Supporting Information

Chemical Synthesis of the EPF-Family of Plant Cysteine-Rich Proteins and Late-Stage Dye Attachment by Chemoselective Amide-Forming Ligations

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1. General methods

1.1 Reagents and solvents and abbreviations

Fmoc-amino acids with suitable side-chain protecting groups, HCTU (O-(1H-6-Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate) and HATU(1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazole[4,5-b]pyridinium 3-oxid hexafluorophosphate) were purchased from Merck KGaA (Darmstadt, Germany). HPLC grade CH$_3$CN from Kanto Chemical Co., Inc. (Tokyo, Japan) and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) was used for analytical and preparative HPLC purification. DMF from FUJIFILM Wako Pure Chemical Corporation was directly used without further purification for solid phase peptide synthesis. Protected Fmoc-Val-α-ketoacid,$^{[1]}$ Boc-(S)-5-oxaproline,$^{[2]}$ and Fmoc-Orn HA$^{[3]}$ were prepared as previously reported by our group. Gly-Ser isoacyl dipeptide was prepared according to reported procedure.$^{[4]}$ All other chemicals were of the highest-grade commercially available and used as received. Merck KGaA (Darmstadt, Germany), Kanto Chemical Co., Inc. (Tokyo, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

List of the building blocks used for this work:

![Protected Fmoc-Val-α-ketoacid](image1)

![Boc-(S)-5-oxaproline](image2)

![Protected Fmoc-Orn ha](image3)

![Gly-Ser isoacyl dipeptide](image4)

List of abbreviations used:

Acm: acetamidomethyl

Boc-Opr: (S)-2-(tert-butoxycarbonyl) isoxazolidine-3-carboxylic acid

Boc: tert-butoxycarbonyl

COMU: (1-cyano-2-ethoxy-2-oxo-ethylidenaminooxy)-dimethylaminomorpholinocarbenium hexafluorophosphate

DIC: N,N'-diisopropylcarbodiimide

DMF: N,N-dimethylformamide

DMSO: dimethylsulfoxide

DODT: 2,2-(ethylenedioxy)diethanethiol
DTT: dithiothreitol
Fmoc: 9-fluorenlymethyloxycarbonyl
Gdn•HCl: guanidine hydrochloride
GSH: glutathione
GSSG: glutathione disulfide
HATU: O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HCTU: O-(1H-6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HOBt: hydroxybenzotriazole
NMM: N-methylmorpholine
NMP: N-methyl-2-pyrrolidone
SPPS: solid phase peptide synthesis
TCEP: tris(2-carboxyethyl)phosphine
TFA: trifluoroacetic acid
TIPS: triisopropylsilane
Trt: trityl group

1.2 Peptide synthesis
a) Solid phase peptide synthesis (SPPS)

Peptides were synthesized on a CS Bio 136X synthesizer using Fmoc SPPS chemistry. The following Fmoc amino acids with side-chain protecting groups were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Acm)-OH Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(1-Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, Fmoc-Nle-OH.

SPPS was performed on aminomethyl polystyrene resin or HMPB-ChemMatrix resin or 2-chlorotrityl polystyrene resin. Manual loading of the first amino acid residue onto the resin and subsequent Fmoc-SPPS followed established standard protocols. A summary of the utilized synthesis protocols: Fmoc-deprotections were performed with 20% piperidine in DMF (8 min ×2). Couplings were performed with Fmoc-amino acid (4.0 equiv relative to resin substitution), HCTU (3.8 equiv) and NMM (8.0 equiv) in DMF for 60 min. If required, the coupling step was repeated (double coupling) and LiCl washes (0.8 M LiCl in DMF) were performed before Fmoc-deprotection and coupling. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride and 10% NMM in DMF for 10 min. Amino acid residues prone to epimerization such as cysteine were coupled using preformed HOBt esters. In a typical procedure,
Fmoc-Cys(Acm)-OH (4.0 equiv relative to resin loading) was dissolved in DMF, and HOBt (4.0 equiv) and DIC (4.0 equiv) were added. The mixture was added to the resin and allowed to react for 2 h.

b) Manual coupling of special amino acids

Protected Fmoc-Val-α-ketoacid, Boc-(S)-5-oxoproline, Fmoc-Orn HA, and Gly-Ser isoacyl dipeptide were coupled manually. The monomer (1.5 equiv) was dissolved in a minimal amount of DMF (minimal concentration of monomer: 0.1 M), HATU (1.5 equiv) and NMM (3.0 equiv) were added. After a brief period of preactivation (2 min), the solution was added to the resin and allowed to react for 2 h. If required, the coupling was repeated with 1.0 equiv of monomers, 1.0 equiv of HATU, and 2.0 equiv of NMM.

c) Mutations and protecting groups

Norleucine Substitution: All methionine residues (Met) were substituted by norleucine (Nle) residues in the protein sequence, to avoid oxidation while handling, storage, and refolding.

1.3 General HPLC analysis and purification

Peptides and protein segments were analyzed and purified by reverse phase high performance liquid chromatography (RP-HPLC) on Jasco analytical and preparative instruments equipped with dual pumps, a mixer, an in-line degasser, and variable wavelength UV detector (simultaneous monitoring of the eluent at 220 nm, 254 nm, and 301 nm) or on a Gilson preparative instrument fitted with a 10 mL injection loop. If required, the columns were preheated using a column heater or a water bath. The mobile phase for RP-HPLC were Milli-Q water containing 0.1% TFA and HPLC grade CH$_3$CN containing 0.1% TFA. In the described HPLC analysis and purifications, TFA was always used as solvent modifier.

Analytical RP-HPLC: Analytical HPLC was performed on a Shiseido Capcell Pak MG-II (5 µm, 120 Å pore size, 4.6 mm I.D. × 250 mm), or on a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 4.6 mm I.D. × 250 mm), or on Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 4.6 mm I.D. × 250 mm) at a flow rate of 1 mL/min.

Preparative RP-HPLC: Preparative HPLC was performed on a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm), on a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 30 mm I.D. × 250 mm), on a Phenomenex Jupiter C18 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm), on a
Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 30 mm I.D. × 250 mm), or on a Phenomenex Jupiter C4 (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm). The following type of method was used: the column was pre-equilibrated at starting solvent composition for typically 10 min. After injection of the sample, the solvent composition was run to the final solvent composition (e.g., 50% CH₃CN). After the gradient run time, the solvent composition was changed to 95% CH₃CN within 1 min and the column was flushed for 5–7 min. Within 1 min, the solvent composition was changed to 10% CH₃CN and the run ended. For the sake of simplicity, only the gradient time, the starting and end composition of the eluent will be stated at the individual experiments, although all experiments included the full cycle as described above.

1.4 Characterization

MALDI-MS data were obtained on a Bruker Microflex MALTI-TOF spectrometer using 4-hydroxy-α-cyanocinnamic acid as the matrix. High-resolution mass spectra were recorded by the Molecular Structure Center at ITbM, Nagoya University on Thermo Scientific™Exactive™ Plus Orbitrap Mass Spectrometer.

2. Chemical synthesis of EPFL9 proteins

2.1 Synthesis of reduced EPFL9 protein 1a

\[
\text{H}_2\text{N} \xrightarrow{\text{SH}} \text{IGSTAPTCTYNECRGC} \text{SH} \xrightarrow{\text{SH}} \text{RGKRAEQVPVEGNDPIN} \text{SH} \xrightarrow{\text{SH}} \text{AYHYRCVCHR} \xrightarrow{\text{COOH}}
\]

The reduced EPFL9 1a was synthesized on the 2-chloro trityl chloride resin preloaded with Fmoc-Arg-OH (0.5 g, 0.39 mmol loading capacity). After automated SPPS, the resin was washed several times with DMF followed by CH₂Cl₂, dried and subjected to cleavage using a mixture of 95:2.5:2.5 TFA:TIPS:H₂O (20 mL/g resin) for 2 h at room temperature. The crude TFA solution was separated from the resin by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with cooled Et₂O (ca. 25 mL/g resin), centrifuged and the supernatant was removed by decantation. This trituration/washing step was repeated twice (peptide precipitating out). The crude peptide was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–70% CH₃CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing product
were pooled and lyophilized to obtained 358 mg of reduced EPFL9 1a. The m/z calculated for 1a C_{213}H_{338}N_{69}O_{67}S_{6} [M+7H]^{7+}: 732.3350 Da, measured: 732.3338 Da.

2.2 Synthesis of reduced EPFL9 protein 1b

The reduced EPFL9 1b was synthesized on the 2-chloro trityl chloride resin preloaded with Fmoc-Arg-OH (0.5 g, 0.39 mmol loading capacity). After automated SPPS, the resin was washed several times with DMF followed by CH_{2}Cl_{2}, dried and subjected to cleavage using a mixture of 95:2.5:2.5 TFA:TIPS:H_{2}O (20 mL/g resin) for 2 h at room temperature. The crude TFA solution was separated from the resin by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with cooled Et_{2}O (ca. 25 mL/g resin), centrifuged and the supernatant was removed by decantation. This trituration/washing step was repeated twice (peptide precipitating out). The crude was purified by preparative RP-HPLC using Phenomenex Jupiter C_{18} column (5 \mu m, 300 Å pore size, 30 mm I.D. \times 250 mm) with a gradient of 20–70% CH_{3}CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing product were pooled and lyophilized to obtained 207 mg of reduced EPFL9 1b. The m/z calculated for 1b C_{232}H_{364}N_{75}O_{74}S_{6} [M+7H]^{7+}: 792.6464 Da, measured: 792.6448 Da.
3. Chemical synthesis of cysteine protected EPF2 proteins

3.1 Synthesis of Cys(Acm) protected thioester peptide 3a

The peptide thioester 3a was synthesized on 2-chlorotrityl hydrazine polystyrene resin prepared by reported procedure.[5] The Fmoc-Ser(Trt)-OH (1.0 equiv) was coupled manually to the resin using HATU (1.0 equiv) and NMM (2.0 equiv) in anhydrous DMF at room temperature for 2 h and then the loading capacity was identified. After automated Fmoc SPPS, the resin was washed several times with DMF followed by CH₂Cl₂, dried and subjected to cleavage using a mixture of 95:2.5:2.5 TFA:TIPS:H₂O (20 mL/g resin) for 2 h at room temperature. The crude TFA solution was separated from the resin by filtration and the filtrate was concentrated under reduced pressure.

The residue was triturated with cooled Et₂O (ca. 25 mL/g resin), centrifuged and the supernatant was removed by decantation. This trituration/washing step was repeated twice. The crude peptide hydrazide was dried and dissolved in aqueous CH₃CN with 0.1% TFA for RP-HPLC purification.

The peptide hydrazide (1.0 equiv) was dissolved in phosphate buffer containing 0.2 M NaH₂PO₄, 6 M Gdn•HCl, pH 3.0. The mixture was cooled to −15 °C (ice + NaCl) and maintained for 20 min. The aqueous NaNO₂ (10 equiv) was added dropwise and stirred at −15 °C for another 30 min. The β-mercaptopropionic acid (25 equiv) was added to the reaction mixture at −15 °C and slowly bring the reaction mixture to room temperature. The pH of the reaction mixture was adjusted to 6.8–7.0 and maintained until the reaction was complete. The reaction progress was
monitored by analytical RP-HPLC. The crude peptide thioester 3a was purified by preparative RP-HPLC using Phenomenex Jupiter® C4 column (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–70% CH₃CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing desired product were pooled and lyophilized to give 3a (62 mg, 60% yield corresponding from 100 mg of hydrazide peptide). The m/z calculated for 3a C₁₅₃H₂₄₈N₄₁O₅₄S₆ [M+3H]⁺: 1238.5409 Da, measured: 1238.5476 Da.

![Graph](image)

(a) Thioester formation at 0 h; b) Thioester formation at 30 min; c) Purified peptide thioester 3a

3.2 Synthesis of Cys(Acm) protected thioester peptide 3b

The peptide thioester 3b was synthesized on 2-chlorotrityl hydrazine polystyrene resin prepared by reported procedure.[⁴] The Fmoc-Ser(Trt)-OH (1.0 equiv) was coupled manually to the resin using HATU (1.0 equiv) and NMM (2.0 equiv) in anhydrous DMF at room temperature for 2 h and then the loading capacity was identified. After automated Fmoc SPPS, Fmoc-Orn HA (1.1 equiv, 0.33 mmol) was manually coupled using HATU (1.1 equiv 0.33 mmol) and NMM (2.2 equiv, 0.66 mmol) in anhydrous DMF for 2 h at room temperature. The resin was washed several times with DMF followed by CH₂Cl₂, dried and subjected to cleavage using a mixture of 95:2.5:2.5 TFA:TIPS:H₂O (20 mL/g resin) for 2 h at room temperature. The crude TFA solution
was separated from the resin by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with cooled Et₂O (ca. 25 mL/g resin), centrifuged and the supernatant was removed by decantation. This trituration/washing step was repeated twice. The crude peptide hydrazide was dried and dissolved in aqueous CH₃CN with 0.1% TFA for RP-HPLC purification.

The peptide hydrazide (1.0 equiv) was dissolved in phosphate buffer containing 0.2 M NaH₂PO₄, 6 M Gdn•HCl pH 3.0. The mixture was cooled to −15 °C (ice + NaCl) and maintained for 20 min. The aqueous NaNO₂ (10 equiv) was added dropwise and stirred at −15 °C for another 30 min. The β-mercaptotriopionic acid (25 equiv) was added to the reaction mixture at −15 °C and slowly bring the reaction mixture to room temperature. The pH of the reaction mixture was adjusted to 6.8–7.0 and maintained until the reaction was complete. The reaction progress was monitored by analytical RP-HPLC. The crude peptide thioester 3b was purified by preparative RP-HPLC using Phenomenex Jupiter® C4 column (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–70% CH₃CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing desired product were pooled and lyophilized to give 3b (45 mg, 46% yield corresponding from 100 mg of hydrazide peptide). The m/z calculated for 3b C₁₇₂H₂₇₄N₄₅O₆₁S₆ [M+3H]³⁺: 1379.2677 Da, measured: 1379.2747 Da.

![Retention time vs. m/z](image)

- a) Thioester formation at 0 h; b) Thioester formation at 30 min; c) Purified peptide thioester 3b

### 3.3 Synthesis of Cys(Acm) protected peptide 4

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H₂N | O
--- |---|
| H  | S(Acm)
| S(Acm) | S(Acm)
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H₂N SVIYRTG CRGYYHVSPRA COOH
The cysteine peptide 4 was prepared on 2-chloro trityl chloride resin resin preloaded with Fmoc-Ala-OH (0.41 mmol/g loading). After the automated SPPS procedure, the resin was subjected to cleavage using a mixture of 95:2.5:2.5 TFA:TIPS:H₂O (20 mL/g resin) for 2 h at room temperature. The crude TFA solution was separated from the resin by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with cooled Et₂O (ca. 25 mL/g resin), centrifuged and the supernatant was removed by decantation. This trituration/washing step was repeated twice. The crude peptide was dissolved in aqueous CH₃CN with 0.1% TFA and purified by preparative RP-HPLC using on a Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–70% CH₃CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The pure product fractions were pooled and lyophilized to give 260 mg of 4 (obtained from 1 g of dried resin after SPPS). The m/z calculated for 4 C₁₀₈H₁₇₂N₃₆O₂₉S₃ [M+3H]⁺: 844.7438 Da, measured: 844.7476 Da.

3.4 Synthesis of Cys(Acm) protected EPF2 protein 5a by native chemical ligation

The cysteine peptide 4 (15 mg, 5.9 µM, 1.1 equiv) and the peptide thioester 3a (20 mg, 5.4 µM, 1.0 equiv) were dissolved in 2.5 mL of ligation buffer containing 6 M Gdn•HCl, 200 mM Na₂HPO₄, 100 mM TCEP, 100 mM sodium ascorbate, 3% (v/v) thiophenol, pH 7.4, and the reaction mixture was stirring at room temperature for 16 h. The progress of the reaction was monitored by analytical RP-HPLC. After completion of the reaction, the crude ligated peptide 5a
was purified by preparative RP-HPLC using Phenomenex Jupiter® C4 column (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing the desired product were pooled and lyophilized to give 5a (22 mg, 67% yield). The m/z calculated for 5a C₂₅₈H₄₁₄N₇₇O₈₁S₈ [M+5H]⁵⁺: 1228.5676 Da, measured: 1228.5675 Da.

![Retention time (min)](image)

(a) NCL at 0 h; b) NCL at 16 h; c) Purified linear protein 5a

3.5 Synthesis of Cys(Acm) protected EPF2 protein 5b by native chemical ligation

The cysteine peptide 4 (15 mg, 5.9 μM, 1.1 equiv) and the peptide thioester 3b (20 mg, 4.8 μM, 1.0 equiv) were dissolved in 2.5 mL of ligation buffer containing 6 M Gdn•HCl, 200 mM Na₂HPO₄, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.4, and the reaction mixture was stirring at room temperature. The progress of the reaction was monitored by analytical RP-HPLC. After completion of the reaction, the crude ligated peptide 5b was purified by preparative RP-HPLC using Phenomenex Jupiter® C4 column (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing the desired product were pooled and lyophilized to give 5b (21.5 mg, 68% yield). The m/z calculated for 5b C₂₇₇H₄₃₉N₈₁O₈₈S₈ [M+4H]⁴⁺: 1641.0028 Da, measured: 1641.0009 Da.
4. Chemical synthesis of cysteine protected EPF1 proteins

4.1 Synthesis of Cys(Acm) protected α-ketoacid peptide 8a

The α-ketoacid peptide 8a was synthesized on aminomethyl polystyrene resin preloaded with Fmoc-protected-Val-α-ketoacid (0.25 mol/g loading) by automated Fmoc SPPS through peptide synthesizer. Gly-Ser isoacyl dipeptide and amino acid light after the Gly-Ser isoacyl dipeptide (Ala1 and Cys12) were introduced manually in-between the peptide sequence using HOBT (0.3 mmol, 1.0 equiv) and DIC (0.3 mmol, 1.0 equiv) coupling reagents for 2 h at room temperature.

After completion of the SPPS, the resin was dried and placed in a glass vial and mixture of 95:2.5:2.5 TFA:DODT:H2O (20 mL/g resin) was added, the suspension was shaken at room temperature. After 2 h, the crude TFA solution was separated from resin by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with cooled Et2O (ca. 15 mL/g resin), centrifuged and the supernatant was removed by decantation (peptide precipitating out). The trituration/washing step was repeated twice. The crude peptide dissolved in aqueous CH3CN with 0.1% TFA and purified by preparative RP-HPLC using a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm) at room temperature with a gradient of 20–80% CH3CN (with 0.1% TFA) in 30 min, flow rate 40 mL/min and re-purified using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 30 mm I.D × 250 mm) with a gradient of 20–70%
CH₃CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing the desired product were pooled and lyophilized to give 102 mg of peptide 8a (obtained from 1 g of dried resin after SPPS). The m/z calculated for 8a C₁₄₁H₂₃₄N₄₃O₄₅S₅ [M+3H]³⁺: 1136.5310 Da, measured: 1136.5355 Da.

4.2 Synthesis of Cys(Acm) protected α-ketoacid peptide 8b

The α-ketoacid peptide 8b was synthesized on aminomethyl polystyrene resin preloaded with Fmoc-protected-Val-α-ketoacid (0.25 mol/g loading) by automated Fmoc SPPS through peptide synthesizer. Gly-Ser isoacyl dipeptide and amino acid light after the Gly-Ser isoacyl dipeptide (Ala1 and Cys12) were introduced manually in-between the peptide sequence using HOBT (0.3 mmol, 1.0 equiv) and DIC (0.3 mmol, 1.0 equiv) coupling reagents for 2 h at room temperature.

After automated Fmoc SPPS, Fmoc-Orn HA (1.1 equiv, 0.33 mmol) was manually coupled using HATU (1.1 equiv 0.33 mmol) and NMM (2.2 equiv, 0.66 mmol) in anhydrous DMF for 2 h at room temperature. The resin was dried and placed in a glass vial and mixture of 95:2.5:2.5 TFA:DODT:H₂O (20 mL/g resin) was added, the suspension was shaken at room temperature. After 2 h, the crude TFA solution was separated from resin by filtration and the filtrate was
concentrated under reduced pressure. The residue was triturated with cooled Et₂O (ca. 15 mL/g resin), centrifuged and the supernatant was removed by decantation (peptide precipitating out). The trituration/washing step was repeated twice. The crude peptide dissolved in aqueous CH₃CN with 0.1% TFA and purified by preparative RP-HPLC using a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm) at room temperature with a gradient of 20–80% CH₃CN (with 0.1% TFA) in 30 min, flow rate 40 mL/min and re-purified using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–70% CH₃CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing the desired product were pooled and lyophilized to give 180 mg of peptide 8b (obtained from 1 g of dried resin after SPPS). The m/z calculated for 8b C₁₅₁H₂₅₇N₄₆O₅₅S₅ [M+5H]^{5+:} 752.5501 Da, measured: 752.5533 Da.

4.3 Synthesis of Cys(Acm) protected 5-oxaproline peptide 9

![Retention time (min)](image)

5-Oxaproline peptide 9 was prepared on HMPB-ChemMatrix resin preloaded with Fmoc-Pro-OH (0.30 mmol/g loading). After automated Fmoc SPPS, Boc-Opr (1.5 equiv) was coupled at N-terminus using HATU (1.4 equiv) and NMM (3.0 equiv) in dry DMF for 4 h at room temperature to complete the SPPS. The resin was washed several times with DMF followed by CH₂Cl₂, dried and subjected to cleavage using a mixture of 95:2.5:2.5 TFA:TIPS:H₂O (20 mL/g resin) for 2 h at room temperature. The crude TFA solution was separated from the resin by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with cooled Et₂O (ca.
25 mL/g resin), centrifuged and the supernatant was removed by decantation. This trituration/washing step was repeated twice (peptide precipitating out). The crude peptide was dissolved in aqueous CH$_3$CN with 0.1% TFA and purified by RP-HPLC using a Shiseido Capcell pak C18 UG80 (50 mm I.D. × 250 mm) at room temperature with a gradient of 15–55% CH$_3$CN (with 0.1% TFA) in 20 min, flow rate 40 mL/min and re-purified by a Phenomenex Jupiter C18 column (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–70% CH$_3$CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The pure product fractions were cooled and lyophilized to obtain 160 mg of desired 5-oxaproline peptide 9 (obtained from 1 g of dried resin after SPPS). The purity and identity of peptide 9 was confirmed using analytical HPLC and ESI-HRMS. The m/z calculated for peptide 9 C$_{115}$H$_{182}$N$_{29}$O$_{37}$S$_{3}$ [M+3H]$^{3+}$: 885.7466 Da, measured: 885.7502 Da.

4.4 Synthesis of Cys(Acm) protected EPF1 protein 11a by KAHA ligation

5-Oxaproline peptide 9 (23.4 mg, 8.8 μmol, 1.5 equiv) and α-ketoacid peptide 8a (20 mg, 5.9 μmol, 1.0 equiv) were weighed into a glass vial and dissolved in a mixture of 9:1 DMSO/H$_2$O (20 mM peptide concentration of 8a) with 0.1 M oxalic acid. The mixture was heated to 60 °C for 30 h. After 30 h, the reaction was subjected to O to N acyl shift by dilution to 10-fold volume with 6.0 M Gdn•HCl solution set to pH 9.6 and mixture was stirred at room temperature for 4 h. The reaction mixture was purified by preparative RP-HPLC using Phenomenex Jupiter C18 column (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–70% CH$_3$CN (with
0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing the desired product were pooled and lyophilized to give pure 11a (26 mg, 54% yield). m/z calculated for 11a C_{255}H_{416}N_{72}O_{80}S_{8} [M+6H]^{6+}: 1003.8072 Da, measured: 1003.8108 Da.

![ retention time (min) ]

(a) KAHA Ligation at 0 h; b) KAHA Ligation at 30 h; c) O to N acyl shift at 4 h; d) Purified linear protein 11a

4.5 Synthesis of Cys(Acm) protected EPF1 protein 11b by KAHA ligation

5-Oxoproline peptide 9 (23.4 mg, 8.8 μmol, 1.5 equiv) and α-ketoacid peptide 8b (20 mg, 5.3 μmol, 1.0 equiv) were weighed into a glass vial and dissolved in a mixture of 9:1 DMSO/H_{2}O (20 mM peptide concentration of 8b) with 0.1 M oxalic acid. The mixture was heated to 60 °C for 30 h. After 30 h, the reaction was subjected to O to N acyl shift by dilution to 10-fold volume with 6.0 M Gdn•HCl solution set to pH 9.6 and mixture was stirred at room temperature for 4 h. The reaction mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 30 mm I.D. × 250 mm) with a gradient of 20–70% CH_{3}CN (with 0.1% TFA) in 30 min with 20 mL/min flow rate. The fractions containing the desired product were pooled and lyophilized to give pure 11b (21 mg, 62% yield). m/z calculated for 11b C_{271}H_{437}N_{75}O_{80}S_{8} [M+6H]^{6+}: 1062.3310 Da, measured: 1062.3295 Da.
5. Deprotection of Acm group and protein folding

5.1 General procedure of Acm deprotection

Cysteine Acm protected proteins were dissolved in 50% aq. acetic acid (v/v) containing 1% (w/v) AgOAc (1 mM of the linear protein concentration) and the mixture was stirred at 45 °C for 2 h. The mixture was quenched with 10% DTT in 50% aq. acetic acid (w/v/v), and the precipitation was separated by centrifugation. The precipitate was washed with 50% aq. acetic acid solution (v/v) and the combined supernatant was purified by preparative RP-HPLC.

5.2 Synthesis of reduced EPF2 protein 6a

\[
\begin{align*}
\text{H}_2\text{N} – &\text{TGSSLPDYCAGACSPCKRV-Nle-} \text{ISFECSVAE} – \text{HO} – \text{VIYRCTCGRGYYVPSRA} – \text{COOH}
\end{align*}
\]

The reduced peptide 6a was synthesized according to the general procedure 5.1 using Cys(Acm) Protected linear protein 5a (10 mg, 1.63 μM, 1.0 equiv). at 45 °C. The obtained solution was purified by preparative HPLC using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. x 250 mm) preheated to 60 °C, with a gradient of 30–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired peptide were pooled and lyophilized to give pure 6a (6.5 mg, 70% yield). The m/z calculated for 6a C_{237}H_{379}N_{70}O_{74}S_{8}[M+5H]^{5+}: 1129. 1157 Da; measured: 1129.1156 Da.
5.3 Synthesis of reduced EPF2 protein 6b

The reduced peptide 6b was synthesized according to the general procedure 5.1 using Cys(Acm) Protected linear protein 5b (10 mg, 1.0 equiv) at 45 °C. The obtained solution was purified by preparative HPLC using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) preheated to 60 °C, with a gradient of 30–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired peptide were pooled and lyophilized to give pure 6b (6.6 mg, 72% yield). The m/z calculated for 6b C₂₅₆H₄₀₄N₇₄O₈₁S₈[M+4H]⁺⁺: 1516.6878 Da; measured: 1516.6868 Da.
5.4 Synthesis of reduced EPF1 protein 12a

The reduced protein 12a was synthesized according to the general procedure 5.1 using Cys(Acm) protected linear peptide 11a (10 mg, 1.6 μmol, 1.0 equiv). The obtained mixture was purified by preparative HPLC using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 30–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired peptide were pooled and lyophilized to give pure peptide 12a (5.9 mg, 65% yield). The m/z calculated for 12a C_{231}H_{376}N_{64}O_{72}S_{6}[M+6H]^{6+}: 909.0910 Da; measured: 909.0894 Da.
5.5 Synthesis of reduced EPF1 protein 12b

The reduced protein 12b was synthesized according to the general procedure 5.1 using Cys(Acm) protected linear peptide 11b (10 mg, 1.72 μmol, 1.0 equiv). The obtained mixture was purified by preparative HPLC using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 30–80% CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the desired peptide were pooled and lyophilized to give pure peptide 12b (6.6 mg, 70% yield). The m/z calculated for 12b C247H397N67O78S9[M+6H]+: 967.6149 Da; measured: 967.6135 Da.

5.6 General procedure of protein folding

The Acm-deprotected, reduced linear protein was denatured using denatured buffer containing 6 M Gdn•HCl + 0.1 M Tris•HCl buffer, pH 6.8 (0.5 mM peptide concentration) stirred at room temperature for 1 h open to air. After 1 h, the mixture was diluted with 8-fold volume of folding buffer containing 5.0 mM of reduced glutathione and 2.5 mM oxidized glutathione. The reaction was incubated in a shaker with slow movement at 4 °C for 30 h. The folding progress was
monitored by analytical RP-HPLC. The resulting solution was acidified with aqueous HCl adjust to pH 4–5 and purified by preparative HPLC.

5.7 Synthesis of EPFL9 protein 2a

The folded EPFL9 protein 2a was synthesized according to general procedure 5.6 using reduced linear protein 1a (10 mg, 1.9 µM). The folding buffer pH 8.0 was used. The resulting reaction mixture was purified by preparative HPLC using a Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. x 250 mm) with the gradient 10–60% CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing desired product were pooled and lyophilized to give pure folded EPFL9 2a (5.8 mg, 58% yield). The m/z calculated for 2a C213H332N68O67S6 [M+7H]7+: 731.4711 Da, measured: 731.4706 Da.

a) Folding at 0 h; b) Folding at 30 h; c) Purified Folded EPFL9 Protein 2a

5.8 Synthesis of EPFL9 protein 2b

The folded EPFL9 protein 2b was synthesized according to general procedure 5.6 using reduced linear protein 1b (10 mg, 1.8 µM). The folding buffer pH 8.0 was used. The resulting reaction mixture was purified by preparative HPLC using a Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. x 250 mm) with the gradient 10–60% CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing desired product were pooled and lyophilized to give pure folded EPFL9, 2b (4.7 mg, 47% yield). The m/z calculated for 2b C232H358N73O74S6 [M+7H]7+: 791.7826 Da, measured 791.7817 Da.
**5.9 Synthesis of EPF2 protein 7a**

Folded EPF2 protein 7a was synthesized according to the general procedure 5.6 using reduced protein 6a (2 mg, 0.35 μM). The folding buffer pH 7.0 was used. The resulting solution was purified by preparative RP-HPLC using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing desired product were pooled and lyophilized to give pure folded EPF2 7a (1.3 mg, 55% yield). The m/z calculated for folded EPF2 protein 7a C₂₃₇H₃₇₁N₇₀O₇₄S₈ [M+5H]⁺: 1127.5032 Da; measured: 1127.5030 Da.

**5.10 Synthesis of EPF2 protein 7b**

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a) Folding at 0 h; b) Folding at 30 h; c) Purified protein 2b

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a) Folding at 0 h; b) Folding at 30 h; c) Purified EPF2 Protein 7a
Folded EPF2 protein 7b was synthesized according to the general procedure 5.6 using reduced protein 6b (2 mg, 0.33 μm). The folding buffer pH 7.0 was used. The resulting solution was purified by preparative RP-HPLC using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing desired product were pooled and lyophilized to give pure folded EPF2 7a (1.2 mg, 60% yield). The m/z calculated for folded EPF2 protein 7b C_{25q}H_{39q}N_{7q}O_{81}S_{8} [M+4H]^{4+}: 1514.6722 Da; measured: 1514.6682 Da.

a) Folding at 0 h; b) Folding at 30 h; c) Purified EPF2 Protein 7b

5.11 Synthesis of EPF1 protein 13a

Folded EPF1 protein 13a was synthesized according to the general procedure 5.6 with 1 mg of reduced linear protein 12a. The folding buffer pH 8.0 was used. The resulting mixture was purified by preparative HPLC using a Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the folded protein were pooled and lyophilized to give pure folded EPF1 13a (0.55 mg, 55% yield). The m/z calculated for folded EPF1 protein 13a C_{23q}H_{36q}N_{6q}O_{7q}S_{8} [M+6H]^{6+}: 907.7472 Da; measured: 907.7461 Da.
5.12 Synthesis of EPF1 protein 13b

Folded EPF1 protein 13b was synthesized according to the general procedure 5.6 with 1 mg of reduced linear protein 12b. The folding buffer pH 8.0 was used. The resulting mixture was purified by preparative HPLC using a Phenomenex Jupiter C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) CH$_3$CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing the folded protein were pooled and lyophilized to give pure folded EPF1, 13b (0.6 mg, 62% yield). The m/z calculated for folded EPF1 protein 13b C$_{247}$H$_{389}$N$_{67}$O$_{78}$S$_8$ [M+6H]$^{6+}$: 966.2711 Da; measured: 966.2699 Da.

6. Synthesis of potassium acyltrifluoroborates (KATs) compounds

6.1 Synthesis of Azido-KAT 15
The sodium hydride (60% disperse in mineral oil, 9.6 mmol, 2.0 equiv) was added to the solution of 2-(2-azidoethoxy)ethan-1-ol (4.8 mmol, 1.1 equiv) in anhydrous DMF (15.0 mL) stirred at room temperature for 30 minutes. The 4-fluoro Phenyl KAT (4.34 mmol, 1.0 equiv) was added to the reaction mixture and stirred at 60 °C. After 16 h, saturated aqueous KF (0.5 mL) was added dropwise to the reaction mixture and stirred for 30 min at room temperature. The reaction mixture was filtered and washed with CH$_2$Cl$_2$ (3 × 50 mL) and acetone (3 × 50 mL). The remaining pale-yellow precipitate (containing 15 and inorganic salts) was washed multiple times with DMF (typically 200 – 250 mL) until the precipitate become colorless. The filtrate containing CH$_2$Cl$_2$, acetone and DMF was concentrated under reduced pressure to yield the product 15 as a yellow solid. (1.35 g, 91 %yield). $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ = 8.07 (d, $J$ = 8.4 Hz, 2H), 6.93 (d, $J$ = 9.2 Hz, 2H), 4.22 (t, $J$ = 4.8 Hz, 2H), 3.88 (t, $J$ = 5.2 Hz, 2H), 3.76 (t, $J$ = 5.2 Hz, 2H), 3.42 (t, $J$ = 4.8 Hz, 2H). $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ = 161.4, 130.6, 113.4, 70.0, 69.3, 67.5, 50.5. $^{19}$F NMR (376 MHz, acetone-$d_6$): $\delta$ = -144.5. HRMS (ESI neg.): Exact mass calcd for C$_{11}$H$_{12}$BF$_3$N$_3$O$_3$ [M–K$^-$]: 302.0929, found: 302.0926.
$^1$H NMR (400 MHz, acetone-$d_6$)

$^{13}$C NMR (100 MHz, acetone-$d_6$)
**19F NMR (376 MHz, acetone-\textit{d6})**

**ESI-HRMS**
6.2 General procedure of Dye-KAT synthesis by Huisgen cycloaddition

Alkyne functionalized dyes 14a–e (1.0 equiv), azido ethoxy ethyl KAT 15 (1.0 equiv), copper iodide (1.0 equiv) was dissolved in 50% aqueous CH$_3$CN and triethylamine (3.0 equiv) was added, stirred at 65 °C. After 16 h, the reaction mixture cooled to room temperature and aqueous KF solution (0.5 mL) was added, stirred another 15 min at room temperature. The crude mixture was diluted with brine solution and extracted with CH$_2$Cl$_2$ or ethyl acetate. The organic extracts were collected, dried with Na$_2$SO$_4$, and evaporated in vacuo. The crude residue was purified by column chromatography on silica gel (eluting with acetone/CH$_2$Cl$_2$ or CH$_3$CN/water) to give 16a–e as solid.

6.3 Synthesis of dansyl-KAT 16a

![Chemical structure of dansyl-KAT 16a]

The product 16a was synthesized according to the general procedure 6.2 using 5-(dimethylamino)-N-(prop-2-yn-1-yl)naphthalene-1-sulfonamide$^{[6,7]}$ 14a (100 mg, 0.35 mmol, 1.0 equiv) and (4-(2-(2-azidoethoxy)ethoxy)phenyl)(trifluoro-λ$_4$-boraneyl)methanone, potassium salt 15 (118 mg, 0.35 mmol, 1.0 equiv), CuI (66 mg, 0.35 mmol, 1.0 equiv) and Et$_3$N (97.5 μL, 0.7 mmol, 2.0 equiv) dissolved in 3.5 mL of 50% aqueous CH$_3$CN. White solid, isolated yield 120 mg (0.19 mmol, 55%). $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ = 8.52 (d, $J$ = 8.8 Hz, 1H), 8.36 (d, $J$ = 8.8 Hz, 1H), 8.19 (dd, $J$ = 7.6, 1.2 Hz, 1H), 8.06 (d, $J$ = 9.2 Hz, 2H), 7.57–7.46 (m, 3H), 7.24 (d, $J$ = 1.5 Hz, 1H), 7.24 (d, $J$ = 9.2 Hz, 2H), 7.37 (t, $J$ = 5.6 Hz, 2H), 4.18–4.12 (m, 4H), 3.81–3.75 (m, 4H), 2.85 (s, 6H). $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ = 161.8, 151.7, 143.7, 136.3, 131.7, 130.9, 129.8, 129.7, 129.1, 127.9, 123.4, 123.1, 119.7, 115.3, 114.3, 113.6, 69.3, 69.2, 67.3, 49.6, 44.8, 38.5. $^{19}$F NMR (376 MHz, acetone-$d_6$): $\delta$ = 145.2. HRMS (ESI neg.): Exact mass calcd for C$_{26}$H$_{28}$BF$_3$N$_2$O$_5$S [M–K]$^-$: 509.1867, found 590.1863.
$^1$H NMR (400 MHz, acetone-$d_6$)

$^{13}$C NMR (100 MHz, acetone-$d_6$)
$\text{${}^{19}\text{F NMR (376 MHz, acetone-}d_6\text{)}$}$

![Chemical structure]

**ESI-HRMS**

![Mass spectrum]

**N**: parts per Million; **Fluorine**$^{19}$
6.4 Synthesis of coumarin-KAT 16b

The product 16b was synthesized according to the general procedure 6.2 using 2-oxo-N-(prop-2-yn-1-yl)-2H-chromene-3-carboxamide[8] 14b (79.5 mg, 0.35 mmol, 1.0 equiv) and (4-(2-azidoethoxy)ethoxy)phenyl)(trifluoro-λ4-boraneyl)methanone, potassium salt 15 (118 mg, 0.35 mmol, 1.0 equiv), CuI (66 mg, 0.35 mmol, 1.0 equiv) and Et₃N (97.5 µL, 0.7 mmol, 2.0 equiv) dissolved in 3.5 mL of 50% aqueous CH₃CN. White solid, isolated yield 160 mg (0.28 mmol, 81%). ¹H NMR (400 MHz, acetone-d6): δ = 9.13 (s, 1H), 8.92 (s, 1H), 8.07 (d, J = 8.8 Hz, 2H), 7.95–7.93 (m, 2H), 7.73 (dd, J = 8.8, 1.4 Hz, 2H), 7.45 (t, J = 8.0 Hz, 2H), 6.89 (d, J = 9.2 Hz, 2H), 4.66 (d, J = 5.6 Hz, 2H), 4.59 (t, J = 4.8 Hz, 2H), 4.17 (t, J = 4.8 Hz, 2H), 3.96 (t, J = 5.6 Hz, 2H), 3.84 (t, J = 4.8 Hz, 2H). ¹³C NMR (100 MHz, acetone-d6): δ = 161.3, 154.6, 148.1, 134.1, 130.5, 130.3, 125.2, 116.3, 113.4, 69.5, 69.4, 67.2, 49.9, 49.9, 35.2. ¹⁹F NMR (376 MHz, acetone-d6): δ = -144.1. HRMS (ESI neg.): Exact mass calcd C₂₄H₂₁BF₃N₄O₆[M–K]⁻: 529.1516, Found 529.1505.

¹H NMR (400 MHz, acetone-d6)
$^{13}$C NMR (100 MHz, acetone-$d_6$)

$^{19}$F NMR (376 MHz, acetone-$d_6$)
6.5 Synthesis of pyrene-KAT 16c

The product 16c was synthesized according to the general procedure 6.2 using 1-ethynylpyrene 14c (79.0 mg, 0.35 mmol, 1.0 equiv) and (4-(2-(2-azidoethoxy)ethoxy)phenyl)(trifluoro-λ4-boraneyl)methanone, potassium salt 15 (118 mg, 0.35 mmol, 1.0 equiv), CuI (66 mg, 0.35 mmol, 1.0 equiv) and Et₃N (97.5 μL, 0.7 mmol, 2.0 equiv) dissolved in 3.5 mL of 50% aqueous CH₃CN. White solid, isolated yield 142 mg (0.25 mmol, 72%). ¹H NMR (400 MHz, acetone-d₆): δ = 8.93 (d, J = 7.6 Hz, 1H), 8.53 (s, 1H), 8.29–8.24 (m, 5H), 8.17–8.13 (m, 2H), 8.06–8.02 (m, 3H), 6.89 (J = 7.6 Hz, 2H), 4.80 (t, J = 4.0 Hz, 2H), 4.23 (t, J = 4.0 Hz, 2H), 4.11 (t, J = 4.8 Hz, 2H), 3.93 (t, J = 4.0 Hz, 2H). ¹³C NMR (100 MHz, DMSO-d₆): δ = 160.9, 146.6, 130.4, 127.9, 127.0, 125.7, 125.6, 125.4, 125.3, 113.9, 69.3, 67.6, 67.4, 50.2. ¹⁹F NMR (376 MHz, acetone-d₆): δ = 143.5. HRMS (ESI neg.): Exact mass calcd for C₂₉H₂₂BF₃N₃O₃ [M–K]⁻: 528.1717, found 528.1707.
$^{19}$F NMR (376 MHz, acetone-$d_6$)

ESI-HRMS
The product 16d was synthesized according to the general procedure 6.2 using 5,5-difluoro-1,3,7,9-tetramethyl-10-(4-(prop-2-yn-1-yloxy)phenyl)-5H-4λ4,5λ4-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine[9] 14d (132.0 mg, 0.35 mmol, 1.0 equiv) and (4-(2-(2-azidoethoxy)ethoxy)phenyl)(trifluoro-λ4-boraneyl)methanone, potassium salt 15 (118 mg, 0.35 mmol, 1.0 equiv), CuI (66 mg, 0.35 mmol, 1.0 equiv) and Et₃N (97.5 μL, 0.7 mmol, 2.0 equiv) dissolved in 3.5 mL of 50% aqueous CH₃CN. Green solid, isolated yield 175 mg (0.26 mmol, 70%).

**1H NMR (400 MHz, DMSO-d6):** δ = 8.2 (s, 1H), 7.83 (d, J = 8.8 Hz, 1H), 7.23 (d, J = 8.4 Hz, 2H), 7.16 (d, J = 8.4 Hz, 2H), 6.85 (d, J = 9.2 Hz, 1H), 6.13 (s, 2H), 5.12 (s, 2H), 4.55 (t, J = 5.2 Hz, 2H), 4.06 (t, J = 4.8 Hz, 2H), 3.86 (t, J = 4.8 Hz, 2H), 3.73 (t, J = 4.8 Hz, 2H), 2.41 (s, 6H), 1.36 (s, 6H).

**13C NMR (100 MHz, DMSO-d6):** δ = 160.9, 159.3, 155.2, 143.3, 142.7, 131.6, 130.4, 129.7, 126.8, 125.7, 121.8, 116.0, 113.9, 69.3, 69.2, 67.4, 61.7, 49.9, 14.7.

**19F NMR (376 MHz, DMSO-d6):** δ = −141.2, −143.6; HRMS (ESI neg.): Exact mass calcd for C₃₃H₃₃B₂F₅N₅O₄ [M–K]⁻: 680.2655, found 680.2648.
**19F NMR (376 MHz, DMSO-d6)**

ESI-HRMS

**X**: parts per Million | **Fluorine 19**
6.7 Synthesis of Rhodamine-KAT 16e

The product 16e was synthesized according to the general procedure 6.2 using prop-2-yn-1-yl 2-(3-(diethyl-\(\lambda_4\)-azaneylidene)-6-(diethylamino)-3\(H\)-xanthen-9-yl)benzoate\cite{10} 14e (168.0 mg, 0.35 mmol, 1.0 equiv) and (4-(2-(2-azidoethoxy)ethoxy)phenyl)(trifluoro-\(\lambda_4\)-boraneyl)methanone, potassium salt 15 (118 mg, 0.35 mmol, 1.0 equiv), CuI (66 mg, 0.35 mmol, 1.0 equiv) and Et\(_3\)N (97.5 \(\mu\)L, 0.7 mmol, 2.0 equiv) dissolved in 3.5 mL of 50% aqueous CH\(_3\)CN. Red solid, isolated yield 183 mg (0.26 mmol, 64%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 8.21\) (dd, \(J = 8.0, 1.2\) Hz, 1H), 7.92–7.72 (m, 5H), 7.48 (d, \(J = 8.8\) Hz, 1H), 7.00 (dd, \(J = 8.4, 1.6\) Hz, 2H), 6.95–6.90 (m, 4H), 6.84–6.81 (m, 2H), 5.04 (s, 2H), 4.48 (t, \(J = 5.2\) Hz, 2H), 4.07 (t, \(J = 4.4\) Hz, 2H), 3.83 (t, \(J = 4.8\) Hz, 2H), 3.72 (t, \(J = 4.8\) Hz, 2H), 3.63 (q, \(J = 7.6\) Hz, 8H), 1.21 (t, \(J = 7.6\) Hz, 12H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta = 165.0, 160.8, 157.9, 157.6, 155.6, 141.2, 135.7, 133.8, 131.3, 131.0, 130.3, 129.7, 125.4, 115.0, 114.7, 113.8, 113.4, 96.4, 69.1, 67.4, 58.7, 49.8, 45.8 12.9; \(^{19}\)F NMR (376 MHz, DMSO-\(d_6\)): \(\delta = -145.19\). HRMS (ESI neg.): Exact mass calcd for C\(_{42}\)H\(_{45}\)BF\(_3\)KN\(_3\)O\(_6\)[M]\(^+\): 822.3054, found 822.3036.
$^1$H NMR (400 MHz, DMSO-\textit{d6})

$^{13}$C NMR (100 MHz, DMSO-\textit{d6})
**19F NMR (376 MHz, DMSO-d6)**

![Chemical structure image]

**ESI-HRMS**

![Mass spectrometry image]
6.8 UV absorption spectra of Dye-KATs

The UV absorption was measured in 50% aqueous CH$_3$CN at room temperature with 50 μM potassium acyltrifluoroborates 15, 16a–e. The observed intense absorption for compound 15 at $\lambda$ (max) = 275 nm, 16a at $\lambda$ (max) = 266 nm, 16b at $\lambda$ (max) = 286 nm, 16c at $\lambda$ (max) = 350 nm, 16d at $\lambda$ (max) = 499 nm, 16e at $\lambda$ (max) = 562 nm.

7. Protein functionalization by KAT ligation

7.1 General Experimental Procedure for Fluorescent Labelling of EPF Proteins by KAT Ligation

Functionalized EPF proteins 2b, 7b, and 13b (1.0 equiv) was dissolved in 50% aqueous CH$_3$CN mixture with 0.1% TFA. The reaction mixture was placed in a closed UV chamber and irradiated with 365 nm UV light at room temperature under dark to deprotect the photo labile group. The photo deprotection was monitored by analytical RP-HPLC. After completion of the deprotection, the dye potassium acyltrifluoroborate (KATs) 15, 16a–e (1.2 equiv) was directly added to the reaction mixture stirred at room temperature for 20 min. The dye conjugated folded EPFs 17a–f, 18a–f and 19a–f were identified in analytical RP-HPLC, and MALDI-TOF. The crude mixture was purified by RP-HPLC.

7.2 Synthesis of dansyl-EPFL9 17a

The dansyl conjugated EPFL9 17a was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPFL9 2b (1 mg, 0.18 μM) and dansyl KAT 16a (0.14 mg, 0.22 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex
Jupiter® C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH$_3$CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 17a (0.54 mg, 52% yield). The purity and identity of the 17a was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 17a C$_{244}$H$_{366}$N$_{76}$O$_{73}$S$_{7}$ [M+4H]$^{4+}$: 1438.1322 Da, measured: 1438.1313 Da.

7.3 Synthesis of coumarin-EPFL9 17b

The coumarin conjugated EPFL9 17b was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPFL9 2b (1 mg, 0.18 µM) and coumarin KAT 16b (0.12 mg, 0.22 µM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH$_3$CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 17b (0.62 mg, 60% yield). The purity and identity of the 17b was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 17b C$_{242}$H$_{359}$N$_{75}$O$_{74}$S$_{6}$ [M+4H]$^{4+}$: 1422.8734 Da, measured 1422.8705 Da.
7.4 Synthesis of pyrene-EPFL9 17c

The pyrene conjugated EPFL9 17c was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPFL9 2b (1 mg, 0.18 μM) and pyrene KAT 16c (0.12 mg, 0.22 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 17c (0.72 mg, 70% yield). The purity and identity of the 17c was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 17c C247H360N70O71S6 [M+4H]4+: 1422.6284 Da, measured: 1422.6269 Da.
7.5 Synthesis of BODIPY-EPFL9 17d

The BODIPY conjugated EPFL9 17d was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPFL9 2b (1 mg, 0.18 µM) and BODIPY KAT 16d (0.16 mg, 0.22 µM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 17d (0.8 mg, 85% yield). The purity and identity of the 17d was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 17d C₂₅₁H₃₇₁BF₂N₇₆O₇₂S₆ [M+4H]⁴⁺: 1460.6524 Da, measured 1460.6505 Da.

7.6 Synthesis of Rhodamine-EPFL9 17e

The rhodamine-B conjugated EPFL9 17e was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPFL9 2b (1 mg, 0.18 µM) and rhodamine-B KAT 16e (0.18 mg, 0.22 µM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 17e (0.90 mg, 84% yield). The purity and identity of the 17e was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 17e C₂₆₀H₃₈₃N₇₆O₇₄S₆ [M+4H]⁵⁺: 1189.1368 Da, measured: 1189.1352 Da.
7.7 Synthesis of Azido-EPFL9 17f

The azido conjugated EPFL9 17f was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPFL9 2b (1 mg, 0.18 μM) and azido KAT 15 (0.07 mg, 0.22 μM). The crude mixture was preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 17f (0.74 mg, 75% yield). The purity and identity of the 17f was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 17f C₂₂₉H₃₅₂N₇₄O₇₁S₆ [M+6H]⁶⁺: 911.0750 Da, measured 911.0786 Da.
7.8 Synthesis of dansyl-EPF2 18a

The dansyl conjugated EPF2 18a was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF2 7b (1 mg, 0.165 μM) and dansyl KAT 16a (0.12 mg, 0.198 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 18a (0.71 mg, 68% yield). The purity and identity of 18a was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 18a C₂₆₈H₄₀₄N₇₆O₈₀S₉ [M+4H]⁴⁺: 1567.9403 Da, measured 1567.9398 Da.

7.9 Synthesis of coumarin-EPF2 18b

The coumarin conjugated EPF2 18b was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF2 7b (1 mg, 0.165 μM) and coumarin KAT 16b (0.11 mg, 0.198 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 18b (0.74 mg, 72% yield). The purity and identity of 18b was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 18b C₂₆₆H₄₀₀N₇₆O₈₁S₈ [M+4H]⁴⁺: 1552.6815 Da, measured 1552.6817 Da.
**7.10 Synthesis of pyrene-EPF2 18c**

The pyrene conjugated EPF2 18c was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF2 7b (1 mg, 0.165 μM) and pyrene KAT 16c (0.11 mg, 0.198 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 18c (0.61 mg, 60% yield). The purity and identity of 18c was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 18c C271H402N75O78S8 [M+4H]4+: 1552.6885 Da, measured 1552.6838 Da.
7.11 Synthesis of BODIPY-EPF2 18d

The BODIPY conjugated EPF2 18d was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF2 7b (1 mg, 0.165 μM) and BODIPY KAT 16d (0.14 mg, 0.198 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 18d (0.76 mg, 72% yield). The purity and identity of 18d was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 18d C₂₇₅H₄₁₂BF₂N₇₇O₇₉S₈ [M+4H]⁴⁺: 1590.4606 Da, measured: 1590.4617 Da.

7.12 Synthesis of Rhodamine-EPF2 18e

The rhodamine-B conjugated EPF2 18e was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF2 7b (1 mg, 0.165 μM) and rhodamine-B KAT 16e (0.16 mg, 0.198 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 18e (0.8 mg, 75% yield). The purity and identity of the 18e was confirmed using analytical RP-HPLC and ESI-HRMS. m/z calculated for 18e C₂₈₄H₄₁₂N₇₇O₈₁S₈ [M+3H]⁴⁺: 1615.9773 Da, measured: 1615.9709 Da.
7.13 Synthesis of Azido-EPF2 18f

The azido conjugated EPF2 18f was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF2 7b (1 mg, 0.165 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 18f (0.7 mg, 67% yield). The purity and identity of the 18f was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 18f C253H393N75O78S8 [M+6H]6+: 997.6137 Da, measured: 997.6176 Da.
7.14 Synthesis of dansyl-EPF1 19a

The dansyl dye conjugated EPF1 19a was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF1 13b (1 mg, 0.173 μM) and dansyl KAT 16a (0.13 mg, 0.207 μM). The crude reaction mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 19a (0.68 mg, 65% yield). The purity and identity of 19a was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 19a C₂₅₉H₃₉₀N₇₀O₇₇S₉ [M+4H]⁴⁺: 1502.1711 Da, measured: 1502.1710 Da.

![Graph](image)

(a) Photo-deprotection at 0 min; b) Photo-deprotection at 30 min; c) KAT Ligation at 20 min; d) Purified 19a

7.15 Synthesis of coumarin-EPF1 19b

The coumarin dye conjugated EPF1 19b was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF1 13b (1 mg, 0.173 μM) and coumarin dye KAT 16b (0.12 mg, 0.207 μM). The crude reaction mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 19b (0.58 mg, 56% yield). The purity and identity of 19b was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 19b C₂₅₇H₃₉₁N₆₉O₇₈S₈ [M+4H]⁴⁺: 1486.9124 Da, measured 1486.9123 Da.
7.16 Synthesis of pyrene-EPF1 19c

The pyrene conjugated EPF1 19c was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF1 13b (1 mg, 0.173 μM) and pyrene dye KAT 16c (0.12 mg, 0.207 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 19c (0.59 mg, 57% yield). The purity and identity of 19c was confirmed using analytical RP-HPLC and ESI-HRMS. m/z calculated for 19c C₂₆₂H₃₉₂N₆₈O₇₅S₈[M+4H]⁴⁺: 1486.6674 Da, measured: 1486.6658 Da.
7.17 Synthesis of BODIPY-EPF1 19d

The BODIPY conjugated EPF1 19d was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF1 13b (1 mg, 0.173 µM) and BODIPY dye KAT 16d (0.15 mg, 0.207 µM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 19d (0.68 mg, 65% yield). The purity and identity of 19d was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 19d C₂₆₆H₄₀₃BF₂N₇₆O₇₆S₈ [M+4H]⁴⁺: 1524.6914 Da, measured 1524.6903 Da.

7.18 Synthesis of Rhodamine-EPF1 19e

The rhodamine-B conjugated EPF1 19e was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF1 13b (1 mg, 0.173 µM) and rhodamine-B dye KAT 16e (0.17 mg, 0.207 µM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 µm, 300 Å pore size, 21.2 mm I.D. × 250 mm) with a gradient of 20–80% CH₃CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 19e (0.64 mg, 60% yield). The purity and identity of 19e was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 19e C₂₇₅H₄₁₅N₇₀O₇₈S₈ [M+4H]⁴⁺: 1550.4601 Da, measured: 1550.4518 Da.
7.19 Synthesis of Azido-EPF1 19f
The azido conjugated EPF1 19f was synthesized according to general procedure 7.1 using folded photo-protected hydroxylamine EPF1 13b (1 mg, 0.173 μM) and azido KAT 15 (0.07 mg, 0.207 μM). The crude mixture was purified by preparative RP-HPLC using Phenomenex Jupiter® C18 column (5 μm, 300 Å pore size, LC column 250 × 21.2 mm) with a gradient of 20–80% CH3CN with 0.1% TFA in 30 min, flow rate 10 mL/min. The fractions containing compounds pooled and lyophilized to give pure 19f (0.61 mg, 62% yield). The purity and identity of 19f was confirmed using analytical RP-HPLC and ESI-HRMS. The m/z calculated for 19f C_{244}H_{383}N_{68}O_{75}S_{8} [M+5H]^{5+}: 1144.3197 Da, measured: 1144.3240 Da.
8. Bioassay

8.1 Plant materials and growth conditions

*Arabidopsis thaliana* accession Columbia (Col) was used for bioassays. Plants were grown at 22 °C under continuous light (36 \( \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)) for 7 days.

8.2 Peptide treatment

Evaluation of peptides was performed as previously described,\[^{[11]}\] in which all peptides and fluorophores were dissolved in DMSO. Arabidopsis Col-0 seeds were sown in 96-well plates (TL5003; True Line) containing 95 \( \mu \text{L} \) of 1/2 Murashige and Skoog (MS) medium[^{[12]}\] with rotary shaking at 140 rpm under continuous light at 22 °C. Five \( \mu \text{L} \) of peptides (or fluorophores) dissolved in DMSO at 1 mM were diluted with liquid 1/2 MS media to 100 \( \mu \text{M} \), and dropped on 1-day-old seedlings (final concentration, 5 \( \mu \text{M} \)). The abaxial epidermis cotyledons of 7-day-old seedling was imaged using confocal microscopy (see below). For visualizing BODIPY-EPFL9 17d, Col-0 seeds were grown for 7 days in liquid 1/2 MS medium, and transferred into new 1/2 MS medium containing 0.6 \( \mu \text{M} \) FM4-64 and 6 nM BODIPY-EPFL9 17d and with or without 6 nM folded-EPFL9 2a for 10 min. with rotary shaking at 40 rpm.

8.3 Confocal microscopy

The Zeiss LSM800 inverted confocal microscope (Oberkochen, Germany) was used for imaging. Cell peripheries were visualized by staining with propidium iodide (PI) (P4170; Sigma-Aldrich) using the following settings: 561 nm laser was used to excite PI. The emission filter was 582–617 nm for PI. For qualitative image presentation, Adobe Photoshop 2021 was used to trim and uniformly adjust the contrast/brightness. For visualization of BODIPY-EPFL9 and FM4-64 (T13320; ThermoFisher), 488 nm laser was used to excite BODIPY and 561 nm laser was used to excite FM4-64. The emission filter was 410–546 nm for BODIPY and 579–617 nm for FM4-64.

8.4 Quantitative analysis of stomatal density

Abaxial cotyledons from 7-day-old seedlings of respected genotypes or treatments were subjected to clearing solution. Specifically, samples were fixed a mixture of ethanol and acetic acid (9:1, \( \text{v/v} \)) at 4 °C and rehydrated in a graded ethanol series (70%, 50% and 30%) for 30 min in each solution, and transferred to clearing solution (a mixture of 8 g of chloral hydrate, 1 mL of glycerol, 2 mL of water) for at least overnight at 4 °C. The cleared samples were spread onto the slide glass
and observed under the microscope (Carl Zeiss AXIO Imager A2.) equipped with differential interference contrast (DIC) optics and the ZEN imaging software (ZEN2.3 Lite). The central regions overlying the distal vascular loop were imaged and numbers of stomata were quantified. For each genotype or chemical treatment, sample size of 7 to 10 was used and over thousand epidermal cells were counted to provide statistical robustness. Statistical analysis (ANOVA after Tukey’s HSD test) was performed using RStudio (www.rstudio.com) version 1.4.1717 for stomatal density.

8.5 Quantitative analysis of bioactivity of fluorophores on stomatal formation

**Figure S1.** Fluorophores do not affect stomatal development. (A) representative confocal images of cotyledon abaxial epidermis from the 7-day-old Arabidopsis wild type Col-0 seedlings treated with mock, 5 μM fluorophore-KAT 16a–e, For the BODIPY-KAT 16d treatment, the image for 10 μM treatment is also shown. Scale bar = 50 μm. (B) quantitative analysis of the number of stomata shown as a box plot. Dots, individual data points. Median values are shown as lines in
the boxplot. ANOVA after Tukey’s HSD test was performed for comparison of samples treated with the mock and each fluorophore. Number of leaves analyzed, n=8, 9, 10, 8, 8, 8, 9 for treatment with mock, 5 μM dansyl-KAT 16a, 5 μM coumarin-KAT 16b, 5 μM pyrene-KAT 16c, 5 μM BODIPY-KAT 16d, 10 μM BODIPY-KAT 16d, 5 μM rhodamine B-KAT 16e, respectively. All the same letters indicate no significant difference (P > 0.05).

9. References

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