Peptide 2-formylthiophenol esters do not proceed through a Ser/Thr ligation pathway, but participate in a peptide aminolysis to enable peptide condensation and cyclization

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Materials and Methods. All commercial materials (Aldrich, Chemimpex and GL Biochem) were used without further purification. All solvents were reagent grade or HPLC grade (RCI or DUKSAN). The following Fmoc amino acids were purchased from GL Biochem and Chemimpex and used in the solid phase synthesis: Fmoc-Ala-OH, Fmoc-d-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Pro-OH, Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Trp(Boc)-OH. All separations involved a mobile phase of 0.05 % TFA (v/v) in acetonitrile and 0.05 % TFA (v/v) in water. HPLC separations were performed with Waters HPLC system equipped with photodiode array detector (Waters 2996) using Vydac 218TP C18 column (5µm, 300 Å, 4.6 x 250 mm) at a flow rate of 0.6 ml/min for analytical HPLC and XBridge Prep C18 10 µm OBD column (10 μ m, 300 Å, 30 x 250 mm) at a flow rate of 15 ml/min for preparative HPLC. Mass spectral analyses were performed with Water 3100 mass spectrometer. ¹H and ¹³C NMR spectra were recorded on Bruker Advance DRX 300 FT-NMR spectrometer at 300 MHz for ¹H NMR and 75 MHz for ¹³C NMR.



Figure S1. The synthesis of 2-mercaptobenzyaldehyde.

Synthesis of 2,2'-dithiodibenzaldehyde. Oxidation of 2-mercaptobenzyl alchohol 1 with PCC was done according to a literature reported with modifications.¹ In a 100 mL round-bottomed flask with a magnetic stirrer and filled with argon, PCC (11.53 g, 53.50 mmol) was added in dry CH_2Cl_2 (50 mL). A solution of alcohol 1 (3.00 g. 21.40 mmol) in dry CH_2Cl_2 (10 mL) is added slowly to the stirred mixture. After stirring for 4 h at room temperature, the black gum was washed with CH_2Cl_2 (3 x 15 mL). The combined organic solution was passed through a short pad of Celite. Solvent was

condensed at room temperature under reduced pressure gave 2 and was directly used in the next step without further purification.

Synthesis of 2-mercaptobenzaldehyde: In a round-bottomed flask with a magnetic stirrer and filled with argon, crude 2,2'-dihiodibenzaldehyde 2 in DMF (50 mL) was added. MeOH (50 mL) and water (30 mL) were added to the stirred solution. Ph₃P (8.42 g 32.10 mmol) was added in portion and the mixture was stirred for 30 min at room temperature. The mixture was then cooled in an ice-water bath with stirring for 0.5 h. The supernatant was extracted twice with Et₂O. The combined Et₂O layer was washed with cold water twice and was dried with Na₂SO₄. The dried Et₂O solution was concentrated in a rotary evaporator to a volume of 2-3 mL at 0°C. Column chromatography (hexane-ethyl acetate 5:1) of the residue affords **3** (1.66 g, 56 % over two steps).

¹H NMR (300 MHz, CDCl₃) δ 10.06 (s, 1H), 7.73 (d, 1H, J = 7.6 Hz), 7.23-7.73 (m, 3H), 5.50 (br s,1H); ¹³C NMR (300 MHz, CDCl₃) δ 190.30, 133.66, 133.66, 130.88, 128.60, 128.60, 122.43.



Figure S2. Protection of the aldehyde group on 2-mercaptobenzaldehyde.

Synthesis of 2-mercaptobenzaldehyde ethylene acetal.² A solution of 2-mercaptobenzaldehyde (3, 1.66 g, 12.01 mmol) and p-toulenesulfonic acid (1.02 g, 6.01 mmol) in benzene (60 mL) and ethylene glycol (63.46 g, 120.10 mmol) was heated under reflux at 90 degrees for 2 h under nitrogen with the removal of water by a Dean-Stark apparatus. The mixture was then washed with water, dried over Na₂SO₄ and concentrated. Column chromatography on silica gel (hexane-ethyl acetate 5:1) of the residue afford 4 (1.71 g, 78 %).

¹H NMR (300 MHz, CDCl₃) δ 7.52-7.55 (m, 1H), 7.31-7.34 (m, 1H), 7.19-7.22 (m, 2H), 6.01 (s, 1H), 4.02-4.18 (m, 4H), 3.75 (s, 1H); ¹³C NMR (300 MHz, CDCl₃) δ 128.93, 126.89, 126.89, 124.36, 124.36, 123.38, 99.49, 62.80, 62.80.

Solid phase peptide synthesis. Peptide synthesis was performed manually on a 2chlorotrityl chloride resin (GL Biochem) under the standard Fmoc protocol. Removal of Fmoc protecting group was performed using a mixture of 1:4 (v:v) of piperidine/DMF for 20 min. Coupling was performed using Fmoc-amino acids (4 equiv.), HATU (4 equiv.) and DIEA (8 equiv.) for 1 h at room temperature. Upon completion of the synthesis, CH₂Cl₂:TFE:AcOH (8:1:1, v:v:v) was used to cleave the peptide acid from the resin while the side-chains are still protected. Then the solvent was dried under vacuum.

Synthesis of peptide-thiol salicylaldehyde (SAL) esters with C-terminal Gly or Pro. Protected peptide acids (1 equiv.), PyBOP (3 equiv.), DIEA (3 equiv.) were dissolved in anhydrous CH_2Cl_2 (0.3 mL). To this solution, 4 (2 equiv.) in anhydrous CH_2Cl_2 (0.2 mL) was added slowly. The mixture was stirred at room temperature for 2 h. After the reaction was completed, CH_2Cl_2 was removed. TFA (2 mL) was added and the mixture was stirred at room temperature for 30 min. The crude peptide thiol-SAL ester was purified by HPLC.

For model peptides (Ac-ITGEFNAG thiol-*SAL* ester and Ac-ITGEFNAP thiol-*SAL* ester), crude protected peptide acids (60 mg) were coupled with **4** to form peptide thiol-*SAL* esters (15 mg, 35 % and 17 mg, 36 %, respectively) (Figure S3 and S4). For the synthesis model peptide Ac-AFQIG thiol-*SAL* ester, the crude protected peptide acid (30 mg) was coupled with **4** and then treated with TFA followed by RP-HPLC purification to yield 9 mg (43 %) (Figure S5). For the synthesis model peptide Ac-VYAAPYLAGG thiol-*SAL* ester, the crude protected peptide acid (30 mg) was coupled with **4** and then treated with TFA followed by RP-HPLC purification to yield 14 mg (46 %) (Figure S6). For the synthesis model peptide Ac-VYAAPYLAGG thiopehnol ester, the crude protected peptide acid (30 mg) was coupled with thiophenol and then treated with TFA followed by RP-HPLC purification to yield 15 mg (51 %) (Figure S7).



Figure S3. RP-HPLC and MS spectra of Ac-ITGEFNAG thiol-*SAL* ester. (ESI⁺) calcd. for $C_{44}H_{59}N_9O_{14}S$ [M+H]⁺ 969.39, found [M+Na]⁺ 992.18.

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Figure S4. RP-HPLC and MS spectra of Ac-ITGEFNAP thiol-*SAL* ester. (ESI⁺) calcd. for $C_{47}H_{63}N_9O_{14}S$ [M+H]⁺ 1009.42, found [M+Na]⁺ 1032.31



Figure S5. RP-HPLC and MS spectra of Ac-AFQIG *thiol*-SAL ester. (ESI⁺) calcd. for $C_{34}H_{44}N_6O_8S$ [M+H]⁺ 696.29, found [M+H]⁺ 697.06 and [M+Na]⁺ 719.19.



Figure S6. RP-HPLC and MS spectra of Ac-VYAAPYLAGG *thiol*-SAL ester. (ESI⁺) calcd. for $C_{56}H_{74}N_{10}O_{14}S [M+H]^+ 1143.3$, found $[M+H]^+ 1143.4$

Minutes



Figure S7. RP-HPLC and MS spectra of Ac-VYAAPYLAGG *thiophenol* ester. (ESI⁺) calcd. for $C_{55}H_{74}N_{10}O_{13}S$ [M+H]⁺ 1115.3, found [M+H]⁺ 1115.4.

Synthesis of peptide-thiol salicylaldehyde esters with C-terminal Ala.

The peptide-thiol salicylaldehyde esters (Ac-FVGFSDTYGA-thiol SAL ester and Ac-FVGFSDTYG*a* thiol-*SAL* ester) were prepared using the hydrazine displacement method.³ The peptide hydrazine was prepared by treating 5 % hydrazine hydrate to trityl chloride resin for 30 min. Then standard Fmoc SPPS was performed. The resinbound peptide was then treated with TFA/thioanisole/TIPS (9/0.5/0.5) for 1 h and washed with ether. The crude peptide (12 mg) was dissolved in 0.2 M phosphate buffer at pH 6.5 with 6 M GnHCl and cooled to -10 °C. Then 0.5 M NaNO₂ was added and stirred for 15 min. Then pH was adjusted to 2 and 4 (3 equiv.) was added into the sample. The reaction performed for 2 h and treated with TFA for 15 min. The peptide thiol-*SAL* ester was purified by RP-HPLC (L-Ala thiol *SAL* ester 3 mg, 24 % and D-Ala thiol-SAL ester 2.7 mg, 22 %). Co-injection of these two peptide thiol SAL esters was performed to demonstrate that no epimerization took place (Figure S8).



Figure S8. RP-HPLC spectra of Ac-FVGFSDTYGA thiol-*SAL* (left), Ac-FVGFSDTYG*a* thiol-*SAL* (middle) and co-injection of both peptide thiol-*SAL* esters (right).

Investigation on the ligation condition.

Ligation studies under the Ser/Thr ligation condition:

Ac-SPKMVQG thiol-SAL ester was reacted with NH₂-SFAVGA-OH under the standard Ser/Thr ligation condition of Pyr: AcOH (1:1, mol: mol) overnight at 5 mM. Ac-FSATG thiol-*SAL* ester and Ac-FVGFSDTYGA thiol-*SAL* ester were reacted with NH₂-TGFVA-OH and NH₂-SGFVA-OH individually under the same condition None of the above reactions gave the ligation product, with the starting materials intact.

Ligation studies under direct aminolysis conditions:

Ac-AFQIG thiol-*SAL* ester (1 equiv.) was reacted with NH_2 -GLVYA-CO₂H (2 equiv.) in various solvents including DMF, PBS buffer, DMSO and 1-methyl-2-pyrrolidone. Different equivalents of bases were added into the system such as 1-10 equiv. of DIEA and 1-3 equiv. of DBU. The reaction was monitored by RP-HPLC after 6 h.

Ligation of model peptides. Ac-ITGEFNAG thiol-*SAL* ester was dissolved in DMSO (1 mM) and the N-terminal model peptides (NH_2 -**Z**GVFA-CO₂H) were added into the mixture at 1.3 mM. Then 3 equiv. of DIEA was added to initiate the ligation reaction. The reaction was performed at room temperature and quenched by TFA at 4 and 8 h. The reactions were quenched by TFA and water then monitored by analytical LCMS. The conversion was calculated by the ratio of the product area over the product and the hydrolyzed peptide (Figure S9).



Figure S9. LCMS spectra of Ac-ITGEFNAG thiol *SAL* ester ligated with different N-terminal model peptides of NH₂-ZGVFA-OH, where Z = Asp, Glu, Pro, Ser or Gly. The reaction was completed in 4 h. Label **1** = ligation product, **2** = excess NH₂-ZGVFA-OH.

The peptide model Ac-ITGEFNAP thiol-*SAL* ester was dissolved in DMSO (1.5 mM) and the N-terminal model peptides (NH₂-**Z**GVFA-OH) were added into the mixture at 1 mM. Then 3 equiv. of DIEA was added to initiate the ligation reaction. The reaction was performed at room temperature and quenched by TFA at 4 and 8 h (Figure S10).



Figure S10. LCMS spectrum of Ac-ITGEFNAP-thiol salicylaldehyde ester ligated with different N-terminal model peptides of NH₂-ZGVFA-OH, where Z = Asp, Glu, Pro, Ala or Gly. The reaction was completed in 8 h. Label **1** = ligation product, **2** = remained NH₂-ZGVFA-OH.

Synthesis of natural cyclic peptides. The peptide-thiol salicylaldehyde esters were dissolved in DMSO (1 mM) and 3 equiv. of DIEA was added. The reaction performed for 6 to 8 h and monitored by analytical LCMS, followed by purification using preparative HPLC.

1. Phakellistatin-13

Boc-FGPT(*t*Bu)LW(Boc)P-OH was synthesized by the general Fmoc-SPPS protocol. The crude protected peptide acid (30 mg) was coupled with **4** using the above method and was then deprotected with TFA. The peptide thiol salicylaldehyde ester was obtained after preparative RP-HPLC (7 mg, 27 %) and was cyclized at 1 mM for 6 hours. Then the final product (2.5 mg, 41 %) was obtained after preparative RP-HPLC. (ESI⁺) calcd. for $C_{42}H_{55}N_8O_8$ [M+H]⁺ 799.9, found [M+H]⁺ 799.2.



2. Antamanide

Boc-AFFPPFFVPP-OH was synthesized by the general Fmoc-SPPS protocol. The crude protected peptide acid (30 mg) was coupled with **4** using the above method and was then deprotected with TFA. The peptide thiol salicylaldehyde ester was obtained after preparative RP-HPLC (10 mg, 46 %) and was cyclized at 1 mM for 6 hours. Then the final product (4.3 mg, 48 %) was obtained after preparative RP-HPLC. (ESI⁺) calcd. for $C_{64}H_{79}N_{10}O_{10}$ [M+H]⁺ 1147.4, found [M+H]⁺1147.4 and [M+Na]⁺ 1169.3.



3. Stalladelin D

Boc-VPS(*t*Bu)PY(*t*Bu)FPAAIG-OH was synthesized by the general Fmoc-SPPS protocol. The crude protected peptide acid (30 mg) was coupled with **4** using the above method and was then deprotected with TFA. The peptide thiol salicylaldehdye ester was obtained after preparative RP-HPLC (8.5 mg, 30 %) and was cyclized at 1 mM for 8 hours. Then the final product (3.4 mg, 45 %) was obtained after preparative RP-HPLC. (ESI⁺) calcd. for $C_{55}H_{78}N_{11}O_{13}$ [M+H]⁺ 1100.3, found [M+H]⁺ 1100.4 and [M+Na]⁺ 1122.4.



4. Cyclosqumosin D

Boc-GVVS(*t*Bu)Y(*t*Bu)Y(*t*Bu)PG-OH was synthesized by the general Fmoc-SPPS protocol. The crude protected peptide acid (30 mg) was coupled with 4 using the above method and was then deprotected with TFA. The peptide thiol salicylaldehdye ester was obtained after preparative RP-HPLC (6.7 mg, 26 %) and was cyclized at 1 mM for six hours. Then the final product (2.8 mg, 49 %) was obtained after preparative RP-HPLC. (ESI⁺) calcd. for $C_{40}H_{55}N_8O_{11}$ [M+H]⁺ 823.4, found [M+H]⁺ 823.2.



5. Dichotomin G

Boc-LPS(*t*Bu)T(*t*Bu)FPPIP-OH was synthesized by the general Fmoc-SPPS protocol. The crude protected peptide acid (30 mg) was coupled with mercaptobenzyaldehyde using the above method and was then deprotected with TFA. The peptide thiol salicylaldehdye ester was obtained after preparative RP-HPLC (11 mg, 18 %) and was cyclized at 1 mM for six hours. Then the final product (4.8 mg, 50 %) was obtained after preparative RP-HPLC. (ESI⁺) calcd. for C₄₈H₇₂N₉O₁₁ [M+H]⁺ 950.5, found [M+H]⁺ 950.2 and [M+Na]⁺ 972.5.



References:

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NMR spectrums ¹H NMR spectrum of Compound **3**:





¹³C NMR spectrum of Compound **3**:



¹H NMR spectrum of Compound **4**:

