Electronic Supplementary Material (ESI) for Molecular BioSystems

Chemical ligation of epoxide-containing fusicoccins and peptide fragments guided by 14-3-3 protein

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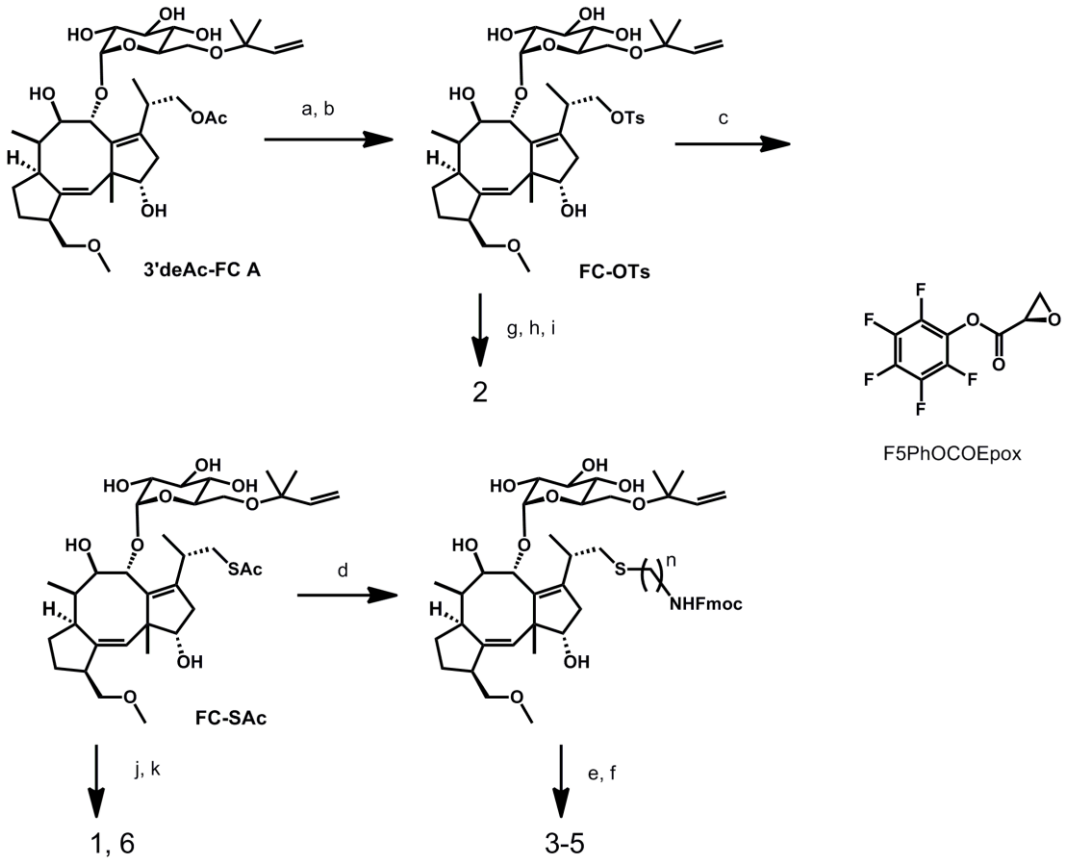
Electronic supplementary information

General Methods. Reagents and solvents were obtained from commercial sources without further purification unless otherwise noted. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-LA 400 spectrometer. Chemical shifts were reported in δ (ppm) relative to tetramethylsilane. All coupling constants were described in Hz. Flash column chromatography was performed on silica gel (40-63 μm) under a pressure of about 4 psi. Synthesized final compounds were checked for purity by analytical HPLC, which was performed using a JASCO PU-2086 and a JASCO UV-2075 detector with a GL Science Inertsil 150 mm x 4.6 mm, 5 μm C-18 column or TOSO TSKgel Amide-80 eluted with gradient 10% to 90 % of acetonitrile in 0.1 % TFA in water in 30 min. High-resolution mass data (HRMS) and low-resolution mass data (LRMS) of the compounds were analyzed by Nagasaki University Instrument Center under the guidance of Profs. T. Maki and N. Yamaguchi. MALDI-TOF mass spectra were measured by Shimadzu KRATOX AXIMA using 2',4',6'-trihydroxyacetophenone as a matrix. 3'-Deacetyl fusicoccin A was obtained from a large scale culture of *Phomopsis amygdali*, and the experimental details were reported in the literature. [1] Peptides used in this study were prepared by Fmoc-solid-phase-peptide-synthesis using 2-chlorotrityl chloride resin (Novabiochem). Recombinant 14-3-3ζ protein was expressed and purified by a procedure previously reported in the literature. [2]

General procedure for synthesis of fusicoccin derivatives (Scheme S1).

19-OTs fusicoccin (FC-OTs). A solution of 3'-deacetyl-fusicoccin A (3.97 g, 6.22 mmol) and K_2CO_3 (1.72 g, 12.4 mmol) in methanol (50 mL) was stirred at r.t. for 2.5h, and the product was extracted with chloroform (400 mL) after acidification with 10% citric acid. The organic layer was washed with brine, dried (anhydrous sodium sulfate), and concentrated to give the 19-hydroxyl fusicoccin as a pale yellow amorphous (3.57 g, 100%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.80 (dd, 1H, J = 10.8, 17.6Hz), 5.33 (br, s, 1H),5.13(dd, 1H, J = 1.2, 10.8Hz), 5.12 (dd, 1H, J = 1.2, 10.8Hz), 5.05 (d, 1H, J = 3.8Hz), 3.98 (dd, 1H, J = 4.4, 10.2Hz), 3.89 (d, 1H, J = 10.2Hz), 3.81 (dd, 1H, J = 9.6, 11.7Hz), 3.76 (dd, 1H, J = 44., 5.9Hz), 3.55-3.65 (m, 3H), 3.48 (dd, 1H, J = 4.8Hz), 3.46 (dd, 1H, J = 7.8, 9.8Hz), 3,44 (dd, 1H, J = 11.7,

Scheme S1. Synthesis of fusicoccin-based modules (**1-6**)



Reagents and conditions: a) K_2CO_3, MeOH, quant.; b) TsCl, 55%; c) AcSK, 88%; d) NaOH, $NaBH_4$, $Br(CH_2)nNHFmoc$; e) Et_2NH; f) $F_5PhOCOEpox$; g) NaN_3, 90%; h) 10% Pd-C, quant.; i) F5PhOCOEpox, 65%; j) NaOH, $NaBH_4$, (S)-epichlorohydrin for **1**; k) NaOH, $NaBH_4$, S-(-)-glycidol for **6**, 92%.

11.7Hz), 3.38-3.46 (m, 2H), 3.38 (dd, 1H, *J* = 9.2, 9.2Hz), 3.36 (s, 3H), 3.15-3.27 (m,1H), 2.75-2.85 (m, 1H), 2.34 (dd, 1H, *J* = 6.0, 16.0Hz), 2.18 (dd, 1H, *J* = 4.4, 16.0Hz), 1.84-2.06 (2H, m), 1.46-1.78 (4H, m), 1.28 (s, 3H), 1.28 (s, 3H), 1.21 (s, 3H), 1.08 (d, 3H, *J* = 7.1Hz), 0.87, (d, 3H, *J* = 7.1Hz).

This compound (1.00 g, 1.68 mmol) was reacted with tosyl chloride (1.47 g, 7.70 mmol) in the presence of pyridine (610 mg, 7.71 mmol) in 1,2-dichloroethane (23 mL) at 60 °C for 4h. The product was extracted with ethyl acetate (250 mL) from 10% citric acid (50 mL), and the organic layer was washed with brine, and dried (anhydrous sodium sulfate). After concentration, the residual pale yellow solid (2.12 g) was purified by SiO_2 column chromatography (chloroform : acetone: EtOH = 100:15:3) to give a white solid (692 mg, 55 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, 2H, *J* = 8.4Hz), 7.39 (d, 2H. *J* = 8.4Hz), 5.86 (dd, 1H, *J* = 11.0, 17.6Hz), 5.31(dd, 1H, *J* = 1.8, 1.8Hz), 5.14(dd, 1H, *J* = 1.2, 17.6Hz), 5.13 (dd, 1H, *J* = 1.2, 11.0Hz), 5.12 (d, 1H, *J* = 4.0Hz), 3.94 (dd, 1H, *J* = 4.4, 9.8Hz), 3.89 (d, 1H, *J* = 9.8Hz), 3.81-3.90 (m, 3H), 3.72-3.79 (m, 1H), 3.68 (dd, 1H, *J* = 3.3, 5.6Hz), 3.68 (dd, 1H, *J* = 4.0, 9.6Hz), 3.52-

3.58 (m, 2H), 3.49 (dd, 1H, *J* = 7.8, 9.8Hz), 3.45 (dd, 1H, *J* = 6.0, 9.0Hz), 3.40 (dd, 1H, *J* = 9.0, 9.0Hz), 3.39-3.44 (m, 1H), 3.37 (s, 3H), 2.73-2.8 (m, 1H), 2.47 (s, 3H), 2.14 (dd, 1H, *J* = 5.8, 16.0Hz), 2.03 (dd, 1H, *J* = 3.3, 16.0Hz), 1.84-2.06 (2H,m), 1.46-1.78 (4H, m), 1.30 (s, 3H), 1.30 (s, 3H), 1.22 (s, 3H), 0.97 (d, 3H, *J* = 6.9Hz), 0.90 (d, 3H, *J* = 7.0Hz).

19-Thioacetyl fusicoccin (FC-SAc). A solution of FC-OTs (500 mg, 0.67 mmol), potassium thioacetate (380 mg, 3.33 mmol) in DMF (23 mL) was stirred at 70 °C for 8h under argon atmosphere. After concentration, the residue was dissolved in ethyl acetate (250 mL) and 10% citric acid (50 mL), and the organic layer was washed with brine, and dried (anhydrous sodium sulfate). Filtration and evaporation of the solvent gave a crude solid (443 mg), which was purified by SiO_2 column chromatography (chloroform: acetone: EtOH = 100:15:3) to give the desired product a white solid (384 mg, 88 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.80 (dd, 1H, *J* = 11.0, 17.6 Hz), 5.32(dd, 1H, *J* = 1.8, 1.8 Hz), 5.14(dd, 1H, *J* = 1.2, 17.6 Hz), 5.13 (dd, 1H, *J* = 1.2, 11.0 Hz), 5.02 (d, 1H, *J* = 4.0 Hz), 3.91 (dd, 1H, *J* = 4.4, 10.2 Hz), 3.86 (d, 1H, *J* = 10.2 Hz), 3.84 (dd, 1H, *J* = 9.6, 11.7 Hz), 3.75-3.82 (m, 2H), 3.60 (dd, 1H, 4.0 Hz, 9.6 Hz), 3.53 (dd, 1H, *J* = 4.8, 9.8 Hz), 3.42 (dd, 1H, *J* = 6.0, 9. 2Hz), 3.38 (dd, 1H, *J* = 9.2, 9. 2Hz), 3.36 (s, 3H), 3.35-3.55 (m, 2H), 3.13-3.26 (m, 1H), 3.03 (dd, 1H *J* = 6.1, 12.8 Hz), 2.88 (dd, 1H, *J* = 9.1, 12.8 Hz), 2.74-2.84 (m, 1H), 2.49 (dd, 1H, *J* = 5.9, 15.8 Hz), 2.36 (s, 3H), 2.16 (dd, 1H, *J* = 4.4, 12.8 Hz), 1.84-2.06 (m, 2H), 1.46-1.78 (m, 4H), 1.28 (s, 3H), 1.28 (s, 3H), 1.21 (s, 3H), 1.08 (d, 3H, 6.9Hz), 0.87 (d, 3H, *J* = 7.1 Hz).

Epoxide-containing Fusicoccin (1). To a solution of FC-SAc (60 mg, 0.09 mmol) and sodium hydroxide (7 mg, 0.19 mmol) in ethanol (6 mL) was added sodium borohydride (7 mg, 0.19 mmol), and the mixture was stirred at 0 °C for 1h under argon atmosphere. This solution was slowly added to a solution of (-) -epichlorohydrin (254 mg, 2.75 mmol) in ethanol (3 mL) with cannula at 0 °C (2 ml/min), and the resulting mixture was stirred at 0 °C for 12h under argon atmosphere. The reaction mixture was dissolved in ethyl acetate (150 mL), and the organic layer was washed with H_2O and brine, and dried (anhydrous sodium sulfate). Concentration and SiO_2 column chromatography (chloroform: acetone: EtOH = 100:20:4) gave the product as a white solid (21 mg, 34%). ^{1}H NMR (400 MHz, $CDCl_3$): 5.79 (dd, 1H, *J* = 18, 12 Hz), 5.32 (s, 1H), 5.15 (s, 1H), 5.12 (d, 1H, *J* = 6.4 Hz), 5.05 (t, 1H, *J* = 3.2 Hz), 4.29 (d, 1H, *J* = 16 Hz), 4.01 (m, 1H), 3.95-3.78 (m, 4H), 3.68 (m, 1H), 3.60 (m, 1H), 3.52-3.30 (m, 11H), 3.20 (m, 2H), 2.97-2.57 (m, 7H), 2.53-2.45 (m, 1H), 2.20-2.16 (m, 1H), 2.01 (m, 1H), 1.92 (m, 1H), 1.79-1.68 (m, 2H), 1.65-1.51 (m, 2H), 1.28 (s, 3H), 1.27 (s, 3H), 1.19 (s, 2H), 1.14 (d, 3H, *J* = 6.0 Hz), 0.88 (d, 3H, *J* = 7.2 Hz): ESI-TOFMS (*m/z)* calcd for $C_{35}H_{56}O_{10}S_1$ $[M+Na]^+$, 691.35; found, 691.26.

Epoxide-containing Fusicoccin (2). To a solution of FC-OTs (500 mg, 0.67 mmol) in dimethylformamide (18 mL) was added sodium azide (150 mg, 2.31 mmol), and the mixture was stirred at 80 C for 24h. After concentration, the residue was dissolved in ethyl acetate (250 mL) and 10% citric acid (50 mL), and the organic layer was washed with brine, and dried (anhydrous sodium sulfate).

Filtration, concentration, and SiO_2 column chromatography (chloroform: acetone: EtOH = 100:20:4) afforded 19-azide derivative (384 mg, 88%). ESI-TOFMS (*m/z)*: calcd for $C_{32}H_{51}N_3O_9$ $[M+Na]^+$, 644.35; found, 644.30.

This azide derivative (80 mg, 0.048 mmol) was hydrogenated by stirring in the presence of 10% palladium hydroxide (6 mg) in ethanol (3 ml) containing a drop of pyridine under hydrogen atmosphere for 30 min. The mixture was filtered and concentrated. The resulting residue was dissolved in dimethylformamide (3 mL) and diisopropylethylamine (20 μL, 0.12 mmol), and pentafluorophenyl glycidate (13.4 mg, 0.053 mmol) was added to the solution, which was stirred at rt for 2h. The product was extracted with ethyl acetate (100 mL) and H_2O (50 mL), and the organic layer was washed with brine, and dried (anhydrous sodium sulfate). The residual oil was purified by chromatography (chloroform:acetone:MeOH:isopropanol = 100:40:8:1) to obtain a white solid (32 mg, 37%). ^{1}H NMR (400 MHz, $CDCl_3$): 5.33 (brs, 1H), 5.07 (d, 1H, *J* = 3.7 Hz), 4.00 (dd, 1H, *J* = 9.8, 9.8 Hz), 3.92 (dd, 1H), 3.89 (dd, 1H, *J* = 10.2, 10.2 Hz), 3.80 (dd, 1H, *J* = 44., 5.6 Hz), 3.67 (1H, dd, *J* = 4.2, 9.6 Hz), 3.58-3.65 (m, 2H), 3.56 (dd, 1H, *J* = 4.8, 4.8 Hz), 3.47 (dd, 1H, *J* = 7.8, 9.8 Hz), 3.49 (dd, 1H, *J* = 2.4, 5.4 Hz), 3,44 (dd, 1H, *J* = 6.0, 9.2 Hz), 3.40 (dd, 1H, *J* = 9.2, 9.2 Hz), 3.21-3.22 (m,1H), 3.02 (dd, 1H, *J* = 5.4, 5.4 Hz), 2.73-2.85 (m, 3H), 2.40 (dd, 1H, *J* = 5.6, 15.8 Hz), 2.18 (dd, 1H, *J* = 4.4, 15.8 Hz), 1.83-2.09 (m, 3H), 1.46-1.78 (m,3H), 1.49 (2H, dd *J* = 2.2, 7.4 Hz), 1.21 (s, 3H), 1.13 (s, 3H), 1.13 (s, 3H), 1.04 (d, 3H, *J* = 6.6 Hz), 0.89 (d, 3H, *J* = 7.0 Hz), 0.84 (t, 3H, *J* = 7.4 Hz): ESI-TOFMS (*m/z)* calcd for $C_{35}H_{57}N_1O_{11}$ $[M+Na]^+$, 690.82; found, 690.81.

Epoxide-containing Fusicoccin, n=1 (3). To a solution of FC-SAc (100mg, 0.15 mmol) and sodium hydroxide (12 mg, 0.30 mmol) in methanol (5 mL) was added sodium borohydride (12 mg, 0.32 mmol), and the mixture was stirred at 0 °C for 1h under argon atmosphere. This solution was slowly added to a solution of Fmoc-2-aminoethyl-1-bromide (317 mg, 0.92 mmol) in THF (5 mL) with cannula at 0 °C (2 ml/min), and the resulting mixture was stirred at 0 °C for 14h under argon atmosphere. The reaction mixture was dissolved in ethyl acetate (300 mL) and 10% citric acid (30 mL), and the organic layer was then washed with 5% sodium bicarbonate (30 mL) and brine, and dried (anhydrous sodium sulfate). Concentration and SiO_2 column chromatography (chloroform: acetone: EtOH = 100:20:4) gave Fmoc-protected fusicoccin as a white solid (80 mg, 58%). This compound was immediately used for the next reaction.

Deprotection of Fmoc group was performed by stirring a mixture of the Fmoc-protected fusicoccin (60 mg, 0.092 mmol) and 20% diethylamine in dichloromethane (5 mL) at rt for 6h. After concentration, the residue was purified by preparative liquid chromatography (gradient: 0 to 100% of acetonitrile in 0.1% trifluoroacetic acid in H_2O in 28 min) followed by lyophilization to afford the free amine as a white powder. This free amine was reacted with pentafluorophenyl s-(-)-glycidate (9 mg, 0.034 mmol) in the presence of triethylamine (5 mg, 0.05 mmol) in dimethyl formamide (3 mL) at rt for 3h. After the

reaction, the solvent was removed under reduced pressure, and the residual oil was dissolved in ethyl acetate (300 mL) and 10% citric acid (30 mL). The organic layer was then washed with 5% sodium bicarbonate (30 mL), and brine, and dried (anhydrous sodium sulfate). After filtration and concentration, the residue was purified by preparative liquid chromatography (gradient: 0 to 100% of acetonitrile in 0.1% trifluoroacetic acid in H_2O in 28 min), and a fraction at 6 min was lyophilized to give the desired product as a white powder (20 mg, 31% for 2 steps): ^{1}H NMR (400 MHz, $CDCl_3$): 5.78 (dd, 1H, *J* = 11.0, 17.4 Hz), 5.32 (brs, 1H), 5.15 (dd, 1H, *J* = 1.2, 11 Hz), 5.14 (dd, 1H, *J* = 1.2, 17.4 Hz), 5.03 (d, 1H, *J* = 3.8 Hz), 3.96-4.08 (m, 2H), 3.93 (dd, 1H, *J* = 4.4, 10 Hz), 3.84 (d, 1H, *J* = 10.1 Hz), 3.77 (dd, 1H, *J* = 4.4, 5.7 Hz), 3.73-3.80 (m,1H), 3.63 (dd, 1H, *J* = 4.0, 9.6 Hz), 3.60-3.68 (m,1H), 3.53 (dd, 1H, *J* = 4.8, 9.8 Hz), 3.47-3.60 (m, 3H), 3.43 (dd, 1H, 9.2, 9.2Hz), 3.39 (dd, 1H, *J* = 6.2, 9.2Hz), 3.37 (s, 3H), 3.10-3.21 (m,1H), 3.06 (dd, 1H, *J* = 5.5, 5.5 Hz), 2.85 (dd, 1H, 2.5, 5.5Hz), 2.37-2.84 (m, 1H), 2.62-2.70 (m, 3H), 2.42-2.51 (m, 1H), 2.50 (dd, 1H, *J* = 6.0, 15.8 Hz), 2.28 (dd, 1H, *J* = 4.4, 15.8 Hz), 1.84-2.04 (m, 2H), 1.46-1.78 (m, 3H), 1.28 (s, 3H), 1.27 (s, 3H), 1.19 (s, 3H), 1.14 (d, 3H, *J* = 6.7Hz), 0.89 (d, 1H, *J* = 6.9 Hz): ESI-TOFMS (*m/z)*: [M] calcd for $C_{37}H_{59}N_1O_{11}S_1$ $[M+Na]^+$, 748.37; found, 748.38.

Epoxide-containing Fusicoccin, n=3 (4). This compound was derived from FC-SAc by a similar method to that described for **3** except for using Fmoc-4-aminobutyl-1-bromide: ^{1}H NMR (400 MHz, $CDCl_3$): 6.47 (t, 1H, *J* = 6.4Hz), 5.80 (dd, 1H, *J* = 17.6 and 10.4 Hz), 5.33 (s, 1H), 5.15 (d, 1H, *J* = 2.4 Hz), 5.12 (d, 1H, *J* = 2.4 Hz), 5.04 (d, 1H, *J* = 3.6 Hz), 4.03-3.94 (m, 2H), 3.89-3.78 (m, 1H), 3.75-3.63 (m, 1H), 3.54-3.40 (m, 6H), 3.37 (s, 3H), 3.20 (m, 1H), 3.02 (t, 1H, *J* = 5.0 Hz), 2.86-2.77 (m, 3H), 2.20-1.90 (m, 2H), 1.74-1.53 (m, 4H), 1.28 (s, 3H), 1.27 (s, 3H), 1.20 (s, 3H), 1.12 (d, 3H, *J* = 6.8 Hz), 0.88 (d, 3H, *J* = 6.8 Hz): ESI-TOFMS (*m/z)*: calcd for $C_{39}H_{63}N_1O_{11}S_1$ $[M+Na]^+$, 776.97; found, 776.41.

Epoxide-containing Fusicoccin, n=5 (5). This compound was derived from FC-SAc by a similar method to that described for **3** except for using Fmoc-6-aminohexyl-1-bromide. ^{1}H NMR analysis was not possible due to the insufficient amount of the product obtained, thus the product was directly used in the assay after the mass analysis: ESI-TOFMS (*m/z)*: calcd for $C_{41}H_{67}N_1O_{11}S_1$ $[M+Na]^+$, 804.43; found, 804.45.

Diol-containing Fusicoccin, (6). FC-SAc (100 mg, 0.16 mmol) was hydrolyzed by treatment with NaOH (6.2 mg, 0.16 mmol) in ethanol (2 mL) in the presence of sodium borohydride (6 mg, 0.16mmol). After stirring the mixture at rt for 50min under argon atmosphere, the solution was transferred to s-(-)-glycidol (11.5 mg, 0.16mmol) in ethanol (1 mL) dropwise at 0 °C. The solution was further stirred at r.t. overnight under argon atmosphere. After concentration, the crude oil was purified by column chromatography to give the product as a colorless amorphous (97 mg, 92%): ^{1}H NMR (400 MHz, $CDCl_3$): 5.78 (dd, 1H, *J* = 17.0 and 10.4 Hz), 5.44 (br s, 1H), 5.33 (s, 1H), 5.30 (br s, 2H), 5.14 (d, 1H, *J* = 2.4 Hz), 5.10 (d, 1H, *J* = 2.4 Hz), 5.01 (d, 1H, *J* = 4.0 Hz), 4.91 (br s, 1H), 4.70 (s, 1H), 4.66 (s, 1H), 4.39 (br s, 1H), 4.01-3.69 (m, 8H), 3.59-3.41 (m, 10H), 3.36 (s, 3H), 3.20 (m, 1H), 2.79 (m, 2H), 2.70-

2.58 (m, 4H), 2.47 (m, 4H), 2.14 (dd, 1H, *J* = 15.6 and 4.4 Hz), 1.99-1.89 (m, 3H), 1.69 (m, 1H), 1.57 (m, 2H), 1.27 (s, 3H), 1.26 (s, 3H), 1.20 (s, 3H), 1.11 (d, 3H, *J* = 6.8 Hz), 0.89 (d, 1H, *J* = 6.8 Hz), 0.86 (d, 3H, *J* = 4.8 Hz): ESI-TOFMS (*m/z*): calcd for $C_{35}H_{58}O_{11}S_1$ $[M+Na]^+$, 709.88; found, 709.88.

Stability of FC-based modules

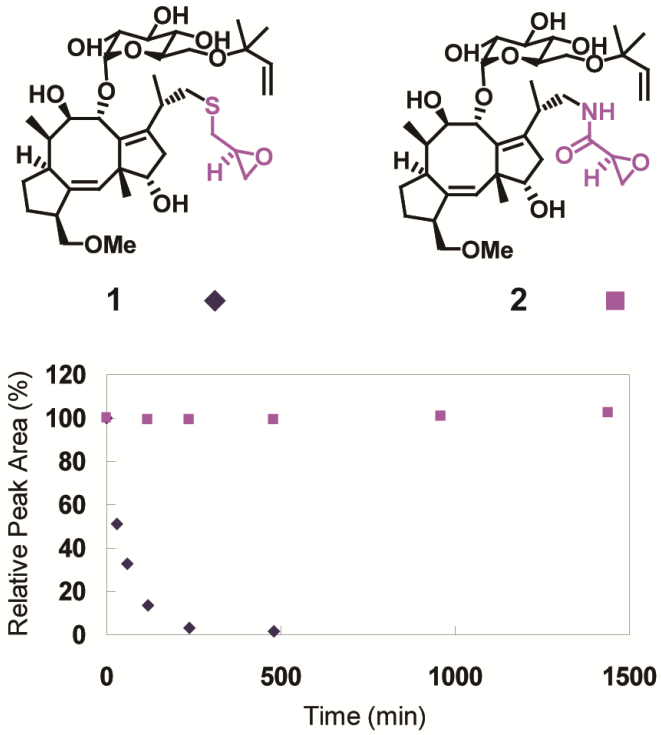


Fig. S1 FC-based modules (**1** and **2**, 1 mM) were monitored in Tris HCl (20 mM, pH 7.3) at 25 °C.

MALDI-TOF analysis of the FC-peptide conjugates.

Compound	Calcd[M]+	Found
2-QSYDC	1281.59	1281.7
3-QSYDC	1339.58	1341.0
4-QSYDC	1367.61	1393.1
5-QSYDC	1395.64	- [a)]

a) The MALDI-TOF analysis for 5-QSYDC gave no detectable peaks.

Stability of protein holding of 14-3-3 under pH 9.0

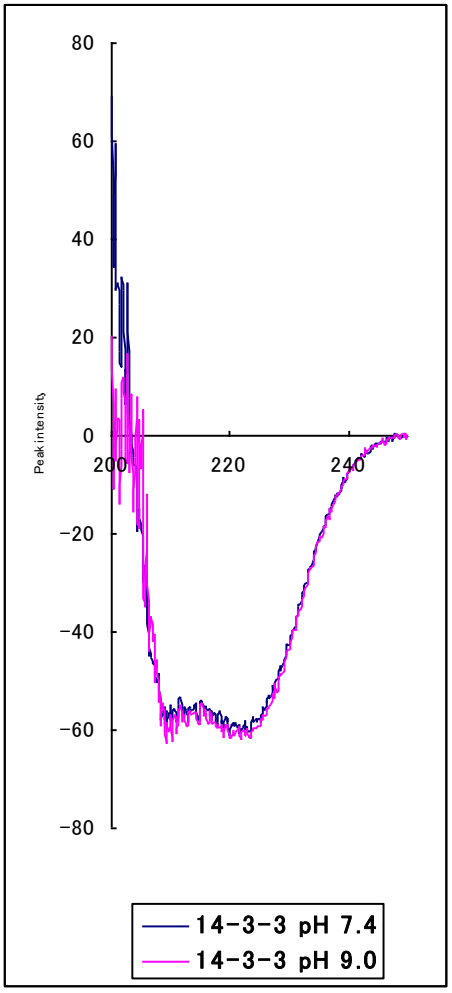


Fig. S2 CD spectra of 14-3-3 under pH. 7.4 (blue) and pH 9.0, respectively.

References:

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[2] M. Takahashi, A. Kawamura, N. Kato, T. Nishi, I. Hamachi, J. Ohkanda, *Angew. Chem. Int. Ed.* 2012, **51**, 509-512.