Chapter 12

Covalent Cross-Linking of Kinases with Their Corresponding Peptide Substrates

Alexander V. Statsuk and Kevan M. Shokat

Abstract

Protein phosphorylation represents the most dominant and evolutionary conserved posttranslational modification for information transfer in cells and organisms. The human genome encodes >500 protein kinases, and thousands of phosphorylation sites are present in mammalian proteome. To develop a global view of phosphorylation network, there is a need to map the connectivity between kinases and phosphoproteome. We developed a chemical kinase–substrate cross-linker **1** that converts transient kinase–substrate interactions into a covalently linked kinase–substrate complex in vitro and in the presence of cell lysates. The method can be applied to identify unknown upstream kinases responsible for phosphorylation events in cell lysates.

Key words: Kinase, Substrate, Kinase inhibitor, Thiophene-2,3-dialdehyde, Covalent cross-link

1. Introduction

The human genome encodes more than 500 kinases, and thousands of protein phosphorylation sites have been identified. Given the spacial and temporal regulation of protein phosphorylation, mapping the connectivity between kinases and the phosphoproteome represents a daunting task. To this end, we and others have developed methods that enable the identification of protein kinase substrates (1–8). The inverse problem, however, that is identification of unknown kinases responsible for known phosphorylation events, has proven to be much more challenging. Examples of phosphorylation sites with unknown upstream kinases include the following: Ser-170 of the protein BAD which enhances its anti-apoptotic activity (9), Ser-497 of the natriuretic peptide receptor,

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which is responsible for desensitization of the receptor's antihypertensive activity (10), and Ser-325 of the tumor suppressor LKB1, which is phosphorylated in response to the biguanide antidiabetic agent metformin (11, 12). These examples, as well as the thousands of other phosphorylation sites in the mammalian proteome (13), highlight the need to develop new tools for mapping phosphorylation networks. We have previously reported that Akt1 kinase and cysteine-containing fluorescent substrate peptide can be covalently cross-linked by an adenosine-based *o*-phthaldialdehyde reagent in vitro shown in Fig. 1 (14). However, the initially developed adenosine based *o*-phthaldialdehyde reagent failed to cross-link the kinase with the cysteine-containing fluorescent substrate peptide in the presence of cell lysate. To improve the selectivity of the cross-linker, we replaced the weak, kinase-binding adenosine moiety with a promiscuous kinase inhibitor scaffold, and replaced the highly reactive *o*-phthaldialdehyde moiety with the less-reactive

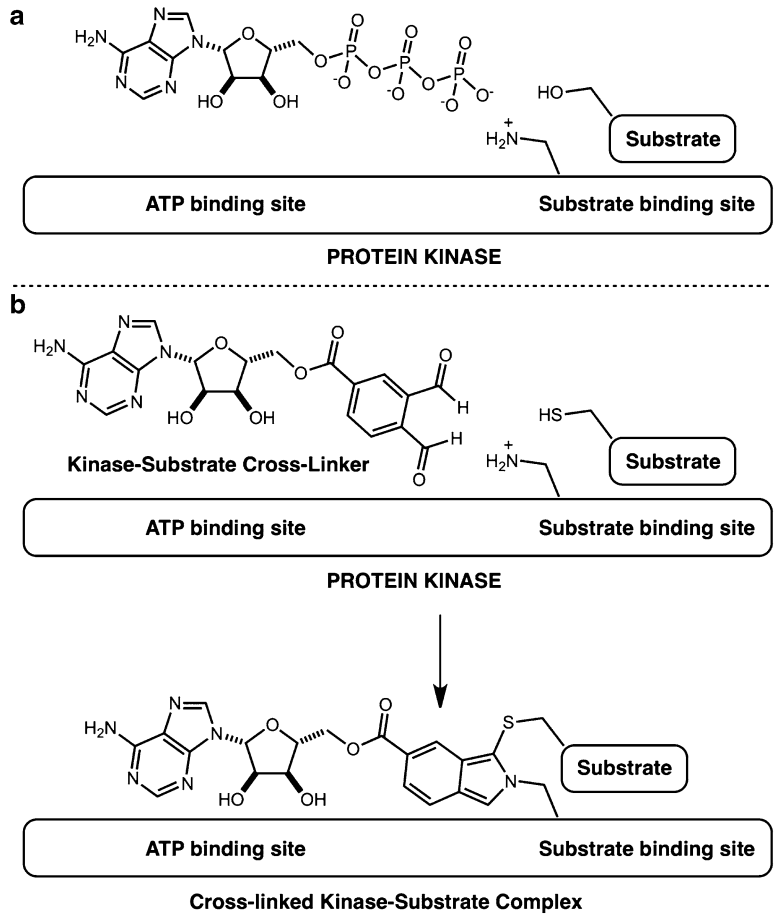

Fig. 1. Kinase–substrate cross-linking strategy. (**a**) Phosphorylation of the protein substrate by an upstream kinase. (**b**) The serine of the kinase is replaced with a reactive cysteine moiety that facilitates kinase–substrate cross-link by cross-linker.

thiophene-2,3-dialdehyde fragment. The combination of those two structural modifications that we describe in this chapter led to the development of a new reagent **1** capable of cross-linking Akt1 kinase with its substrate peptide **2** (Fluorescein-ZZRPRTSCF-OH, Z = 6-aminohexanoic acid) both in vitro and in the presence of competing cellular lysate proteins (15).

2. Materials

2.1. Synthesis of Cross-Linker 1

1. 2,4-Dichloropyrimidine: ≥98% purity.
2. 3-Cyclopropyl-1H-pyrazol-5-amine.
3. *p*-Phenylenediamine: ≥97% purity.
4. 2,3-Thiophenedicarboxaldehyde.
5. Ethylene glycol: ≥99% purity.
6. *n*-Butyllithium: 2.5 M solution in hexane.
7. *N*,*N*-Dimethyl formamide (DMF): anhydrous.
8. Sodium cyanoborohydride: 95% purity.
9. Trifluoroacetic acid (TFA): 98% purity.
10. Benzene: ≥99% purity.
11. Tetrahydrofuran (THF): anhydrous, ≥99.9% purity, inhibitor free.
12. Methanol: ≥99% purity.
13. Acetonitrile: ≥99.9% purity.

2.2. Synthesis of Akt1 Substrate Peptide Fluorescein-ZZRPRTSCF-OH, **2**

1. Fmoc-Phe-Wang resin: 100–200 mesh.
2. Fmoc-Cys(Trt)-OH.
3. Fmoc-Ser(tBu)-OH.
4. Fmoc-Thr(tBu)-OH.
5. Fmoc-Arg(Pbf)-OH.
6. Fmoc-e-Ahx-OH.
7. 5-Carboxyfluorescein.
8. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphat (HBTU).
9. *N*,*N*-Diisopropylethylamine.

2.3. Reagents and Equipment for Cross-Linking Experiments

1. Recombinant active Akt1/PKBα.
2. Fluorescein-ZZRPRTSCF-OH (Z = 6-aminohexanoic acid, peptide is prepared via solid phase synthesis).
3. Cross-linker **1** (prepared via synthesis).

4. Flourescent imager: e.g., Typhoon 9400, variable mode imager, 490 nm excitation band, 520 nm emission band.
5. Buffer for kinase–substrate cross-linking experiments: 25 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)), pH 6.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol.

2.4. SDS-Polyacrylamide Gel Electrophoresis

1. Running buffer (1×): 0.025 M Tris base, 0.19 M Glycine, 0.004 M SDS, pH 8.5.
2. Polyacrylamide gel: 12% acrylamide, Tris–HCl, 50-μL well, 10 wells.
3. Prestained molecular-weight markers.
4. Laemmli loading buffer (5×): 1.5 g SDS, 3.75 mL of 1 M tris(hydroxymethyl)aminomethane (Tris–HCl) pH 6.8, 0.015 g of bromophenol blue, 1.16 g of DTT, 7.5 mL of water, and 7.5 mL of glycerol.

2.5. Equipment for Chromatographic Purifications

An automated chromatography purification system (Intellyflash 280) was used throughout our experiments to purify chemical intermediates. Alternatively, any other such device or regular glass chromatography columns may be used for chromatographic purifications.

3. Methods

The covalent cross-linking reaction of Akt1 kinase with the fluorescently labeled cysteine-containing substrate peptide is very rapid and is generally completed within 20 min at room temperature. The extent of the cross-linking reaction can be monitored by visualizing the covalently cross-linked fluorescent kinase–substrate complex by direct in-gel fluorescence scanning. Our studies indicate that pH < 7 is optimal for the cross-linking reaction (see Fig. 2a). The cross-linking reaction of Akt1 kinase with the cysteine-containing substrate peptide is resistant toward competing external sulfur nucleophiles, tolerating up to 1,000-fold excess of β-mercaptoethanol relative to Akt1 substrate peptide **2** (see Fig. 2b). Boiling the cross-linked Akt1-peptide substrate complex induces its decomposition, thus it is not recommended to boil samples containing covalently cross-linked kinase–substrate complex prior to loading on polyacrylamide gel (see Fig. 1c). The kinase–substrate cross-linker **1** should always be added at the very last step to the reaction mixture. Cross-linker **1** cross-linked Akt1 kinase with the fluorescent, cysteine-containing substrate peptide in the presence of HeLa cell lysates (see Fig. 3).

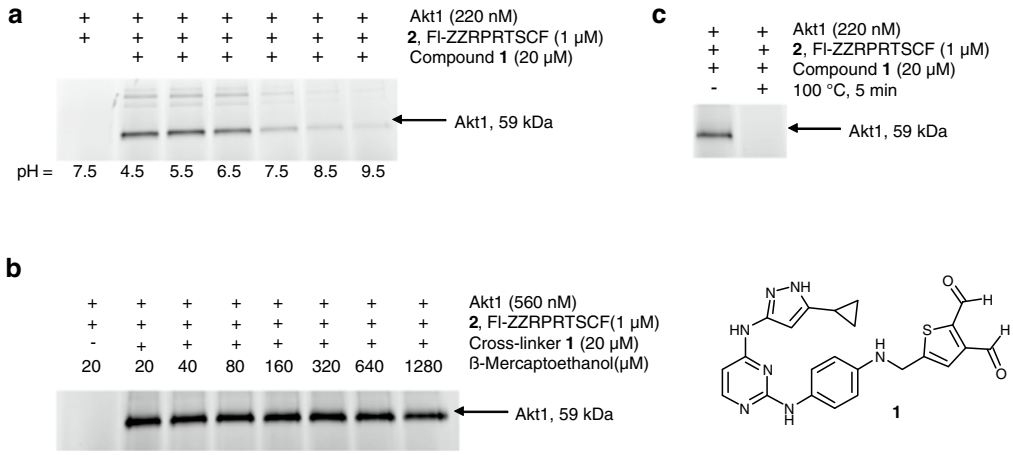

Fig. 2. Cross-linking of kinase Akt and a model peptide substrate 2. (**a**) Cross-linking of Akt1 and fluorescent substrate peptide Fl-ZZRPRTSCF-OH (2) with cross-linker 1 at different pH values. Akt1 (220 nM) and fluorescent peptide 2 (1 μM) are treated with cross-linker 1 (20 μM) for 20 min at r.t. at different pH values, followed by SDS-PAGE and in-gel scanning fluorescence. (**b**) Akt1 (560 nM) and fluorescent peptide 2 (1 μM) were treated with cross-linker 1 (20 μM) for 20 min at r.t. in the presence of increasing concentrations of β-mercaptoethanol, followed by SDS-PAGE and in-gel fluorescence scanning. (**c**) Decomposition of the cross-linked product upon heating. Akt1 (220 nM) and fluorescent peptide 2 (1 μM) were treated with cross-linker 1 (20 μM) for 20 min at r.t., then boiled for 5 min, followed by SDS-PAGE and in-gel scanning fluorescence.

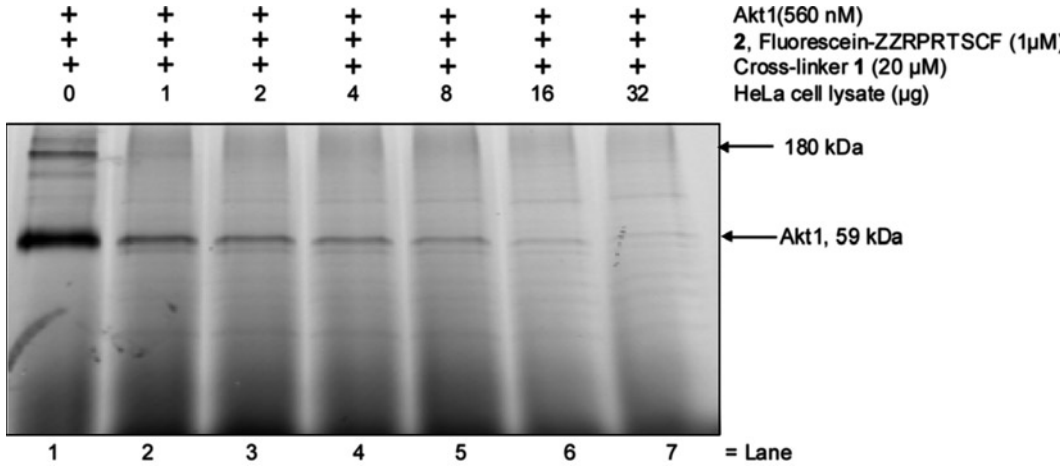

Fig. 3. Cross-linking of Akt1 and fluorescent peptide substrate 2 with cross-linker 1 in the presence of increasing amounts of HeLa cell lysate proteins. Akt1 and substrate peptide 2 were treated with cross-linker 1 for 20 min at r.t., followed by SDS-PAGE and in-gel scanning fluorescence.

3.1. Synthesis of Chemical Cross-Linker 1

Unless specified otherwise, all reactions were performed in dry solvents and under nitrogen atmosphere.

3.1.1. Synthesis of Compound 4

1. Charge a 500-mL round-bottom flask equipped with a reflux condenser and a Dean-Stark trap with 2,3-thiophene dicarboxaldehyde **3** (5.35 g, 38 mmol), p-toluenesulfonic acid monohydrate (0.1 g), ethylene glycol (11.73 g, 190 mmol), and benzene (80 mL).
2. Reflux the resulting mixture for 2 h with simultaneous removal of water into the Dean-Stark trap.
3. Quench the reaction mixture with a 5% aqueous solution of Na_2CO_3 (100 mL).
4. Separate organic layers and extract the aqueous layer with ethyl acetate (100 mL).
5. Wash combined organic fractions with saturated sodium chloride solution (100 mL), dry over $MgSO_4$, filter, and evaporate.
6. Purify the resulting oily residue on the Intelliflash 280 using disposable SF65-400g columns (Gradient, Hexane–ethyl acetate = 100:0 → 0:100, over 30 min, flow rate = 100 mL/min) to afford 2,3-Bis(1,3-dioxolan-2-yl)thiophene **4** as the major elution peak (7 g, 85% yield). Alternatively, purification can be done using regular glass chromatography columns and 300 g of regular silica gel. It is recommended in this case to use Hexane: Ethyl acetate = 2:1 as a solvent system. ^{1}H NMR ($CDCl_3$) 4.03 (t, 2H),4.11 (t, 2H), 6.05 (s, 1H), 6.33 (s, 1H), 7.11 (d, J = 5.2 Hz, 1H), 7.27 (d, J = 5.55 Hz, 1H) (Fig. 4).

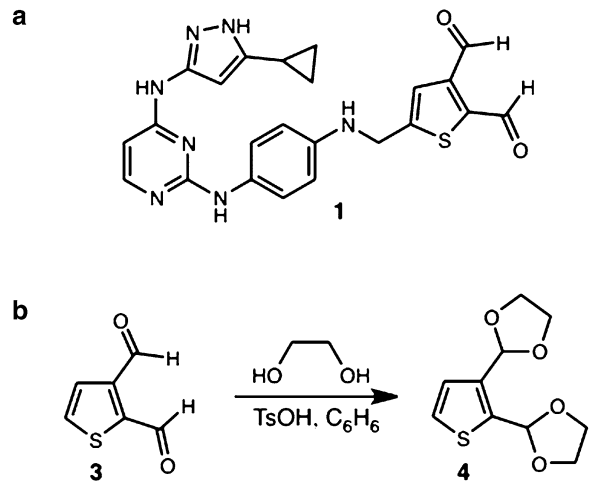

Fig. 4. Synthesis of compound 4. (**a**) Structure of the kinase–substrate cross-linker 1. (**b**) Synthesis of compound 4.

*3.1.2. Synthesis of Compound **5***

1. Charge a 250 mL Round-bottom flask with 2,3-Bis(1,3-dioxolan-2-yl)thiophene **4** (2.6 g, 11.40 mmol) and tetrahydrofurane (50 mL), and cool down to –78°C.
2. Add *n*-BuLi (5 mmol) dropwise at –78°C.
3. Raise the temperature to 0°C and stir the reaction mixture for an additional 30 min.
4. Add dimethyl formamide (DMF) (5 mmol, 0.971 mL) to the reaction mixture at 0°C.
5. Stir the reaction mixture at ambient temperature for 30 min followed by quenching with water (50 mL) (see Note 1).
6. Separate organic layers and extract the aqueous layer with ethyl acetate (100 mL). Wash the combined organic extracts with saturated sodium chloride solution (100 mL), dry over $MgSO_4$, filter, and evaporate. Purification of the resulting residue on Intelliflash 280 using disposable SF25-40g columns (Gradient, Hexane–ethyl acetate = 100:0 → 0:100, over 30 min, flow rate = 40 mL/min) affords aldehyde **5** (2.2 g, 75% yield) as the major elution peak. Alternatively, purification can be done using regular glass chromatography columns charged with 60 g of regular silica gel. It is recommended in this case to use Hexane–Ethyl acetate (1:1) as a solvent mixture. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.80 (s, 1H), 7.74 (s, 1H), 6.32 (s, 1H), 5.98 (s, 1H), 4.10-4.05 (m, 4H), 4.05-3.95 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.4, 150.5, 142.2, 138.8, 136.1, 98.8, 98.6, 65.8, 65.5. MS calculated for $C_{11}H_{12}O_5S$ 256.04, found 257.03 (Fig. 5).

*3.1.3. Synthesis of Compound **8***

1. Charge a round-bottom flask equipped with a reflux condenser with 2,4-dichloropyrimidine **6** (3.2 g, 21.6 mmol), 5-cyclopropyl-2H-pyrazol-3-ylamine 7 (2.65 g, 21.5 mmol), KOAc (30 eq., 64 g) and dissolve in THF–water 1:1 (140 mL).
2. Keep the resulting reaction mixture at 55°C for 48 h.
3. After 48 h, remove heating and separate organic layers. Evaporate the organic layer and dissolve residue in CH_2Cl_2 (30 mL) and keep at –20°C for 3 h. Collect the precipitated pyrimidine chloride **8** by filtration. To collect additional amounts of pyrimidine chloride, evaporate filtrates, redissolve

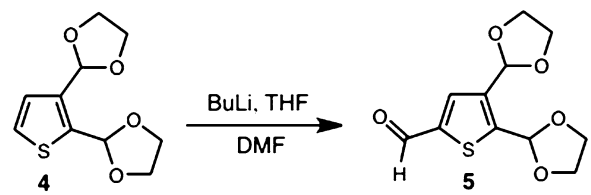

Fig. 5. Synthesis of compound 5.

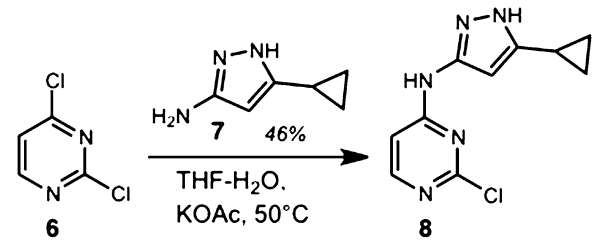

Fig. 6. Synthesis of compound 8.

in CH_2Cl_2 (25 mL) and keep at –20°C for another 3 h followed by filtration. Purification of the combined solids on Intelliflash 280 using disposable SF25-80g columns (Gradient, $CHCl_3$–CH_3OH (100:0→92:8), over 30 min, flow rate = 85 mL/min) affords compound **8** as the major elution peak (46% yield, 2.32 g). Alternatively, purification can be done using regular glass chromatography columns charged with 60 g of regular silica gel. It is recommended in this case to use $CHCl_3$–CH_3OH (9:1) as a solvent mixture. ^{1}H NMR (400 MHz, DMSO) δ 12.14 (s, 1H), 10.23 (s, 1H), 8.10 (s, 1H), 1.84 (m, 1H), 0.88 (m, 2H), 0.64 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 161.4, 160.7, 160.0, 153.9, 148.6, 147.8, 146.8, 8.4, 8.2. MS calculated for $C_{10}H_{10}ClN_5$ 235.06 (M^+), found 236.15 (M^+) (Fig. 6).

*3.1.4. Synthesis of Compound **10***

1. Charge a round-bottom flask equipped with a reflux condenser with pyrimidine monochloride **8** (1.6 g, 6.8 mmol), *p*-phenylenediamine **9** (0.74 g, 6.8 mmol) and *n*-BuOH (60 mL). Treat the resulting mixture with concentrated HCl (0.1 mL) and keep at 100°C overnight.
2. Collect purple precipitates by filtration, wash with 20 mL of *n*-BuOH and dry at 50°C under vacuum overnight, to afford amine **10** (1.5 g, 73% yield), which is used in the next step without further purification (see Note 2). MS calculated for $C_{16}H_{17}N_7$ 307.15, found 308.17 (Fig. 7).

*3.1.5. Synthesis of Compound **11***

1. Charge a round-bottom flask with amine **10** (0.06 g, 195 μmol), aldehyde **5** (0.048 g, 187 μmol), and CH_3OH (5 mL).
2. Treat the resulting mixture with solid $NaBH_3CN$ (0.01 g, 168 μmol), stir at ambient temperature overnight and evaporate. Purification on Intelliflash 280 using disposable SF10-4g columns (Gradient, CH_2Cl_2 (1% NEt_3)– CH_3OH (1% NEt_3) (100:0→92:8), over 30 min, flow rate = 18 mL/min) affords compound **11** as the major elution peak (0.064 g, 63% yield). Alternatively, purification can be done using regular glass chromatography columns charged with 10 g of regular silica gel. It is recommended in this case to use $CHCl_3$–CH_3OH (9:1) containing 1% NEt_3 as a solvent mixture. ^{1}H NMR (400 MHz,

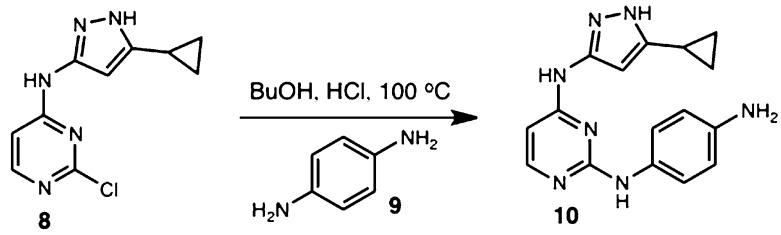

Fig. 7. Synthesis of compound 10.

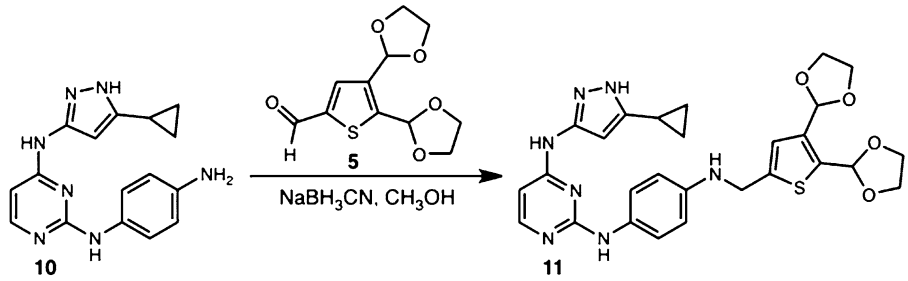

Fig. 8. Synthesis of compound 11.

$CDCl_3$) δ 9.13 (bs, 1H), 7.78 (m, 2H), 7.17 (m, 2H), 6.90 (s, 1H), 6.51 (m, 2H), 6.15 (m, 1H), 5.73 (m, 1H), 4.27 (s, 2H), 3.99 (m, 4H), 3.87 (m, 4H), 1.74 (m, 1H), 0.80 (m, 2H), 0.56 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.2, 155.6, 144.3, 143.9, 143.6, 139.1, 138.9, 137.5, 136.1, 130.2, 125.0, 124.3, 123.7, 113.6, 99.1, 98.6, 97.5, 65.5, 65.4, 46.5, 9.0, 8.3. MS calculated for $C_{27}H_{29}N_7O_4S$ 547.2, found 548.07 (Fig. 8).

*3.1.6. Synthesis of Cross-Linker **1***

1. Charge a round bottom flask with compound **11** (0.035 g, 64 μmol) and dissolve in a 1:1 mixture of CH_3CN–H_2O (3 mL) followed by treatment with trifluoroacetic acid (50 μL, 10 eq.).
2. Stir the reaction mixture overnight (see Note 3) and purify directly *via* reverse phase HPLC CH_3CN/H_2O containing 0.1% TFA (Gradient, CH_3CN (0.1% TFA)–H_2O (0.1% TFA) = 2:98 → 98:2, over 40 min, flow rate = 20 mL/min) followed by lyophilization to afford cross-linker **1** (13 mg, 45% yield). ^1H NMR (400 MHz, DMSO-d_6) δ 11.94 (bs, 1H), 10.38 (s, 1H), 10.34 (s, 1H), 9.54 (bs, 1H), 8.73 (bs, 1H), 7.81 (m, 1H), 7.53 (s, 1H), 7.31 (s, 1H), 7.21 (s, 1H), 6.54 (s, 1H), 6.52 (s, 1H), 4.49 (s, 2H), 1.76 (bs, 1H), 0.83 (m, 2H), 0.59 (m, 2H). MS calculated for $C_{23}H_{21}N_7O_2S$ 459.15, found 459.99.
3. Dissolve the resulting cross-linker **1** in DMSO to make a 20 mM DMSO stock solution, and store at −80°C until further use. It is recommended to avoid freeze–thaw cycles, since the cross-linker **1** may decompose (Fig. 9).

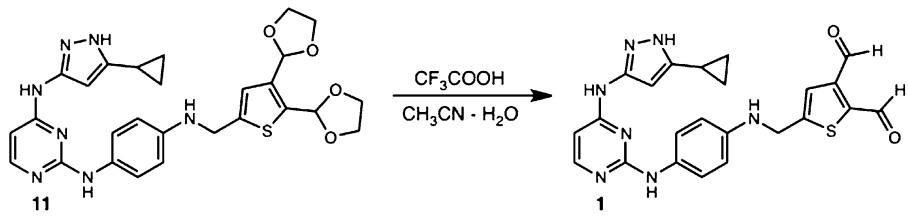

Fig. 9. Synthesis of kinase–substrate cross-linker 1.

3.2. Solid Phase Synthesis of Fluorescent Cysteine Containing Akt1 Peptide Substrate Fluorescein-ZZRPRTSCF-OH, 2

3.2.1. Fluorescein-Ahx-Ahx-Arg-Pro-Arg-Thr-Ser-Cys-Phe-OH (2)

1. Prepare the substrate peptide **2** i according to the general solid phase peptide synthesis procedure using Fmoc-Phe-Wang resin. Perform each coupling step for 2 h with 2 mmol of corresponding amino acid dissolved in 5 mL of DMF, in the presence of HBTU (2 mmol, 0.758 g) and iPr_2NEt (4 mmol, 0.695 mL).
2. Carry out Fmoc deprotection in 20% piperidine/DMF for 20 min at room temperature.
3. Cap the N-terminus of the peptide by agitating the resin overnight in the presence of 6-carboxyfluorescein (0.5 mmol), HBTU (0.5 mmol, 0.189 g), and $i\text{-}Pr_2NEt$ (1 mmol, 0.173 mL) overnight.
4. Prior to cleavage, wash the resin with DMF (3 × 5 mL), CH_2Cl_2 (3 × 5 mL), CH_3OH (3 × 5 mL), and CH_2Cl_2 (3 × 5 mL).
5. Cleave the peptide from the resin by treatment with 94:2:2:2 TFA–1,2-ethanedithiol–H_2O–triisopropylsilane for 3 h. Remove the solvent in vacuo and purify the resulting crude product by C18 reverse-phase HPLC (CH_3CN/H_2O/0.1% TFA). Following lyophilization, the peptide is obtained as a yellow solid. MS (ESI), *m/z* calcd for $C_{69}H_{91}N_{15}O_{18}S$: 1,449.6. Found: *m/z* 1,450.8 $(M+H)^+$. Dissolve the resulting solid peptide in water to prepare a 1 mM stock solution and store at –80°C until further use.

3.3. General Protocol for Cross-Linking of AKT1 Substrate Peptide 2 with AKT1

Final concentrations of reaction components are the following: cross-linker **1** 20 μM, substrate peptide **2** 1 μM, Akt1 kinase 400 ng/30 μL.

1. Prepare a 600 μM stock solution of cross-linker **1** in water.
2. Prepare a 30 μM stock solution of Akt1 substrate peptide **2** in water.
3. Dissolve 400 ng of Akt kinase (0.4 mg/mL) in 30 μL of reaction buffer (25 mM HEPES, pH = 6.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol).
4. Add 1 μL of Akt1 substrate peptide **2** (30 μM stock solution in water) and mix well by pipetting it up and down.
5. Add 1 μL of cross-linker **1** (600 μM stock solution in water), mix well with the reaction mixture by pipetting up and down (see Note 4).

6. Incubate reaction mixture for 20 min at room temperature.
7. After 20 min, quench the reaction with 6 μL of 5× Laemmli buffer. Remember not to boil the sample.
8. Load the samples (30 μL) onto polyacrylamide gels, followed by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and direct in-gel scanning fluorescence measurements (490 nm excitation band, 520 nm emission band).
9. The same procedure should be used to cross-link Akt1 kinase with the substrate Akt1 peptide **2** in the presence of HeLa cell lysate. In this case, one should premix Akt1 kinase with the substrate peptide **2** and 5 μg of HeLa cell lysate in the reaction buffer, adding chemical cross-linker **1** last. Representative cross-linking experiments with or without HeLa cell lysates are presented in Fig. 3.

4. Notes

1. Chemical transformations that involve organolithium intermediates are usually quenched with acidic aqueous media such as 1 M HCl. In the case of compound **5**, however, this must be avoided because the 1,3-dioxolane protective group is easily removed under acidic conditions. This will liberate a dialdehyde moiety that will interfere with the subsequent reductive amination step (Subheading 3.1.2).
2. The LCMS trace of compound **11** should show one elution peak. Thus no additional purification should be needed. Compound **11** is very polar and insoluble in a variety of solvents, such as $CH_3OH/CH_3CN/DMSO$, which makes chromatographic purifications hard to perform (see Subheading 3.1.4).
3. The final deprotection step in the synthesis of cross-linker **1** is usually completed within 10–14 h, but it is important to check the progress of the deprotection reaction by LCMS before purifying it. In the case of incomplete conversion, the reaction mixture should be evaporated on a rotary evaporator, redissolved in $CH_3CN–H_2O$ (1:1), and treated with trifluoroacetic acid (10 eq., 50 μL) and stirred until full conversion of the starting material **12** (see Subheading 3.1.5).
4. When performing the cross-linking reaction between Akt1 kinase and the peptide substrate **2**, cross-linker **1** should be added at the very last step. We have noticed that Akt1 kinase can be nonspecifically cross-linked with the cross-linker **2** in the absence of the peptide **2**, forming nonspecifically cross-linked higher molecular weight intermediates (see Subheading 3.3).

Acknowledgments

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