Photocleavable Linker for Protein Affinity Labeling to Identify the Binding Target of KCN-1

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PHOTOCLEAVABLE LINKER FOR PROTEIN AFFINITY LABELING TO IDENTIFY THE BINDING TARGET OF KCN-1

by

HANG T. TRAN

Under the Direction of Binghe Wang

ABSTRACT

KCN-1 is known to reduce tumor growth 6-fold in mice implanted with LN229 glioma cells. Although this inhibitor is effective, the mechanism of action for KCN-1 is not well understood. Based on preliminary studies, KCN-1 reduces tumor growth by disrupting the HIF-1 (hypoxia-induced factor-1) pathway. The binding target of KCN-1 needs to be investigated in order to develop KCN-1 or its analogs for therapeutic applications. In this research, a molecule was designed and synthesized for the identification of the binding target of KCN-1. Specifically, this molecule contains the inhibitor (KCN-1), a photocleavable linker,
beads, and the affinity label (L-DOPA). When UV light shines on the linker, the \textit{trans}-alkene isomerizes to \textit{cis}-alkene and undergoes intramolecular ring-closing reaction, which helps cleave the immobilized bead from the linker. The immobilized bead is used to separate the binding fragment attached to the photocleavable linker from the solution after enzyme digestion. The affinity label (L-DOPA) reacts with a nucleophile from the binding target and creates a covalent bond. If the design is successful, this method is able to analyze the mass of the peptide sequence and determine the binding target of KCN-1.

INDEX WORDS: Photocleavable linker, Affinity label, KCN-1, HIF-1 pathway
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by

HANG T. TRAN

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1. INTRODUCTION

1.1 Hypoxia

Hypoxia is a condition of oxygen deprivation. When oxygen level decreases, an adaptive response occurs that is mediated by hypoxia-inducible factor-1 (HIF-1). HIF-1 activates transcription of genes that help cells survive in low oxygen conditions. These genes code for proteins that promote angiogenesis to form new blood vessels, glucose metabolism to provide more energy, and cell proliferation.

Hypoxia commonly occurs in solid tumors because oxygen is limited in the interior of the tumor mass. The blood vessel is too far from the hypoxic area to deliver adequate amount of oxygen. Thus, there is an increase of HIF-1 activity and stabilization in those areas. As a result, the activation of genes helps solid tumor cells survive and expand. Hypoxia is a major driving force of tumor progression because cells undergo adaptive genetic changes that prevent hypoxia-induced cell death and necrosis.

1.2 HIF-1 Pathway

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that responds to changes in oxygen and is up-regulated in hypoxia. It is a heterodimeric protein that consists of HIF-1α and HIF-1β subunits. HIF-1α is oxygen-sensitive consisting of an oxygen-dependent degradation (ODD) domain. HIF-1β completes the heterodimeric protein and binds with HIF-1α for the translocation to the nucleus.
Figure 1.1: A comparison of hypoxia-inducible factor-1 pathway in normoxia and hypoxia⁴.

Under normoxia, as shown in Figure 1.1, HIF-1α subunit has an exceptionally short half-life because it is subjected to rapid turnover and degradation by the ubiquitin proteasome pathway⁵. Since oxygen is available, the oxygen-dependent degradation (ODD) domain undergoes hydroxylation by prolyl hydroxylases (PHDs) and factor inhibiting HIF-1 (FIH-1). The PHDs and FIH-1 are iron-dependent enzymes requiring oxygen to hydroxylate prolines and asparagines, respectively, in the ODD domain. As a result of the hydroxylated prolines, the von Hippel-Lindau protein (VHL) binds to the HIF-1α subunit, which leads to ubiquitination. Since
the asparagines are hydroxylated, the transcription co-activator (CBP/p300) cannot bind to the HIF-1α subunit, which leads to transcriptional repression. Thus, transcriptional activity is reduced and HIF-1α subunit undergoes proteasomal degradation.

Under hypoxic condition, as shown in Figure 1.1, HIF-1α subunit cannot be hydroxylated because of decreased oxygen availability. This means that VHL cannot bind to HIF-1α subunit and cannot lead to proteosomal degradation. HIF-1α subunit is abundant and binds to the transcription co-activator (CBP/p300), which initiates the transcriptional activity. Then, HIF-1β subunit binds to the HIF-1α subunit and the heterodimeric protein translocates to the nucleus for the transcription of genes. These genes include erythropoiesis (Epo) to produce red blood cells, vascular endothelial growth factor A (VEGF) to form more blood vessels, and glucose metabolism (Glut1) to provide energy for cells5.

1.3 KCN-1 Inhibitor

HIF-1 is overly expressed in human cancer. Since more genes are expressed, as well as altered, many tumors undergo genetic changes that facilitate adaptation and survival of the cells2. Thus, these adaptations and genetic changes make tumor cells more resistance to conventional treatments, such as chemotherapy2. Therefore, inhibition of HIF-1 leads to anti-tumor effects and can be a target for drug development.

![Molecular structure of KCN-1](image)

Figure 1.2: Molecular structure of KCN-1.

The interest of finding small molecules that disrupt the HIF-1 pathway led to an inhibitor known as KCN-1. KCN-1, shown in Figure 1.2, is a lead compound identified by screening a
10,000 member combinatorial library from Nicolaou. It was chosen for evaluation in Dr. Van Meir’s laboratory at Emory University. Preliminary in vivo experiments of KCN-1 showed significant anti-tumor activity. Nude mice were implanted with LN229 glioma tumor cells on both hind flanks. After one week, one group of mice was injected with KCN-1 (60 mg/kg; 5 days/week) and the vehicle. The other group received the vehicle alone, the control. The mice injected with KCN-1 showed 6-fold less in tumor mass than the control. Based on the results, KCN-1 reduces and slows tumor growth. In addition, the KCN-1 injected mice displayed no apparent signs of toxicity or other negative effects.

Although KCN-1 is a potent inhibitor that reduces tumor growth dramatically, the binding target of KCN-1 is not known. However, there is evidence to support that KCN-1 binds to component of the HIF-1 pathway. Based on other preliminary studies done in collaboration with the Van Meir lab, KCN-1 may bind to transcription co-activator p300. This study was performed by Oregon Green fluorophore modified KCN-1 for cell imaging. Although results showed that KCN-1 binds to transcription co-activator p300, further experiments are needed to confirm this mechanism of KCN-1 interacting in tumor cells. Therefore, protein affinity labeling experiments will be used to identify the binding target of KCN-1.

1.4 Affinity Labeling

There are many techniques for tagging proteins. One of these techniques is affinity labeling. This method uses the property of recognition between two different molecules to react and form a chemical bond. It is widely used to identify proteins, localize binding sites, and determine targets of drugs. The affinity label molecule binds specifically and reversibly to the target protein. However, when it is photo-activated or chemically activated, it forms a highly reactive intermediate and reacts irreversibly with the nucleophilic residue of the target protein.
In recent years, the Kodadek laboratory developed a novel type of chemically activated molecule that formed an extremely reactive electrophilic intermediate. This method was used to determine the binding target of free ubiquitin (Ub). The molecule contained ubiquitin tagged with a biarsenical fluorescein derivative (F1AsH), a biotin moiety, and a catechol of the dihydroxyphenylalanine (L-DOPA). The most important aspect of this molecule was the catechol of L-DOPA as shown in Figure 1.3. The catechol ring of the L-DOPA subunit can be oxidized to an ortho-quinone selectively with sodium periodate (NaIO₄). At close proximity between the ubiquitin and its binding target protein, the electrophilic quinone intermediate reacts with the nucleophilic residue of the protein. The results showed that the proteasome function was not affected by the chemical activator NaIO₄. Since the Kodadek method was very effective in their studies, it will be used for the affinity labeling work in this research. The affinity label (L-DOPA) will be utilized to investigate the binding of KCN-1 to its target protein.
1.5 Purpose of the Study

Figure 1.4: Photocleavable linker attached to the immobilized bead, L-DOPA, and KCN-1.

The purpose of this study is to design a small molecule that will identify the binding target of KCN-1. Thus, the objective is to synthesize the molecule shown in Figure 1.4. This molecule contains inhibitor KCN-1, a chemically activatable affinity label L-DOPA, the bead, and a photocleavable linker.
The proposed mechanism of this project consists of KCN-1 binding to a protein at a specific site. As mentioned above, the L-DOPA is the chemically activatable affinity label molecule. It is used to form a covalent bond with the binding target. When the target protein and the KCN-1 are closely associated, the chemical activator NaIO₄ converts the catechol of L-DOPA to a very reactive electrophile. As shown in Figure 1.5, sodium periodate (NaIO₄) oxidizes the catechol of L-DOPA to form 1,2-benzoquinone. Thus, a nucleophile from the receptor of the target protein reacts with this electrophilic quinone. This reaction converts the quinone back to the catechol. Overall, this procedure forms a covalent bond between the affinity label molecule L-DOPA and the binding target.
After the protein undergoes enzyme digestion, as shown in Figure 1.6, the fragment covalently bound to the photocleavable linker is pulled out through the use of the bead, which is insoluble so it can easily separate from the solution and other fragments. The linker and immobilized bead are cleaved from the rest of the molecule once separated from the solution. This process occurs when UV light of 365 nm excites the *trans*-alkene, leading to isomerization and formation of the *cis*-alkene\textsuperscript{10}. The linker then undergoes intramolecular cyclization reaction. The carbonyl, adjacent to the double bond carbon, reacts with the hydroxyl, on the aromatic ring, to form a chromanone and cleaves the KCN-1, L-DOPA, and the residue covalently bond to the L-DOPA\textsuperscript{10}. Then, the covalently linked residue is analyzed through mass spectroscopy by ESI or MALDI. The molecular weight is used to determine the sequence of the binding target. If the design is successful, this method is able to analyze the mass of the binding sequence and identify the target protein.
Figure 1.7: The retrosynthetic analysis of the photocleavable linker.

The retrosynthesis of the photocleavable linker is shown in Figure 1.7. Amidation is the key component, which makes this synthetic route possible. Deprotection of Boc- and MOM-group make it possible for the amidation with the immobilized bead. Compound 11 is formed by reacting KCN-1 free amine 12d and carboxylic acid of the l-DOPA 10. The l-DOPA fragment is commercially available and is added by yet another amidation with the linker 8.
2. DISCUSSION

2.1 Synthesis of Compound 11

Scheme 1. Synthetic route of compound 11

The synthesis of the photocleavable linker was accomplished in 3.6% overall yield, as shown in Scheme 1. The first step was the iodination of commercially available \( m \)-hydroxybenzoic acid 1 by the use of iodine and potassium iodide in aqueous ammonia. The
aromatic carboxylic acid 2 was reduced with borane in tetrahydrofuran to generate primary alkyl benzylic alcohol 3. Protection of the hydroxyl was needed for the conversion of the alcohol 4 to azide 5. Thus, the hydroxyl on the aromatic ring 3 was protected with chloromethyl methyl ether (MOMCl). The next step was azidation of the primary alkyl benzylic alcohol 4 using diphenylphosphoryl azide (DPPA) in the presence of strong organic base diazabicycloundecane (DBU). This reaction was preceded by O-phosphorylation followed by S<sub>N</sub>2 displacement to generate the azide compound 5. This compound was reduced to amine by a mild method of Staudinger reaction, which used triphenylphosphine as the reducing agent<sup>11</sup>. The final reaction yielded triphenylphosphine oxide as the side product and the amine compound 6. The amine needed to be preserved for the attachment with the bead at the end. Thus, the Boc-protected compound 7 was generated using di-tert-butyl dicarbonate in the presence of base triethylamine in methanol. The next step was to create the photochemical esterase. This step was a simple Heck reaction using palladium catalyze carbon-carbon coupling between aryl iodide and activated alkene, acrylic acid, in the presence of K<sub>2</sub>CO<sub>3</sub> base<sup>12</sup>. The carboxylic acid 8 was activated with N-hydroxysuccinimide (NHS) and coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDCI) in dichloromethane (DCM). The activated ester 9 reacted with commercially available (S)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid (l-DOPA) and triethylamine in DMF to generate compound 10. Compound 11 was successfully synthesized by amidation of compound 10 with KCN-1 amine 12d in the presence of coupling agents EDCI and hydroxybenzotriazole (HOBt). The final protected compound 11 was purified by HPLC.
2.2 Synthesis of KCN-1 amine 12d

Scheme 2. Synthetic route of compound 12d (KCN-1 amine).

The KCN-1 amine 12d was synthesized as shown in Scheme 2. The KCN-1 12a was made available by the Van Meir Laboratory at Emory University. The methoxy on the para-position was selectively demethylated with thiophenol and K$_2$CO$_3$ base in N-Methyl-2-pyrrolidone (NMP) at temperature of 150 ºC. The time was extremely important because if the reaction ran more than five minutes, the methoxy on the meta-position would be demethylated also. Then the hydroxyl compound 12b was alkylated with N-(4-bromobutyl) phthalimide and K$_2$CO$_3$ base in dimethylformamide (DMF). The phthalimide KCN-1 12c was reduced to the final KCN-1 amine 12d by refluxing with hydrazine in a mixture of ethanol and dichloromethane.
2.3 Approaches to Compound 13

Although compound 11 was successfully synthesized, there were many complications for the deprotection of MOM- and Boc- groups, as shown in Scheme 3. The molecule was very fragile to harsh conditions. The trifluoroacetic acid (TFA), pKa of ~0.3, was first used to remove the MOM- and Boc- groups, which was unsuccessful. At 10% TFA in DCM, only the Boc-group was cleaved and MOM-group was intact. At 50% TFA in DCM, the products were too complicated to separate and the residue, left after the reaction, had overlapping peaks on the HPLC. Apparently, TFA was not a good reagent for the removal of the MOM- and Boc- groups. Thus, other methods were considered.

It was reported that trace amount of concentrated HCl in MeOH at 62 ºC for 15 minutes can cleave the MOM-group\textsuperscript{13}. There was also an experiment that reported 4 M HCl in MeOH at room temperature for 30 minutes can cleave the Boc-group\textsuperscript{14}. In addition, water in the
concentrated HCl has been used to prevent double bond isomerization that occur under anhydrous acidic condition\textsuperscript{15}. Therefore, the second route was to use a trace concentrated HCl in MeOH at 60 °C for 15 minutes. Unfortunately, this experiment was not successful either. KCN-1 was found to be sensitive in acidic environment and decomposed easily based on $^1$H NMR.

Since HCl was too acidic for compound 11, the search for better reagents led to boron trihalides. Some boron trihalides, such as boron trichloride (BCl\textsubscript{3}), boron tribromide (BBr\textsubscript{3}), and boron trifluoride (BF\textsubscript{3}) were considered to be good candidates. Test model reactions were conducted to determine the effectiveness of the boron trihalides. These tests consisted of one equivalent of KCN-1, one equivalent of photocleavable linker derivative ester, and two equivalents of the boron trihalides in DCM under -100 °C, as shown in Scheme 4. The products were the deprotected ester linker (P1) and the decomposed KCN-1 (P2). The R in P2 meant that portion of KCN-1 had decomposed. After the reaction was completed, the product was separated by flash chromatography and analyzed by $^1$H NMR.

Scheme 4. Test model reactions of boron trihalides in DCM under -100 °C.
Figure 2.1: Test model reactions monitored by TLC.

Thin layer chromatography (TLC) was used to monitor the overall reaction as shown in Figure 2.1. For boron trichloride, the ester linker was deprotected completely within 30 minutes and KCN-1 decomposed slightly. However, for boron tribromide, the ester linker was not fully deprotected and KCN-1 decomposed entirely at 15 minutes. Boron trifluoride was even worse than BBr₃. For BF₃, KCN-1 had decomposed at 10 minutes. Based on these results, BCl₃ was a better candidate because BF₃ and BBr₃ are too acidic for the reaction. A milligram test of compound 11 will be used to determine the reactivity of BCl₃ soon. Hopefully, the deprotection of compound 11 will be resolved as expected.
3. CONCLUSION

Compound 11 was synthesized in 10 steps with 3.6% overall yield for now, but the step of deprotection seems more difficult than expected. Searching for an effective deprotecting reagent for compound 11 is underway now. If an effective reagent cannot be found in the near future, the design of the research has to be modified to replace the MOM- group with a different protecting group. Hopefully, the deprotection of compound 11 will be resolved soon. Then this research will be complete once compound 11 is deprotected and attached to the immobilized bead. After the small molecule is completely synthesized, it will be sent off for affinity labeling and analysis of the mass of the peptide sequence to determine the binding target of KCN-1.
4. EXPERIMENTAL

4.1 General Methods

NMR spectra were recorded at 400 MHz for $^1$H and 100 MHz for $^{13}$C on a Bruker 400 Avance instrument. Chemical shifts (δ values) and coupling constants (J values) are reported in parts per million (ppm) and hertz (Hz), respectively, using TMS ($^1$H NMR) or deuterated solvent ($^{13}$C NMR) as the internal standard (s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, br = broad). Unless otherwise noted, all reagents and the authentic samples were purchased from commercial sources and were used without further purification. Flash chromatography was carried out on flash silica gel (Sorbent 230–400 mesh). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). Mass spectroscopy was recorded on API 3200 LC/MS/MS system.

4.2 Photocleavable Linker

3-Hydroxy-4-iodobenzoic acid 2. To a mixture of a solution of 3-hydroxybenzoic acid 1 (13.8 g, 0.10 mol) in concentrated aqueous ammonia was slowly added a mixture of I₂ (23.4 g, 0.09 mol) and potassium iodide (18.6 g, 0.11 mol) in de-ionized water (100 mL) (to prevent from generating NI₃). The reaction mixture was stirred at room temperature for 40 minutes, and then acidified by concentrated HCl (180 mL). The precipitate was collected by filtration. The filtrate was extracted with EtOAc (250 mL × 2). The precipitate was combined with the organic layer and the EtOAc was removed under reduced pressure. The crude compound was purified by recrystalization (H₂O/MeOH=5/1) to yield white crystal (9.24 g, 35%). $^1$H NMR (MeOD): δ 7.79 (d, J=8.0 Hz, 1H), 7.46 (d, J=2.0 Hz, 1H), 7.22 (dd, J=8.0, 2.0 Hz, 1H). $^{13}$C NMR (MeOD): δ 169.5, 158.3, 140.6, 133.4, 123.1, 116.3, 93.4. ESI-MS (−): Calcd for C₇H₅I₂O₃: 263.93 m/z. Found: 262.9 m/z [M–H]⁻.
5-(Hydroxymethyl)-2-iodophenol 3. Under N₂ atmosphere at 0 ºC, to a solution of 3-hydroxy-4-iodobenzoic acid 2 (9.30 g, 0.04 mol) in anhydrous THF (100 mL) was slowly added borane in THF (70.0 mL, 0.07 mol). The reaction mixture was stirred at room temperature overnight. Then reaction mixture was quenched with 2 M Na₂CO₃ (10 mL). The aqueous solution was extracted with Et₂O (20 mL × 2). The organic layer was dried with Na₂SO₄ and concentrated under reduced pressure. The crude compound was purified by flash chromatography on silica gel (hexane/EtOAc=5/1) to yield pale yellow crystal (8.60 g, 86%). ¹H NMR (CD3OD): δ 7.61 (d, J=8.0 Hz, 1H), 6.86 (s, 1H), 6.59 (dd, J=8.0 Hz, 1H), 4.50 (s, 2H). ¹³C NMR (CD3OD): δ 158.2, 145.0, 140.3, 120.9, 114.4, 82.9, 64.6. ESI-MS (−): Calcd for C₇H₇IO₂: 249.95 m/z. Found: 248.4 m/z [M−H]⁻.

(4-Iodo-3-(methoxymethoxy)phenyl)methanol 4. To a mixture of 5-(hydroxymethyl)-2-iodophenol 3 (5.00 g, 0.02 mol) and K₂CO₃ (5.60 g, 0.04 mol) in acetone (4 mL) was added methoxymethyl chloride (4 mL, 0.53 mol) and the resulting mixture was stirred at room temperature overnight. The reaction mixture was filtered and washed with acetone. The filtrate was concentrated under reduced pressure. The residue was added H₂O (20 mL) and EtOAc (20 mL). Then the aqueous solution was further extracted with EtOAc (20 mL × 2). The organic layer was dried with Na₂SO₄ and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (hexane/EtOAc=5/1) to yield pale yellow crystal (8.60 g, 86%). ¹H NMR (CD3OD): δ 7.61 (d, J=8.0 Hz, 1H), 6.86 (s, 1H), 6.59 (dd, J=8.0 Hz, 1H), 4.50 (s, 2H). ¹³C NMR (CD3OD): δ 158.2, 145.0, 140.3, 120.9, 114.4, 82.9, 64.6. ESI-MS (−): Calcd for C₉H₁₁IO₃: 293.98 m/z. Found: 292.9 m/z [M−H]⁻.

4-(Azidomethyl)-1-iodo-2-(methoxymethoxy)benzene 5. Under N₂ atmosphere at 0 ºC, to a solution of (4-iodo-3-(methoxymethoxy)phenyl) methanol 4 (104 mg, 0.35 mmol) in
anhydrous THF (5 mL) was added diphenyl phosphorazidate (DPPA) (91 µL, 0.42 mmol) and the resulting mixture was stirred for ten minutes. Then diazabicycloundecene (DBU) (64 µL, 0.42 mmol) was added into the solution. The reaction was warmed to room temperature and stirred overnight under N\textsubscript{2}. Then reaction mixture was concentrated under reduced pressure. The residue was added H\textsubscript{2}O (10 mL) and EtOAc (10 mL). The aqueous solution was further extracted with EtOAc (10 mL \(\times\) 2). The organic layer was dried with Na\textsubscript{2}SO\textsubscript{4} and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (hexane/EtOAc=20/1) to yield yellow liquid (78 mg, yield 70%). 1H NMR (CDCl\textsubscript{3}): \(\delta\) 7.74 (d, \(J\=8.0\) Hz, 1H), 7.01 (d, \(J\=2.0\) Hz, 1H), 6.71 (dd, \(J\=8.0, 2.0\) Hz, 1H), 5.23 (s, 2H), 4.27 (s, 2H), 3.49 (s, 3H). 13C NMR (CDCl\textsubscript{3}): \(\delta\) 156.3, 139.7, 137.3, 123.2, 114.4, 94.9, 86.8, 56.5, 54.2.

ESI-MS (−): Calcd for C\(_9\)H\(_{10}\)IN\(_3\)O\(_2\): 318.98 m/z. Found: 317.7 m/z [M−H]\(^−\).

\textbf{(4-iodo-3-(methoxymethoxy)phenyl)methanamine} 6. To a solution of 4-(azidomethyl)-1-iodo-2-(methoxymethoxy)benzene 5 (1.60 g, 4.9 mmol) and triphenylphosphine (PPh\(_3\)) (2.60 g, 9.9 mmol) in THF (49 mL, 10 mL per mmol) was added de-ionized water (2 mL, 0.04 mL per mmol) and the reaction was stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure. The residue was added brine (10 mL) and EtOAc (10 mL). The aqueous solution was further extracted with EtOAc (15 mL \(\times\) 2). The organic layer was dried with Na\textsubscript{2}SO\textsubscript{4} and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (hexane/EtOAc=20/1 (200 mL) to elute out PPh\(_3\), then pure EtOAc (100 mL) once the triphenylphosphine oxide came out, finally pure MeOH (300 mL) for the product) to yield yellow liquid (1.35 g, yield 94%). ESI-MS (+): Calcd for C\(_9\)H\(_{12}\)INO\(_2\): 292.99 m/z. Found: 293.9 m/z [M+H]\(^+\).
**tert-Butyl 4-iodo-3-(methoxymethoxy)benzylcarbamate 7.** To a solution of (4-iodo-3-(methoxymethoxy)phenyl)methanamine 6 (25 mg, 0.08 mmol) and di-tert-butyl dicarbonate (23 mg, 0.10 mmol) in MeOH (5 mL) was added triethylamine (14 µL, 0.10 mmol) and the resulting mixture was stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (hexane/EtOAc=10/1) to yield yellowish white crystal (17 mg, yield 53%). \(^1\)H NMR (D\(_2\)O): \(\delta\) 7.71 (d, \(J=8.0\) Hz, 1H), 6.98 (d, \(J=2.0\) Hz, 1H), 6.71 (d, \(J=8.0\) Hz, 1H), 5.23 (s, 2H), 4.89 (s, 1H), 4.25 (d, \(J=8\) Hz, 2H), 3.51 (s, 3H), 1.46 (s, 9H). \(^1^3\)C NMR (D\(_2\)O): \(\delta\) 156.4, 156.1, 141.4, 139.7, 122.8, 114.1, 95.2, 85.7, 79.9, 56.7, 44.5, 28.6, 27.6. ESI-MS (+): Calcd for C\(_{14}\)H\(_{20}\)INO\(_4\): 393.04 m/z. Found: 394.4 m/z [M+H]^+.

**(E)-3-(4-((tert-Butoxy carbonylamino) methyl)-2- (methoxymethoxy) phenyl) acrylic acid 8.** Under N\(_2\) atmosphere at room temperature, to a mixture of (\(t\)-Bu)\(_4\)N\(^+\) Br (188 mg, 0.58 mmol), Pd(OAc)\(_2\) (5.4 mg, 0.02 mmol), and K\(_2\)CO\(_3\) (642 mg, 4.7 mmol) was added a solution of tert-butyl 4-iodo-3-(methoxymethoxy)benzylcarbamate 7 (183 mg, 0.47 mmol) in DMF (2 mL). Then acrylic acid (48 µL, 0.70 mmol) was added into the mixture. The reaction mixture was heated to 60\(^\circ\) C and stirred overnight. Then the reaction mixture was cooled to room temperature. Cold H\(_2\)O (5 mL) and 1 M HCl (1 mL) were added to the mixture. The reaction mixture was added brine (10 mL) and EtOAc (15 mL). The aqueous solution was further extracted with EtOAc (15 mL \(\times\) 2). The organic layer was dried with MgSO\(_4\) and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (hexane/EtOAc=20/1 (200 mL), 4/1 (400 mL), 1/1 (200 mL)) to yield yellow crystal (81 mg, 51%). \(^1\)H NMR (D\(_2\)O): \(\delta\) 8.09 (d, \(J=16.0\) Hz, 1H), 7.51 (d, \(J=8.0\) Hz, 1H), 7.08 (s, 1H), 6.95 (d, \(J=8.0\) Hz, 1H), 6.51 (d, \(J=16\) Hz, 1H), 5.26 (s, 2H), 4.96 (s, 1H), 4.31 (d, \(J=8\) Hz, 2H), 3.51 (s,
3H), 1.47 (s, 9H). $^{13}$C NMR (D$_2$O): $\delta$ 172.5, 156.6, 156.2, 143.8, 141.9, 129.2, 123.0, 120.9, 117.9, 113.8, 94.8, 80.0, 56.6, 44.7, 28.6. ESI-MS (−): Calcd for C$_{17}$H$_{23}$NO$_6$: 337.15 m/z. Found: 336.2 m/z [M−H]$^−$.

$(E)$-2,5-Dioxopyrrolidin-1-yl 3-(4-((tert-butoxycarbonylamino)methyl)-2-(methoxymethoxy)phenyl)acrylate 9. Under N$_2$ atmosphere at room temperature, to a solution of $(E)$-3-(4-((tert-butoxycarbonylamino)methyl)-2-(methoxymethoxy)phenyl)acrylic acid 8 (70 mg, 0.21 mmol) and EDCI (39 mg, 0.21 mmol) in anhydrous DCM (3 mL) was added N-hydroxysuccinimide (24 mg, 0.21 mmol) dissolved in anhydrous DCM (1 mL) and the resulting mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure. The residue was added brine (10 mL) and EtOAc (10 mL). The aqueous solution was further extracted with EtOAc (10 mL × 2). The organic layer was dried in MgSO$_4$ and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (hexane/EtOAc=4/1 (100 mL), 3/1 (400 mL), and 1/1 (300 mL)) to yield colorless crystals (57 mg, 63%). ESI-MS (+): Calcd for C$_{21}$H$_{26}$N$_2$O$_8$: 434.17 m/z. Found: 435.1 m/z [M+H]$^+$.  

$(E)$-2-(3-(4-((tert-Butoxycarbonylamino)methyl)-2-(methoxymethoxy)phenyl) acrylamido) -3-(3,4-dihydroxyphenyl)propanoic acid 10. To a solution of $(E)$-2,5-dioxopyrrolidin-1-yl 3-(4-((tert-butoxycarbonylamino)methyl)-2-(methoxymethoxy)phenyl)acrylate 9 (57 mg, 0.13 mmol) and (S)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid (L-DOPA) (22 mg, 0.11 mmol) in anhydrous DMF (1.5 mL) was added triethyl amine (NEt$_3$) (18 µL, 0.13 mmol) and the reaction was stirred at room temperature overnight. Then the pH was adjusted from 8 to 3 by adding 1 M HCl (~10 mL) to the mixture. The reaction mixture was added brine (10 mL) and EtOAc (15 mL). The aqueous solution was further extracted with EtOAc (10 mL × 2). The organic layer was dried in MgSO$_4$ and concentrated under reduced pressure. The crude was
purified by flash chromatography on silica gel (DCM/MeOH=14/1 (200 mL), 10/1 (400 mL), and 5/1 (100 mL)) to yield yellow crystals (46 mg, 68%). $^1$H NMR (MeOD): $\delta$ 7.82 (d, $J$=16 Hz, 1H), 7.47 (d, $J$=8 Hz, 1H), 7.07 (s, 1H), 6.89 (d, $J$=8 Hz, 1H), 6.70 (d, $J$=16 Hz, 1H), 6.67 (s, 1H), 6.66 (d, $J$=8 Hz, 1H), 6.57 (d, $J$=8 Hz, 1H), 5.22 (s, 2H), 4.71 (dd, $J$=7.2, 5.6 Hz, 1H), 4.19 (s, 2H), 3.43 (s, 3H), 3.10 (dd, $J$=14, 6 Hz, 1H), 2.91 (dd, $J$=14, 6 Hz, 1H), 1.45 (s, 9H). $^{13}$C NMR (MeOD): $\delta$ 175.9, 168.8, 158.7, 157.3, 146.2, 145.2, 144.6, 137.0, 130.1, 129.3, 124.4, 121.9, 121.8, 121.6, 117.5, 116.4, 114.6, 95.8, 80.4, 56.9, 56.1, 44.9, 38.2, 28.9. Calcd for C$_{26}$H$_{32}$N$_{2}$O$_{9}$: 516.21 m/z. Found: 515.1 m/z [M–H].

(E)-tert-Butyl 4-(3-(3,4-dihydroxyphenyl)-1-(4-(4-((2,2-dimethyl-2H-chromen-6-yl)methyl)-N-phenylsulfamoyl)-2-methoxyphenoxy)butylamino)-1-oxopropan-2-ylamino)-3-oxoprop-1-enyl)-3-(methoxymethoxy)benzylcarbamate 11. Under N$_2$ atmosphere at room temperature, to a solution of (E)-2-(3-(4-((tert-butoxycarbonylamino) methyl)-2-(methoxy methoxy)phenyl) acrylamido)-3-(3,4-dihydroxy phenyl) propanoic acid 10 (21 mg, 0.04 mmol), EDCI hydrochloride (8, 0.04 mmol), and HOBt (5 mg, 0.04 mmol) in anhydrous DCM (2 mL) was added KCN-1 amine 12d (20 mg, 0.04 mmol) dissolved in anhydrous DCM (0.2 mL) by a syringe. Two equivalent of anhydrous DCM (0.2 mL) was used to wash the vial and syringe used to add the KCN-1 amine. The reaction was stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (DCM/MeOH=20/1 (100 mL), 10/1 (100 mL), 5/1 (150 mL), pure MeOH (50 mL)) to yield white crystal (19 mg, 47%). $^1$H NMR (MeOD): $\delta$ 7.85 (d, $J$=16 Hz, 1H), 7.51 (d, $J$=8 Hz, 1H), 7.29 (m, 2H), 7.23 (m, 3H), 7.08 (m, 3H), 7.00 (m, 2H), 6.93 (m, 2H), 6.82 (m, 1H), 6.69 (m, 2H), 6.60 (m, 1H), 6.53 (m, 1H), 6.22 (d, $J$=10 Hz, 1H), 5.62 (d, $J$=10 Hz, 1H), 5.25 (s, 3H), 4.61 (s, 3H), 4.21 (s, 2H), 4.02 (t, 2H), 3.69 (s, 3H), 3.44 (s,
3H), 3.14 (dd, J=12, 6 Hz, 1H), 2.96 (dd, J=12, 6 Hz, 1H), 2.86 (dd, J=12, 6 Hz, 1H), 1.65 (m, 5H). $^{13}$C NMR (MeOD): $\delta$ 173.7, 168.8, 157.5, 153.9, 150.6, 146.4, 145.4, 140.6, 137.3, 132.2, 131.1, 130.6, 129.9, 129.1, 127.9, 124.5, 123.2, 122.6, 121.75, 117.6, 117.1, 116.6, 114.7, 113.2, 111.9, 95.9, 80.5, 77.5, 70.0, 57.1, 56.7, 55.1, 49.8, 48.5, 45.0, 40.0, 38.9, 30.9, 28.9, 28.3, 27.3, 27.0. ESI-MS (−): Calcd for C$_{55}$H$_{64}$N$_4$O$_{13}$S: 1020.42 m/z. Found: 1019.8 m/z [M−H]$^−$.

4.3 KCN-1 AMINE (12d)

$N$-((2,2-Dimethyl-2H-chromen-6-yl)methyl)-4-hydroxy-3-methoxy-$N$-phenyl benzene sulfonamide 12b. Under N$_2$ atmosphere at 150 °C, to a mixture of K$_2$CO$_3$ (149 mg, 1.07 mmol) in N-Methyl-2-pyrrolidone (NMP) (2 mL) was added thiophenol (PhSH) (0.11 mL, 1.07 mmol) and the reaction mixture was stirred for 15 minutes. Then a solution of $N$-((2,2-dimethyl-2H-chromen-6-yl)methyl)-3,4-dimethoxy-$N$-phenylbenzene-sulfonamide 12a (100 mg, 0.21 mmol) in NMP (1 mL) was added into the reaction mixture. The reaction was stirred for 5 minutes (no longer than 5 minutes) and immediately poured into cold de-ionized H$_2$O (25 mL). The aqueous solution was extracted with EtOAc (50 mL × 4). Brine (~10 mL) was added as needed for better separation. The organic layer was washed with de-ionized water (20 mL × 6) to get rid of most of NMP. The organic layer was dried in MgSO$_4$ and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (DCM/EtOAc=50/1 (500 mL), 30/1 (200 mL)) to yield white crystal (57 mg, 60%). ESI-MS (−): Calcd for C$_{25}$H$_{25}$NO$_5$S: 451.15 m/z. Found: 450.1 m/z [M−H]$^−$.

$N$-((2,2-Dimethyl-2H-chromen-6-yl)methyl)-4-((4-(1,3-dioxoisindolin-2-yl)butoxy)-3-methoxy-$N$-phenylbenzenesulfonamide 12c. Under N$_2$ atmosphere at 50 °C, a solution of 2-(4-bromobutyl) isoindoline-1,3-dione (90 mg, 0.32 mmol), $N$-((2,2-dimethyl-2H-chromen-6-yl)methyl)-4-hydroxy-3-methoxy-$N$-phenyl benzene sulfonamide 12b (130 mg, 0.29 mmol), and
K₂CO₃ (44 mg, 0.32 mmol) in (DMF) (2 mL) was stirred overnight. The reaction mixture was added de-ionized H₂O (20 mL) and EtOAc (20 mL). The aqueous solution was further extracted with EtOAc (100 mL × 2). The organic layer was dried in MgSO₄ and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (DCM/EtOAc=50/1) to yield colorless solid (89 mg, 47%). ¹H NMR (MeOD): δ 7.75 (m, 2H), 7.65 (m, 2H), 7.32 (m, 1H), 7.27 (m, 3H), 6.99 (m, 2H), 6.97 (m, 4H), 6.59 (d, J=8 Hz, 1H), 6.21 (d, J=8 Hz, 1H), 5.55 (d, J=8 Hz, 1H), 4.59 (s, 2H), 4.12 (m, 3H), 3.88 (m, 2H), 3.73 (s, 3H), 2.04 (s, 1H), 1.92 (s, 4H), 1.27 (s, 6H), 1.24 (m, 2H). ESI-MS (+): Calcd for C₃₇H₃₆N₂O₇S: 652.22 m/z. Found: 653.3 m/z [M+H]+.

4-(4-Aminobutoxy)-N-((2,2-dimethyl-2H-chromen-6-yl)methyl)-3-methoxy-N-phenyl benzenesulfonamide (KCN-1 amine) 12d. Under N₂ atmosphere at refluxed, to a solution of N-((2,2-dimethyl-2H-chromen-6-yl)methyl)-4-(4-(1,3-dioxoisoindolin-2-yl)butoxy)-3-methoxy-N-phenylbenzenesulfonamide 12c (48 mg, 0.07 mmol) in EtOH/DCM (75/25) (3 mL) was added hydrazine monohydrate (H₂N-NH₂·H₂O) (7.1 µL, 0.15 mmol) and the resulting mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure and then vacuum filtrate with DCM. The filtrate organic layer was concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (DCM/MeOH=20/1 (200 mL), 10/1 (500 mL), 5/1 (300 mL)) to yield colorless solid (20 mg, 55%). ¹H NMR (MeOD): δ 7.29 (m, 1H), 7.27 (m, 3H), 6.98 (m, 2H), 6.94 (m, 1H), 6.91 (m, 2H), 6.85 (m, 1H), 6.57 (d, J=8 Hz, 1H), 6.20 (d, J=8 Hz, 1H), 5.55 (d, J=8 Hz, 1H), 4.60 (s, 2H), 4.05 (t, 2H), 3.69 (s, 3H), 2.74 (t, 2H), 1.87 (m, 5H), 1.70 (s, 3H), 1.61 (m, 2H), 1.35 (s, 6H). ESI-MS (+): Calcd for C₂₉H₃₄N₂O₅S: 522.22 m/z. Found: 523.3 m/z [M+H]+.
REFERENCE


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**Process Parameters**

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**Chemical Shift**:

- ** Dax**: 0.00000000 ppm
- ** Bax**: 0.00000000 ppm
- ** Eax**: 0.00000000 ppm
- ** Fex**: 0.00000000 ppm

**Additional Information**

- ** Molec**: 5.099 µs
- ** Time**: 10.33 µs
- ** Date**: 22091217

**Acquisition Parameters**

- ** Proton**: 1
- ** Emitter**: 1
- ** Receiver**: 1
- ** Current Data Parameters**

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**Bruker**

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