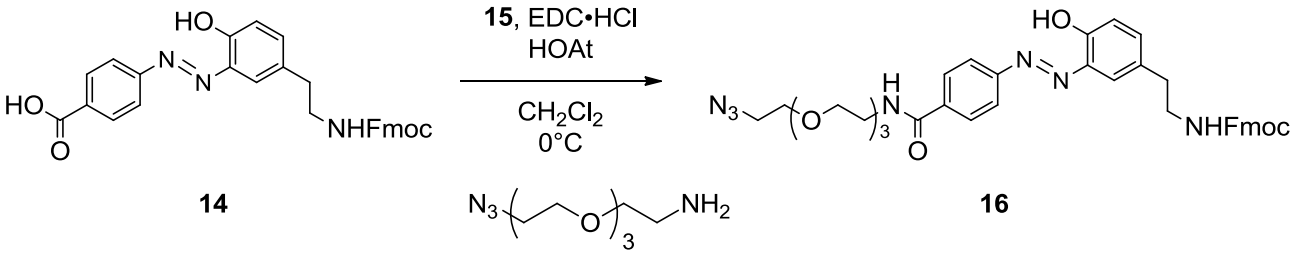


6 **9**

To a solution of **6** (11.7 mg, 25.0 μmol) in dichloromethane (250 μL) was added DAST (33.0 μL, 250 μmol) at -20 °C. The reaction mixture was heated to room temperature and stirred for 15 min at that temperature, then diluted with dichloromethane. A saturated aqueous solution of $NaHCO_3$ and water were added, and the resulting mixture was extracted three times with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give a crude product, which was purified by preparative thin-layer chromatography (eluent: hexane/ethyl acetate = 2/3) to give **9** (tautomeric mixture: 52:48 in $CDCl_3$, 6.6 mg, 56%) as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.19-1.61 (m, 24H), 1.94 (t, *J* = 2.3 Hz, 1H), 2.18 (dt, *J* = 2.8 Hz, 7.4 Hz, 2H), 2.92-3.07 (m, 2H), 4.57-4.83 (m, 5H), 7.26-7.29 (m, 2H), 7.35-7.44 (m, 3H), 10.4 (br, 0.48H), 11.3 (br, 0.52H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 18.36, 27.54, 27.57, 27.67, 27.90, 28.45, 28.73, 29.07, 29.14, 29.17, 29.37, 29.40, 29.47, 29.52, 29.56, 29.67, 29.77, 46.90, 47.20, 68.01, 79.39 (d, *J* = 20.2 Hz), 80.85 (d, *J* = 176.3 Hz), 81.06 (d, *J* = 175.3 Hz), 81.41 (d, *J* = 19.3 Hz), 90.31, 91.84, 127.12, 127.17, 128.61, 128.65, 129.31, 129.34, 134.63, 134.74, 170.65, 174.45, 175.11, 175.58, 190.48 (d, *J* = 5.8 Hz), 195.18 (d, *J* = 4.8 Hz); ^{19}F-NMR (378 MHz, $CDCl_3$) δ -233.57 (t, *J* = 46.2 Hz), -233.94 (t, *J* = 46.2 Hz); HRMS-ESI (*m*/*z*): $[M+Na]^+$ calcd for $C_{29}H_{40}FNNaO_3$, 492.2890; found, 492.2885.

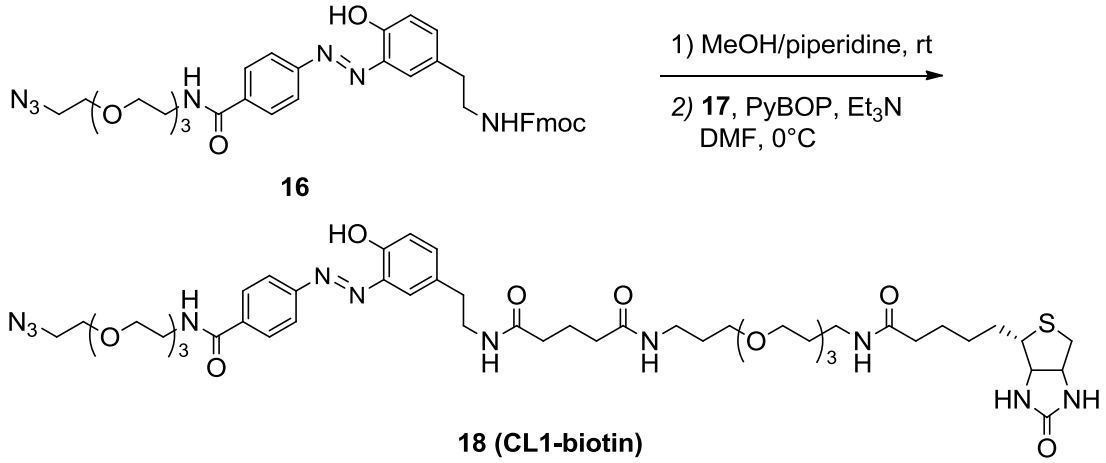
Synthesis of compound **16**



Compounds **14** (250 mg, 493 μmol) and **15** (108 μL, 542 μmol) were dissolved in CH_2Cl_2 (2.7 mL), and then EDC·HCl (104 mg, 542 μmol) and HOAt (80.6 mg, 592 μmol) were added to the solution at 0 °C. The reaction mixture was stirred for 1 h at 0 °C, then diluted with dichloromethane. A saturated aqueous solution of $NaHCO_3$ and water were added, and the resulting mixture was extracted three times with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give a crude product, which was purified by silica gel column chromatography (eluent: chloroform / methanol = 90/1) and by GPC to give **13** (107 mg, 28%) as an orange oil.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 2.88 (t, *J* = 6.4 Hz, 2H), 3.34 (brt, *J* = 6.4 Hz, 2H), 3.34 (t, *J* = 4.9 Hz, 2H), 3.51 (brq, *J* = 6.4 Hz, 2H), 3.61-3.71 (m, 14H), 4.21 (brt, *J* = 6.8 Hz, 1H), 4.41 (brd, *J* = 6.8 Hz, 2H), 4.86 (br, 1H), 6.99-7.02 (m, 2H), 7.20 (brd, *J* = 8.3 Hz, 1H), 7.29 (t, *J* = 7.3 Hz, 2H), 7.38 (t, *J* = 7.3 Hz, 2H), 7.56 (d, *J* = 7.3 Hz, 2H), 7.74 (d, *J* = 7.3 Hz, 2H), 7.81 (brs, 1H), 7.89 (d, *J* = 8.3 Hz, 2H), 7.96 (d, *J* = 8.3 Hz, 2H), 12.65 (s, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 34.86, 39.81, 42.07, 47.12, 50.47, 66.41, 69.58, 69.85, 70.09, 70.37, 70.48, 77.21, 118.33, 119.83, 122.06, 124.88, 126.88, 127.52, 128.21, 130.43, 133.04, 134.43, 136.34, 137.13, 141.14, 143.76, 151.25, 152.01, 153.26, 166.40; HRMS-ESI (*m*/*z*): $[M+Na]^+$ calcd for $C_{38}H_{41}N_7NaO_7$, 730.2965; found, 730.2935.

Synthesis of compound **18**

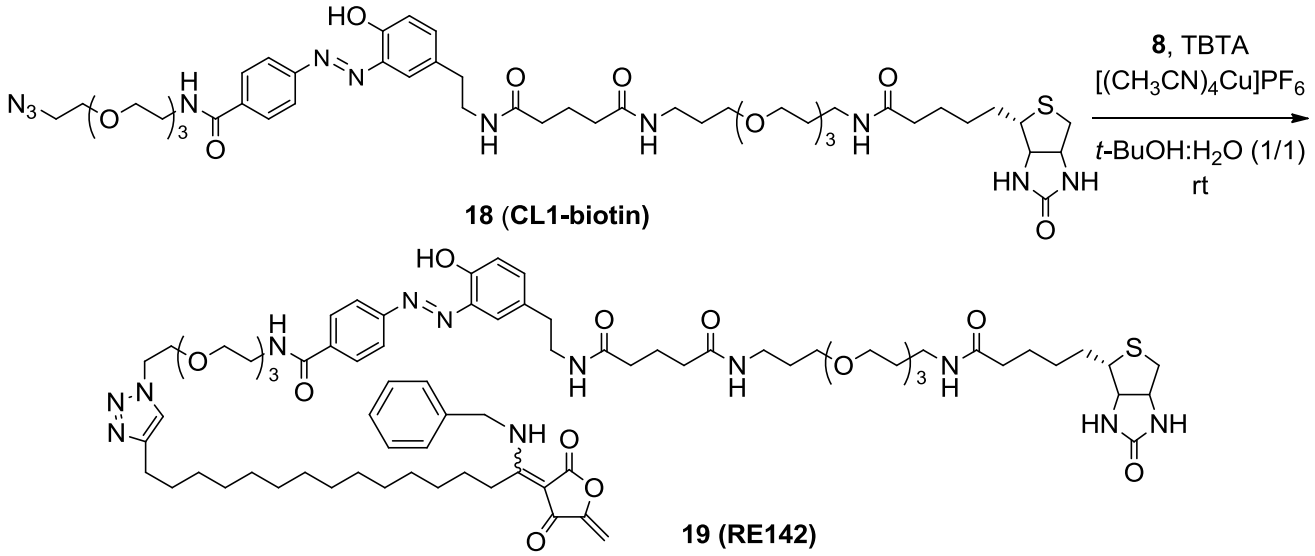


To a solution of **16** (69.5 mg, 98.2 μmol) in methanol (1.0 mL) was added piperidine (50 μL) at 0 °C. The reaction mixture was stirred for 6.0 h at room temperature, then diluted with methanol and hexane. The methanol layer was separated, washed with hexane, diluted with chloroform (saturated with NH_3), and concentrated *in vacuo* to give a crude product.

The crude product (50.8 mg) was taken up in dimethylformamide (1.0 mL), and then **17** (60.6 mg, 108 μmol), PyBOP (56.2 mg, 108 μmol), and triethylamine (27.4 μL, 196 μmol) were added to the solution at 0 °C. The reaction mixture was stirred for 12 h at 0 °C, then concentrated by azeotropic evaporation with toluene *in vacuo*. The residue was purified by silica gel column chromatography (eluent: chloroform / methanol = 50/1) and by GPC (eluent: chloroform) to give **18 (CL1-biotin)** (76.4 mg, 76%, 2 steps) as an orange oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.33-1.41 (m, 2H), 1.56-1.74 (m, 8H), 1.88 (quintet, *J* = 7.4 Hz, 2H), 2.13 (t, *J* = 7.6 Hz, 2H), 2.16 (t, *J* = 6.8 Hz, 2H), 2.21 (t, *J* = 7.4 Hz, 2H), 2.67-2.71 (m, 1H), 2.81-2.85 (m, 3H), 3.07 (m, 1H), 3.25-3.29 (m, 4H), 3.32 (t, *J* = 4.9 Hz, 2H), 3.48-3.67 (m, 28H), 4.24 (m, 1H), 4.43 (m, 1H), 5.80 (br, 1H), 6.41 (br, 1H), 6.78-6.85 (m, 3H), 6.92 (d, *J* = 8.6 Hz, 1H), 7.19 (dd, *J* = 8.6 Hz, 2.2 Hz, 1H), 7.27 (br, 1H), 7.75 (d, *J* = 2.2 Hz, 1H), 7.85 (d, *J* = 8.8 Hz, 2H), 7.94 (d, *J* = 8.8 Hz, 2H), 12.54 (s, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 22.19, 25.71, 28.11, 28.28, 29.04, 34.57, 35.40, 35.58, 35.93, 37.54, 39.97, 40.52, 40.68, 45.70, 46.68, 50.62, 55.64, 60.15, 61.80, 69.56, 69.66, 69.87, 69.91, 69.97, 70.14, 70.30, 70.32, 70.36, 70.43, 70.51, 70.56, 118.12, 121.96, 128.22, 130.64, 132.67, 134.36, 136.29, 137.02, 151.03, 151.90, 163.67, 166.32, 172.62, 172.79, 173.03; MALDI-TOF/MS (*m*/*z*): $[M+Na]^+$ calcd for $C_{48}H_{73}N_{11}NaO_{12}S$, 1050.51; found 1050.67; HRMS-ESI (*m*/*z*): $[M-H]^-$ calcd for $C_{48}H_{72}N_{11}O_{12}S$, 1026.5083; found, 1026.5157.

Synthesis of compound **19**



Compounds **18 (CL1-biotin)** (7.3 mg, 7.10 μmol) and **8** (7.0 mg, 1.56 μmol) were dissolved in *t*-BuOH (173 μL) and H_2O (173 μL), and then $[Cu(CH_3CN)_4]PF_6$ (2.6 mg, 7.10 μmol) and TBTA (3.8 mg, 7.10 μmol) were added to the solution at room temperature. The reaction mixture was stirred for 2.5 h, then further $[Cu(CH_3CN)_4]PF_6$ (2.6 mg, 7.10 μmol) and TBTA (3.8 mg, 7.10 μmol) were added. The mixture was stirred for 4.5 h at room temperature and diluted with chloroform. A saturated aqueous solution of NH_4Cl and water were added, and the resulting mixture was extracted three times with chloroform. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give a crude product, which was purified by preparative thin-layer chromatography (eluent: chloroform/methanol = 4/1) to give **19 (RE142)** (tautomeric mixture: 74:26 in $CDCl_3$, 6.2 mg, 59%) as an orange oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.17-1.37 (m, 18H), 1.37-1.50 (m, 4H), 1.53-1.70 (m, 6H), 1.72-1.80 (m, 6H), 1.92 (m, 2H), 2.17 (t, *J* = 7.1 hz, 2H), 2.19 (t, *J* = 7.3 Hz, 2H), 2.24 (t, *J* = 7.3 Hz, 2H), 2.58-2.73 (m, 3H), 2.84-2.90 (m, 2H), 2.96 (m, 2H), 3.03-3.14 (m, 1H), 3.27-3.34 (m, 4H), 3.48-3.69 (m, 26H), 3.83 (t, *J* = 5.1 Hz, 2H), 4.27-4.30 (m, 1H), 4.45 (t, *J* = 5.1 Hz, 2H), 4.45-4.50 (m, 1H), 4.65-4.68 (m, 2H), 4.92 (brd, *J* = 2.2 Hz, 1H), 5.21 (d, *J* = 2.2 Hz, 0.74H), 5.28 (d, *J* = 2.2 Hz, 0.26H), 5.32 (br, 1H), 6.04 (br, 1H), 6.6 (brm, 1H), 6.77 (brm, 2H), 6.96 (d, *J* = 8.5 Hz, 1H), 7.23 (dd, *J* = 8.5 Hz, 1.7 Hz, 1H), 7.26-7.41 (m, 6H), 7.78 (brd, *J* = 1.7 Hz, 1H), 7.88 (d, *J* = 8.6 Hz, 2H), 7.99 (d, *J* = 8.6 Hz, 2H), 10.1 (br, 0.26H), 11.5 (br, 0.74H), 12.59 (brs, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$, clear signals were observed) δ 22.08, 25.57, 25.65, 27.60, 27.63, 27.84, 27.95, 28.03, 28.10, 28.91, 28.98, 29.14, 29.16, 29.28, 29.30, 29.35, 29.37, 29.41, 29.49, 29.51, 29.54, 29.56, 29.58, 29.67, 29.73, 29.81, 34.54, 35.36, 35.56, 35.83, 37.56, 37.79, 39.91, 39.95, 40.52, 40.59, 47.02, 47.28, 50.01, 55.50, 60.07, 61.79, 69.58, 69.71, 69.72, 69.95, 69.96, 70.00, 70.03, 70.17, 70.19, 70.40, 70.42, 70.47, 70.49, 91.54, 91.65, 92.05, 118.36, 121.65,

122.17, 127.13, 128.45, 128.64, 129.33, 130.86, 133.01, 134.63, 134.66, 136.55, 137.27, 148.28, 151.33, 151.64, 152.22, 152.86, 163.45, 166.58, 167.15, 171.98, 172.80, 173.00, 173.03, 174.91, 175.13, 184.45; MALDI-TOF/MS (*m*/*z*): $[M+H]^+$ calcd for $C_{77}H_{113}N_{12}O_{15}S_1$, 1477.82; found 1477.69; HRMS-ESI (*m*/*z*): $[M+2Na]^{2+}$ calcd for $C_{77}H_{112}N_{12}Na_2O_{15}S_1$, 761.3943; found, 761.3955.

Biochemistry

Enzyme Preparations and Inhibition Assay

Inhibitory activities of RE derivatives for CDC25s were evaluated according to our previous report.[1] Preparation of GST-CDC25A was also described in that report.[1] Preparation of CAC25A catalytic domain (catCDC25A) were conducted similarly, except for the final elution method from Glutathione Sepharose 4B (GE Healthcare Japan Corporation, Tokyo, Japan). Instead of GSH, catCDC25A was eluted with PreScission Protease (GE Healthcare Japan Corporation) according to the manufacturer's protocol.

Sequence of catCDC25A

MH^+(average) = 24205
Sequence:
GPLGSLIGDFSKGYLFHTVAGKHQDLKYISPEIMASVLNGKFANLIKEFVIIDCRYPYEYEGGHIK
GAVNLHMEEEVEDFLLKKPIVPTDGKRVIVVFHCEFSSERGPRMCRYVRERDRLGNEYPKLHYP
ELYVLKGGYKEFFMKCQSYCEPPSYRPMHHEDFKEDLKKFRTKSRTWAGEKSKREMYSRLKK
LEFPGRLERPHRN

Covalent bond formation of compound 5 with catCDC25A (Figure S1)

Purified recombinant human CDC25A catalytic domain (catCDC25A) in buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5) was prepared as described in "Enzyme preparation". Compound **5** (1 mM) or DMSO (as a vehicle control) was incubated with 1.7 μM catCDC25A and 2 mM DTT for 1 h at 30 °C. The reaction mixture was purified on C4 ZipTips to remove free and/or loosely bound **5**. The reaction products were eluted with acetonitrile:0.1% TFAaq. (3:7, 5:5 and 7:3, v/v). One μL of the eluant was mixed with 1 μL of matrix (a saturated solution of sinnapinic acid in acetonitrile:0.1% TFAaq. (1:1, v/v)), and the resulting mixture was put onto a MALDI plate. A Microflex instrument (Bruker Daltonics) was used for the MALDI-TOF/MS analysis, and data were collected in the linear mode.

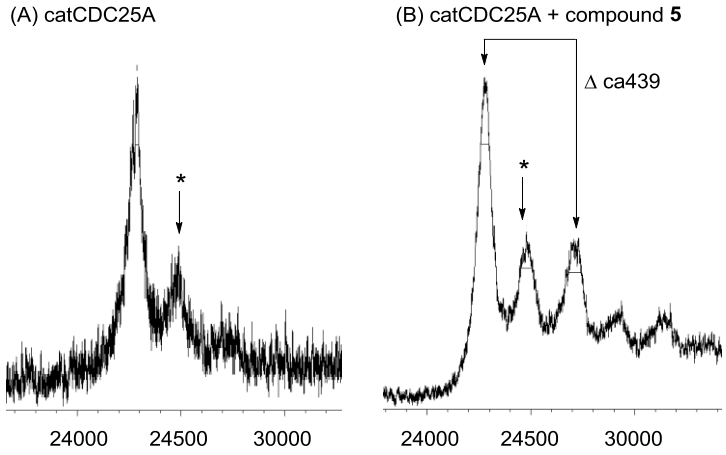


Figure S1. MALDI-TOF/MS analysis of catCDC25A after treatment with compound 5. Peaks with asterisks are probably due to matrix adducts of catCDC25A.

General method for western blotting

Samples were resolved on 10% SDS-PAGE gels, and bands were transferred to PVDF membranes (Millipore, Billerica, MA) and probed with specific antibodies. Detection was done with the ECL Western blotting detection system (Millipore) and a LAS-4000 (Fuji Film, Tokyo, Japan). Primary antibodies included anti-CDC25A (sc-7389; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-GST (sc-138; Santa Cruz Biotechnology, Inc.) and anti-Biotin (B-7653; Sigma, Saint Louis, MO). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG (sc-2004; Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG (170-6516; Bio-Rad, Hercules, CA). Streptavidin-HRP was purchased from Calbiochem (OR03L).

Covalent bond formation of RE derivatives with GST-CDC25A without purification by gel filtration (Figure S2)

GST or GST-CDC25A (300 nM) was incubated with RE derivatives or DMSO (as a vehicle control) for 30 min at 30 °C in Tris-based buffer (100 mM Tris-HCl, 40 mM NaCl, 2 mM DTT, 20% glycerol, pH 8.2). Conjugation with biotin-azide probe was conducted by Cu(I)-mediated azide-alkyne Huisgen reaction (click chemistry) for 1 h at room temperature. Click chemistry conditions were 50 μM biotin-N_3 (**13**), 1 mM TCEP (added as a solution in water), 40 μM TBTA (added as a solution in DMSO/t-BuOH (4:1, v/v)), and 1 mM $CuSO_4$ (added as a solution in water). The reaction products were denatured with 6 M urea, reduced with 10 mM DTT for 15 min at 65 °C, and then alkylated with 40 mM iodoacetamide for 30 min at 37 °C in the dark. The reaction was stopped by adding 6 x SDS sample buffer (0.35 M Tris-HCl, 0.6 M DTT, 10% SDS, 30% glycerol, 0.05% bromophenol blue, pH 6.8), and the resulting mixture was boiled for 5 min. Samples were analyzed by western blotting with streptavidin-HRP or anti-GST antibody.

Under the conditions used for the Huisgen reaction and/or sample preparation for SDS-PAGE in the presence of remaining free RE derivative, non-specific binding of biotin-N_3 to GST was observed (Figure S2).

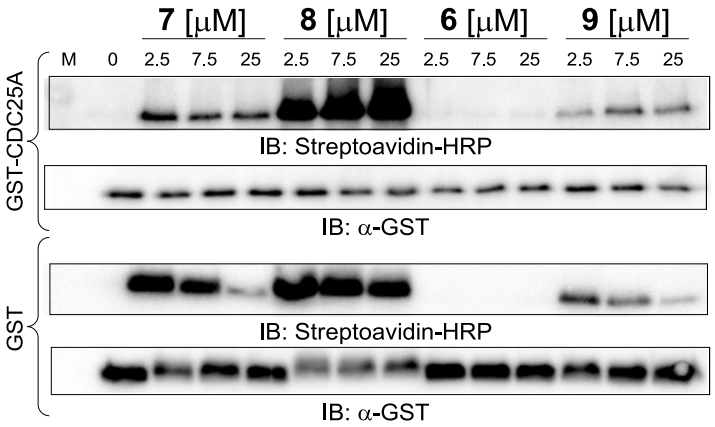


Figure S2. Western blotting analysis of GST and GST-CDC25A after treatment with various concentrations of the alkyne-modified RE derivatives **6**~**9** followed by conjugation with biotin-N_3 (**13**), using Cu(I) mediated click chemistry

Covalent bond formation of RE derivatives with GST-CDC25A with purification by gel filtration (Figure 4B)

GST or GST-CDC25A (300 nM) was incubated with RE derivatives (25 μM) or DMSO (as a vehicle control) for 30 min at 30 °C in Tris-based buffer (100 mM Tris-HCl, 40 mM NaCl, 2 mM DTT, 20% glycerol, pH 8.2). Excess free RE derivatives were removed on a MicroBiospin column 6 (Bio-Rad, Hercules, CA). GST or GST-CDC25A was eluted with Tris-based buffer (100 μL x 4 times). Conjugation with biotin-azide probe for each fraction was conducted by Cu(I)-mediated azide-alkyne Huisgen reaction (click chemistry) for 1 h at room temperature. Click chemistry conditions were 50 μM biotin-N_3 (**13**), 1 mM TCEP (added as a solution in water), 40 μM TBTA (added as a solution in DMSO/t-BuOH (4:1, v/v)), and 1 mM $CuSO_4$ (added as a solution in water). The reaction was stopped by adding 6 x SDS sample buffer (0.35 M Tris-HCl, 0.6 M DTT, 10% SDS, 30% glycerol, 0.05% bromophenol blue, pH 6.8), and the resulting mixture was boiled for 5 min. Samples were analyzed by western blotting with anti-biotin or anti-CDC25A antibody.

Analysis of covalent bond formation of RE142 with catCDC25A by MALDI-TOF/MS (Figure 5A and 5B)

Purified recombinant human CDC25A catalytic domain (catCDC25A) in buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5) was prepared as described in "Enzyme preparation". RE142 (**19**, DMSO solution, final 6.6 mM) or DMSO (as a vehicle control) was incubated with 33 μM catCDC25A and 10 mM DTT for 1 h at 30 °C. The reaction mixture was further purified on C4 ZipTips to remove free and/or loosely bound RE142 (**19**). The reaction products were eluted with acetonitrile:0.1% TFAaq. (3:7, 5:5 and 7:3, v/v). One μl of the eluant was mixed with 1 μL of matrix (a saturated solution of sinnapinic acid in acetonitrile:0.1% TFAaq. (1:1, v/v)), and the resulting mixture was put on a MALDI plate. A Microflex instrument (Bruker Daltonics) was used for the MALDI-TOF/MS analysis, and data were collected in the linear mode.

Analysis of covalent bond formation of RE142 with catCDC25A by western blotting in Tris-based buffer (Figure 5C)

Purified recombinant human CDC25A catalytic domain (33 μM, catCDC25A) in buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5) was diluted with Tris-based buffer (100 mM Tris-HCl, 40 mM NaCl, 2 mM DTT, 20% glycerol, pH 8.2). catCDC25A (400 nM) was incubated with CL1-Biotin (**18**) or RE142 (**19**) at 30 °C for 30 min. Excess free molecules were removed on a MicroBiospin column 6 (Bio-Rad), and samples were analyzed by western blotting with anti-biotin or anti-CDC25A antibody.

Analysis of covalent bond formation of RE142 with catCDC25A by western blotting in phosphate-based buffer (Figure 5D)

Purified recombinant human CDC25A catalytic domain (33 μM, catCDC25A) in buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5) was diluted by adding phosphate-based buffer (8.8 mM KH_2PO_4, 8.8 mM K_2HPO_4, pH 8.0). Diluted catCDC25A (400 nM) was incubated with CL1-Biotin (**18**) or RE142 (**19**) at 30 °C for 30 min. Excess free molecules was removed on a MicroBiospin column 6 (Bio-Rad), and samples were analyzed by western blotting with anti-biotin or anti-CDC25A antibody.

Detection of RE142-modified peptide by LC-MS analysis (sample preparation for Figure 6A)

Purified recombinant human CDC25A catalytic domain (50 μM, catCDC25A) in buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5) was incubated with RE142 (**19**, 1 mM) or DMSO (as a vehicle control) in the presence of CHAPS for 2 h at 30 °C. Then, proteins were precipitated with TCA/acetone to remove unbound RE142 (**19**) and CHAPS. The resulting precipitates were dissolved and denatured in denaturing buffer (1 M Tris-HCl, 7 M guanidine-HCl, 10 mM EDTA, pH 8.5). Proteins were reduced with 10 mM DTT for 30 min at 30 °C, alkylated with 10 mM iodoacetamide for 1 h at room temperature in the dark, and then digested sequentially with 2 μg of Lys-C (Wako Pure Chemical Industries, Osaka, Japan) overnight at 37 °C and 1 μg of trypsin (Promega, Madison, WI) for 9 h at 37 °C. Biotin-modified peptides were then bound to Neutravidin beads (Thermo Fisher Scientific, MA, USA) overnight at 37 °C, and unlabeled peptides were washed out with washing buffer (1 M NaCl, 0.05% SDS, 10% ethanol) three times and phosphate-based buffer (8.8 mM KH_2PO_4, 8.8 mM K_2HPO_4, pH 8.0) three times. RE142 (**19**)-modified peptides were eluted from the beads by reduction of the diazobenzene moiety using phosphate-based buffer containing 0.5 M $Na_2S_2O_4$ and 0.005 % SDS for 5 h at room temperature with shaking. The eluate was purified and concentrated on C18 ZipTips. The reaction products were eluted with acetonitrile in water (20%, 50% and 80%, v/v).

Mass Spectrometry

Chemicals

All solvents for LC-MS analysis, 0.1% (v/v) formic acid (FA)-distilled water, 0.1% (v/v) FA-acetonitrile (MeCN), trifluoroacetic acid (TFA) (HPLC grade), were purchased from Kanto Chemical Co., Inc.. Detergent, n-decyl-β-$_D$-glucopyranoside (DG), was purchased from Sigma.

Liquid chromatography and mass spectrometry

Mass spectra were acquired using a LTQ Orbitrap XL equipped with an electrospray ionization (ESI) source (Thermo Fisher Scientific). Full mass scan was acquired in the FT mode (resolution 60,000) and MS/MS scan (CID) was acquired in the ion-trap (IT) mode. On a nanoflow HPLC system Ultimate 3000 (Thermo Fisher Scientific), a spray tip column (Nikkyo Technos Co., Ltd, Tokyo, Japan) and a ZORBAX 300SB-C18 column (0.3 x 5 mm, Agilent) were used as the analytical column and trap column, respectively. For the analytical column, mobile phase A consisted of 0.1% FA in distilled water; mobile phase B consisted of 80 or 100% MeCN containing 0.1% FA. For the trap column, mobile phase C consisted of 0.1% TFA in distilled water; mobile phase D consisted of 100% MeCN, 0.1% TFA. Enzyme-digested peptides diluted with 0.005-0.01% DG[2,3] were analyzed by nanoLC-MS. The gradient method was used with mobile phase A and mobile phase B at a flow rate of 200 nL/min.

Database search was performed using a peptide sequencing program, Proteome Discoverer 1.0 (Thermo Fisher Scientific) through a database for MS/MS ion search, SEQUEST. Theoretical mass values of peptides were calculated by Qual Browser (Thermo Fisher Scientific).

Prediction of RE142-binding site of human catCDC25A

1. LC-MS/MS analysis of catCDC25A

In order to confirm the sequence coverage of catCDC25A by LC-MS, enzyme-digested catCDC25A, which had been digested with Lys-C and subsequently with trypsin in solution after guanidine hydrochloride denaturation, was analyzed by LC-MS. The acquired data were analyzed using the SEQUEST search engine against the catCDC25A amino acid sequence. Most of the digested peptides were eluted before 45 min and the sequence coverage was 98% (Figure S3).

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[2] K. Kodama, S. Fukuzawa, H. Nakayama, T. Kigawa, K. Sakamoto, T. Yabuki, N. Matsuda, M. Shirouzu, K. Takio, K. Tachibana and S. Yokoyama, *ChemBioChem* 2006, **7**, 134-139.

[3] M. Asanuma, K. Kurokawa, R. Ichikawa, K.-H. Ryu, J.-H. Chae, N. Dohmae, B. L. Lee and H. Nakayama, *FEBS Journal* 2011, **278**, 716-728.

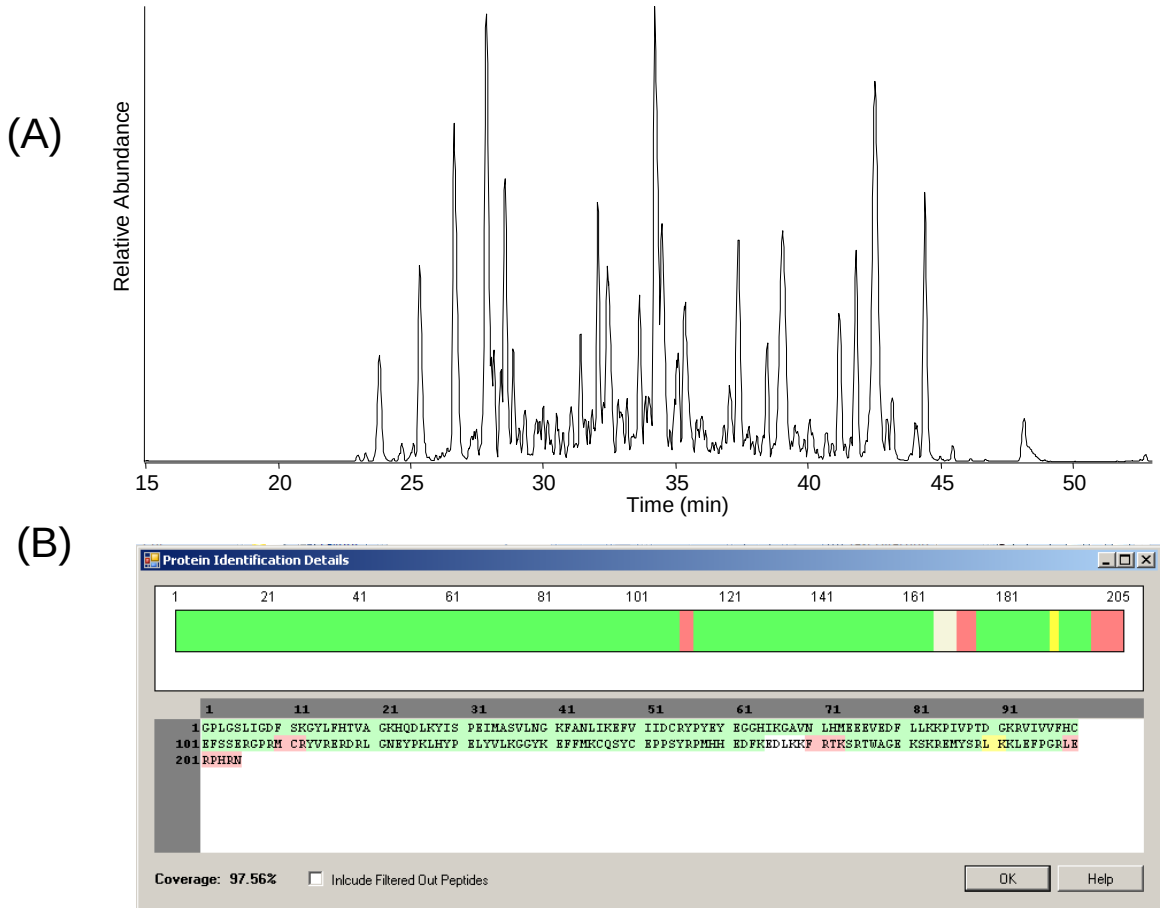


Figure S3. Analysis of non-treated catCDC25A by LC-MS/MS. (A) Base peak chromatogram of proteolytically digested catCDC25A. The micropump gradient method was used, as follows. Mobile phase A: 0.1% FA, mobile phase B: 0.1% FA, 80% MeCN. 0-10 min: 5% B, 10-45 min: 5-60% B, 45.1-50 min: 100% B, 50.1-70 min: 5% B. (B) SEQUEST database search result by Protein Discoverer (Thermo Fisher Scientific). Green, yellow, and red indicate a high, middle, and low hit fidelity, respectively.

2. LC-MS/MS analysis of catCDC25A treated with RE142

After Lys-C/trypsin double digestion of catCDC25A treated with DMSO or RE142 (**19**), RE142-modified peptides were purified and concentrated according to the above-mentioned procedure. The samples eluted from NeutrAvidin beads by cleavage of the diazobenzene linker were analyzed by LC-MS.

Several peptides were detected in the samples treated with DMSO or RE142 (Table S1). Among them, a peak corresponding to the mass value of cRE142-modified peptide [EFVIIDCRYPYEYEGGHIK] was eluted at around 60 min and detected at *m/z* 780.15, the charge state of which was 4 (Figure S4). A comparison of the LC-MS results for DMSO- or RE142-treated catCDC25A showed that the ion peak was specifically detected in RE142-treated catCDC25A (Figure S4). No other peptide modified with RE142 could be detected. Considering that most of the peptides were eluted within 45 min, the RE142 modification appeared to increase the hydrophobicity of the peptide.

The MS/MS spectrum of the peak at *m/z* 780.15, the charge state of which was 4, is shown in Figure S5. Some product ions were assigned to a simulated MS/MS pattern of RE142 (Cys)-modified peptide [EFVIIDCRYPYEYEGGHIK] (Table S2). However, the major peaks at around *m/z* 990 could not be

assigned to any y or b ions. Therefore, they might be due to fragmentation of the cRE142 moiety (insert in Figure S5). The predicted fragmentation was derived from Mass Frontier 6.0 (Thermo Fisher Scientific)

DMSO	RE142	Sequence	Position	Modifications	Charge	MH^+ [Da]	Δ M [ppm]
		GYLFHTVAGK	343-352		2, 3	1092.584	0.2
		FANLIK	372-377		2	705.429	-0.42
		EFVIIDCR	378-385	C7(Carbamidomethyl)	2	1051.525	0.88
		EFVIIDCR	378-385		2	994.503	0.05
N. D.		EFVIIDCRYPYEYEGGHIK	378-396	C7(cRE142)	4	3117.580	0.15
		YPYEYEGGHIK	386-396		2, 3	1355.626	-0.5
		KPIVPTDGK	414-422		2, 3	954.562	-0.29
		KPIVPTDGKR	414-423		2	1110.664	0.53
		ERDRLGNEYPK	446-456		2, 3, 4	1376.690	-0.24
		DRLGNEYPK	448-456		2, 3	1091.548	-0.23
N. D.		LGNEYPK	450-456		2	820.419	-0.6
		LHYPELYVLK	457-466		2, 3	1274.714	0.03
		EFFMK	471-475		2	701.333	-0.35
N. D.		SRTWAGEK	504-511		2	934.474	-0.18
		KLEFPGR	522-528		2	846.483	-0.43
		LEFPGR	523-528		2	718.388	-1.02

Table S1. SEQUEST database search result of DMSO- or RE142 (**19**)-treated catCDC25A fragments. The sequences are fragments of catCDC25A treated with DMSO or RE142 (**19**) observed by LC-MS/MS analyses. After treatment with DMSO or RE142 (**19**), catCDC25A was proteolytically digested and purified on NeutrAvidin beads, followed by cleavage of the diazobenzene linker with $Na_2S_2O_4$. The samples eluted from the beads were analyzed by LC-MS. Green and red colors indicate high and low hit fidelity. N. D. means no detected ion. The ions are shown in the MH^+ [Da] and the ΔM [ppm] columns.

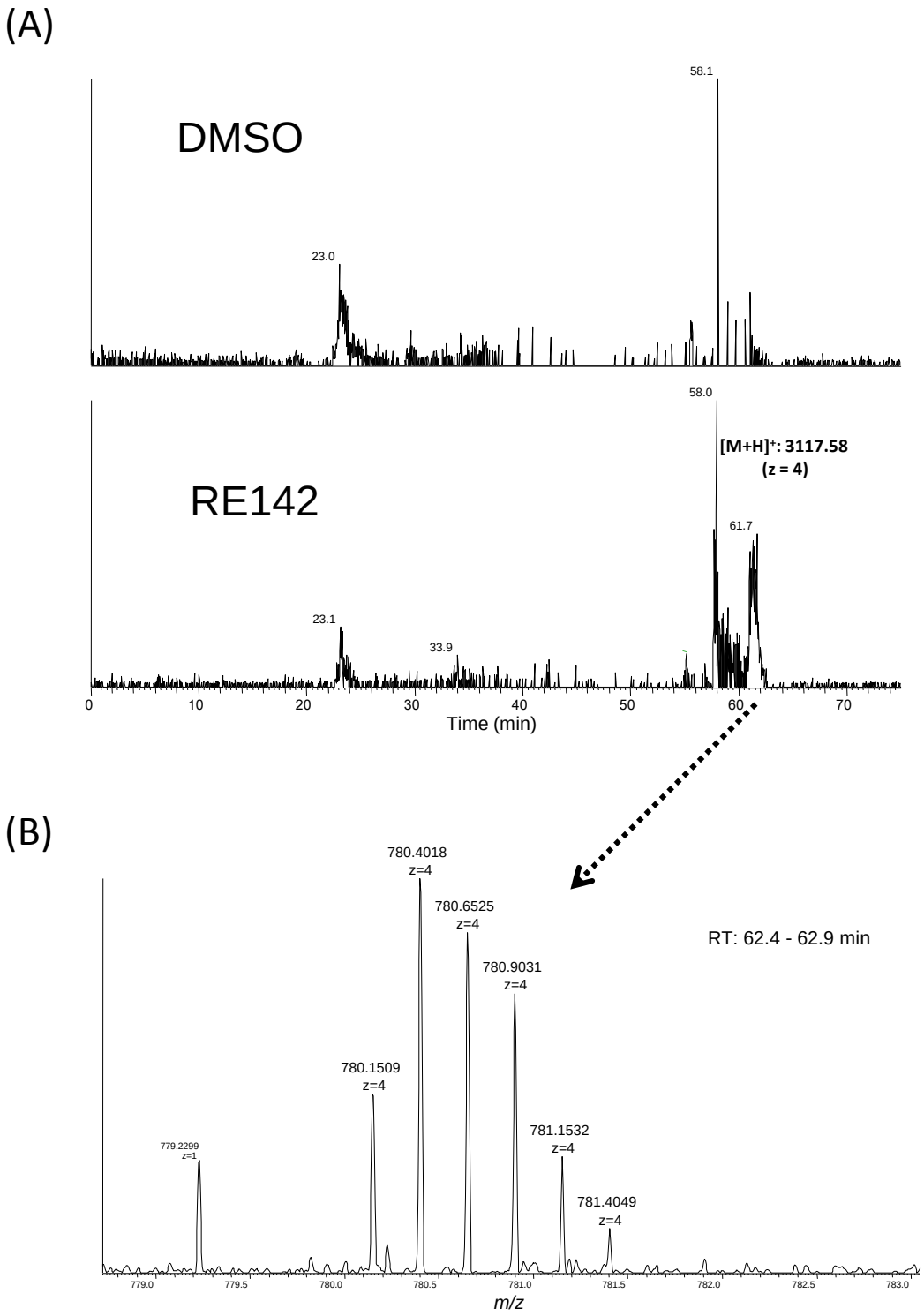


Figure S4. LC-MS analysis of catCDC25A treated with DMSO or RE142 (2nd analysis of Figure 6). The micropump gradient method was used, as follows. Mobile phase A: 0.1% FA, mobile phase B: 0.1% FA, 80% MeCN. 0-15 min: 5% B, 15-50 min: 5-60% B, 50.1-55 min: 100% B, 55.1-75 min: 5% B. (A) Extracted ion current chromatogram at *m/z* 780.60-781.00. (B) Mass spectrum at the retention time of 62.35-62.90 min. The peak was corresponds to the calculated cRE142-modified peptide [EFVIIDCRYPYEYEGGHIK].

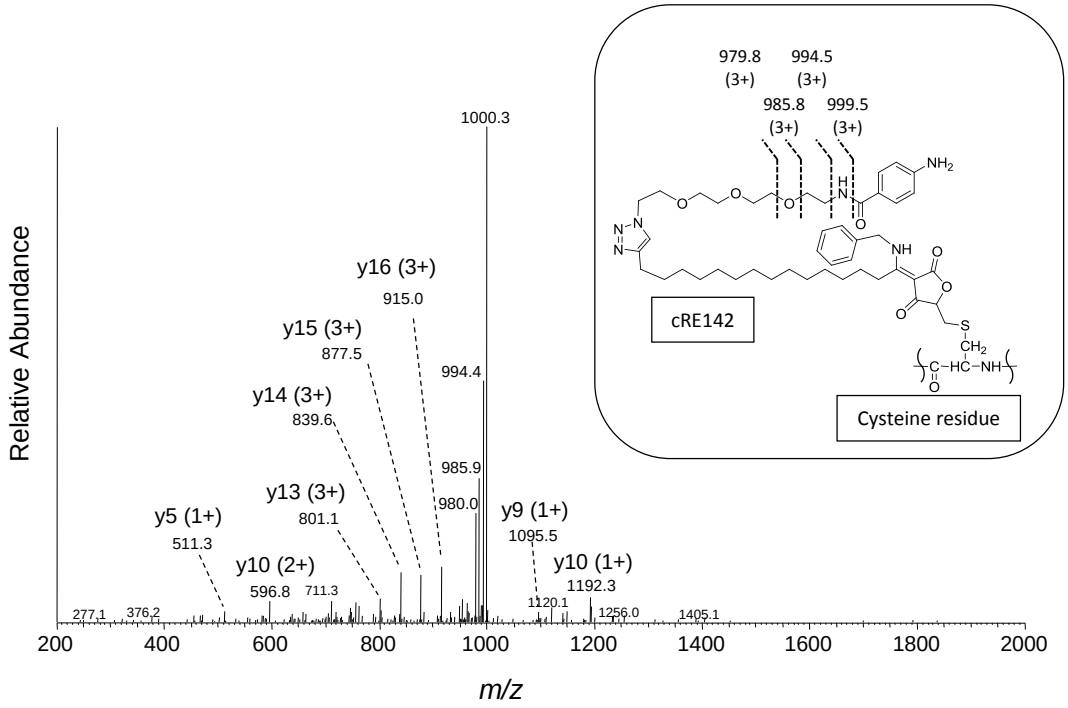


Figure S5. Ion-trap MS/MS (CID) spectrum of precursor ion *m/z* 780.15. The major product ions around *m/z* 990 appear to be derived from the fragmentation of cRE142 (insert). The insert illustrates the predicted fragmentation and the derived mass values.

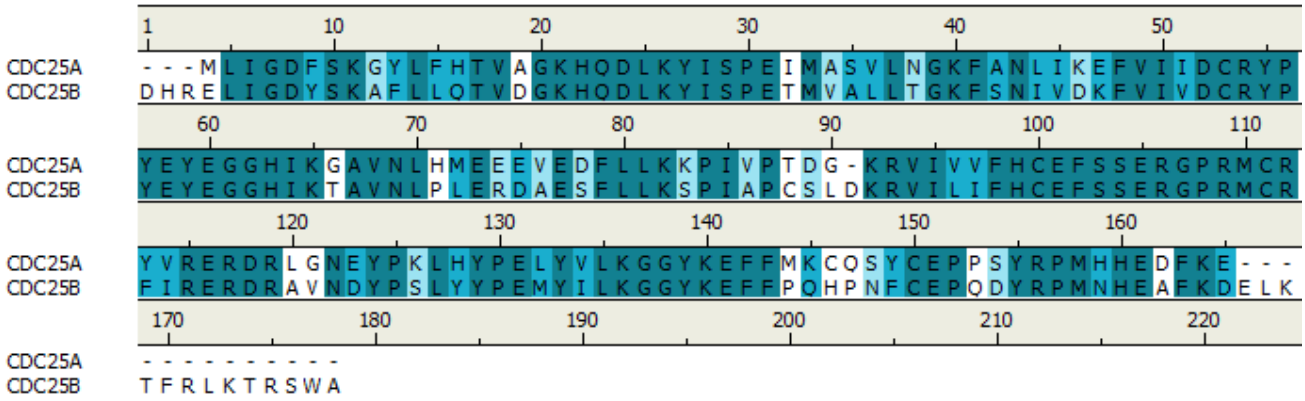
Seq.	y (1+)	y (2+)	y (3+)	y
E				19
F	2988.5	1494.8	996.9	18
V	2841.5	1421.2	947.8	17
I	2742.4	1371.7	914.8	16
I	2629.3	1315.2	877.1	15
D	2516.2	1258.6	839.4	14
C384-cRE142	2401.2	1201.1	801.1	13
R	1511.7	756.4	504.6	12
Y386	1355.6	678.3	452.5	11
P	1192.6	596.8	398.2	10
Y	1095.5	548.3	365.8	9
E	932.4	466.7	311.5	8
Y	803.4	402.2	268.5	7
E	640.3	320.7	214.1	6
G	511.3	256.2	171.1	5
G	454.3	227.6	152.1	4
H	397.3	199.1	133.1	3
I	260.2	130.6	87.4	2
K	147.1	74.1	49.7	1

Table S2. Simulated fragment ion pattern of cRE142(on Cys)-modified peptide [EFVIIDCRYPYEYEGGHIK]. Red color indicates the ions assigned by SEQUEST.

Computational Simulation

Homology Model of CDC25A complex with sulfate ions from crystal structure of CDC25B

The homology model for CDC25A complex with sulfate ions was constructed by using Discovery Studio 3.0, with the default setting and parameters for all calculations. First, sequence alignment of the sequences of catCDC25A crystal structure (PDB:1C25) and catCDC25B crystal structure (PDB:1QB0) was performed with the "Alignment Sequence with Structure" mode in Discovery Studio 3.0. The result of sequence alignment is shown below.



Construction of the homology model based on above sequence alignment was conducted by using "Build Homology Models" in Discovery Studio 3.0. To keep the sulfate ions in the crystal structure of CDC25B, two sulfate ions were selected as "Ligands" in the "Copy from Templates" parameter panel. Calculation in the default setting provided three candidate structures, and we selected one protein structure with the lowest "PDF Total Energy" as the homology model of catCDC25A shown in Figure 3B.

Molecular modeling of compound 5 covalently bound to CDC25A

In order to elucidate the binding mode of the compound using the docking method, the crystal structure of cdc25A (PDB:1C25) was retrieved from the Protein Data Bank (www.rcsb.org). The protein preparation wizard implemented in Maestro[4] was used to prepare the structure. All crystallographic water molecules were removed and hydrogen atoms were added in the protein structure. The side chain of C384 was flipped to the solvent-exposed surface to make it accessible to the ligand. The intrinsically disordered region at the C-terminal of the protein was also trimmed. The structure was then minimized to 0.3Å RMSD difference from the native structure using the OPLS_2005 force field in order to remove steric clashes among the side chain atoms. Similarly, the compound geometry was optimized using the OPLS_2005 force field in MacroModel[5] with a root-mean-square gradient of 0.01 kcal/mol/Å.

For molecular docking simulations, GOLD suite v2.5[6] was used. Active site atoms were considered to be those located within 15Å of the C384 residue. Constrained docking of the optimized ligand in this site was performed. During docking, the distance between the methylene carbon atom of the ligand and the S-atom of the C384 residue was constrained to 1.80 Å. The GOLD PLP scoring function was used to rank the poses. GOLD program uses the genetic algorithm to dock the compound in a selected binding

[4] Maestro, version 9.3, Schrödinger, LLC, New York, NY, 2012.
[5] MacroModel, version 9.9, Schrödinger, LLC, New York, NY, 2012.
[6] G. Jones, P. Willett, R. C. Glen, A. R. Leach and R. Taylor, *J. Mol. Biol.*, 1997, **267**, 727-748

site. After docking simulations, the most appropriate pose was selected and a covalent bond was generated between the protein atom and the ligand. Then the complex containing the ligand atoms lying within 10 Å distance from residues of the protein was subjected to conjugate gradient-based energy minimization using the MMFx94 force-field in order to remove any clashes and geometric distortions.