Harnessing *bis*-Electrophilic Boronic Acid Lynchpin for Azaborolo Thiazolidine (ABT) Grafting in Cyclic Peptides

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I. General Remarks:

Ia. Chemical and cell line

4-Hydroxybenzyl alcohol was purchased from BLD Pharma, India. All the Fmoc-protected amino acids, 2-(1H-benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexaflurophospahte (HBTU) and Rink amide AM-polystyrene resin (loading: 0.67 mmol/g) were purchased from Supra Sciences, India. Trifluoroacetic acid (TFA), N, N-Diisopropylethylamine (DiPEA), and Thioanisole were procured from Spectrochem, India. Bis(pinacolato)diboron (B_2pin_2) was purchased from Avra Synthesis, India. Potassium carbonate was purchased from Loba Chemie, India. N-phenyl-bis(trifluoromethanesulfonimide) ($PhNTf_2$) [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) ($Pd(dppf)Cl_2$), potassium acetate, Di-tert-butyl-dicarbonate, Tris(2-carboxyethyl)phosphine, Sodium sulfate, iodine, anisole were purchased from GLR Innovations, India. Phenol and 1, 2- Ethanedithiol were purchased from SRL and TCI chemicals. General solvents, DMF, DCM, Diethyl ether, Methanol, and HPLC grade acetonitrile were purchased from Finar, India. Iodoacetic acid was purchased from Sigma Aldrich, India.

RPMI media, Trypsin - EDTA Solution 1X, and DPBS were purchased from Gibco. Tris base and Dimethyl sulfoxide (DMSO, Molecular Biology grade) were purchased from Himedia. A549, Mia Paca 2 and HEK293 cells were purchased from the National Centre for Cell Sciences, Pune, India. 96-well plates and cell culture flasks were purchased from Tarson, India, Glass bottom Confocal Petridishes were purchased from Himedia. Annexin-V FITC Apoptosis Kit was purchased from Elabsciences. JC-1 dye and Mito Tracker Red were purchased from Chemscene.

Ib. Instruments:

All the NMR spectra were recorded in a 400 MHz Jeol JNM ECS400 NMR spectrometer. A Waters XEVO G2-XS QTOF mass spectrometer and Waters UPLC-assisted SQD2 collected and generated all the mass spectrometric data. HPLC purification and data generation were done on the Shimadzu Prominence UFLC system. Cell survival studies (MTT Assay) were monitored in a multimode plate reader Tecan Infinite 200 F Plex. AnnexinV-FITC apoptosis studies were performed in Sysmex Cube-6 Flowcytometer.

Ic. General methods for LC-MS analysis for monitoring reaction

Method 1: In typical experiments, Waters Acquity UPLC C18 (1.7 µm, 2.1×50 mm) analytical column was employed for the LC-MS analysis. This method spanned 7 min; the mobile phase acetonitrile-water (0.1% HCOOH) with a flow rate of 0.4 mL/min was used. The gradient was fixed at 5% to 35% acetonitrile in 2.5 min. A gradient from 35% to 95% acetonitrile in 2 min followed by isocratic 95% over a minute and then column equilibration at the initial isocratic gradient of 5% acetonitrile over 1.5 min.

Method 2: This method lasted for 11 min program with flow 0.3 mL/min; the gradient was kept at 5% to 35% acetonitrile over 8 min, along with a gradient from 35% to 95% acetonitrile in 2 min, followed by isocratic 95% over a minute and then column equilibration at the initial isocratic gradient 5% acetonitrile over 1.5 min. All the chromatograms were monitored at 220 nm, employing a D2 lamp as a light source. The kinetics of the reactions were calculated by integrating the peak area. For MS analysis experiments, all samples were subjected to the ESI +ve mode.

Method 3: It is a 16-min long method with a flow rate of 0.25 mL/min for prudent separation; the gradient was kept as 5% to 60% acetonitrile over 11.5 min, along with a gradient from 60% to 90% acetonitrile in 1 min followed by the initial isocratic gradient 5% acetonitrile over 0.5 min. Lastly, an isocratic mode was continued at 5% for 3 min. All the chromatograms were monitored at 220 nm, employing a D2 lamp as a light source. The kinetics of the reactions were calculated by integrating the remaining peak area of linear peptide. For MS analysis experiments, all samples were subjected to the ESI +ve mode.

Id. Analytical HPLC methods for peptides

Method 1:

Waters Reliant C18 (5 µm, 4.6×250 mm, 100 Å) analytical column was used for monitoring the chromatograms, and mobile phase acetonitrile-water (0.05% TFA) with a flow rate of 1 mL/min was used. The gradient was fixed as isocratic 10% acetonitrile over 1 min initially. A gradient from 10% to 50% acetonitrile in 19 min, then a gradient from 50% to 90% acetonitrile over 2 min, followed by isocratic 90% acetonitrile over 3 min, at the end column equilibration at the initial isocratic gradient 10% acetonitrile over 5 min.

Method 2 (for highly polar peptides):

Waters Reliant C18 (5 µm, 4.6×250 mm, 100 Å) analytical column and mobile phase acetonitrile-water (0.05% TFA) with a 1 mL/min flow rate were used. The gradient utilized was isocratic 5% acetonitrile over 1 minute, a gradient from 5% to 25% acetonitrile was continued for 19 min, followed by a gradient from 25% to 90% acetonitrile in 2 min, isocratic 90% acetonitrile in 3 min, and column equilibration at the starting isocratic gradient of 5% acetonitrile in 5 min. The chromatogram was processed at 220 nm using a D2 Lamp.

Ie. Semi-preparative HPLC method for peptide purifications

Method 1:

Shimadzu shim-pack GIST C18 (5 µm, 10 × 250 mm, 100 Å) semi-preparative column was employed with a mobile phase (acetonitrile-water (0.05% TFA)) flow rate of 4 mL/min. The gradient used was isocratic 10% acetonitrile over 2 min, followed by a gradient from 10% to 60% acetonitrile over 26 min, followed by a gradient from 60% to 90% acetonitrile over three min, followed by column equilibration at the initial isocratic gradient 10% acetonitrile over 5 min.

Method 2 (for highly polar peptides):

For highly polar peptides, such as CRGDC, CSPYGRC, iRGD, and Ubiquicidine derivatives, the initial isocratic gradient was used with 5% acetonitrile over 5 min, followed by a gradient from 10% to 25% acetonitrile over 28 min. For BKD1 and BKD2 probes, the initial isocratic gradient was implemented as 10% acetonitrile over 2 min, followed by a gradient from 10% to 30% acetonitrile over 28 min. BKD3 probe was subjected to a program where a continuous gradient was employed from 10% to 95% acetonitrile over 30 min with introductory 2-minute isocratic modes with 10% acetonitrile.

All reactions were monitored at 220 nm in HPLC and LC-MS unless specified.

II. Solid phase peptide synthesis (SPPS)

All peptides have been synthesized on a solid phase deploying Fmoc-protected Rink amide AM resin using conventional Fmoc-chemistry in a glass peptide synthesis vessel with 144 mg (0.1 mmol, 0.67 mmol/g

loading capacity) resin. The systematic reaction orders are ascribed in the following sections from resin swelling to detachment of peptide backbone from the resin.

Resin swelling and Fmoc-deprotection from the resin: Before performing the peptide coupling, the required amount of resin was swelled in the DCM for 30 min. The swelled resin was washed with DMF thrice. Two times, 20% Piperidine in DMF (3mL/0.1 mmol) was subjected to the cleavage of the Fmoc group associated with the resin. After Piperidine treatment, the resin was again washed with an adequate volume of DMF.

Amino acid coupling on the resin: The peptide chain was extended at the N-terminus by coupling the relevant Fmoc-protected amino acids (3 eq.) at room temperature using HBTU (3 eq.) as a coupling agent in DMF by activating with DiPEA (6 eq.)., Fmoc-protected amino acids were activated by adding HBTU and DiPEA for 1 min prior to the charging on resin in every cycle. All coupling reaction on the resin was continued for 30 min. 1 ml capping solution containing acetic anhydride, pyridine, and DMF (1:1:3) was treated with the resin after the very first coupling to avoid truncated byproduct at the end of the full-length peptide synthesis. Fmoc cleavage of the amino acids in every cycle was performed with 20% piperidine in DMF (3mL/0.1 mmol), followed by an adequate DMF wash (3 mL × 3).

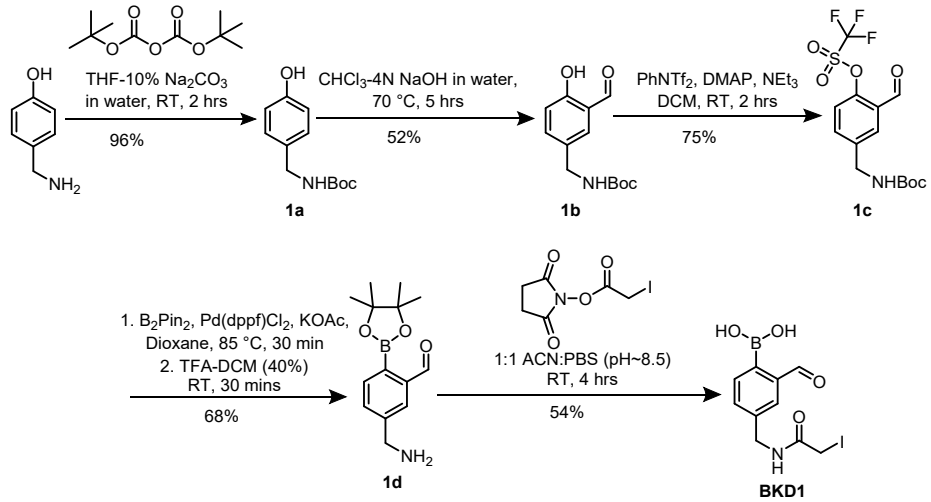
Cleavage of peptide and global deprotection: After the completion of peptide synthesis, the resin was washed six times with DCM (3 mL each) and dried under a vacuum for 30 min. To remove all side chain protecting groups and cleave off the peptide from resin, reagent K (3 mL for 0.1 mmol) was applied under stirring conditions at room temperature for 3 hours, comprising 82.5% TFA, 5% H_2O, 2.5% 1,2-ethanedithiol (EDT), 5% Thioanisole, and 5% phenol. Subsequent resin filtering, the filtrate was precipitated with 35 mL of cold diethyl ether to extract crude peptides. The isolated peptide was further washed with ether 2-3 times, and the crude material was dried entirely with air for 5 mins. Finally, the crude peptide was dissolved in a minimum volume of the required composition of acetonitrile and water in 0.1% TFA, and peptides were purified using semi-prep HPLC.

Table S1. The list of peptides synthesized on-resin and crosslinked with BKD1. Their observed mass and isolated yields are tabulated.

Peptides	Calc. Mass		Obs. Mass		Yield (%)
RGD	$[M+H]^+$	753.2615	$[M+H]^+$	753.1181	73
SPYGR	$[M-2H_2O+2H]^{2+}$	475.1844	$[M-2H_2O+2H]^{2+}$	475.1883	65
Lys-Setmelanotide	$[M-H_2O+3H]^{3+}$	431.1939	$[M-H_2O+3H]^{3+}$	431.2161	61
Vasopressin	$[M-2H_2O+2H]^{2+}$	577.7270	$[M-2H_2O+2H]^{2+}$	577.7459	70
iRGD	$[M-H_2O+2H]^{2+}$	566.7272	$[M-H_2O+2H]^{2+}$	566.7329	62
Ubiquicidine	$[M-2H_2O+5H]^{5+}$	424.8107	$[M-2H_2O+5H]^{5+}$	424.9035	64
Cono-Cy Cono-BiCy	$[M-2H_2O+2H]^{2+}$ $[M-H_2O+2H]^{2+}$	776.2940 713.2544	$[M-2H_2O+2H]^{2+}$ $[M-H_2O+2H]^{2+}$	776.2949 713.2542	58 35
Gom-Cy Gom-BiCy	$[M-H_2O+5H]^{5+}$ $[M-H_2O+5H]^{5+}$	498.6468 469.8288	$[M-H_2O+5H]^{5+}$ $[M-H_2O+5H]^{5+}$	498.6476 498.8310	67 40
P3	$[M+4H]^{4+}$	479.3133	$[M+4H]^{4+}$	479.2468	67
P4	$[M-H_2O+5H]^{5+}$	420.2619	$[M-H_2O+5H]^{5+}$	420.2519	62
P5	$[M-H_2O+3H]^{3+}$	729.4476	$[M-H_2O+3H]^{3+}$	729.4047	59
P6	$[M+2H]^{2+}$	688.7853	$[M+2H]^{2+}$	688.7587	64
P4+P6	$[M+6H]^{6+}$	576.6437	$[M+6H]^{6+}$	576.6401	57

III. Synthesis of probes: **IIIa.**

Synthetic routes of BKD1:



Scheme 1: Synthetic route of BKD1.

Synthesis of 1a: 4-(Aminomethyl)phenol (1 g, 8.13 mmol) was dissolved in a 2:1 ratio of 10% Na2CO3 aqueous solution to THF (20 mL). The temperature of the reaction mixture was then lowered to ~4 °C in an ice bath. Then, Boc-anhydride (1.95 g, 8.94 mmol in 10 mL THF) was added slowly to the reaction mixture during 10-15 min. After adding Boc-anhydride, the reaction was brought to room temperature and extended for 2 hours. The complete conversion of the starting material was monitored via TLC in different time intervals. The THF layer was evaporated under reduced pressure, and the water layer was acidified with 15 mL 1 N HCl solution. The product was extracted from the aqueous layer using EtOAc (3 × 75 mL), and the combined organic layer was washed with brine wash (2 × 50 mL). The organic layer was dried over the anhydrous Na_2SO_4 bed and reduced under vacuum to yield a dark red viscous liquid (96%, 1.74 g), which was directly subjected to the formylation reaction without any purification. The product identity was confirmed by the HRMS.

HRMS-ESI$^+$ (m/z): $[M\text{-}^t\text{Bu}+H]^+$ Calc. 168.0616, Obs. 168.0674.

Synthesis of 1b: 1a (1.5 g, 6.72 mmol) was dissolved in 20 mL 4 N NaOH solution, and the reaction mixture was heated at 100 °C for 10 min, resulting in an orange-colored solution. Then, 10 mL chloroform was added slowly and intermittently to the reaction mixture. The progress of the reaction was investigated from time to time by TLC of the reaction mixture. After 5 hours, the starting material was consumed. Then, the reaction mixture was cooled at room temperature, and the $CHCl_3$ layer was evaporated. The basic aqueous layer was acidified (pH = 3) with a few drops of concentrated HCl. The product was extracted from the aqueous layer using EtOAc (3 × 75 mL). The combined organic layer was washed with brine (2 × 50 mL) and dried under vacuum to get the crude product, which was purified through regular silica gel using 1:4 ethyl acetate/hexane as eluent to afford 52% yield (8.83 g).

^{1}H NMR: (400 MHz, $CDCl_3$) δ 10.96 (s, 1H), 9.87 (s, 1H), 7.49 – 7.41 (m, 2H), 7.12 (d, J = 8.5 Hz, 1H), 6.96 (d, J = 8.5 Hz, 1H), 4.28 (d, J = 5.4 Hz, 4H), 1.46 (s, 18H).

^{13}C NMR: (101 MHz, $CDCl_3$) δ 196.56, 160.98, 155.96, 136.58, 132.53, 130.81, 121.53, 120.43, 118.05, 79.96, 43.74, 29.78.

HRMS-ESI$^+$ (m/z): [M-^{t}Bu+H]$^+$ Calc. 195.0532, Obs. 195.1212.

Synthesis of 1c: 2b (1 g, 3.98 mmol) was allowed to dissolve in anhydrous dichloromethane (10 mL); subsequently, N-Phenyl-bis(trifluoromethanesulfonimide) (1.56 g, 4.38 mmol) and 4-(Dimethylamino)pyridine (DMAP) (48 mg, 0.39 mmol) were added while stirring in an Ar-atmosphere. While cooling the reaction liquid to 0 °C, triethylamine (1.38 mL, 7.96 mmol) was gradually added for 5 min. The reaction mixture was agitated at room temperature for 2 hours in an argon environment. A dark reddish coloration in the reaction mixture was observed during the reaction. After eliminating the DCM layer, the reaction mixture was treated with 100 mL EtOAc. Further, the organic layer was thoroughly washed with 1 N HCl (2 × 50 mL), 10% $NaHCO_3$ (2 × 50 mL), and brine solution (2 × 50 mL). The organic layer was evaporated under a vacuum, and the crude product was purified on a silica gel with EtOAc: hexane (1:4) as eluent (yield = 80%, 1.28 g).

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.26 (s, 1H), 7.89 (d, *J* = 2.1 Hz, 1H), 7.65 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.38 (d, *J* = 8.5 Hz, 1H), 4.40 (d, *J* = 6.0 Hz, 2H), 1.46 (s, 12H).

^{13}C NMR: (101 MHz, $CDCl_3$) δ 186.50, 155.93, 148.97, 140.80, 134.63, 129.11, 128.44, 122.77, 80.37, 43.55, 28.42.

HRMS-ESI$^+$ (m/z): [M+H]$^+$ Calc. 328.0058, Obs. 328.0276.

Synthesis of 1d: For the borylation reaction, a previously reported protocol was followed [1]; anhydrous dioxane (10 mL) was combined with 2c (1.0 g, 2.60 mmol), B_2Pin_2 (1.98 g, 7.82 mmol), Pd(dppf)Cl_2 (106 mg, 0.13 mmol, 5 mol%), and potassium acetate (0.86 g, 8.8 mmol). The reaction mixture was bubbled with argon for 20 minutes before stirring at 85 °C. Upon heating at 85 °C, an orange-to-dark brown coloration evolved with time. After 30 minutes of monitoring with TLC, the reaction was found to be completed. After diluting the reaction mixture with ethyl acetate (80 mL), the solution was filtered through a celite bed. The EtOAc was evaporated at a decreased pressure, and the brownish crude product was quickly purified by flash column on normal silica gel using EtOAc : hexane (2:3) as the eluent (yield = 40%, 377 mg). The resulting colorless viscous oil was subjected to Boc deprotection using 40% TFA-DCM. The excess TFA was evaporated by repeated trituration with hexane, resulting in brown oil.

HRMS-ESI$^+$ (m/z): [M-H_2O+H]$^+$ Calc. 163.0805, Obs. 163.1138.

Synthesis of BKD1:

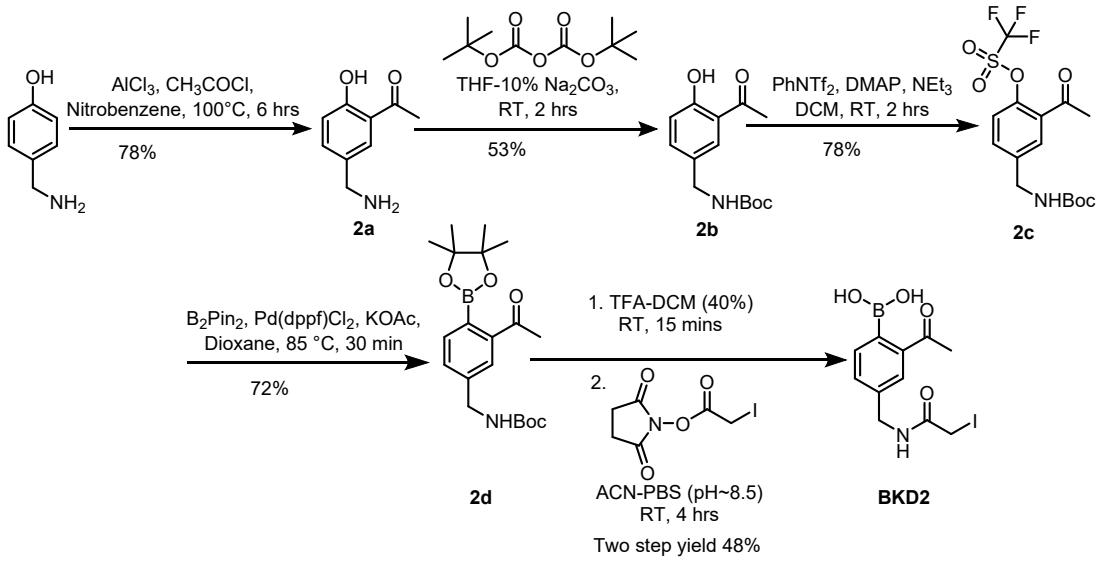
Boronic acid-bearing amine (100 mg, 0.34 mmol) was dissolved in PBS buffer (10 mM, 2 mL), and the pH was adjusted to 8.5. In an ice-cold environment, freshly prepared 2,5-dioxopyrrolidin-1-yl 2-iodoacetate (86 mg, 0.34 mmol) was dissolved in acetonitrile (2 mL) and slowly added to the buffer solution. The completion of the reaction was monitored by employing time-to-time LC-MS analyses. Under low pressure, the reaction volume was decreased, and the mixture was acidified with 0.1% TFA containing acetonitrile-water (1:1). The crude product was purified through semi-preparative HPLC method Ie (Method 1) to yield a clean product (yield = 40%, 53 mg).

^{1}H NMR: (400 MHz, DMSO-d_6) δ 10.09 (s, 1H), 8.81 (t, J = 6.0 Hz, 1H), 7.71 (d, J = 1.1 Hz, 1H), 7.56 – 7.42 (m, 2H), 4.29 (d, J = 6.0 Hz, 2H), 3.65 (s, 2H).

^{13}C NMR: (101 CD_3OD) δ 170.03, 141.31, 138.38, 130.55, 126.78, 124.56, 102.55, 43.04, -3.57.

HRMS-ESI^{+} (m/z): $[M\text{-}H_2O+H]^{+}$ Calc. 319.9055, Obs. 319.9005.

IIIb. Synthetic routes of BKD2



Scheme 2: Synthetic route of BKD2.

Synthesis of 2a: At room temperature, anhydrous aluminum chloride (10.8 g, 81.3 mmol) was gently added to a solution of 4-(aminomethyl)phenol (2 g, 16.26 mmol) in dry nitrobenzene (80 mL). Since the reaction

had an exothermic behavior, the reaction mixture was agitated at room temperature for a few minutes. Acetyl chloride (1.53 g, 19.5 mmol) was added dropwise over 5 min, eliciting a color transition from red to yellow. After cooling to ambient temperature, the reaction mixture was further heated at 100 °C in a vigorous stirring condition for 6 hours, which was transformed into a dark green gel. The thick gel was dissolved in 250 mL of chilled 2 N HCl. The aqueous phase was washed with ethyl acetate (2 × 100 mL). The aqueous layer was concentrated to approximately 50 mL in volume, stored at 4 °C for 14 hours to crystallize the product, and proceeded for the subsequent reaction.

Synthesis of 2b: The whole crystallized material (~17 mmol) was dissolved in a required volume of 10% Na_2CO_3 solution (40 mL) and THF (10 mL). After cooling for 5 min in an ice bath, Boc-anhydride (3.90 g, 17.88 mmol in 10 mL THF) was added slowly over 10 min. The reaction mixture was brought to room temperature and left for 2 hours to complete. The reaction mixture was then acidified to pH 2-3 with 1N HCl, and the product was extracted with ethyl acetate (3 × 100 mL). The mixed organic layer was washed with brine (150 mL) and dried over Na_2SO_4. The Boc-protected product was purified (yield = 45%, 1.93 g) using 1:4 ethyl acetate/hexane as eluent by column chromatography.

^{1}H NMR (400 MHz, $CDCl_3$) δ 12.19 (s, 1H), 7.63 (s, 1H), 7.39 (dd, J = 8.6, 2.1 Hz, 1H), 6.94 (d, J = 8.5 Hz, 1H), 4.87 (d, J = 5.8 Hz, 1H), 2.62 (s, 3H), 1.45 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 204.53, 161.72, 155.99, 136.03, 129.72, 119., 79.84, 44.03, 28.47, 26.82.

HRMS-ESI$^+$ (m/z): $[M\text{-}^tBu+H]^+$ Calc. 210.0722, Obs. 210.0907.

Synthesis of 2c: 2b (1.20 g, 7.27 mmol) was dissolved in anhydrous dichloromethane (10 mL), DMAP (88 mg, 0.72 mmol), and N-Phenyl-bis(trifluoromethanesulfonimide) (2.85 g, 7.99 mmol) was added while stirring under Ar-atmosphere. The reaction mixture was sufficiently cooled to 0 °C, and triethylamine was added slowly (2.53 mL, 14.54 mmol) over 5 min. The reaction mixture was stirred at room temperature for 2 hours in an argon atmosphere. In due course of the reaction, a faint color of the reaction mixture was found to occur. The DCM layer was evaporated, and the reaction mixture was diluted in 100 mL EtOAc. The organic layer was thoroughly washed with 1 N HCl (2 × 50 mL), 10% $NaHCO_3$ (2 × 50 mL), and brine solution (2 × 50 mL). After removing the solvent, the crude product was purified (yield = 80%, 2.52 g) on a silica gel column with EtOAc: hexane (1:4) as the solvent.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (d, *J* = 2.2 Hz, 1H), 7.51 (dd, *J* = 8.5, 2.2 Hz, 1H), 7.27 (dd, *J* = 10.5, 2.0 Hz, 1H), 5.07(s, 1H), 4.36 (d, *J* = 6.1 Hz, 2H), 2.62 (s, 3H), 1.45 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 196.80, 156.08, 145.85, 140.39, 132.43, 132.19, 129.59, 123.00, 120.29, 117.09, 80.28, 43.64,29.61,28.44.

HRMS-ESI$^+$ (m/z): $[M\text{-}^tBu+H]^+$ Calc. 342.0214, Obs. 342.1067.

Synthesis of 2d: 2c (1.5 g, 4.04 mmol), B_2Pin_2 (3.08 g, 12.12 mmol), $Pd(dppf)Cl_2$ (329 mg, 0. 40 mmol, 5 mol%), and potassium acetate (1.19 g, 12.12 mmol) were was mixed in anhydrous dioxane (20 mL) under N_2-atmosphere. The reaction mixture was bubbled with argon for 20 min before heating at 85 °C. Upon heating at 85 °C, an orange-to-dark brown coloration was observed. The completion of the reaction was observed after 30 min of monitoring via TLC. The reaction mixture was diluted with ethyl acetate (80 mL), and the solution was filtered through a celite bed. Under reduced pressure, the EtOAc evaporated, and the brownish crude product was purified on silica gel using EtOAc: hexane (2:3) as the eluent expeditiously. The final product was obtained as a colorless viscous oil (yield = 42%, 595 mg).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (s, 1H), 7.46 (s, 2H), 4.89 (s, 1H), 4.35 (d, J = 6.0 Hz, 2H), 2.60 (s, 3H), 1.46 (s, 9H), 1.43 (s, 12H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 199.84, 155.90, 141.15, 140.14, 132.62, 131.58, 127.65, 83.83, 79.41, 44.44, 28.45, 25.73, 24.95, 24.75.

HRMS-ESI$^+$ (m/z): $[M\text{-}H_2O\text{-}Boc+H]^+$ Calc. 176.0877, Obs. 176.0870.

Synthesis of BKD2: For the deprotection of the amine group, 2d was dissolved in 1 mL TFA and stirred for 10-15 min. After the reaction, the TFA was entirely removed by triturating with hexane, a yellowish liquid that resulted in the end. Finally, boronic acid containing amine (100 mg, 0.51 mmol) was dissolved in PBS buffer (2 mL, 10 mM), adjusting the pH to 8.5. The required amount of 2,5-dioxopyrrolidin-1-yl 2-iodoacetate (87 mg, 0.51 mmol) was dissolved in acetonitrile (2 mL) and slowly added to the buffer solution in a cold condition. Time-to-time LC-MS analyses monitored the completion of the reaction. The reaction volume was reduced under low pressure, and the mixture was acidified with 0.1% TFA containing

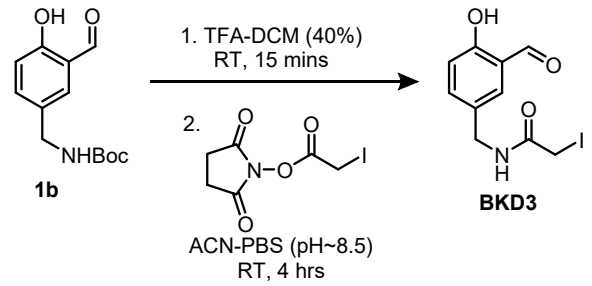
acetonitrile-water (1:1). The acidified solution was purified through semi-preparative HPLC method Ie (Method 1) to obtain the clean product (yield = 55%, 49.8 mg).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.84 (t, 1H), 7.79 (m, 1H), 7.41 (dd,1H), 7.34 (d, 1H), 4.33 (d, 2H), 3.69 (s, 2H), 2.54 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 199.45, 167.98, 140.19, 138.78,138.78 130.48, 127.15, 41.95, 26.45, -1.20.

HRMS-ESI$^+$ (m/z): $[M-H_2O+H]^+$ Calc. 343.9943, Obs. 343.9953.

IIIc. Synthetic routes of BKD3



Scheme 3: Synthetic route of BKD3.

Synthesis of BKD3 from 1b: Amine group of 1b (~80 mg) was deprotected by treating with 40% TFA-DCM (1mL) for 15 mins. For coupling with iodoacetic acid, the previous synthesis procedure for the synthesis of BKD1 and BKD2 was followed. After HPLC purification, the isolated yield was 54%, 42.1 mg.

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.00 (s, 1H), 9.89 (s, 1H), 7.60 – 7.40 (m, 2H), 6.99 (dd, J = 8.5, 1.9 Hz, 1H), 4.46 (dd, J = 14.6, 6.0 Hz, 3H), 3.75 (s, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 196.47, 166.24, 161.24, 136.69, 132.92, 129.23, 121.81, 119.79, 118.33, 43.44, 1.32.

HRMS-ESI$^+$ (m/z): $[M+H]^+$ Calc. 319.9839, Obs. 319.9805.

IV. ^{1}H-NMR analyses of CG and different probes

A short cysteine-containing peptide, CG, was employed in two different equivalents of bis-electrophiles (BKD1-3) to investigate the chemical reactivity of two distinct reactive sites of different bis-electrophiles by recording ^{1}H-NMR spectroscopy. For that, a stock of peptide (50 mM) was prepared in D_2O: PBS buffer (pH

= 7.4) (1:4), and bis-electrophile stock solutions (320 mM) were prepared in DMSO-d_6. In the first titration with 1 equivalent CG, ~4.7 µL of a bis-electrophile stock solution was mixed in the peptide (~ 2mM) to keep a final concentration of bis-electrophile and peptide both at ~2 mM in D_2O: PBS buffer (pH = 7.4) (1:4). The precise equivalency of bis-electrophiles against CG peptide was optimized by ^{1}H-NMR titration from the stock solution of bis-electrophiles, prior to the final experiments and any further experiments performed in our investigation, such as kinetics. Further, one more equivalent of CG (20 µL from the stock) was introduced sequentially in the first titration mixtures. For all NMR experiments, the final volume was kept at ~500-510 µL, and the reaction pH was tuned at 7.4 using a micro pH meter probe. The number of scans was kept at 256 in all attempts. The data for BKD1 titration was shown in the main text, and the data for BKD2-3 were shown in Figure S1-S2.

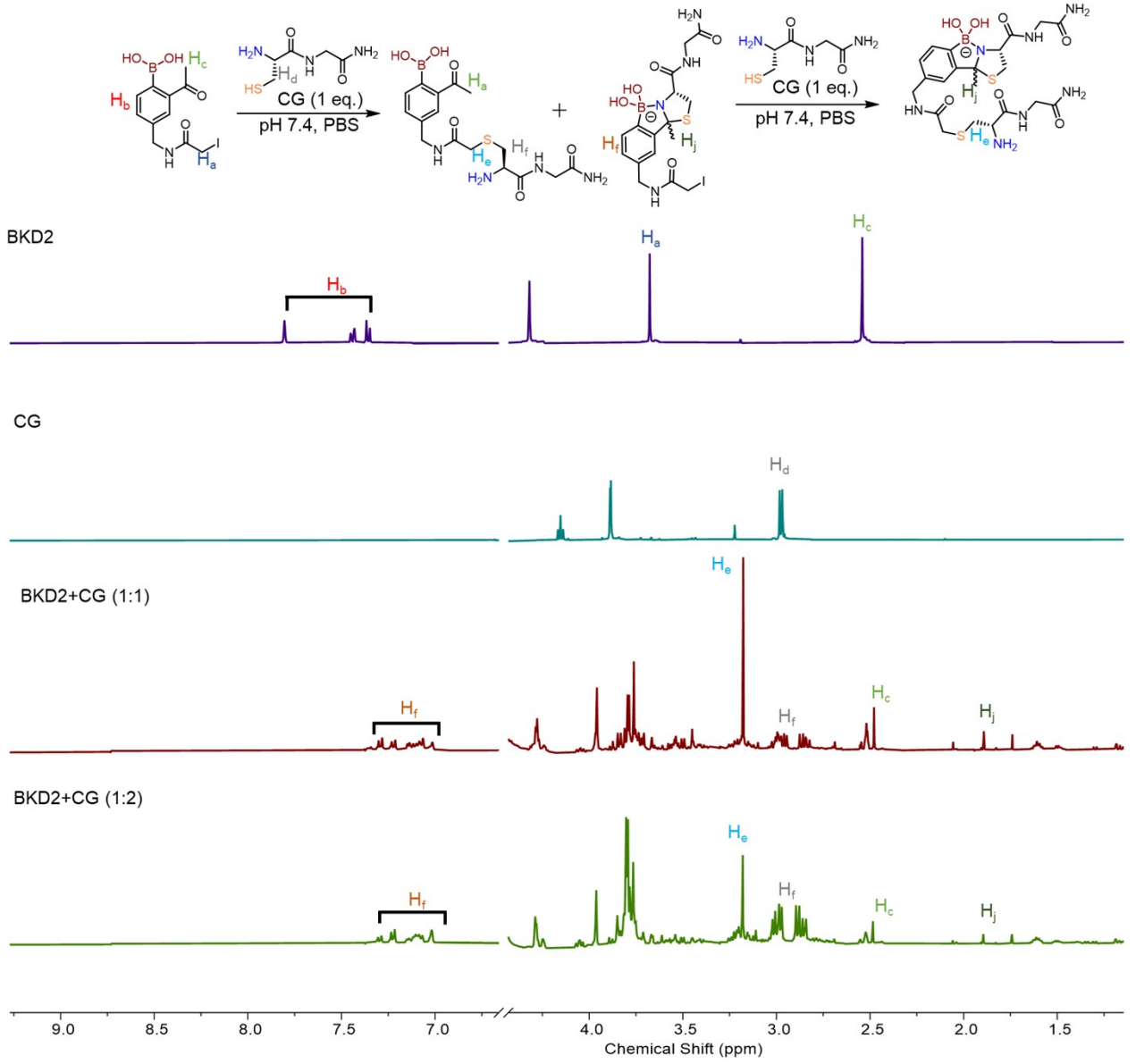


Figure S1. Stacked ^{1}H-NMR spectra of BKD2, CG, and their mixtures in deuterated PBS (pH 7.4, 10 mM) along with the reaction scheme.

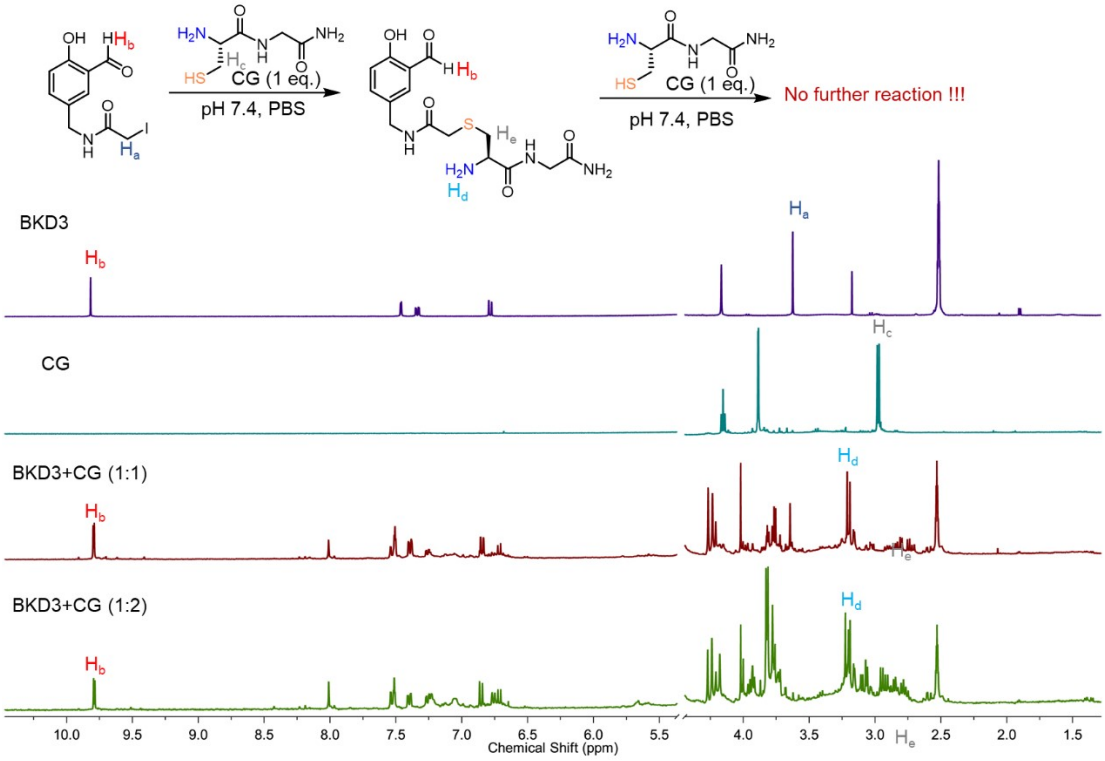


Figure S2. Stacked ^{1}H-NMR spectra of BKD3, CG, and their mixtures in deuterated PBS (pH 7.4, 10 mM) along with the reaction scheme.

V. Reaction of model peptide with BKD1 and BKD2: In a typical method, a required amount of the linear peptide, i.e., CIITRPC, was dissolved in PBS (pH 7.4, 10 mM) buffer to maintain the concentration ~100 µM. To prevent the disulfide formation in the peptide backbone, 1.5 eq. TCEP was added to the solution. Then, 1.05 eq. of BKD1/BKD2 was subjected to the peptide solution, followed by the pH adjustment to 7.4 using 0.5 N NaOH solution. The reaction was continued for 2 to 3 hours at room temperature. LCMS analysis confirmed the near quantitative conversion with BKD1.

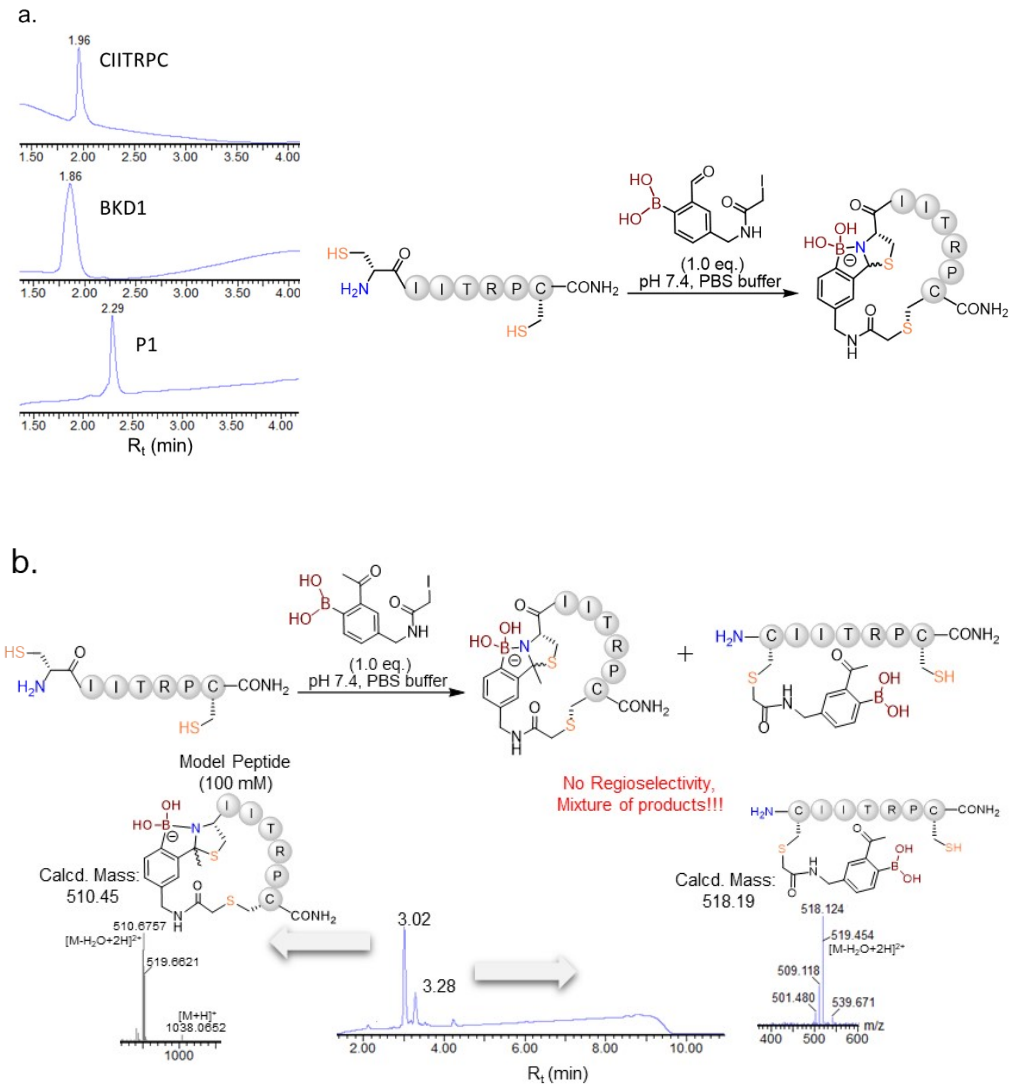


Figure S3. a) Mixing of CIITRPC in BKD1 resulted in a prominent major peak corresponding to the crosslinked product, confirmed by mass. The stacked LC data (Method 1 of section Ic in ESI) collected at 220 nm clearly compare their retention time. b) Treatment of BKD2 to CIITRPC in PBS buffer (pH = 7.4) resulted in two prominent peaks in the LC chromatogram (using Method 2 of section Ic in SI) corresponding to two different masses, implying the two regioselective conjugation of BKD2 with the peptide backbone.

VI. Kinetics studies of peptide crosslinking

The kinetics of peptide crosslinking were investigated in a Waters UPLC-assisted SQD2 LC-MS machine.[2] For this experiment, we used CIITRPC peptide at a concentration of ~100 μM in 1 mL PBS buffer (pH 7.4, 10 mM). The requisite amount of TCEP (1.1 eq.) and BKD1 (1.0 eq.) were added, as previously mentioned, and the pH of the mixture was adjusted to 7.4. At different intervals (Figure S4), 120 μL aliquot of the reaction mixture was taken out, and the ongoing reaction was immediately quenched with 10 mL of 1% TFA/water. For better understanding and sound separation among the reagents and product peaks, samples

were injected using LCMS-method 2 (11 min), where 4 μL of sample was injected every time. All LC data represented in Figure S4 were blank subtracted. The peak area of linear peptide (CIITRPC, RT ~ 2.93 min) was considered to calculate kinetics measurement. Herein, we have observed the decay of peptides (~2.93 min) and BKD1 (~2.38 min) peaks with time progression at 220 nm. The peak of crosslinked peptide (P1) appeared around 3.99 min, which became prominent gradually; however, the broad nature of the product was observed due to the acidic environment of the mobile phase, discussed in the main text.

For this reason, we have monitored the kinetics using the peak area of the linear peptide (CIITRPC) following an exponential decay over time. After 2 hours, the remaining peptide, CIITRPC, was very close to 5%, revealing the complete conversion of the cyclic peptide in the LC spectrum. The experiment was repeated two times, and consistent results were obtained. The kinetic data were fitted to first-order kinetics with the two reactants at equal concentrations. These unique results can be enlightened by the fact that the rapid thiazolidino boronate formation[3] between peptide and BKD1 does not impart any role in the final kinetics, albeit the first ABT formation with the linear peptide undergoes proximity-driven first-order cyclization reaction. The proximity-driven S-alkylation reaction can be considered the rate-determining step since it is kinetically slower (k_2~10^{-1} S^{-1}) than the ABT formation (k_1~10^3 $M^{-1}S^{-1}$). $t_{1/2}$ of the reaction was calculated as 11.17 min by putting 50% conversion in the plot.[4] Based on the $t_{1/2}$ for first-order reactions, the rate constant was calculated to be 0.0621 min^{-1}. The kinetics experiments were repeated twice to check their reproducibility.

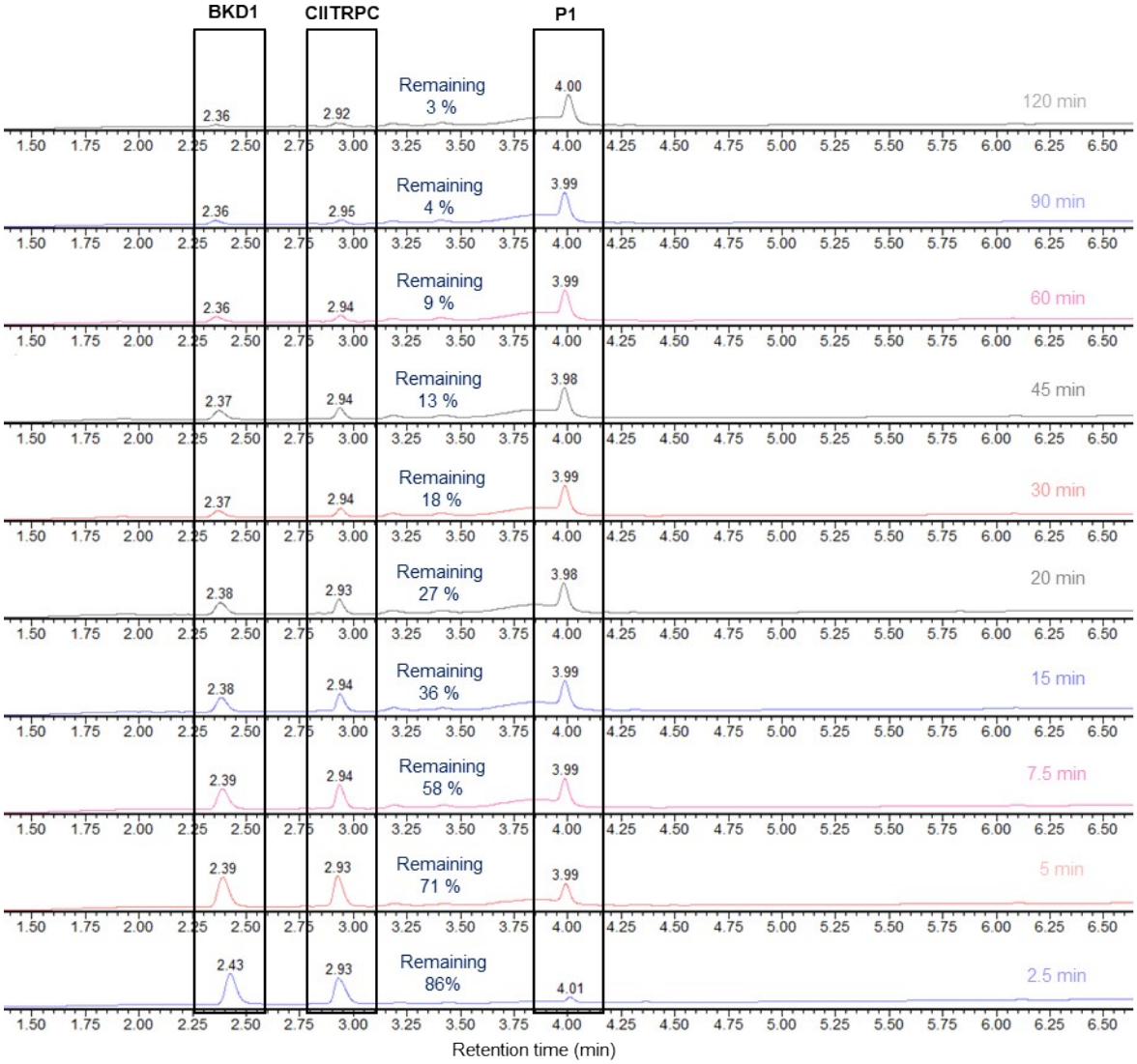


Figure S4. LC chromatograms of crosslinking reaction of CIITRPC with BKD1 at different time intervals.

VII. 2D-NMR structural studies of P2 (BKD1 crosslinked-CAAKAAC)

In this experiment, we have selected a small and simple peptide, CAAKAAC, for the lucid understanding of the chemical shifts at the aliphatic region.[6] We have crosslinked the peptide with BKD1 and purified it via HPLC. COSY and ROESY experiments were performed with cyclic peptide (5 mM in DMSO-d_6) using a 400 MHz Jeol JNM ECS400 NMR spectrometer at 25 °C. The COSY was recorded with the following setting: Scans per t1 Increment 32 and Spinlock Duration 80 ms. The ROESY was recorded using the parameters: Scans per t1 Increment 128 and Spinlock Mixing Time 200 ms. The raw data was processed using MestReNova 10.0.

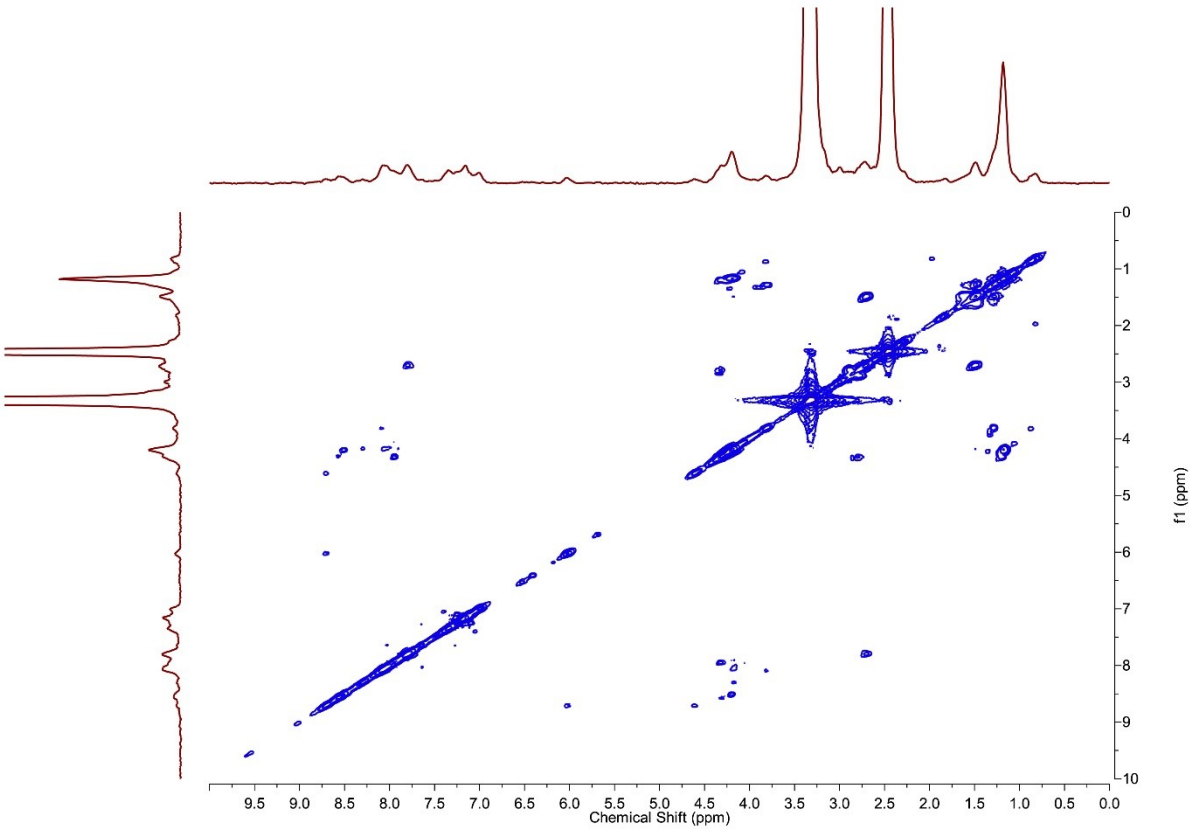


Figure S5. A complete COSY spectrum of P2 was obtained in DMSO-d_6.

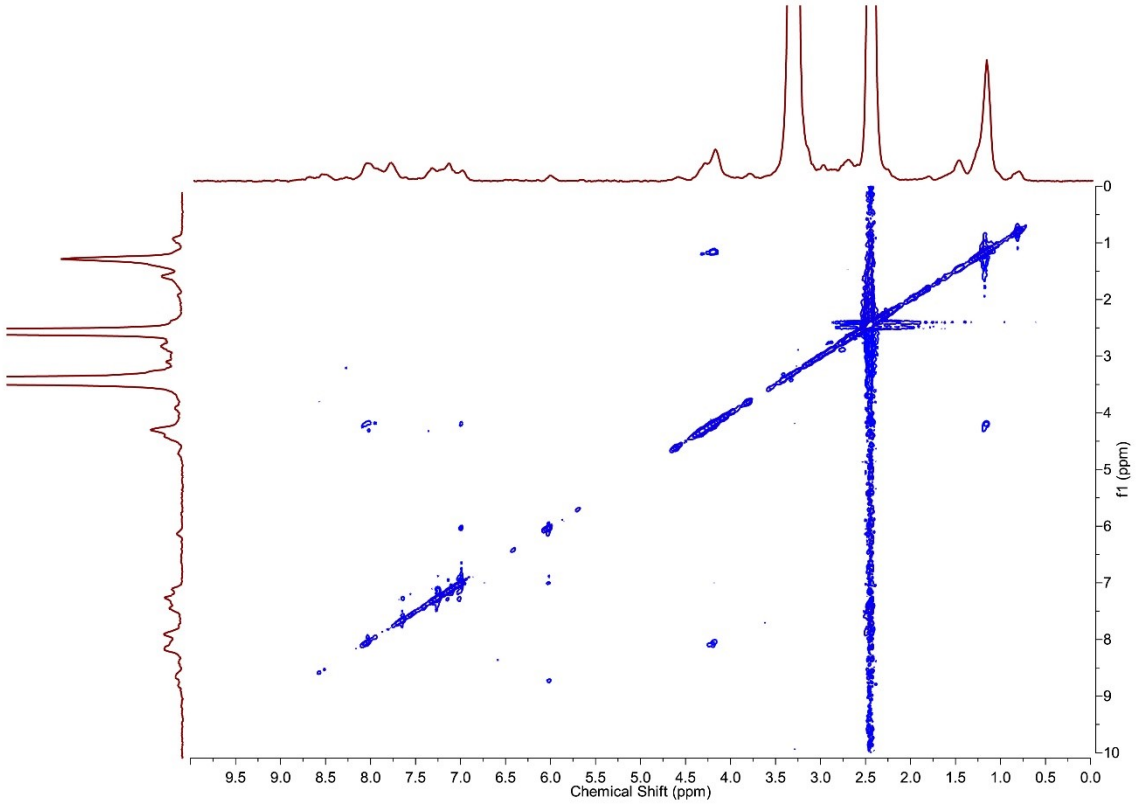


Figure S6. A complete ROESY spectrum of P2 was obtained in DMSO-d_6.

VIII. ^{11}B-NMR spectra of the cyclic peptide and BKD1.

^{11}B-NMR spectra were recorded in DMSO-d_6 utilizing HPLC-purified P1 peptides and BKD1. The concentration was fixed at 5 mM for both peptides within 500 mL DMSO-d_6. In both samples, 1 mM boric acid was doped as an internal standard. Due to the formation of ABT heterocycle in the crosslinked peptide, the boron peak was found to be shifted from ~29.19 ppm to ~8.29 ppm. The reference peak of the boric acid peak was obtained at ~18.9 ppm. The experiments were performed in a 400 MHz Jeol JNM ECS400 NMR spectrometer using a quartz NMR tube. The number of scans was kept at 1000 in all attempts. The raw data was processed using MestReNova 10.0.

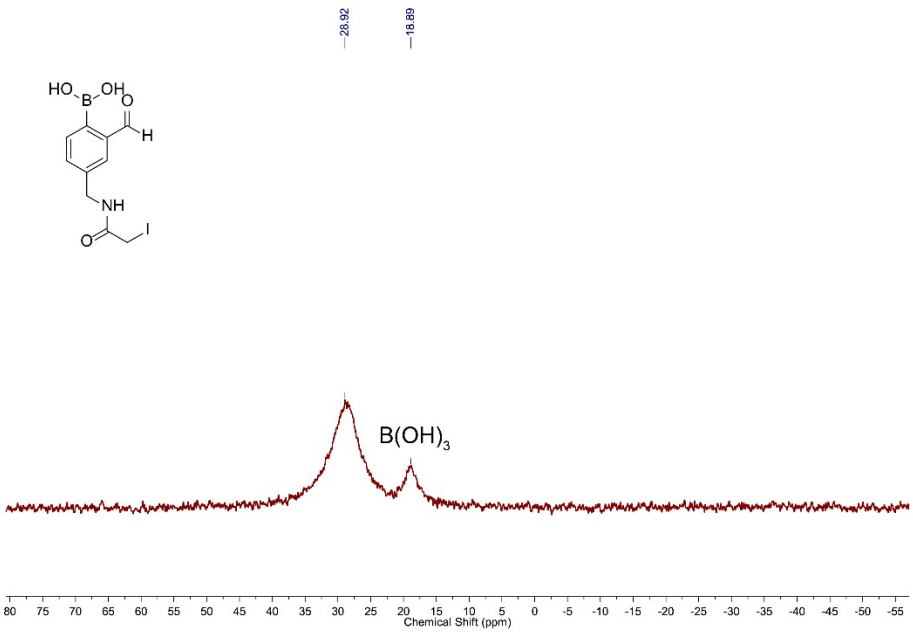


Figure S7. ^{11}B-NMR of BKD1 obtained in DMSO-d_6.

IX. Effect of increasing concentration of the reagents on peptide crosslinking.

To elucidate the optimized reaction concentration, we have varied the concentration of the linear peptides from 500 µM to 2 mM. All the reactions were carried out within 1 mL PBS buffer at pH 7.4 using 1.5 eq. of TCEP and 1.05 eq. of BKD1. All the reactions were kept for 2 hours, stirring at room temperature. LC-MS analyses were performed to monitor the reaction profile at a fixed concentration. All results indicated that increasing concentration resulted in a broadening in peak shape with multiple impurities. The best result was

obtained by keeping the reaction concentrations below 500 µM. Samples were subjected to LCMS-method 3 for better separation. All data were measured in duplicate.

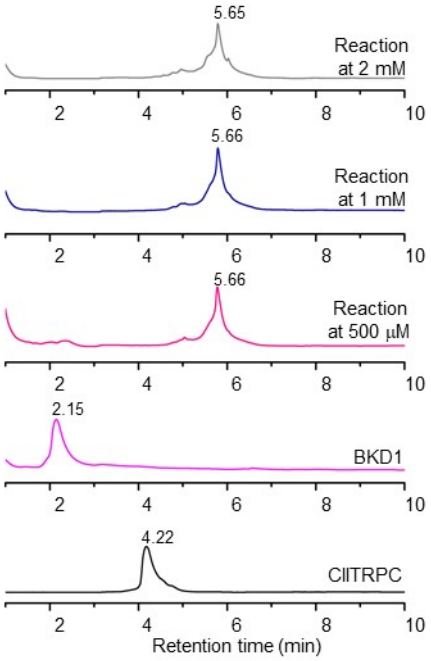


Figure S8. LC-chromatograms of CIITRPC, BKD1, and crosslinking between CIITRPC and BKD1 reaction varying concentrations of CIITRPC from 500 µM to 2 mM; analyzed using Method 3 of section Ic in ESI

X. Substrate scope for monocyclization:

In a typical method, the required amount of the linear peptide (~10 mg) was dissolved in an appropriate amount of PBS (pH 7.4, 10 mM) buffer, maintaining the concentration at 200 mM. To prevent the disulfide formation in the peptide backbone, 1.5 eq. TCEP was added to the solution. After that, 1.05 eq. of BKD1 was subjected to the solution, followed by the pH adjustment to 7.4 using 0.5 N NaOH solution. The reaction was continued for 2 hours at room temperature. Analytical HPLC and LCMS confirmed the near quantitative conversion. Further, the product was purified through semi-preparative HPLC and, upon lyophilization, yielded white powder.

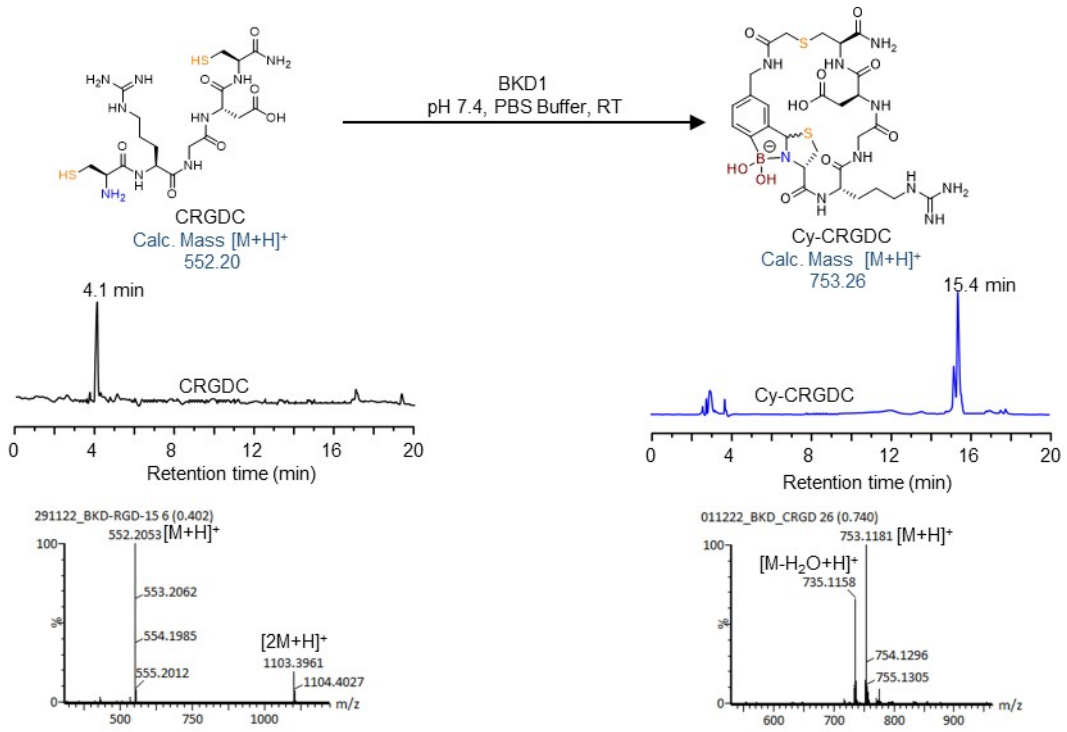


Figure S9. HPLC chromatograms of pure RGD and BKD1 crosslinked peptides were analyzed using Method 2 in section Id in ESI. HRMS data of the linear and crosslinked peptides are shown with their calculated masses.

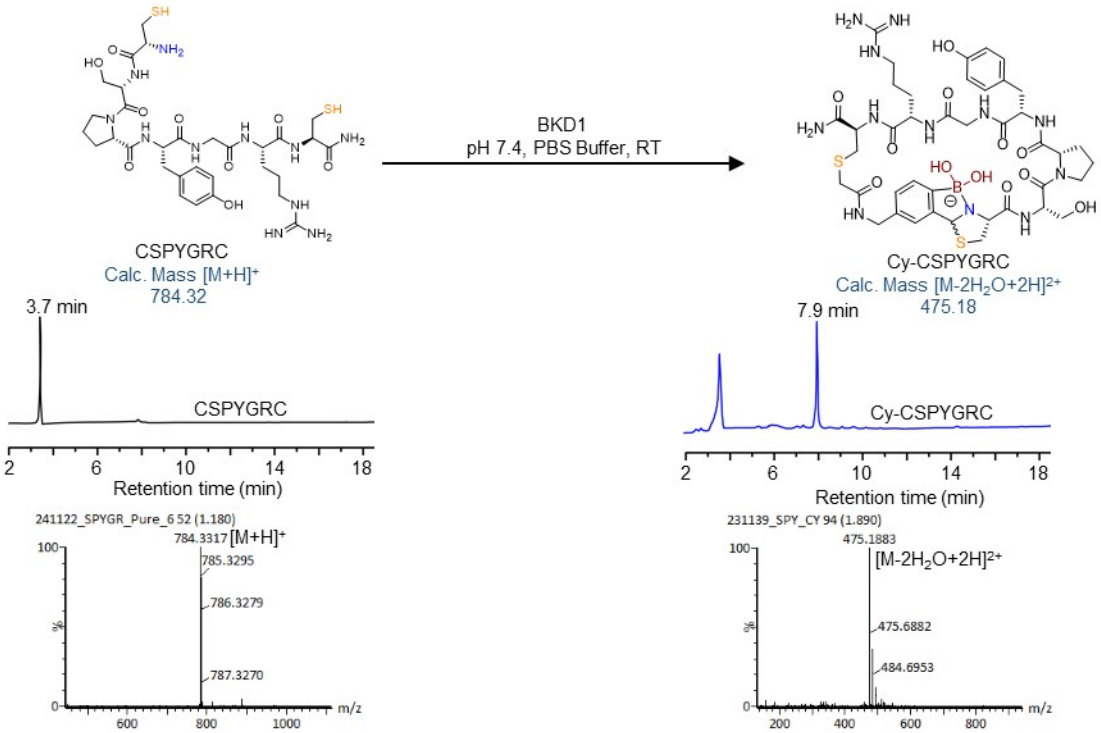


Figure S10. HPLC chromatograms of pure SPYGRC and BKD1 crosslinked peptides were analyzed using Method 2 in section Id in ESI. HRMS data of the linear and crosslinked peptides are shown with their calculated masses.

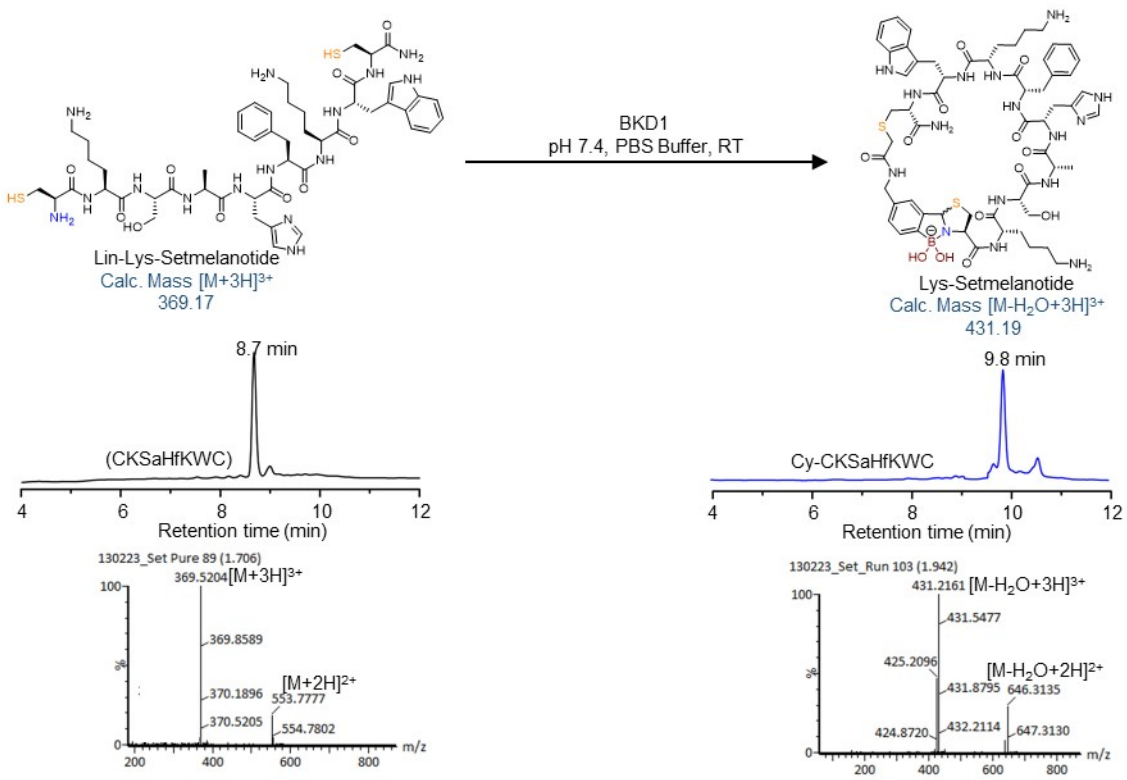


Figure S11. HPLC chromatograms of pure Setmelanotide and BKD1 crosslinked peptides were analyzed using Method 1 of section Id in ESI. HRMS data of the linear and crosslinked peptides are shown with their calculated masses.

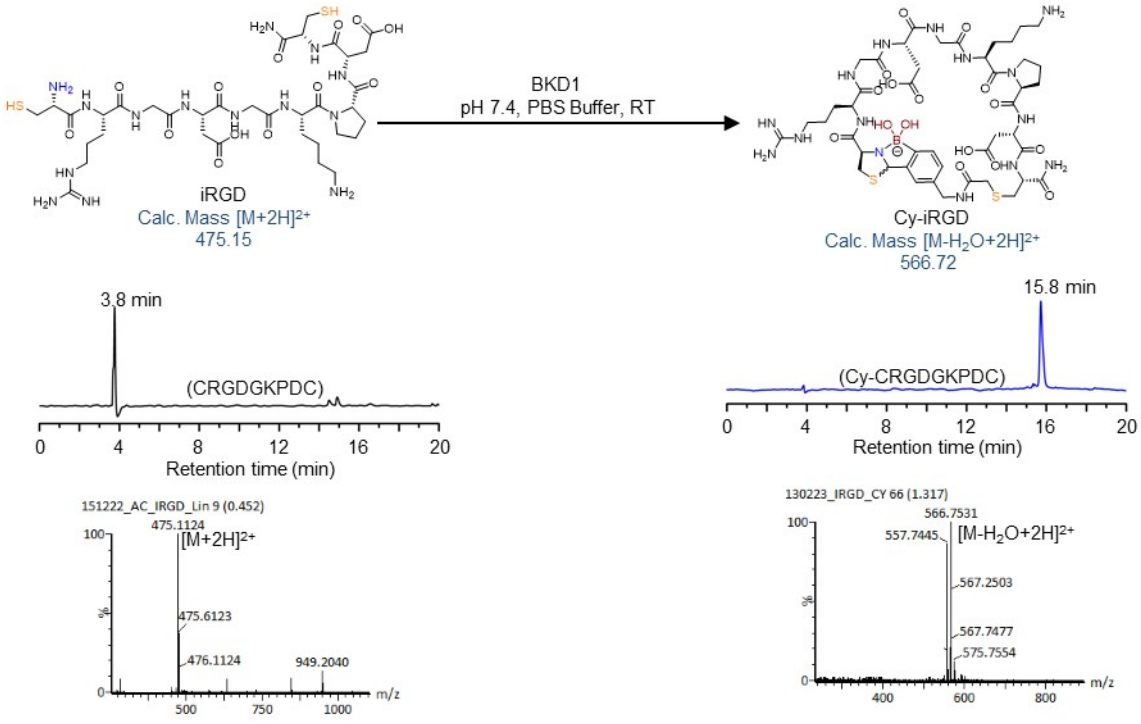


Figure S12. HPLC chromatograms of pure iRGD and BKD1 crosslinked peptides were analyzed using Method 2 in section Id in ESI. HRMS data of the linear and crosslinked peptides are shown with their calculated masses.

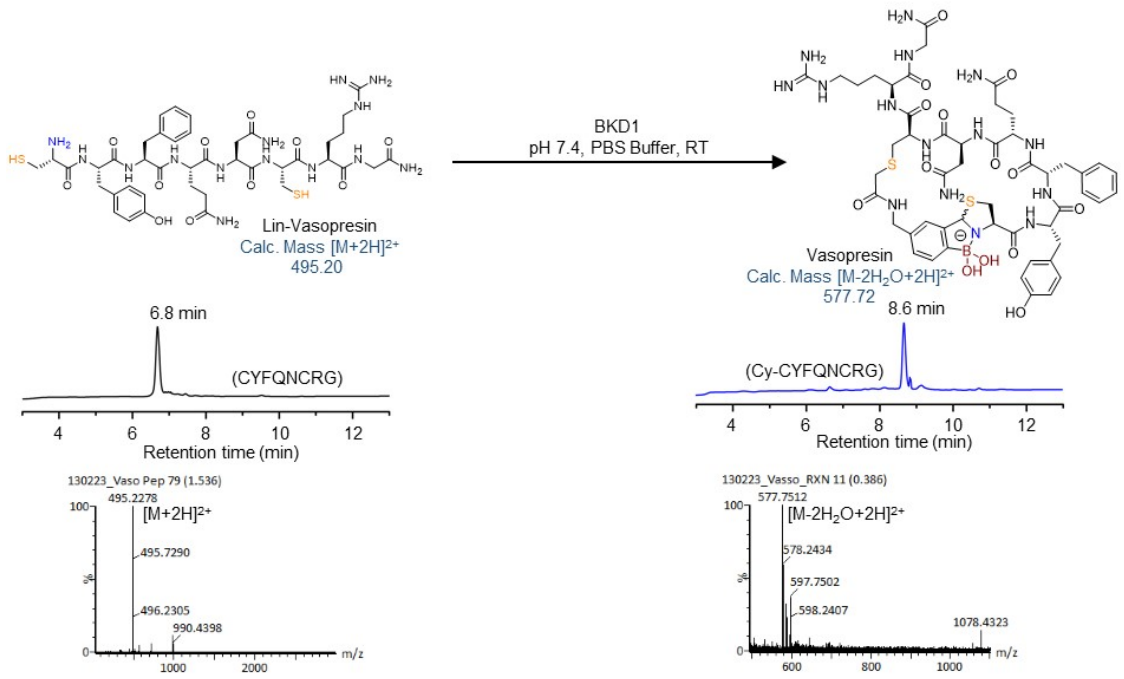


Figure S13. HPLC chromatograms of pure Vasopressin and BKD1 crosslinked peptides were analyzed using Method 1 of section Id in ESI. HRMS data of the linear and crosslinked peptides are shown with their calculated masses.

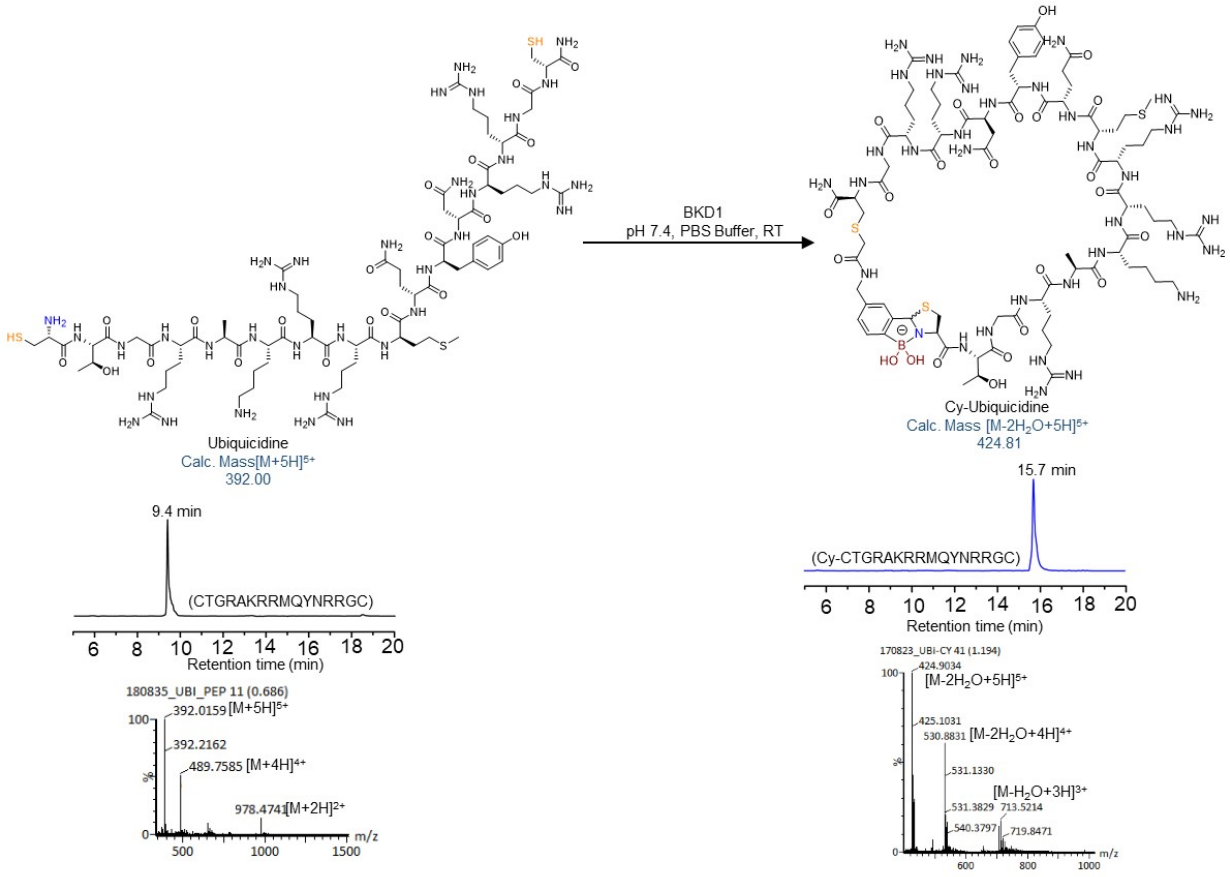


Figure S14. HPLC chromatograms of pure Ubiquicidine and BKD1 crosslinked peptides were analyzed using Method 2 in section Id in ESI. HRMS data of the linear and crosslinked peptides are shown with their calculated masses.

XI. ^{1}H-NMR spectra of the crosslinked peptides

To gain better mechanistic insights into the cyclization reaction, biologically active peptides (CRGDC, CSPYGRC, and Semelanotide) were subjected to ^{1}H-NMR analysis upon incubation with BKD1. Each peptide (200 mM) was cyclized with BKD1 (200 mM) with a higher volume (5 mL, PBS buffer, pH = 7.4). Quantitative conversion of the crosslinked peptide was observed in HPLC. The reaction mixture was lyophilized and dissolved in DMSO (d_6). The crosslinked peptides were submitted for ^{1}H-NMR spectroscopy, with individual native peptides and BKD1 at 2 mM concentration at the final volume of 500 μL. The number of scans was kept at 256 in all attempts.

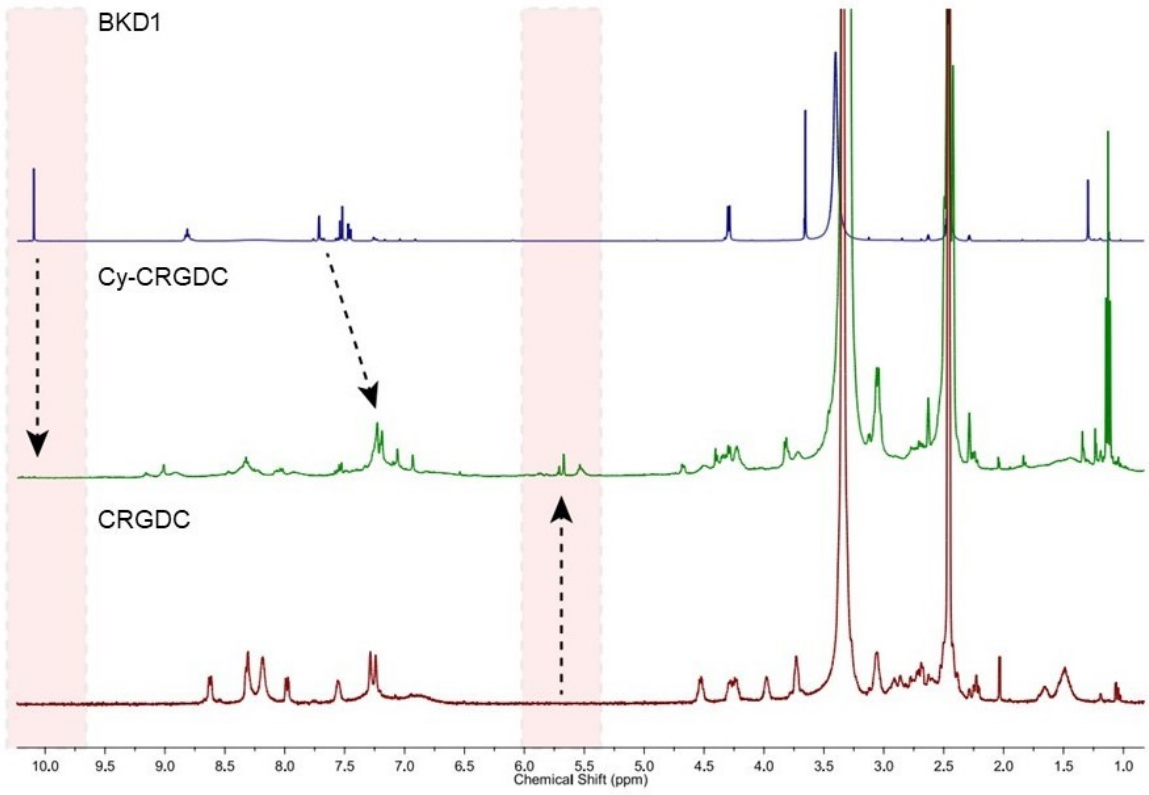


Figure S15. ^{1}H-NMR spectra of CRGDC, crosslinked-CRGDC, and BKD1 recorded in DMSO-d_6.

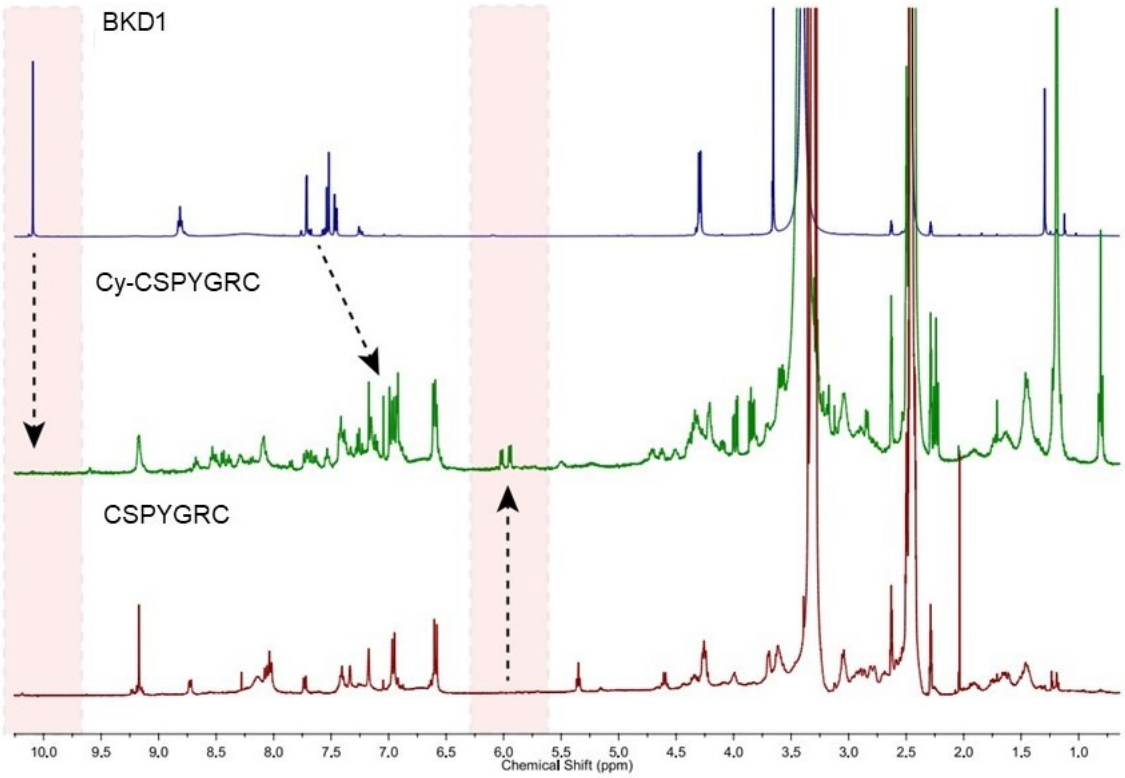


Figure S16. ^{1}H-NMR spectra of CSPYGRC, crosslinked-CSPYGRC, and BKD1 recorded in DMSO-d_6.

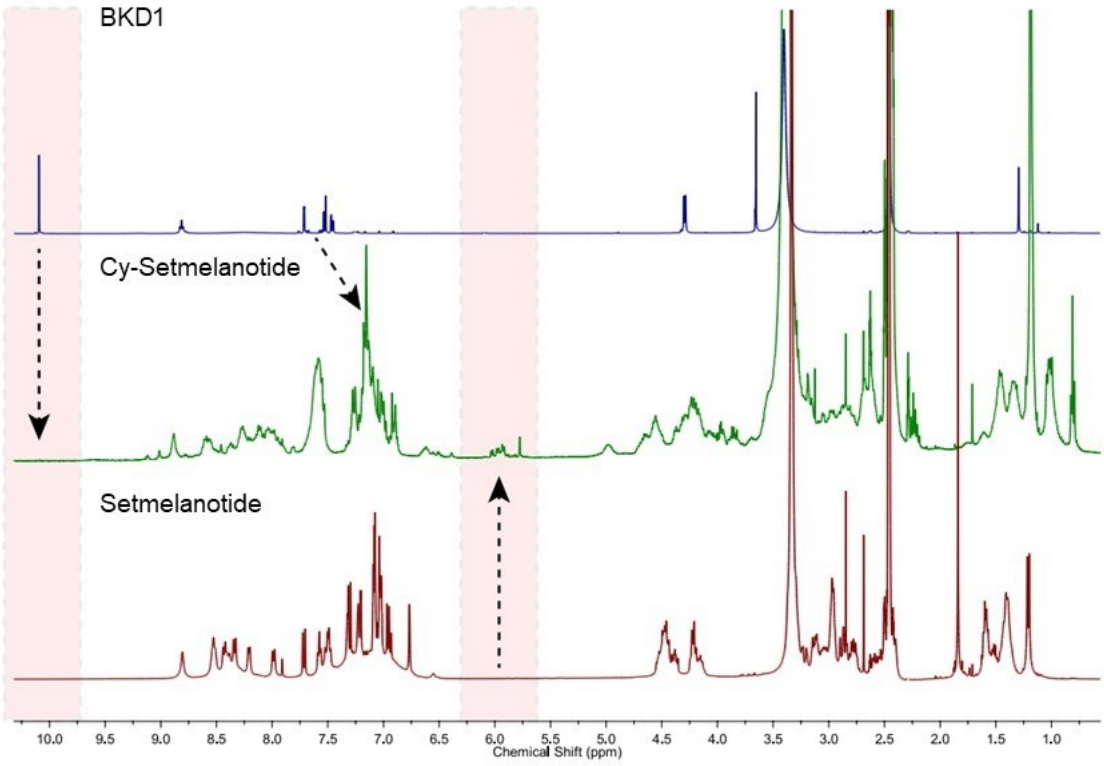


Figure S17. ^{1}H-NMR spectra of setmelanotide, crosslinked setmelanotide, and BKD1 recorded in DMSO-d_6.

XII. General method of bicyclization strategy for μ-conotoxin and gomesin

We followed the general method to perform the ABT crosslinking (SI section X). Further, HPLC purified ABT crosslinked monocyclic peptide (1 mM) was dissolved in 1 mL of 20% MeOH/0.1 N HCl, and a solution of 10 mM I_2 in MeOH was added dropwise until the whole solution turned the yellow color of I_2.[1] The combination was stirred at room temperature for 4 hours. Finally, the crude solution was submitted to HPLC and mass spectrometry without purification. No side products were observed throughout the complete removal of Acm groups and disulfide bond formation.

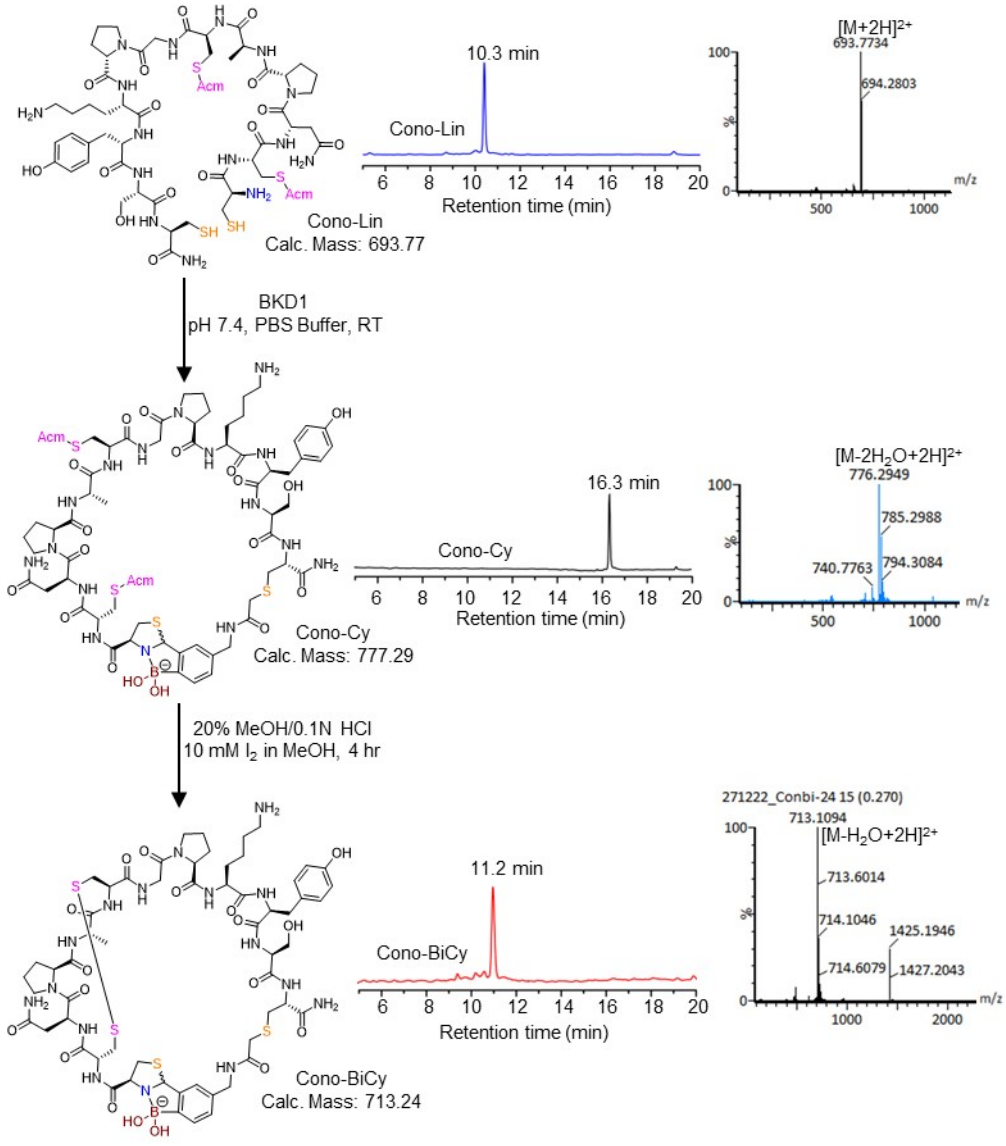


Figure S18. The reaction scheme for bicyclization of gomesin via ABT crosslinking: The corresponding HPLC profile and mass data are shown on the right side of the peptide. HPLC Method 1 in section Id was used for analysis.

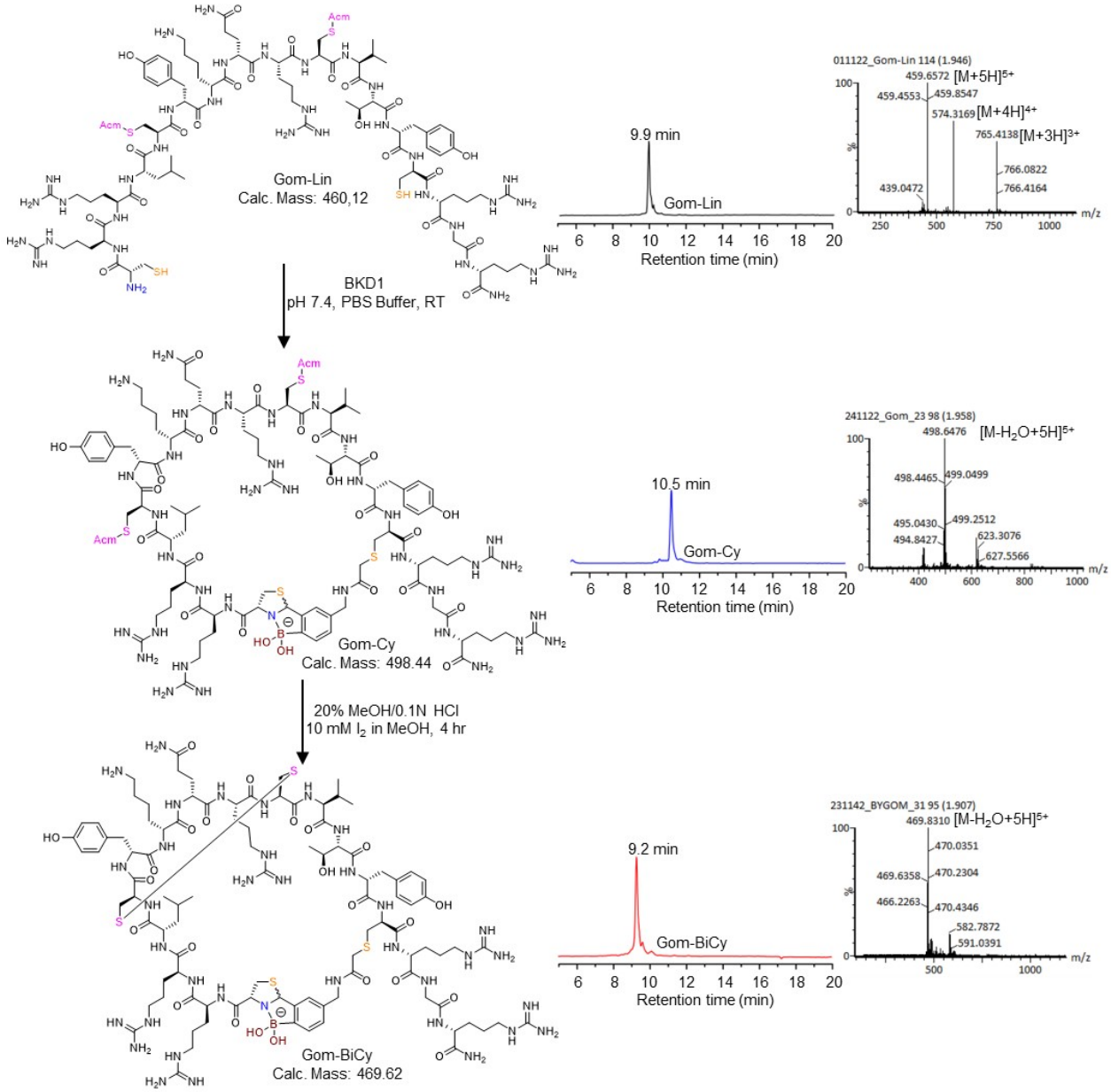


Figure S19. The reaction scheme for bicyclization of μ-conotoxin via ABT crosslinking: The corresponding HPLC profile and mass data are shown on the right side of the peptide structure. HPLC Method 1, shown in section Id, was used for analysis.

XIII. Stability of the cyclic peptide against different pH in NMR

Four sets of HPLC-purified P1 peptides were prepared using 500 μL deuterated PBS buffer (10 mM), where the concentration of P1 was ~2 mM. The pH of the P1 solutions in deuterated PBS was adjusted to 2.0, 4.0, 6.0, and 7.4 for four individual sets. Besides that, two samples were prepared for BKD1 (2 mM, pH 2.0) and CIITRPC (~2 mM, pH 2), and all the samples were submitted. The number of scans was kept at 256 in all attempts.

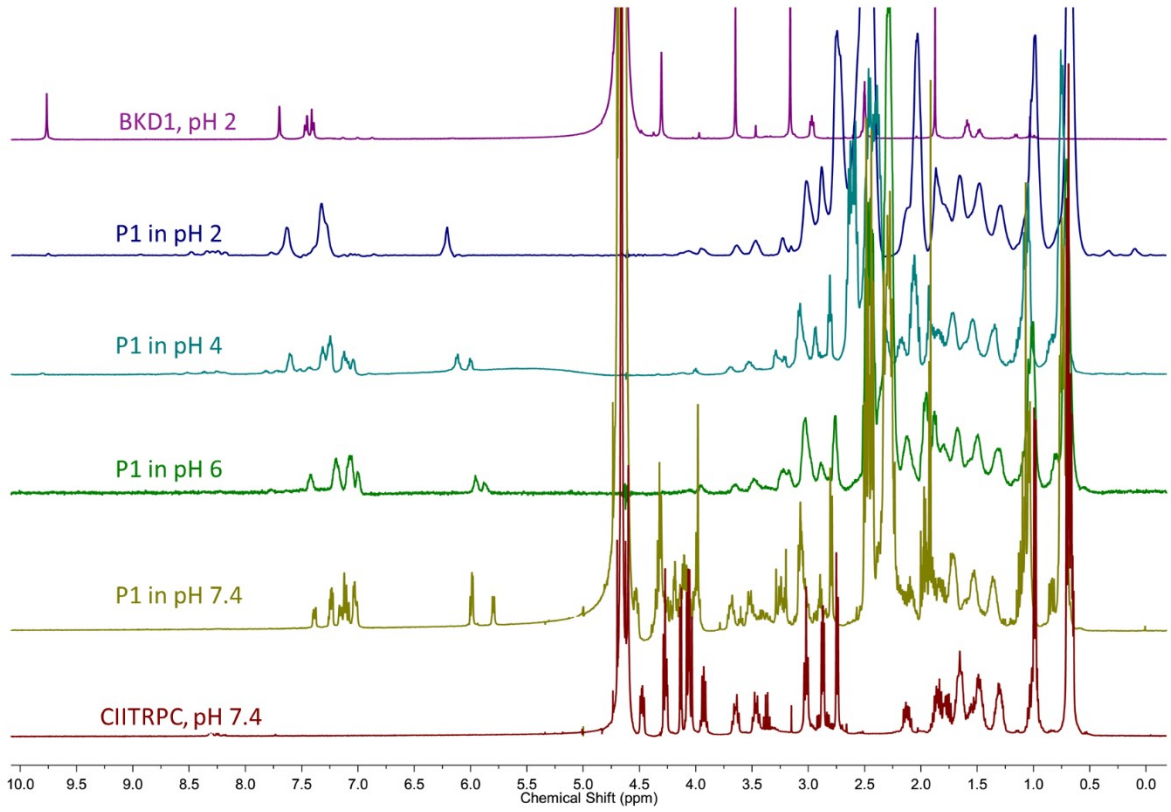


Figure S20. Full ^{1}H-NMR spectra of P1 varying the pH from 7.4 to 2.0 for estimating the stability of the crosslinked peptide in acidic environments.

XIV. Stability of the cyclic peptide in PBS buffer and human serum

The crosslinked peptide P1 was dissolved in PBS buffer (1X, pH 7.4) at a concentration of 200 µM and stored at room temperature. At different time intervals within a month, the reaction mixture was analyzed through LC-MS to check the stability profile of the P1 using method 2. Every time 5 µL was injected from the reaction, no significant change was observed at 3.99 min till 7 days; however, a slight change was observed after 15 days.

The serum stability of crosslinked-UBI was carried out following the reported protocol[1]. Human serum freshly extracted from an individual was diluted to 20% with PBS (10 mM, pH 7.4) and kept at 37 °C. Peptides (5 µL, 10 mM stock in DMSO) were added to 300 µL of the serum and incubated at 37 °C. 50 µL aliquots were withdrawn at 0, 1, 6, 12, and 24 hr intervals and added to 30 µL 1:1 MeOH/10% trichloroacetic acid (TCA) and 100 µL H_2O. The resulting suspension was vortexed and spun at 4000 rpm for 10 min. The supernatant was freeze-dried, and the resulting residue was reconstituted in 1:1 ACN/H_2O. The mixture was again spun down, and the supernatant was subjected to analytical HPLC analysis using a C4 column (4.6 ×

100 mm, 5 µm, 300Å), section 1d method 2. The sample collected at 0 hr was considered the initial standard, against which the peak areas of samples at different time intervals were compared. All data were measured in duplicate. The data is shown in the main text in Figure 4b.

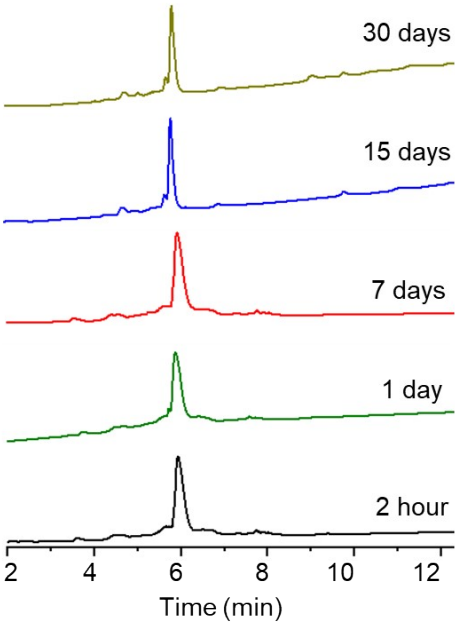


Figure S21. Stability studies of P1 peptide in PBS (pH 7.4, 10 mM) were analyzed using Method 3 of section Ic in ESI.

XV. Stability of the cyclic peptide against ROS environment

Cancer cells possess a certain high ROS concentration, leading to the oxidation of boronic acid. Importantly, as our strategy is wholly focused on ABT-crosslinking, we were keen to know about the fate of our cyclic peptide in the ROS environment.[4] Herein, we have employed H_2O_2 in the aqueous environment to mimic the ROS environment. HPLC-purified P1 peptide was dissolved in PBS buffer to prepare a ~100 µM solution. To that solution, 30 µL of 5 mM H_2O_2 (freshly prepared from 30% v/v H_2O_2) was added to maintain H_2O_2 concentration at ~150 µM. The reaction mixture was incubated for 12 hours by gently shaking. The samples were analyzed at different time intervals via LC-MS-method 1 at 0 hr, 6 hr, and 12 hr by quenching the reaction in 1N HCl. The peak of the cyclic peptide ~2.30 min retained fully in the LC traces of 6 hr and 12 hr, indicating the stability of the cyclic peptide in 150 mM H_2O_2. Data were represented in the main text Figure 4c and ESI S22.

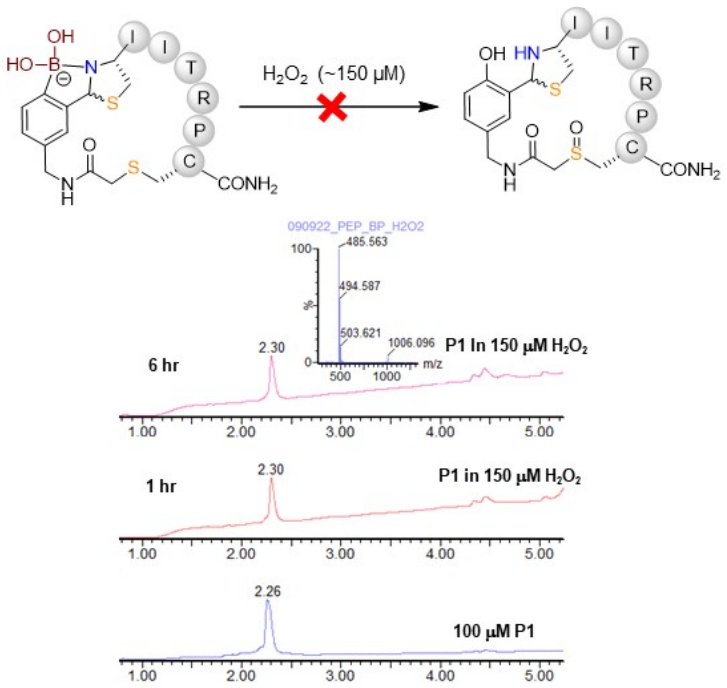


Figure S22. Reaction scheme for the hypothesized boronic acid oxidation with ABT crosslinked peptide in the presence of H_2O_2. The stability profile of the P1 peptide in the presence of ~150 mM H_2O_2 at different time intervals was analyzed using Method 1 in section Ic, which revealed no significant reaction with the crosslinked peptide P1.

XVI. Linearization of the cyclic peptide with phenylhydrazine and semicarbazide derivatives:

Herein, we have shown the scope of the linear peptide back from the cyclic peptide using phenylhydrazine and a biocompatible nucleophile semicarbazide derivative. 1.8 mg HPLC-purified P1 peptide was dissolved in 200 µL PBS buffer (10 mM) to prepare a 8 mM stock. On the other side, a 30 mM stock solution of phenylhydrazine and the derivative of semicarbazide (synthesized by previously reported protocol)[4] in PBS was prepared. Then, 50 µL from the P1 stock solution was incubated with 30 µL of the phenylhydrazine and semicarbazide stock solution separately in PBS (10 mM) with a total volume of 800 µL. After 60 min of incubation, the reaction mixture was analyzed by LC-MS using method 3. The mass data revealed that the cyclic peptide was fully converted to the linear diazaborine derivative of the peptide. The linearization study with semicarbazide was represented in the main manuscript Figure 4d. We also performed ^{1}H-NMR study with phenylhydrazine to understand the linearization of the cyclic peptide P1, whereby 1 mg P1 (2.0 mM) was dissolved in 450 µL deuterated PBS (10 mM). A 50 mM stock solution of phenylhydrazine was prepared in D_2O. 50 µL (4.0 mM, 2 eq.) from the phenylhydrazine stock solution was added to the solution of the cyclic peptide, P1. The final pH of the mixture was tuned to 7.4 and submitted for ^{1}H-NMR after a 1-hour

incubation. D_2O solutions of P1 (2 mM) and phenylhydrazine (2 mM) were also taken for the ^{1}H-NMR for the comparative understanding of diazaborine formation. The results showed that the thiazolidine peak at 6 ppm disappeared entirely due to diazaborine formation. The spectra were obtained using the 400 MHz Jeol JNM ECS400 NMR spectrometer at 25 °C with 64 scans. The data were processed using MestReNova 10.0.

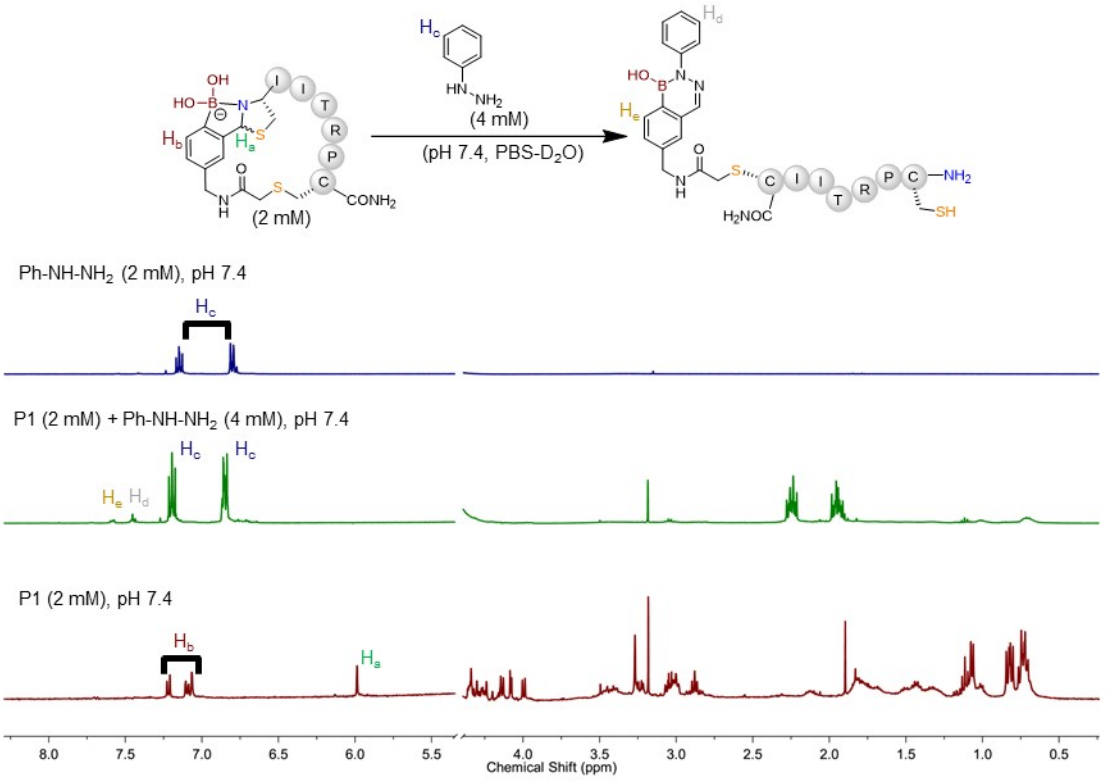


Figure S23. ^{1}H-NMR spectra of P1, phenylhydrazine, and their mixture in deuterated PBS for analyzing the breakdown of thiazolidino linkage and linearisation of the cyclic peptide.

XVII. Cysteine exchange study of the cyclic peptide.

Herein, we envisioned to estimate the stability of the cyclic peptide in the presence of cysteine.[6] Previous reports showed that cysteine could inhibit ABT formation by up to 50%.[3] For the exchange study, 1 mg P1 (2.0 mM) was dissolved in 500 µL deuterated PBS (10 mM), and 0.6 mg L-cysteine (10.0 mM, 5.0 eq.) was added to it. The pH of the final solution was tuned to 7.4 using 0.5 N NaOH and 0.5 N HCl. Separately, a 5 mM solution of L-cysteine and P1 was prepared as controls in 500 µL deuterated PBS (10 mM). Then, the effect of the cysteine exchange was monitored by the ^{1}H-NMR. The results showed that the thiazolidine peak at 6 ppm remains unchanged after 10 hours with the addition of cysteine. The aliphatic peaks of the P1 and cysteine remained almost similar after mixing them, indicating no exchanging propensity between cysteine

and P1. The spectra were obtained using the 400 MHz Jeol JNM ECS400 NMR spectrometer at 25 °C with 64 scans. The data were processed using MestReNova 10.0.

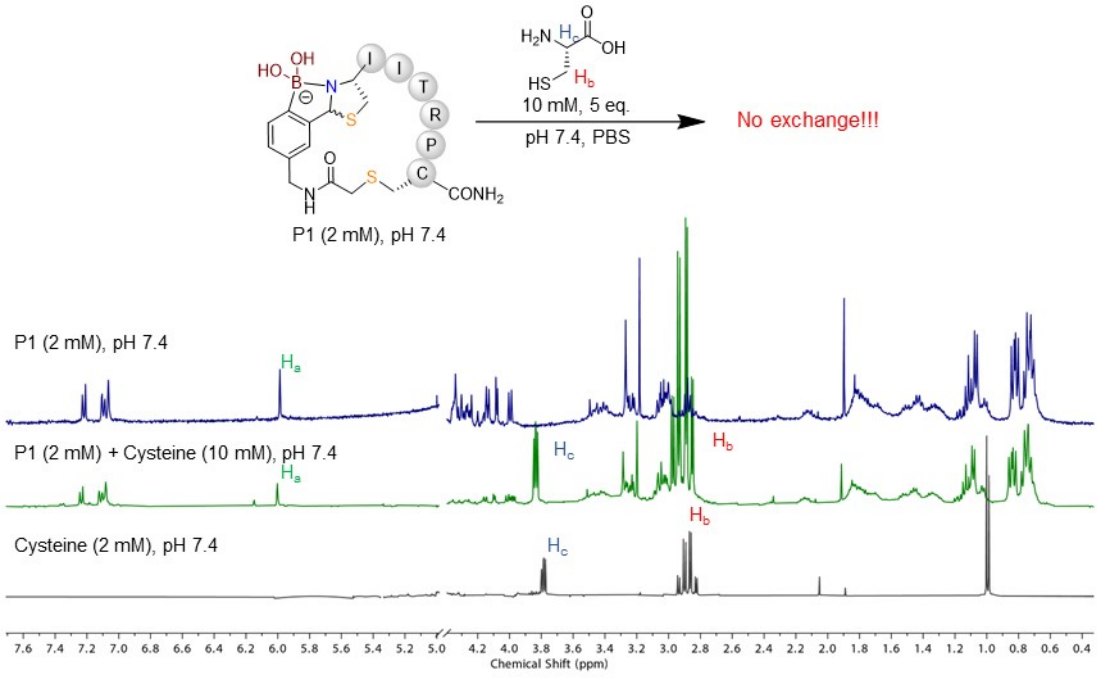


Figure S24. [1]H-NMR spectra of P1, cysteine, and their mixture in deuterated PBS for analyzing the cysteine exchange at physiological pH.

XVIII. Rationale and method for in silico crosslinking of KLA peptide

It has been reported earlier that the antimicrobial peptide KLA exhibits better proapoptotic activity upon preorganized helical conformation. Stapling or crosslinking of a peptide has been shown to improve its secondary structure. Therefore, we used our ABT crosslinking method to fold the KLA peptide into a better helical conformation. We have performed a quick in silico study to understand the requirement of the appropriate spacer length using commonly used amino acid spacers. Initially, we started our investigation with the linear KLA peptide to determine its helicity, which retained ~22% after 20ns MD simulation study, as shown in Figure S25 (a). To improve its helicity, we introduced a spacer or linker and observed improved helicity by ~35%, as shown in Figure S25 (b). Further, by modifying its spacer or linker, the stapled KLA peptide retained ~60% of the helicity within the KLA peptide, as shown in Figure S25 (c). Further, the plot in Figure S25 summarizes the peptide secondary structure elements (SSE) like alpha-helices (in red) and beta-strands (in blue) composition for each trajectory frame throughout the simulation, and the plot at the bottom monitors each residue and its SSE assignment over time.

The linear KLA and stapled KLA peptide (KLAKLAKKLAKLAK) was prepared using Maestro (Schrödinger, LLC, New York, NY, 2021) and processed in a protein preparation wizard. The unfavorable bond lengths and angles were corrected using pre-process and structure analysis tools. The protein structure was then minimized using the Optimized Potential Liquid Simulation 3e (OPLS3e) force field. Further, we ran a small MD simulation of 20 ns (nanoseconds) to obtain a final structure of the stapled KLA peptide. Therefore, Desmond, an MD simulation software, was employed to model the stapled KLA peptide in an aqueous environment. The model constitutes an orthorhombic box of water with a minimum size to contain the model, ensuring a 10 Å distance from the edge of the box. TIP3P was used as the solvent model, and 0.15 M NaCl was added to neutralize it. The system was minimized to 1000 steps, holding all the peptide atoms. The minimized system was subjected to MD simulations using the NPT ensemble and periodic boundary conditions for 20 ns. The Martyna-Tobias-Klein method was used to control the pressure of the system to 1.01 bar. The Nose-Hoover thermostat was applied to control the temperature at 300 K. The trajectories and other parameters were saved every 50 and 1.2 ps (picoseconds), respectively, to return 1000 frames.

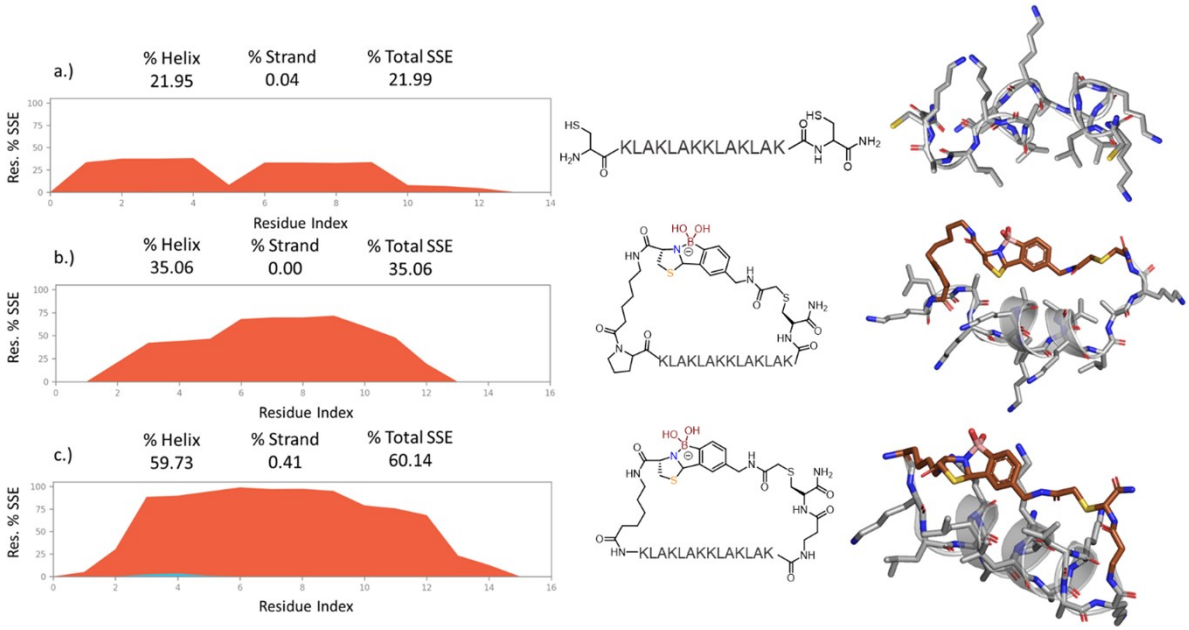


Figure S25: SSE distribution by residue index throughout the simulation in stapled KLA peptide structure. (a.) Distorted helicity of linear KLA peptide. (b.) Distorted helicity of stapled KLA peptide. (c.) Helicity retained in the stapled KLA peptide during MD simulation.

XIX. Synthesis of KLA peptides

Synthesis of P3(Red), P3(Oxi), and P4: The *in-silico*-optimized KLA structures were synthesized for biological studies. The precursor peptide P3 (Red) was synthesized using the SPPS method (section II). P3

(Red) peptide was further subjected to on-resin cyclization to get P3 (Oxi) by using our previously reported protocol[2]. P4 was synthesized using the ABT crosslinking procedure (section X) discussed for monocyclic peptides.

Synthesis of P4-FITC: For imaging, we modified the original P4 sequence by replacing the lysine adjacent to the caproic acid with lysine alloc. To perform on-resin orthogonal removal of the alloc group from lysine, 0.025 mmol of peptidyl resin was swelled in dry dichloromethane (DCM) for 20 minutes under a stream of nitrogen. Subsequently, a solution of phenylsilane (76 μL, 0.625 mmol, 25 eq) in dry DCM (3 mL) and tetrakis(triphenylphosphine)palladium(0) (5 mg, 0.0044 mmol, 0.175 eq) was added to the resin under continuous nitrogen flow. The mixture was stirred in the dark for 40 minutes, and this procedure was repeated once more. Finally, the resin was washed with dimethylformamide (DMF) (5 × 2 min) and DCM (2 × 2 min).

For on-resin FITC attachment to the alloc-deprotected lysine residue, 0.025 mmol of the alloc-deprotected peptidyl resin was swelled in DCM for 15 minutes. Then, a solution of FITC (11 mg, 0.0275 mmol, 1.1 eq) and DiPEA (5 μL, 0.025 mmol, 1 eq) in dry DMF (400 μL) was added to the resin, and the mixture was stirred in the dark for 2 hours, which was further crosslinked with BKD1 using the protocol of section X.

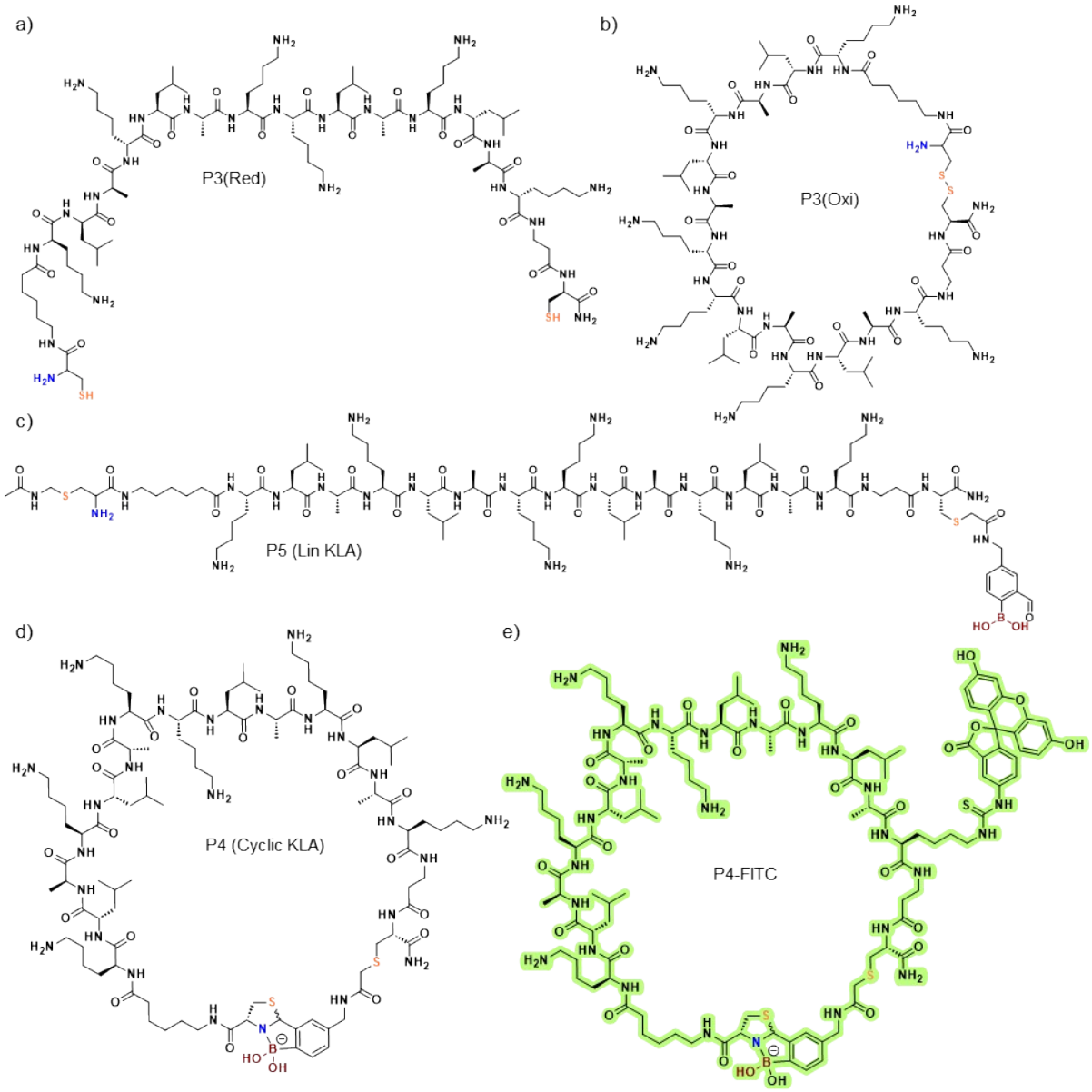


Figure S26. Chemical structures of KLA peptides. a) P3 (Red); b) P3 (Oxi); c) P5; d) P4; e) P4-FITC

Synthesis of P5: P3 peptide (4 mg) having N-terminal Cys protected with Acm was synthesized and was dissolved in 1 mL PBS (pH 7.4, 10 mM) buffer to prepare a 1.43 mM peptide solution. 1.2 eq. TCEP was added to the solution to prevent the formation of disulfide. The pH of the solution was adjusted to 7.4 using 0.5 N NaOH solution. After that, 2.2 eq. of BKD1 (3.16 mM) was added to the solution, followed by the pH adjustment to 7.4. The reaction was continued for 2 to 3 hours at room temperature, monitored by time-to-time LC-MS analyses. The pure peptide was obtained after HPLC purification.

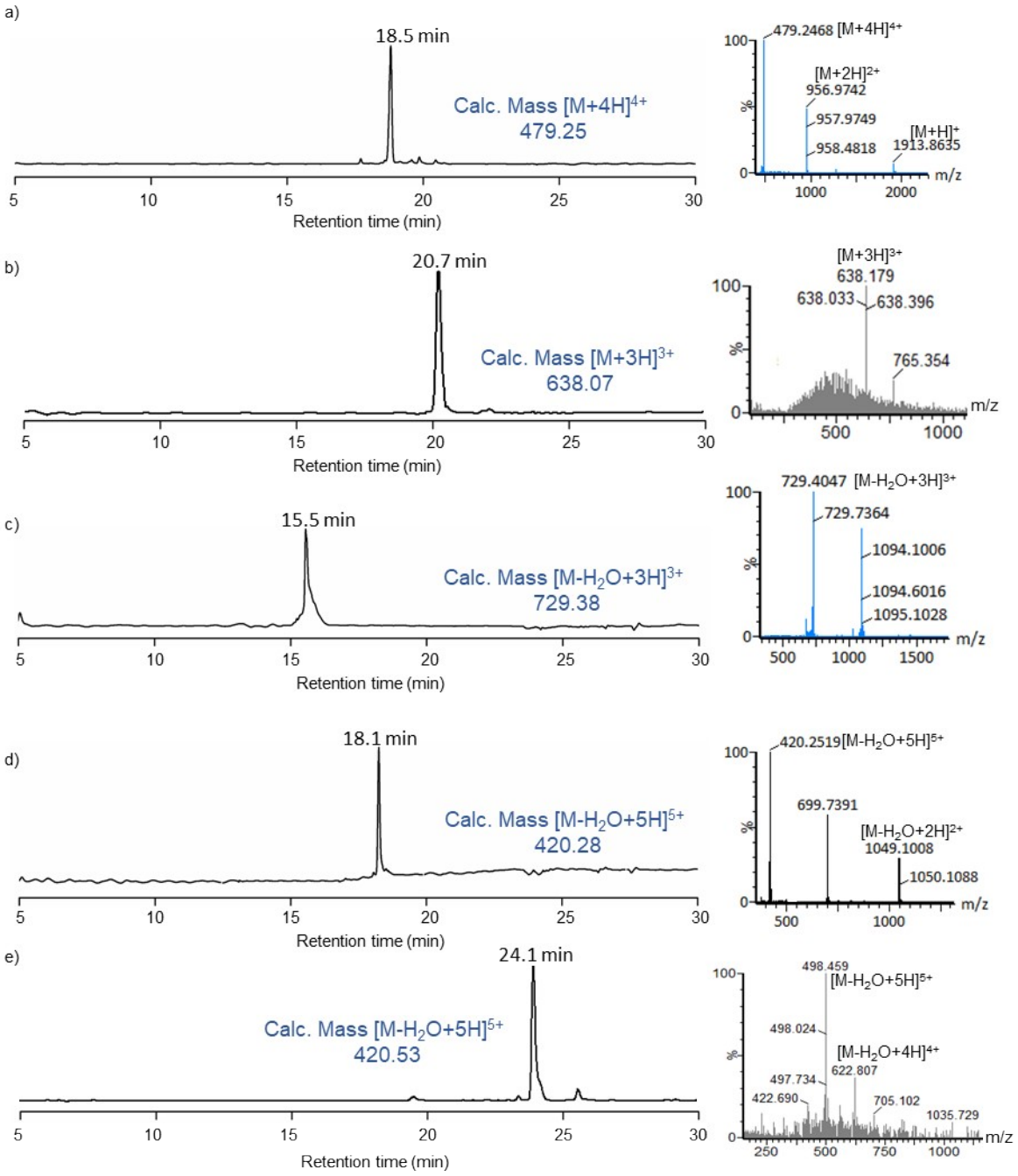


Figure S27. LC profiles and HRMS data of KLA peptides. a) HPLC and HRMS profiles of P3 (reduced), namely P (Red); b) HPLC and HRMS profiles of P3 (oxidized), namely P (Oxi); c) HPLC and HRMS profiles of P5; d) HPLC and HRMS profiles of P4; e) HPLC and HRMS profiles of P4-FITC;

XX. Circular dichroism of KLA Peptides

CD spectra of KLA peptides were recorded on a JASCO J-1500 Circular Dichroism Spectrometer, Easton, MD, USA, using a quartz cell with a 1 mm path length between 200 and 250 nm at room temperature. The peptide concentration was 50 μM in TFE/PBS (10 mM, pH 7.4) mixed solvent (1:1, v/v). Three scans with a scan speed of 50 nm/min were averaged for each measurement. CD spectra were expressed as the mean

residue ellipticity. The percent helicity was calculated from the mean residue ellipticity at 222 nm using the following equation[7].

$$\%\ Helicity = \frac{[\theta]_{222}}{[\theta]_{max}} \times 100$$

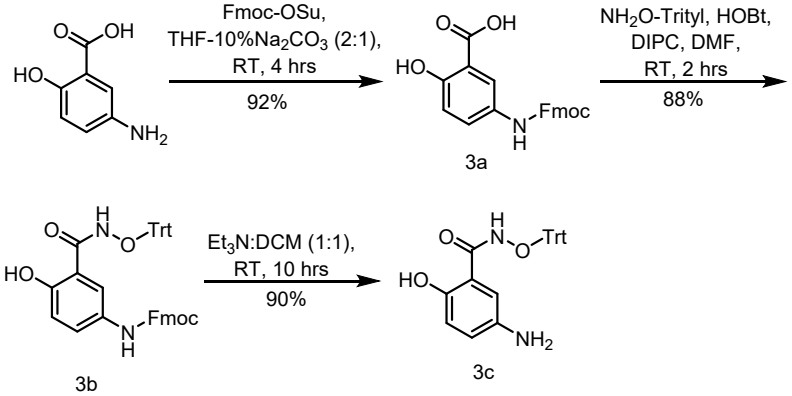
$$[\theta]_{max} = -40{,}000 \times \left(1 - \frac{4}{n}\right)$$

Where $[\theta]_{222}$ is the mean residue ellipticity, $[\theta]_{max}$ is the maximal mean residue ellipticity, and n is the number of amino acids.

Peptide	$[\theta]_{222}$	% Helicity
P3(Oxi)	-4679	15.3
P3(Red)	-9280	29.75
P4	-24170	77.69
P5	-16850	54.16

XXI. Synthesis of Salicylhydroxamic acid (SHA) unit

SHA building block was synthesized as follows.



Scheme 4: Synthetic route of LAN-SHA.

Synthesis of **3a**

5-amino-2-hydroxybenzoic acid (1 g, 6.53 mmol) was first dissolved in 22 mL 10% Na_2CO_3 aqueous solution and was kept in stirring condition in an ice bath. After 10 min, a solution of Fmoc-Osu (2.64 g, 7.84 mmol) in 22 mL THF was added slowly to the stirring solution. After addition, the reaction mixture was allowed to continue at RT for 4 hrs. The progress of the reaction was monitored by performing the TLC of the reaction

mixture. After the completion of the reaction, THF was evaporated under vacuum, and the reaction mixture was acidified with 30 mL 1N HCl. The product was extracted from the aqueous layer using EtOAc (3 × 50 mL). The combined organic layer was washed with brine (2 × 15 mL). Upon evaporation of the organic layer, the crude product was purified via column chromatography to obtain 5.92 g of **3a** (92% yield) as a brownish solid.

^{1}H NMR: (400 MHz, DMSO-d_6) δ 9.61 (s, 1H), 7.94 (s, 1H), 7.87 (d, J = 7.6 Hz, 2H), 7.70 (d, J = 7.4 Hz, 2H), 7.51 (s, 1H), 7.39 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.3 Hz, 2H), 6.85 (d, J = 9.1 Hz, 1H), 4.42 (d, J = 6.6 Hz, 2H), 4.26 (t, J = 6.7 Hz, 1H).

^{13}C NMR: (101 MHz, DMSO-d_6) δ 172.26, 154.11, 144.31, 141.34, 128.24, 127.68, 125.67, 120.74, 117.81, 113.02, 47.15.

HRMS-ESI$^+$ (m/z): $[M+H]^+$ Calc. 376.1180 Obs. 376.1194

Synthesis of **3b**

3a (800 mg, 2.13 mmol) and HOBt (288 mg, 2.13 mmol) were first dissolved in 2.2 mL DMF and cooled to 0 ^{o}C at stirring conditions. After 10 min, DIPC (336 µL, 2.13 mmol) was added in that stirring solution dropwise. This reaction mixture was allowed to stir for 15-20 min. After that, 700 mg of NH_2O-Trityl (700 mg, 2.55 mmol) was added to the reaction mixture, and the reaction was allowed to stir at RT for another 2 hrs. TLC confirmed reaction completion. The reaction mixture was taken in 25 mL chilled 1N HCl, and the product was extracted within EtOAc (3 × 50 mL) from that aqueous layer. The combined organic layer was washed with brine (2 × 15 mL). Finally, the product was purified via column chromatography to obtain 1.18 g of **3b** (88% yield) as a yellowish viscous liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H), 7.78 (d, J = 7.6 Hz, 2H), 7.59 (s, 2H), 7.50 (d, J = 7.6 Hz, 4H), 7.45 – 7.25 (m, 17H), 7.08 (s, 1H), 6.82 (d, J = 8.8 Hz, 1H), 4.50 (d, J = 6.4 Hz, 2H), 4.24 (s, 1H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 143.72, 142.98, 141.53, 141.47, 129.03, 128.80, 128.26, 128.12, 127.98, 127.87, 127.25, 124.95, 120.22, 118.91, 93.91, 66.99, 47.17.

HRMS-ESI$^+$ (m/z): $[M+H]^+$ Calc. 633.2384 Obs. 633.2379

Synthesis of **3c**

To a solution of 3b (657 mg, 1.6 mmol) in 8 mL in DCM, 8 mL triethylamine was added at room temperature. This reaction was allowed to stir for 10-12 hrs, and the reaction completion was confirmed by TLC. After completion of the reaction, DCM was first evaporated under vacuum and acidified with 30 mL 1N HCl. The product was extracted with EtOAc (3 × 50 mL) from the aqueous layer. The combined organic layer was washed with brine (2 × 15 mL). The product was further purified via column chromatography to get the compound **3c** (365 mg) as a pinkish-white solid with a yield of 85%, which was further subjected to coupling with peptide.

XXII. Coupling SHA unit with LAN to get P6

Lanreotide (LAN) was synthesized (0.1 mmol) using our previous protocol.[2] Late beta-alanine was coupled at the N-terminus of the LAN peptide as a spacer. Subsequently, Fmoc removal from the N-terminus using 20% piperidine in DMF and coupling of 2-chloroacetic acid (3 eq.) using DIC (3 eq.) and HOBT (3 eq.) in DMF were performed. The coupling reaction was performed for 15 minutes, and the resin was thoroughly washed thrice with DMF. Finally, the SHA-building block (**3c**, 3 eq.) dissolved in DMF and DiPEA (3 eq.) was charged on the chloroacetamide derivatized peptidyl resin for SHA unit installation. The SHA installation reaction was monitored through LC-MS, and conversion was completed within 30 minutes. Later, the resin was thoroughly washed thrice with DMF and DCM. The peptide was cleaved from the resin using reagent K and precipitated in cold ether. Finally, the pure peptide was obtained after HPLC purification (yield = 64%).

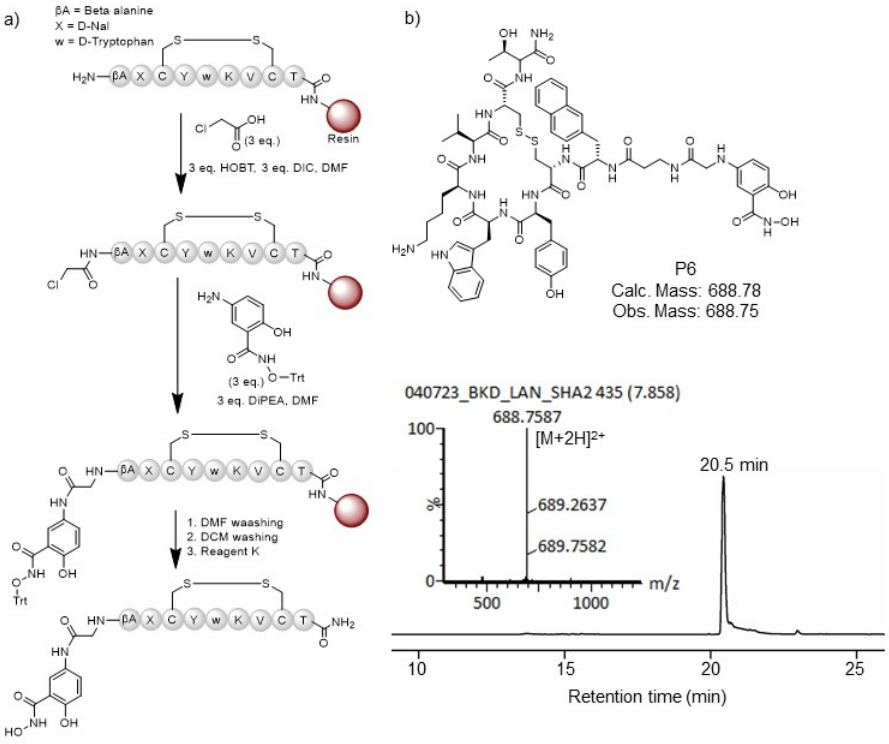


Figure S28. A) Reaction scheme of SHA coupling on peptide. b) Chemical structures of P6 along with HRMS data and HPLC chromatogram of pure P6.

XXIII. Conjugation of P6 and KLA peptides (P4 and P5)

In this study, SHAB 'click' chemistry was employed to conjugate peptide P6 with KLA peptides P4 and P5 under neutral pH conditions, ensuring that the conjugation is working prior to applying it in biological systems. Initial stock solutions of 1 mM for all three peptides were prepared in PBS (pH 7.4). Subsequently, individual incubation of KLA peptides P4 and P5 with P6 occurred at a concentration of 20 mM in PBS (pH 7.4) for 10 minutes. Following the conjugation reactions, the resulting PBS solutions were subjected to High-Resolution Mass Spectrometry (HRMS) analysis at pH 8 (1:1 carbonate buffer:ACN). This confirms the success of the conjugation by the corresponding mass of the desired peptide conjugate (P4+P6) shown in Figure S29. Similarly, the other peptide conjugate (P5+P6) was confirmed.

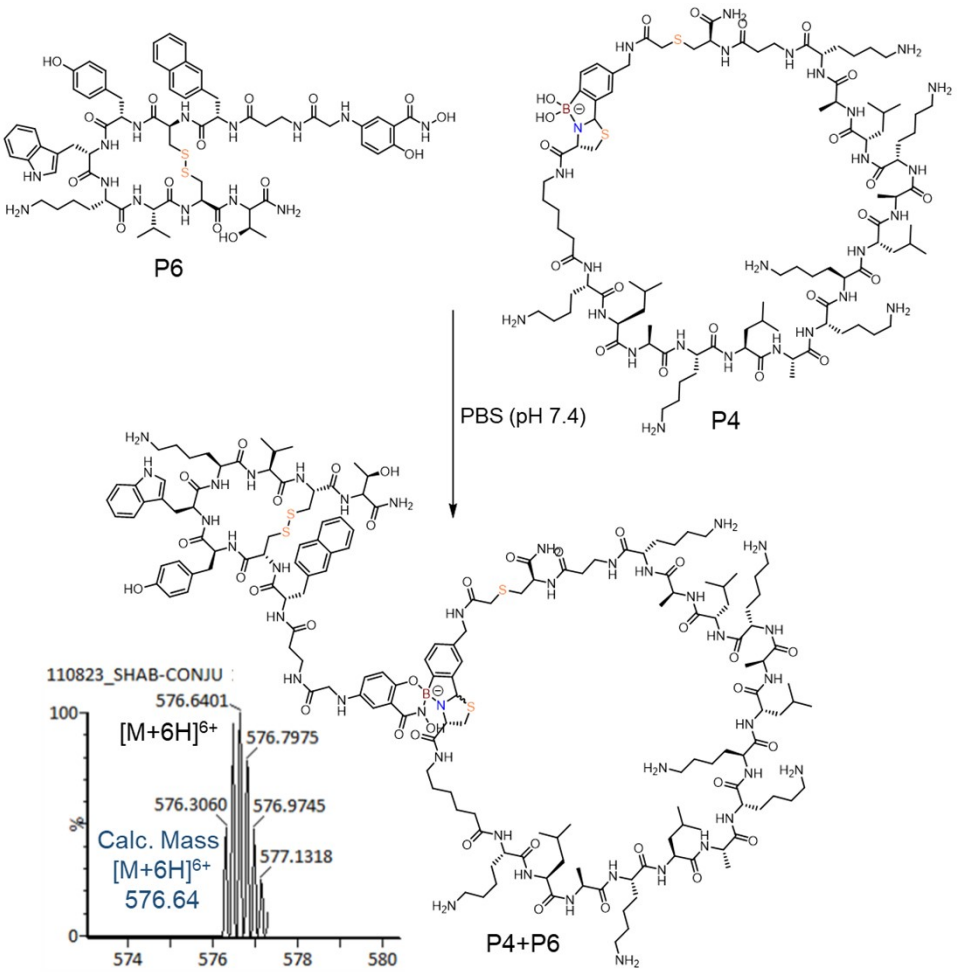


Figure S29. Chemical structures of P4+P6 SHAB conjugate with mass data.

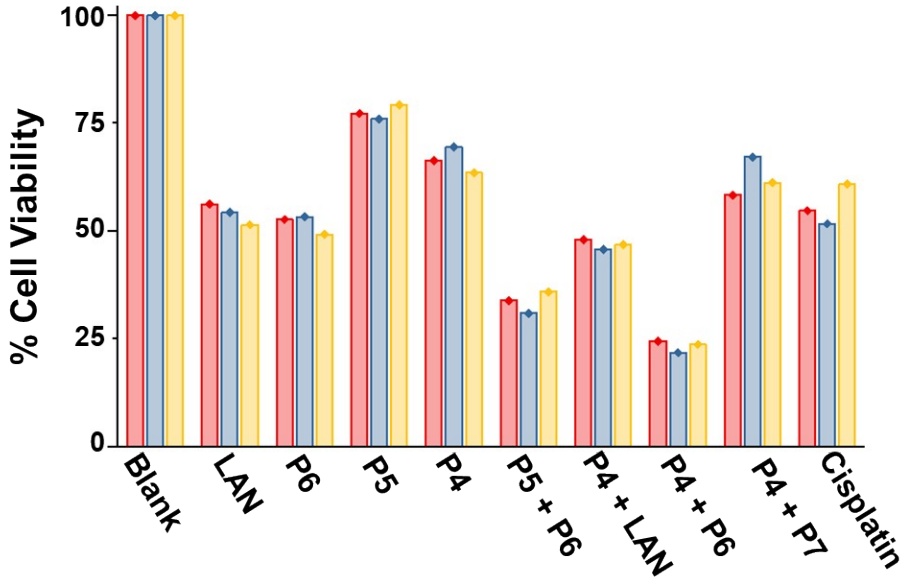
XXIV. MTT Assay:

A549 cells (grown in complete RPMI 1640 media with 10% fetal bovine serum (FBS), 1% Pen/Strep) were grown to about 70% confluency before removal from the surface of a 25 cm^2 tissue culture flask with Trypsin EDTA (2 minutes, 37 ^oC, 5% CO_2). Cells were pelleted (1000 rpm, 5 minutes, 37 ^oC) and diluted in fresh complete RPMI 1640 media to generate aliquots of 10,000 cells per well (100 µL) in a 96-well plate. The cells were allowed to adhere to the surface, and then, the cells were incubated for 12 hours with Cisplatin (Positive Control), LAN (Positive Control), P6, P5, P4, P5+P6, P4+LAN, P4+P6, and P4+P7. Non-treated cells were also considered blank. All samples were incubated at 20 µM and Cisplatin at 50 µM in RPMI 1640 media.

A stock solution at the same concentration for all peptides and conjugates was freshly prepared in DPBS and diluted to the working concentration (20 µM) in RPM1-1640. For each reagent, samples were set up as three replicates for 12 hours, along with the cells that had not received any treatment. After incubation for 12 hr, media was removed and washed with PBS before being incubated for 4 hr with a 0.4 mg/mL concentration of 3-(4,5-Dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in serum-free RPMI media. A 100 µL MTT solution was added to each well and incubated at 37°C for 4 h in an incubator. The MTT assay was quenched with 100 µL DMSO and allowed to incubate for 3-4 hr before absorbance at 570 nm was recorded in the TECAN Infinity F Plex Microplate reader. Each data was performed in triplicate (Figure S30). The same experiment was repeated one more time (i.e., n = 2) to check the consistency of the results. The results of the averages and standard deviations of the wells determined after normalization to the DMSO control are displayed in the main text **Figure 5e**.

To check the P4+P6 selective delivery through SSRT2 receptors, Mia Paca 2, an epithelial cell line derived from tumor tissue of the pancreas, was selected which does not express SSRT2 receptor over the cell surface. HEK293 cells, a non-cancerous normal epithelial cell line, were also tested to understand the selectivity of P4+P6 over cancerous cells. With all these three cell lines, we performed a cytotoxicity assay (MTT) with 20 µM P4+P6 and Cisplatin at 50 µM as positive control. Non-treated cells were considered blank. All the protocols were the same as described above for A549, but for Mia Paca 2 and HEK293, the only change was DMEM media was used instead of RPMI 1640. The results are represented in Figure S30b.

a)



b)

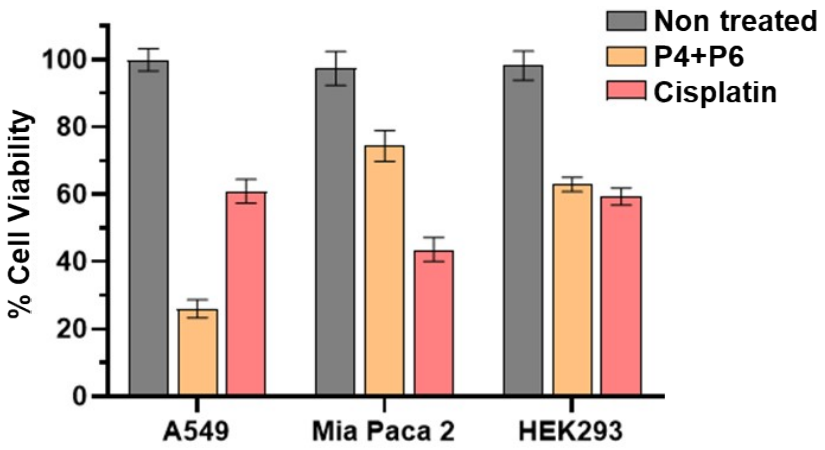


Figure S30: a) The outcome of triplicate MTT assay on the A549 cell line for an experiment is shown in the bar graph. b) Cytotoxic effect of P4+P6 over Mia Paca2 (SSRT2 absent) and HEK293 (normal cell line)

XXV. Apoptosis Assay via Flow-Cytometry

A549 cells (grown in complete RPMI 1640 media with 10% fetal bovine serum (FBS), 1% Pen/Strep) were grown to about 70% confluency before removal from the surface of a 25 cm^2 tissue culture flask with Trypsin EDTA (2 minutes, 37 ^oC, 5% CO_2). Cells were pelleted (1000 rpm, 5 minutes, 37 ^oC). Then, cells were diluted in fresh, complete RPMI 1640 media to generate aliquots of 1×10^5 cells per well (1 mL) in a 12-well plate to adhere to the surface. The cells were incubated for 24 hrs with Cisplatin (Positive Control), LAN (Positive Control), P6, P5, P4, P5+P6, P4+LAN and P4+P6. Non-treated cells were also considered blank. All samples were incubated at 20 µM and Cisplatin at 50 µM in RPMI 1640 media.

For each reagent, samples were set up as three replicates for 24 hours, along with the cells that had not received any treatment. After incubation for 24 hours, the media was removed, washed with PBS, and trypsinized. Cells were taken in a 1.5 mL micro-centrifuge tube and centrifuge at 300 ×g for 5 min and discarded the supernatant. PBS was added to wash the cells, and finally, the cell pellets were incubated into 100 µL of 1×Annexin V Binding Buffer. 2.5 µL of Annexin V-FITC from the apoptosis kit (Elabsciences) was added to the cell suspensions and gently vortexed and incubated at room temperature for ~20 min in the dark. After the incubation of Annexin V-FITC, 400 µL of 1×Annexin V Binding Buffer was added to the tube and mixed gently. Flow cytometry analysis was carried out in a Sysmex Cube 6 system in which the laser wavelength was 488 nm, the detector channel was FL1, and the bandpass filter wavelength was 533/30 nm. Sample analyses were conducted with FSC voltage 250, SSC voltage 350, and FL1 voltage 550. Thirty

thousand events were collected in all samples. Fluorescence readout was measured based on a forward scatter threshold. Data analysis was performed with Sysmex Cube 6 built-in software, applying constant gating on unstained cells for experiments, from which the median fluorescence intensities of the stained cells were extracted and plotted. The gating strategy was kept conserved with all samples. Each data was taken in triplicate. The whole experiment was repeated again (i.e., n = 2) to understand the results' consistency. The median values are represented as a bar graph in the main text **Figure 5f** after normalization of the median value of non-treated non-stained cells, mainly generated due to the autofluorescence of cells.

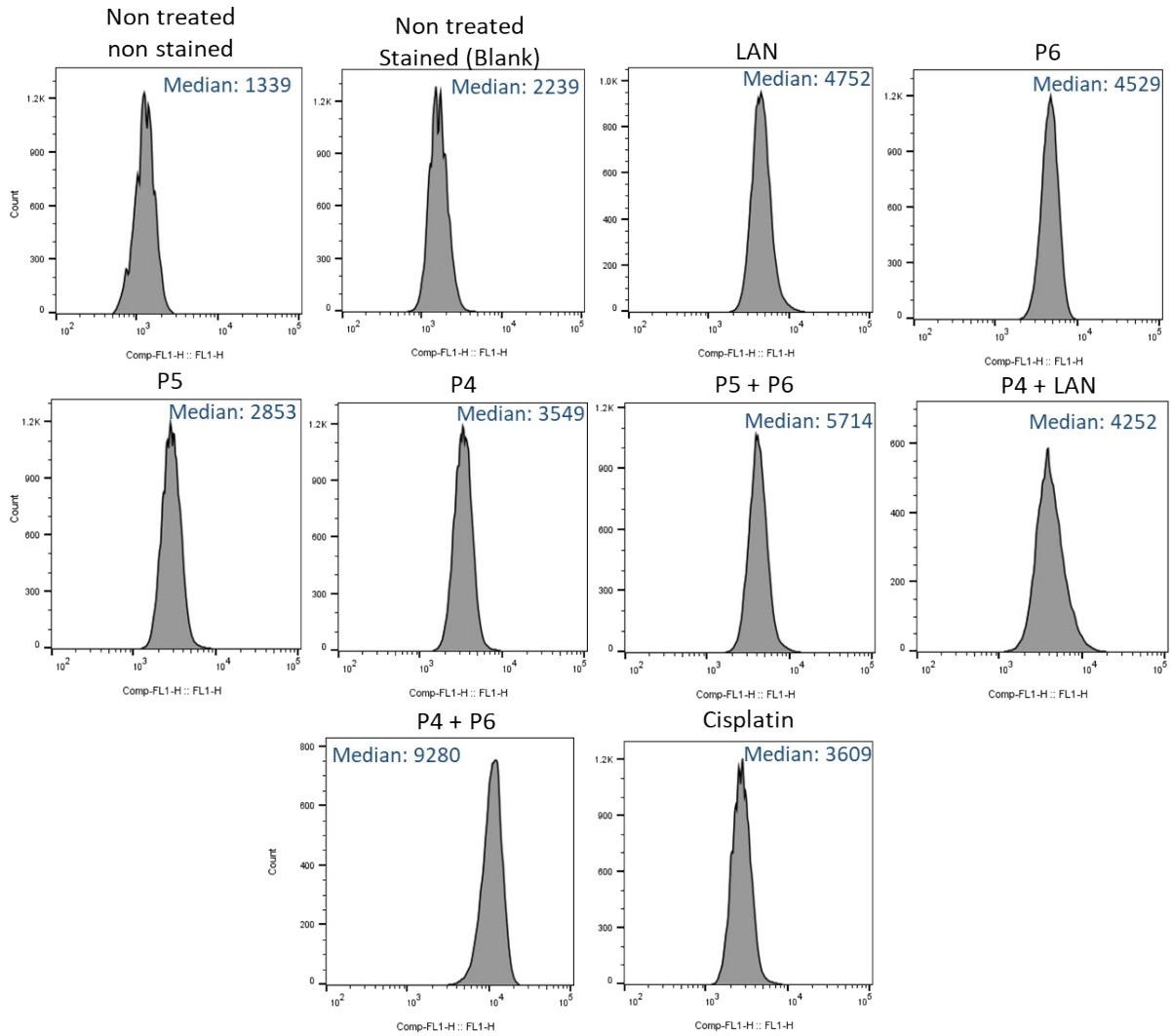


Figure S31: A set of histograms for flow cytometry data of apoptotic A549 cells treated with Annexin V-FITC. The median values are shown at the top corner for each experiment.

XXVI. Confocal laser scanning microscopy.

The cancer cell lines of A549 and Mia Paca2 and the normal cell line of HEK293 were seeded respectively in a glass bottom dish at a density of 1×10^5 cells/ well for 24 h. Thereafter, for A549, 20 μM of P4, P4+P6, P4+P7 were dispersed in RPMI 1640 medium with 10% FBS along with 1% antibiotics were added. The cells were further incubated at 37 ^{o}C for another 24 h. In cases of Mia Paca2 and HEK293, cells were only treated with 20 μM P4+P6 using DMEM instead of RPMI 1640. After 24 h, the mitochondria were stained with JC-1 (10 μg/mL) in fresh media for another 30 min. After washing with PBS 3 times, the cells were observed under a Carl Zeiss LSM 880 (laser scanning confocal microscopy). Acquired images were processed using ZEISS ZEN 3.10 software. The images are represented in **Figure 6d** and Figure S32.

LSCM Parameters: The images were acquired in LSM 880, AxioObserver, with Bit depth at 16 Bits and scaling per pixel set at 0.09 μm. The lens objective was set as Plan-Apochromat 40×/1.3 Oil DIC UV-IR M27. For bright field images, the beam splitter was set as MBS 488, MBS_InVis as Plate, DBS1 as Mirror, and the MainBeam SplitterNonDescanned as Rear. 488 nm lasers at 2% were used with the calibration marker positions set at 0.00 μm. The fluorescence contrast method was used with the pinhole set at 0.79 AU, scan mode as Frame, scan zoom of X and Y as 1.0, rotation at 0°, pixel time 3.62 μs, and line time at 30.00 μs. Channel 1 was set at excitation wavelength of 488 nm and emission wavelength of 562 nm with detection wavelength of 493-630 nm, effective NA at 1.3, depth of focus for T PMT channel at 0.99 μm, binning mode at 1×1, detector type as PMT, gain at 328, detector offset set at 0, digital gain for channel 1 and PMT channel at 0.3 and 1.0 respectively, scan direction as unidirectional and airy can mode off. Keeping all other parameters the same, the red channel was set at an excitation wavelength of 561 nm, an emission wavelength of 640 nm with a detection wavelength of 568-712 nm, a gain of 686.0, and a digital gain of 1.0. The green channel was set at an excitation wavelength of 488 nm, an emission wavelength of 548 nm, with a detection wavelength of 490-606 nm, a gain of 645.0, and a digital gain of 1.0. The blue channel was set at an excitation wavelength of 355 nm, an emission wavelength of 498 nm with a detection wavelength of 410-585 nm, a gain of 623.0, and a digital gain of 1.0, pinhole at 1.85 AU.

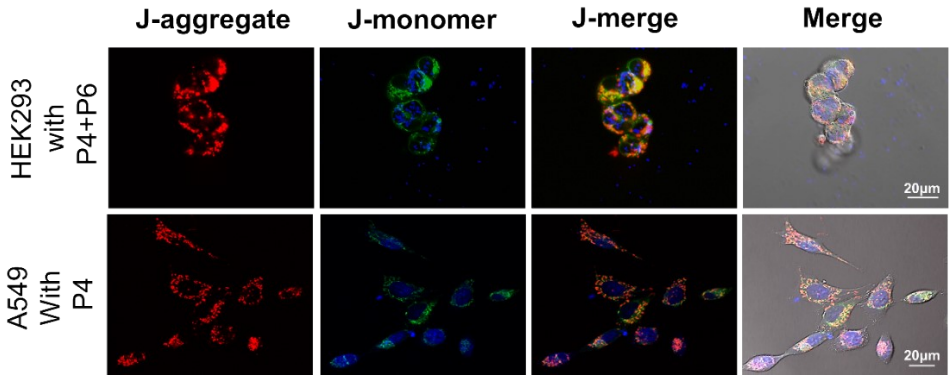


Figure S32: Comparison of mitochondrial membrane potentials (JC-1 assay) in A549 cells and HEK293 cells by LSCM after incubation with P4 (20 μM) and P4+P6 (20 μM), respectively.

To observe the specific mitochondria targeting ability of P4+P6, the peptide was tagged with FITC to prepare P4-FITC (Synthesis in section XIX). A549 cells were seeded in a glass bottom dish at a density 1×10^5 cells/ well for 24 h. After 24 h, the cells were incubated with 20 μM P4-FITC+P6 in complete RPMI 1640 at 37 ^{o}C for another 4 h. Subsequently, 100 nM Mito Tracker Red CM-H2XRos in RPMI 1640 (without FBS to prevent the oxidization of Mito Tracker Red CM-H2XRos) was added to stain the mitochondria for 30 min. After washing with PBS three times, the cells were imaged under Carl Zeiss LSM 880 using green and red channels.

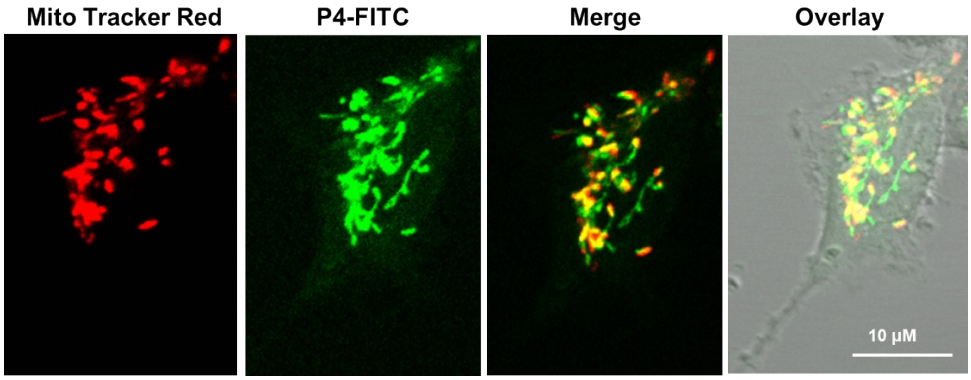


Figure S33: LSCM images for A549 treated with P4-FITC+P6 and Mito Tracker Red CM-H2XRos.

XXVII. NMR Spectra:

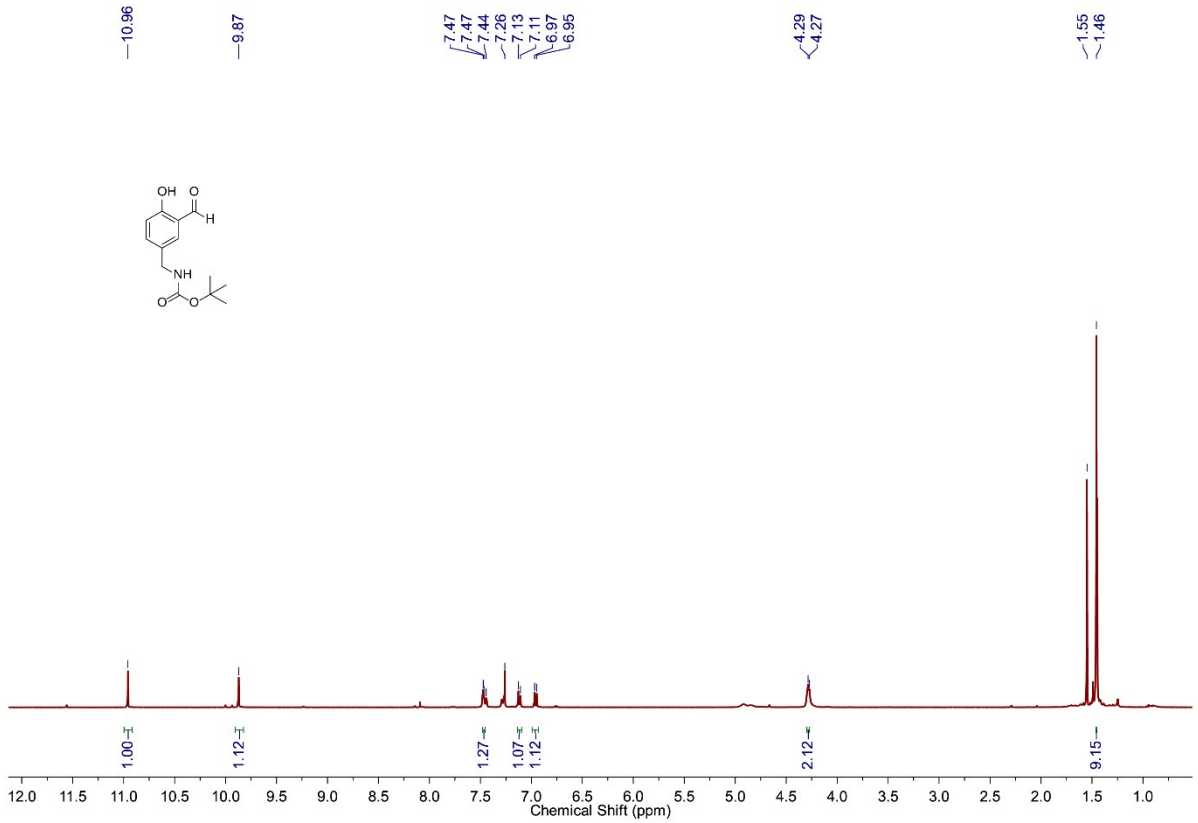


Figure S34. ^{1}H-NMR (400 MHz) spectrum of 1b recorded in $CDCl_3$.

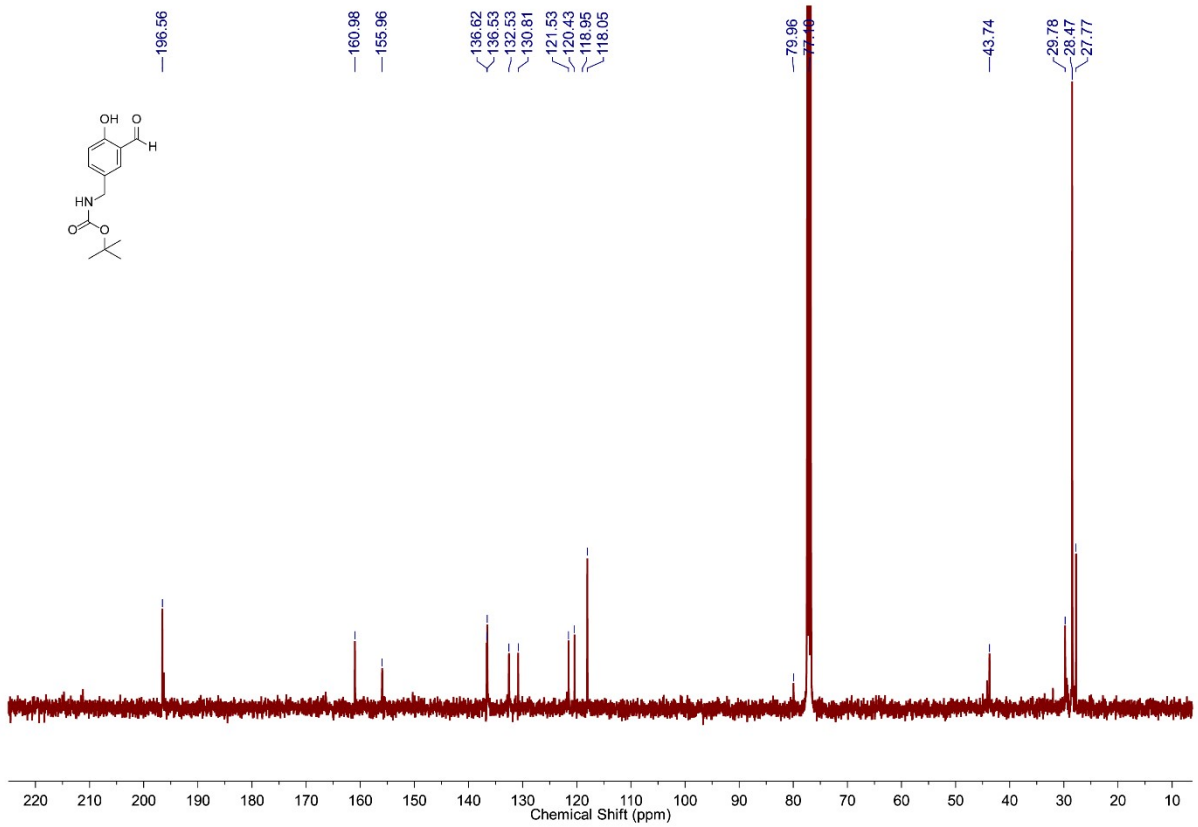


Figure S35. ^{13}C-NMR (101 MHz) spectrum of 1b recorded in $CDCl_3$.

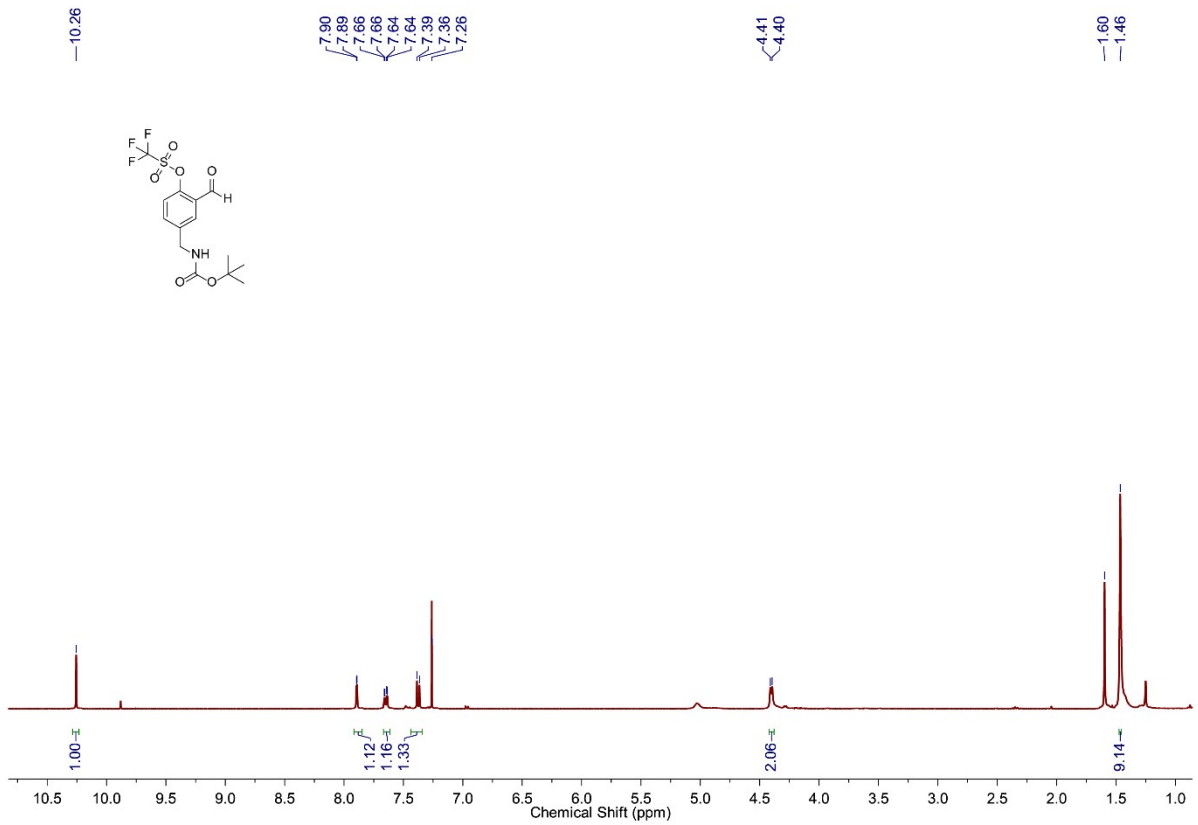


Figure S36. ^{1}H-NMR (400 MHz) spectrum of 1c recorded in $CDCl_3$.

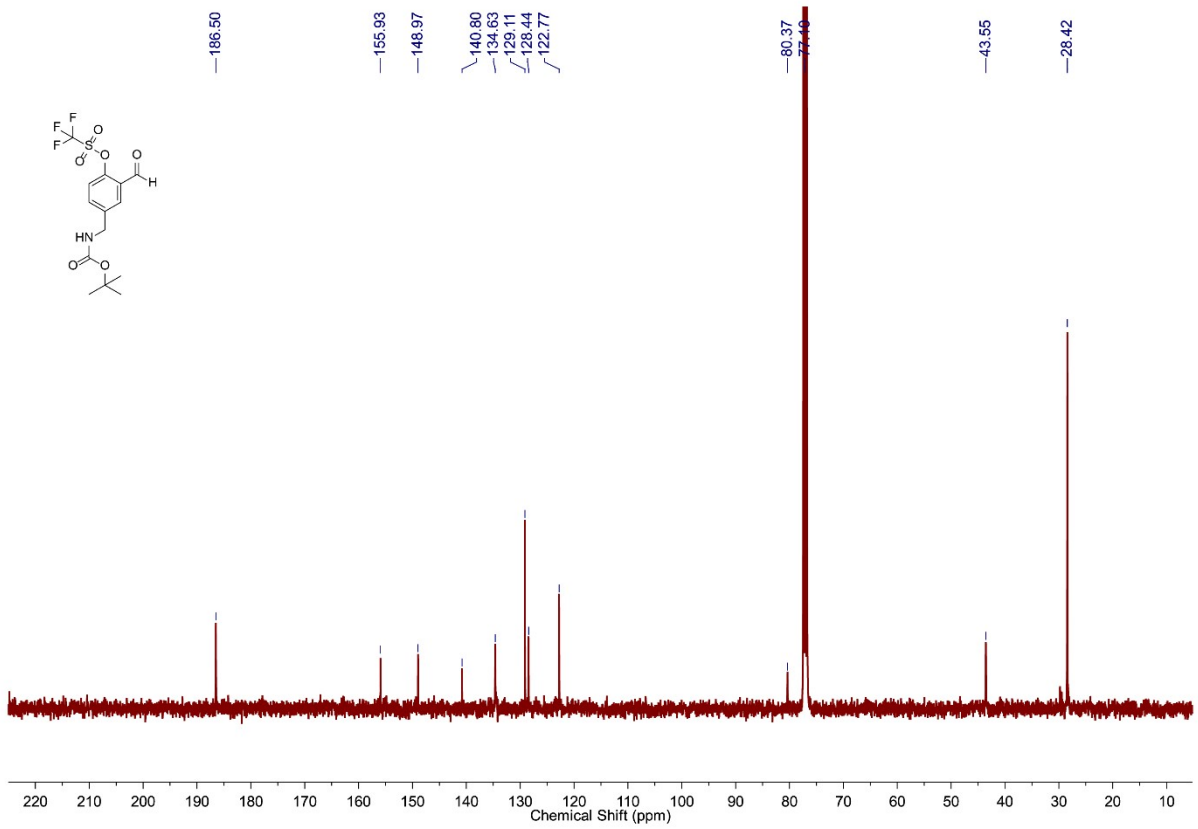


Figure S37. ^{13}C-NMR (101 MHz) spectrum of 1c recorded in $CDCl_3$.

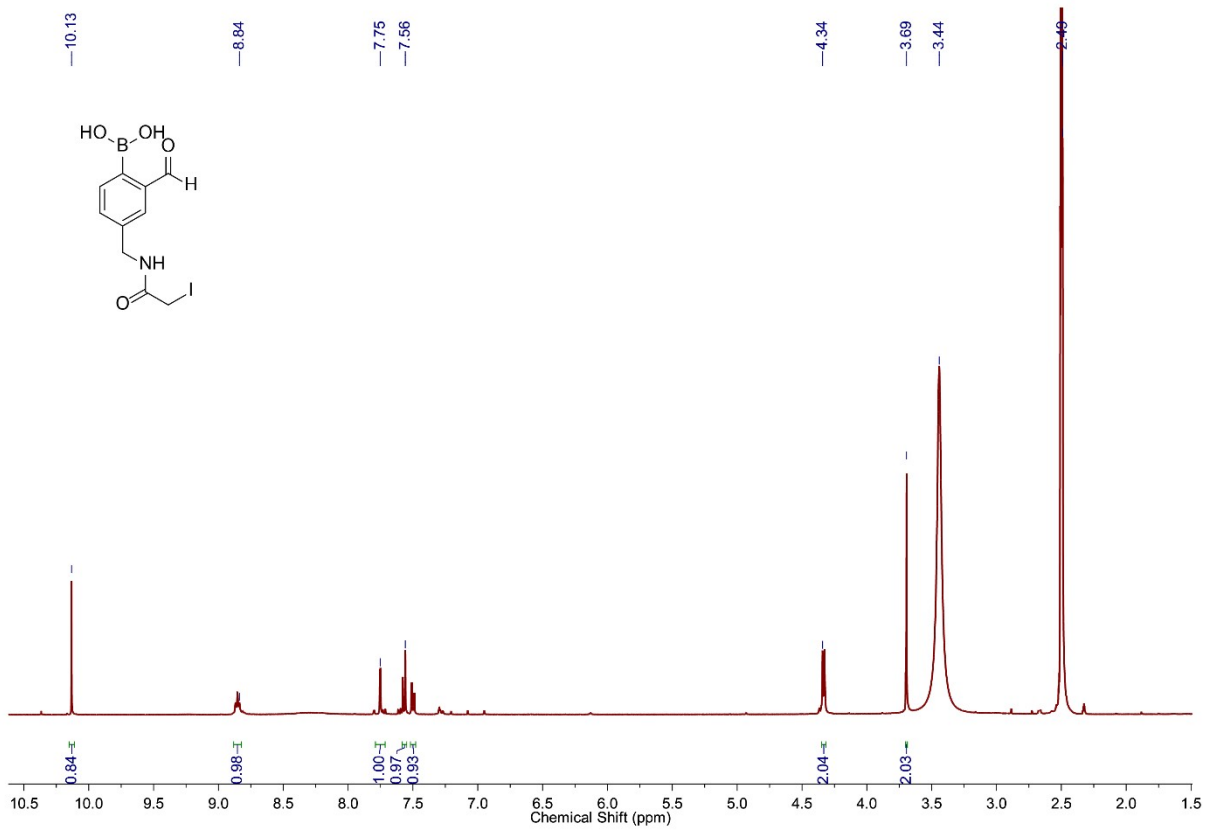


Figure S38. [1]H-NMR (400 MHz) spectrum of BKD1 recorded in DMSO-d_6.

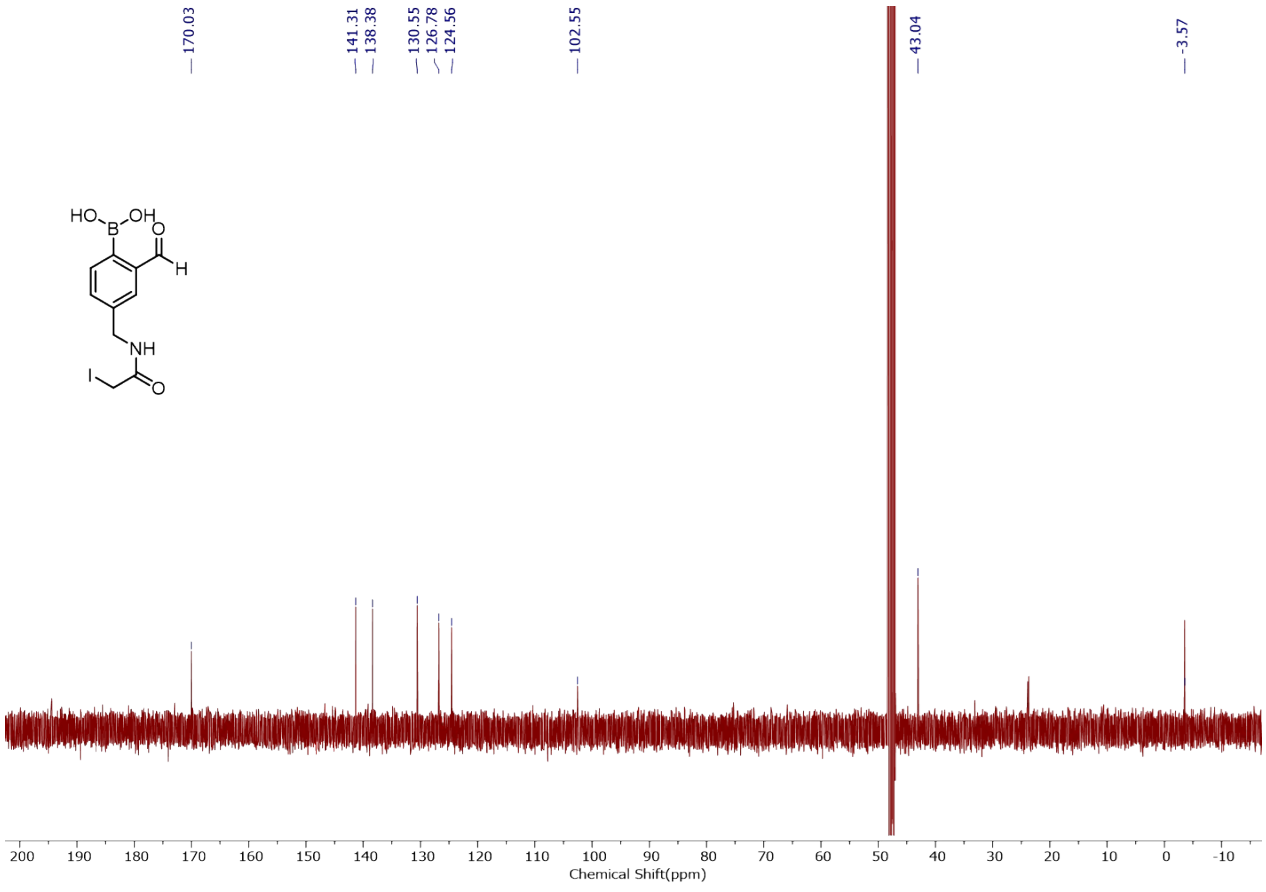


Figure S39. [13]C-NMR (101 MHz) spectrum of BKD1 recorded in CD_3OD.

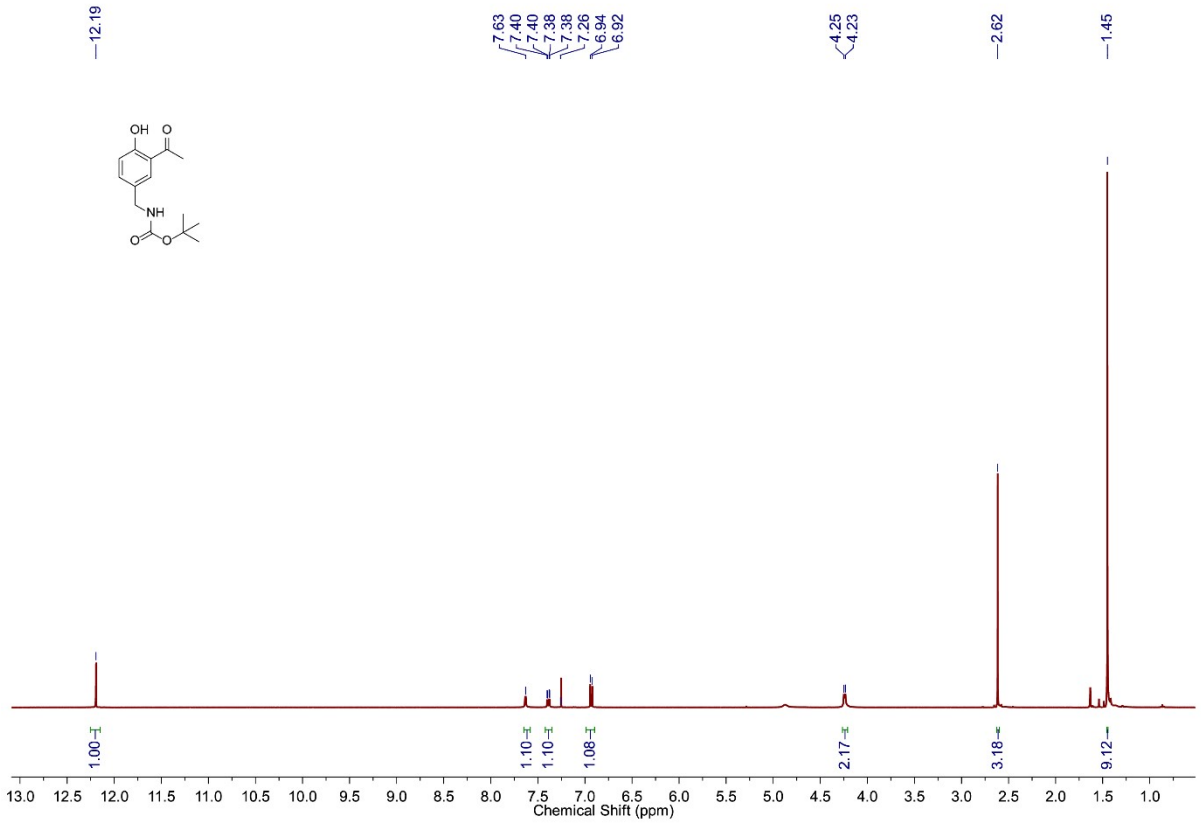


Figure S40. ^{1}H-NMR (101 MHz) spectrum of 2b recorded in $CDCl_3$.

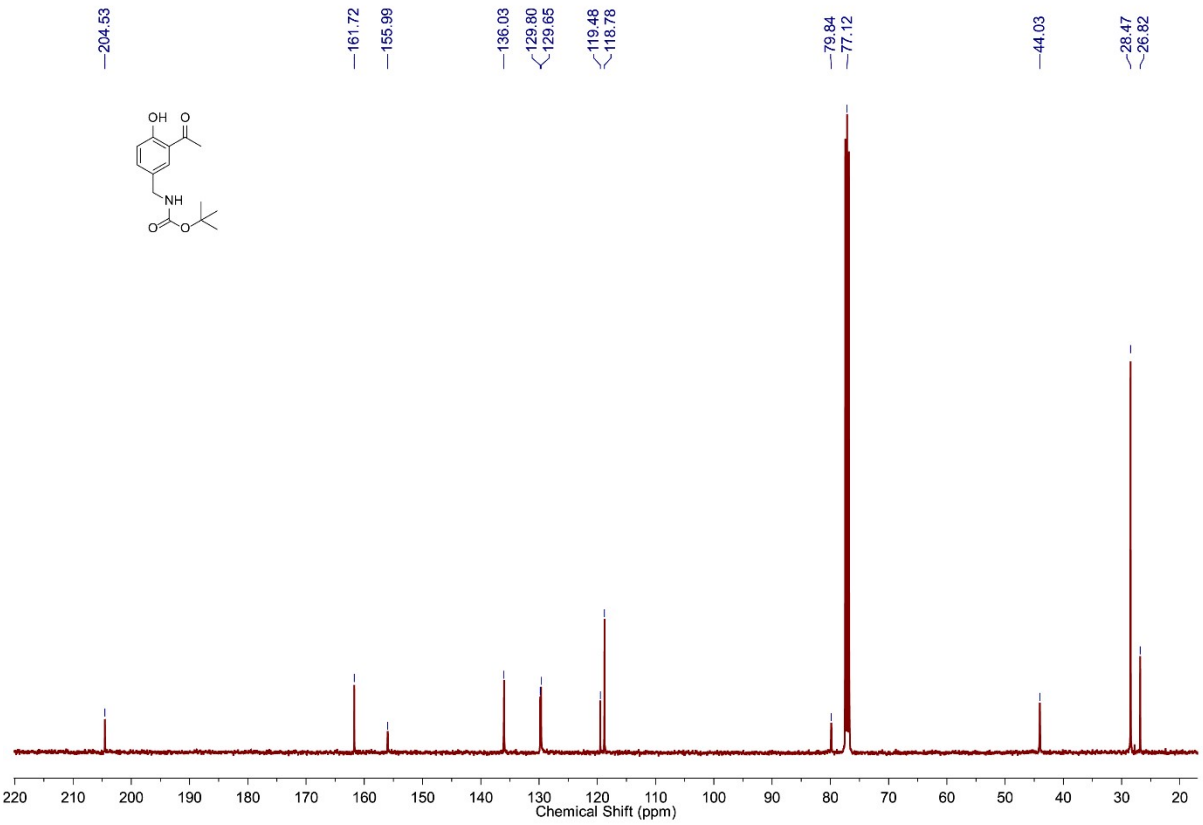


Figure S41. ^{13}C-NMR (101 MHz) spectrum of 2b recorded in $CDCl_3$.

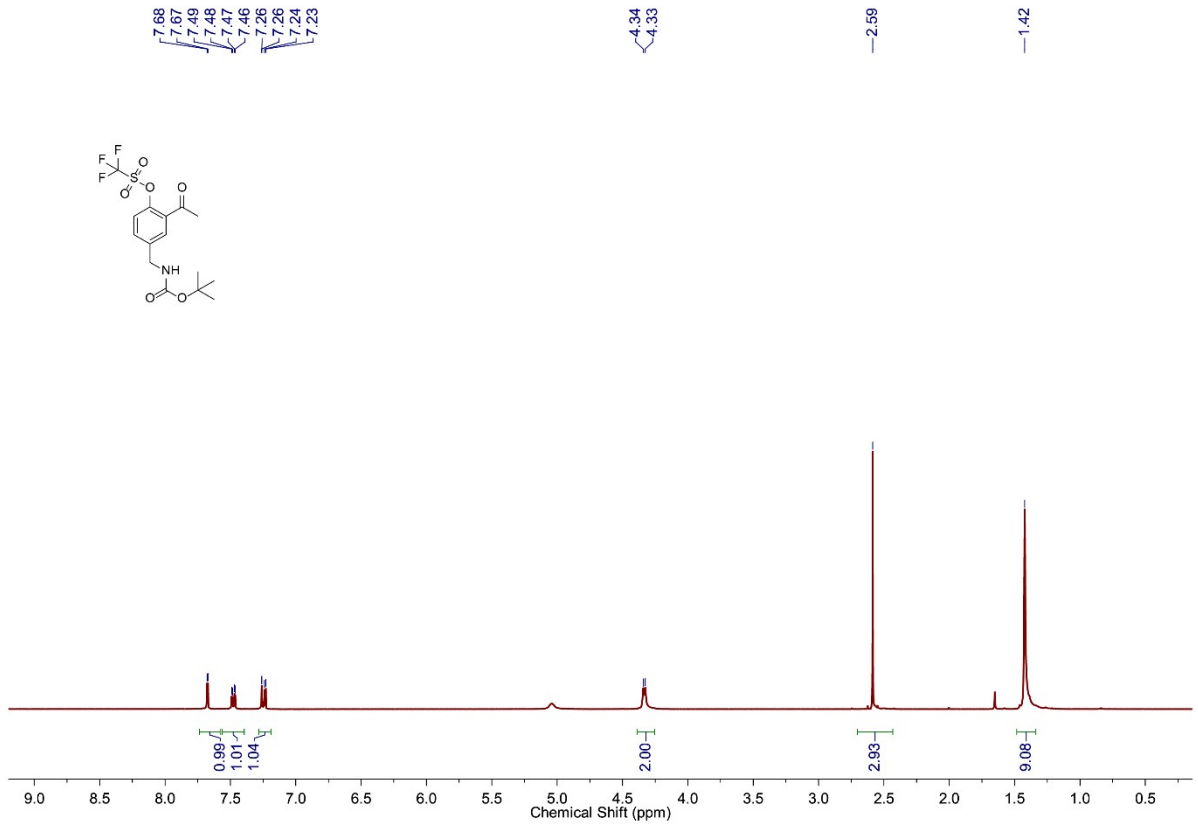


Figure S42. ^{1}H-NMR (400 MHz) spectrum of 2c recorded in $CDCl_3$.

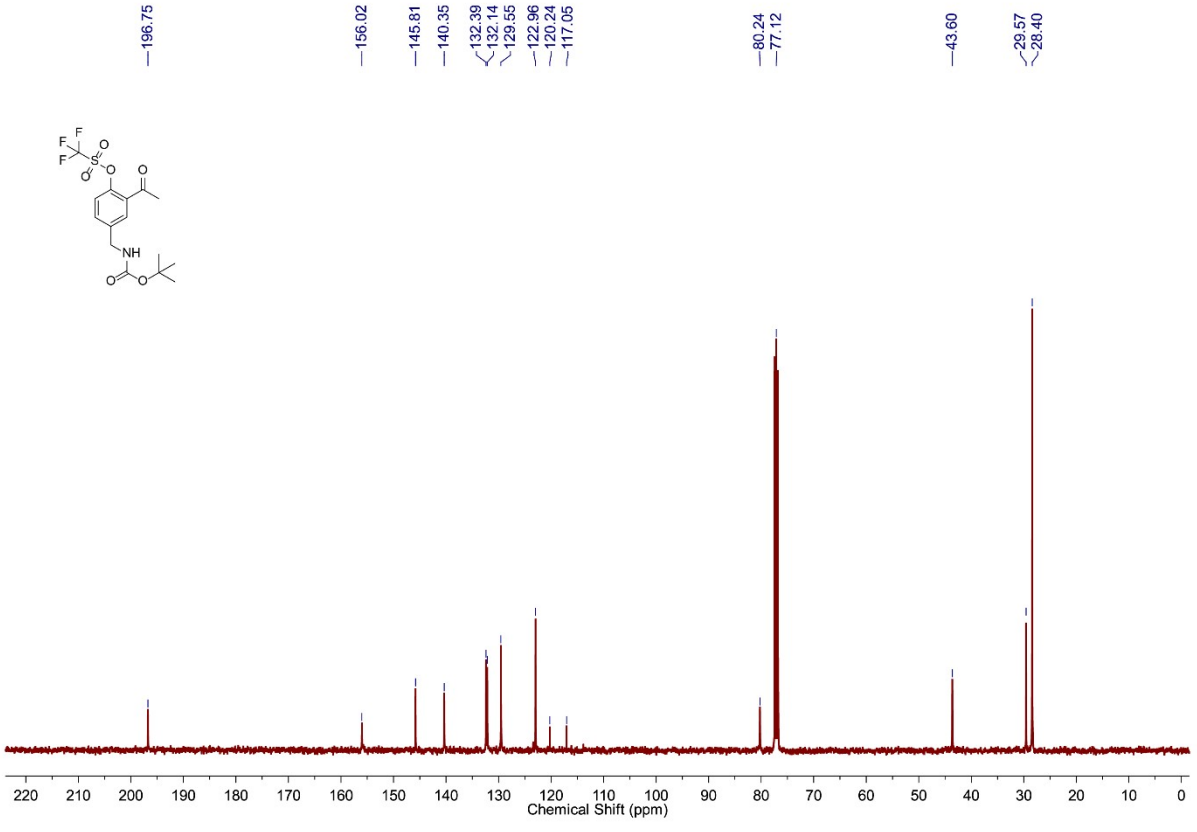


Figure S43. ^{13}C-NMR (101 MHz) spectrum of 2c recorded in $CDCl_3$.

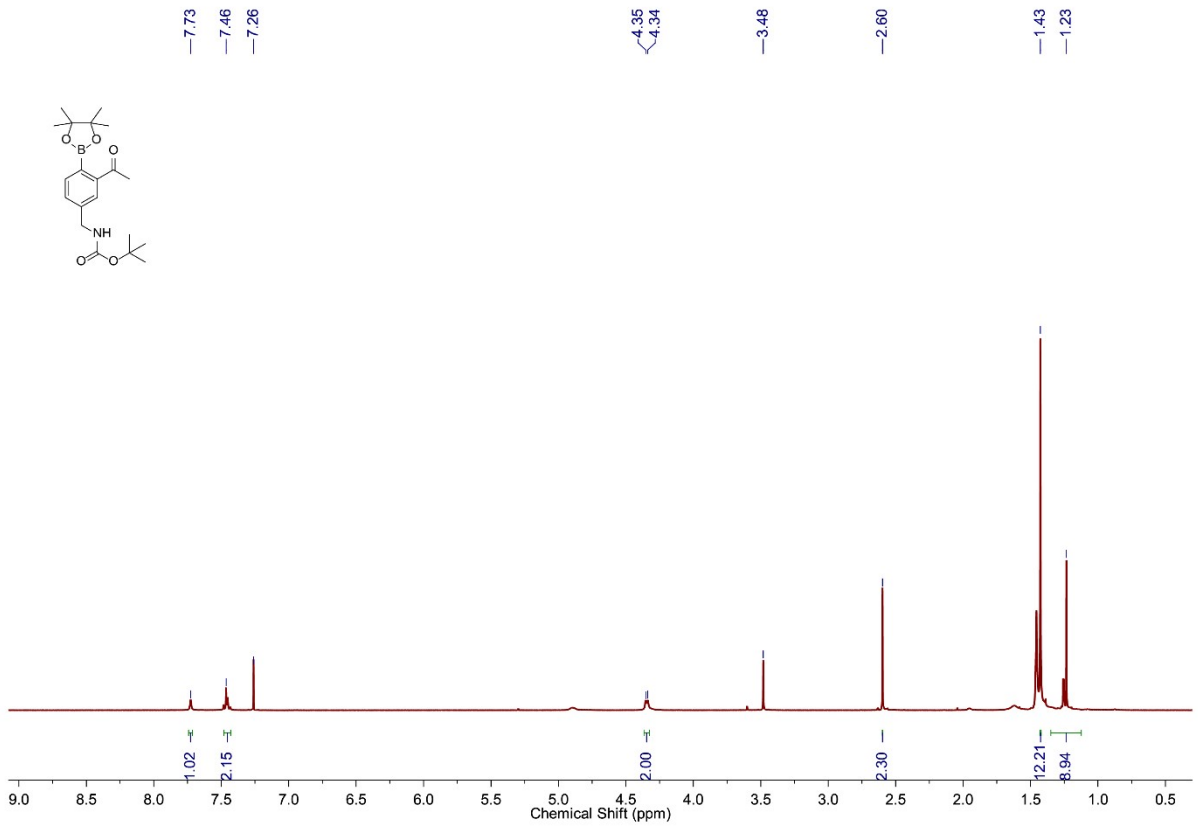


Figure S44. ^{1}H-NMR (400 MHz) spectrum of 2d recorded in $CDCl_3$.

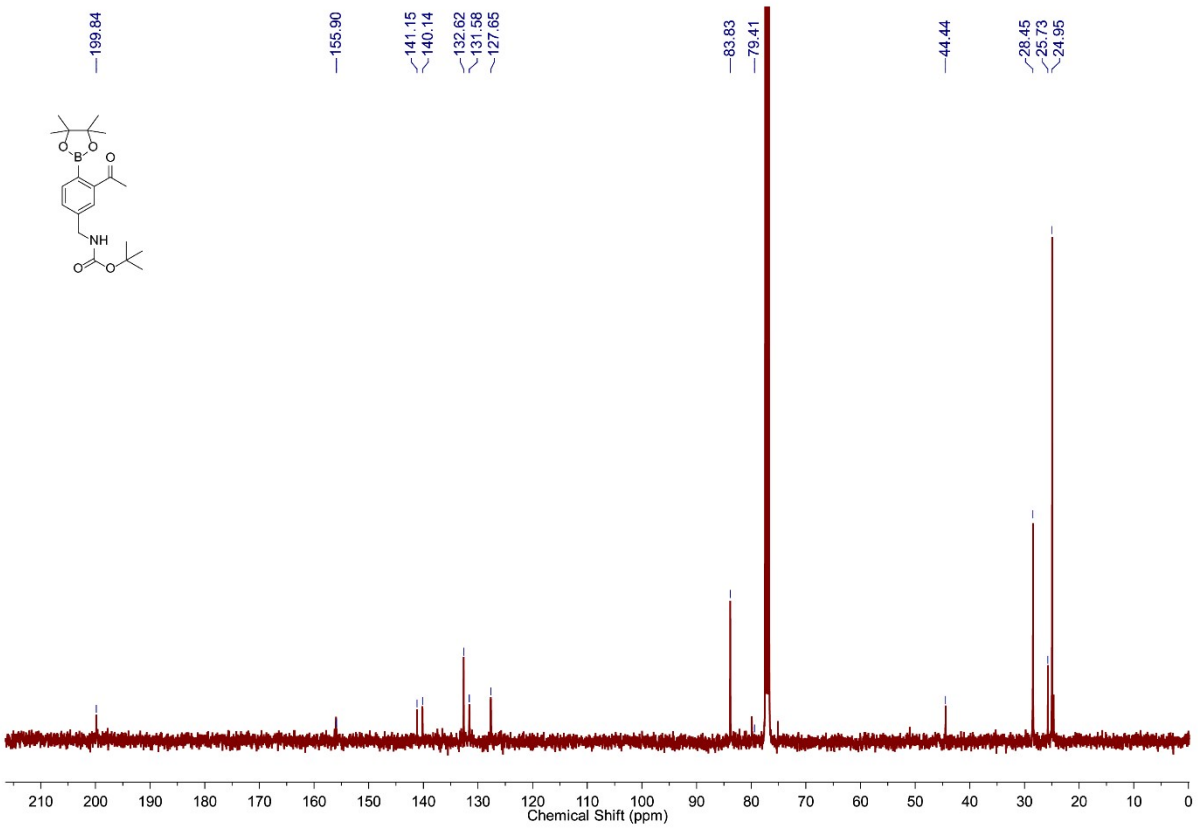


Figure S45. ^{13}C-NMR (101 MHz) spectrum of 2d recorded in $CDCl_3$.

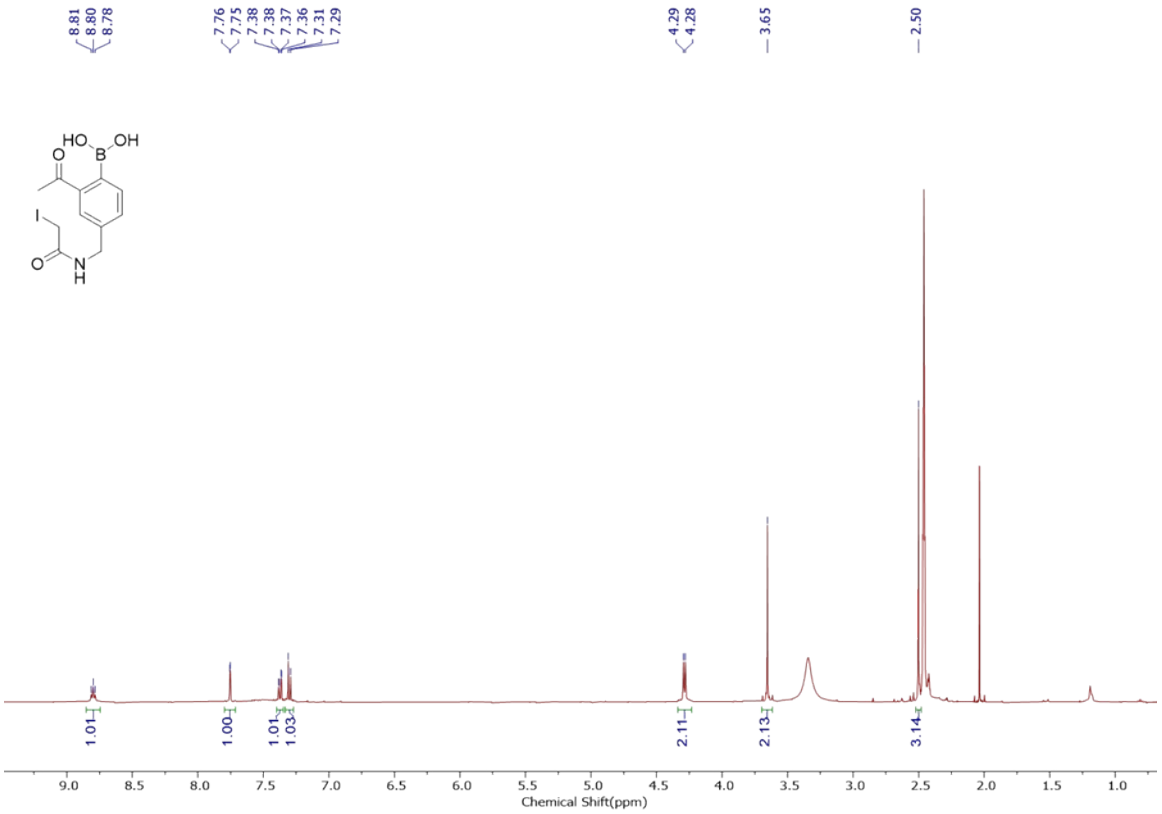


Figure S46. [1]H-NMR (400 MHz) spectrum of BKD2 recorded in DMSO-d_6.

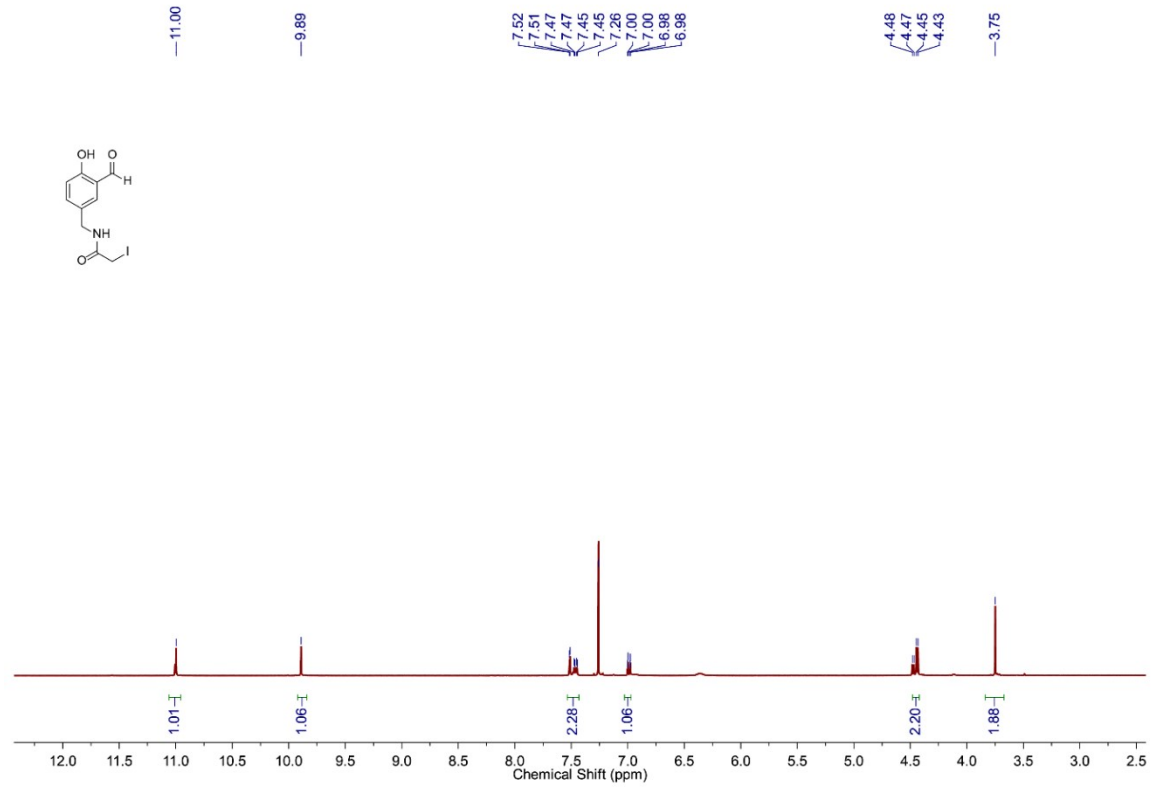


Figure S47. [1]H-NMR (400 MHz) spectrum of BKD3 recorded in $CDCl_3$.

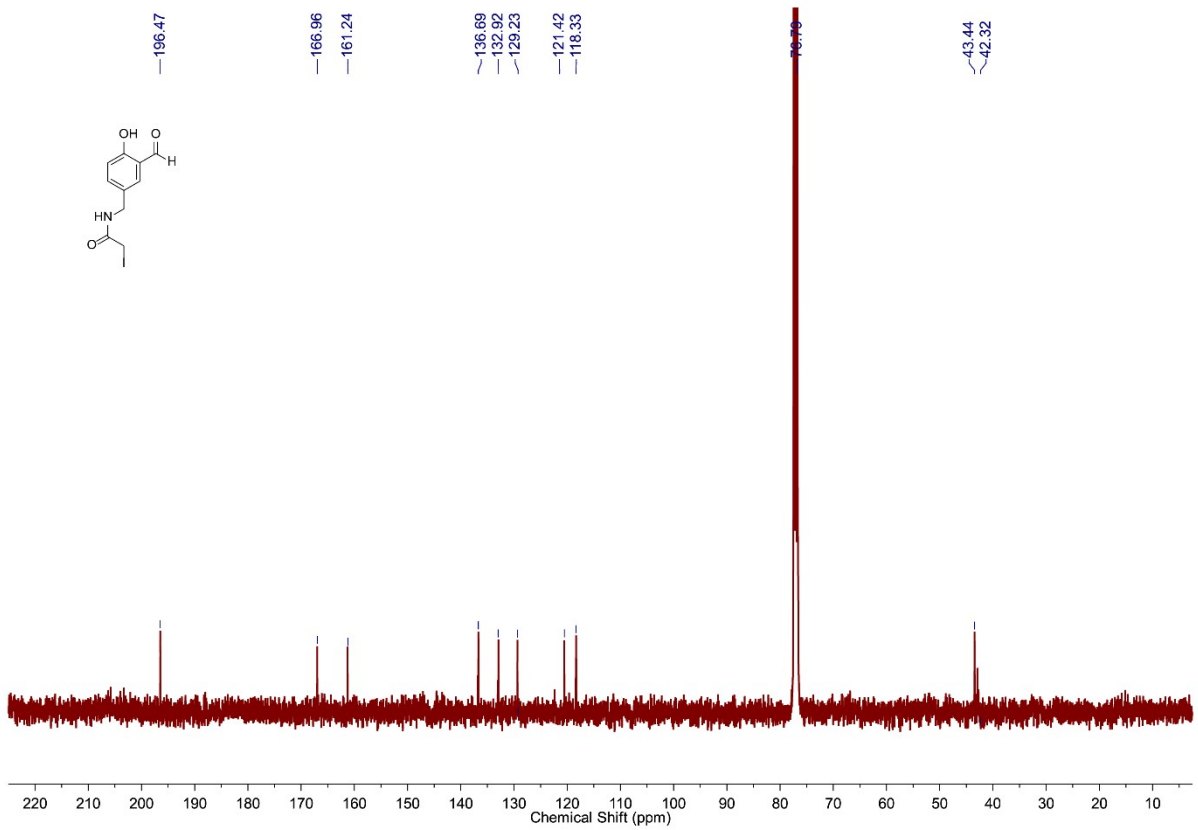


Figure S48. ^{13}C-NMR (101 MHz) spectrum of BKD3 recorded in $CDCl_3$.

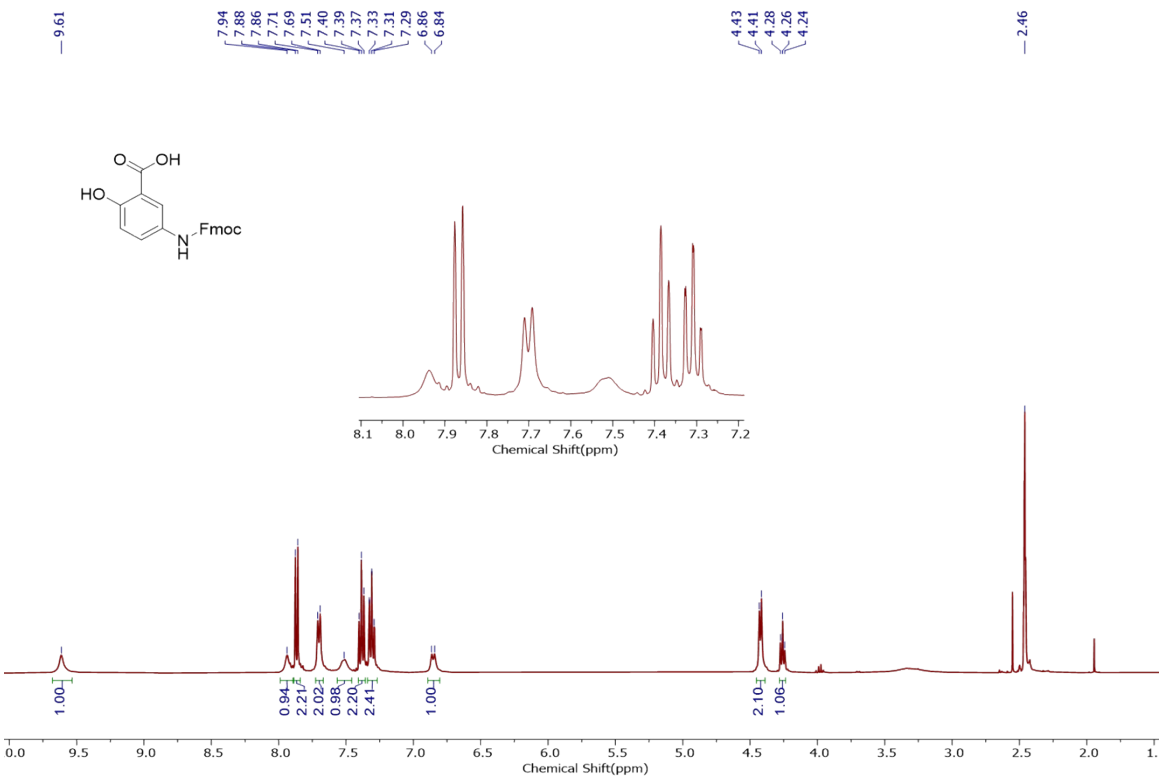


Figure S49. ^{1}H-NMR (400 MHz) spectrum of 3a recorded in DMSO-d_6.

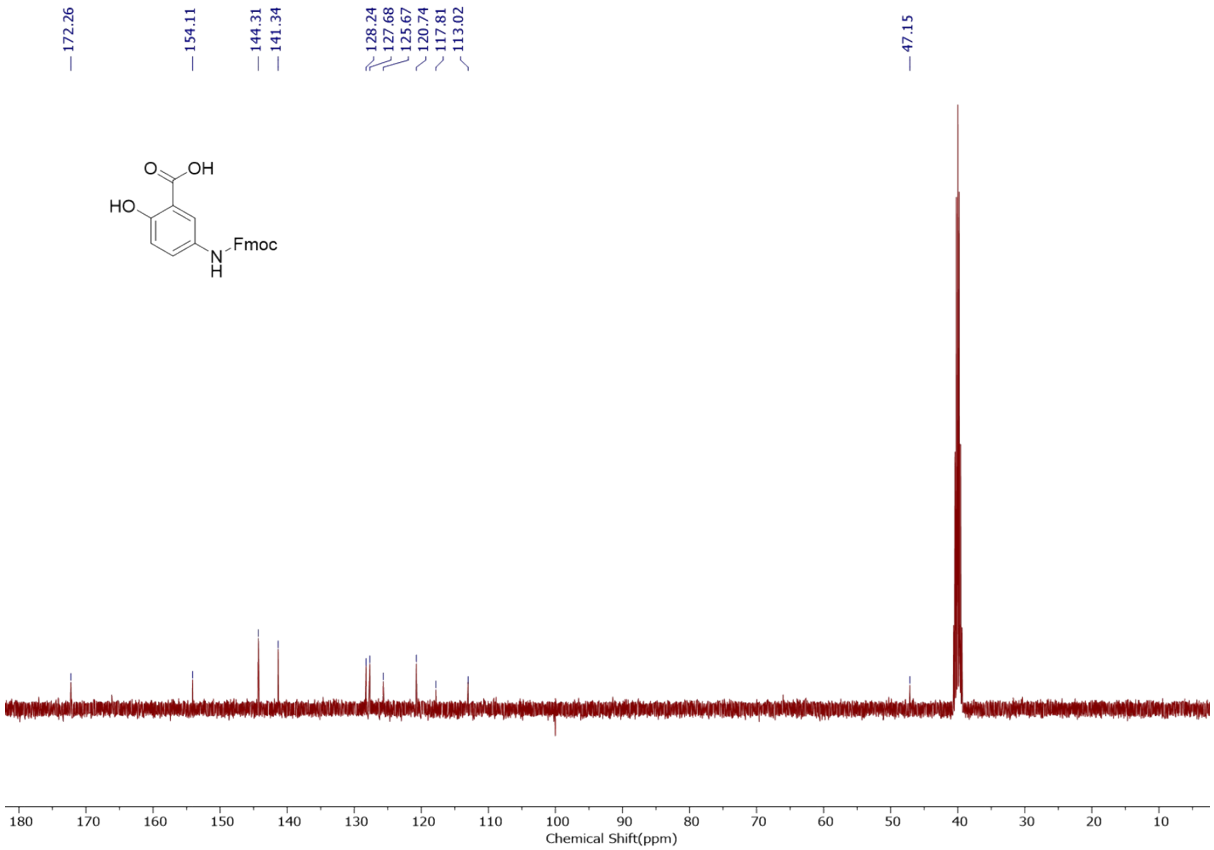


Figure S50. ^{13}C-NMR (101 MHz) spectrum of 3a recorded in DMSO-d_6.

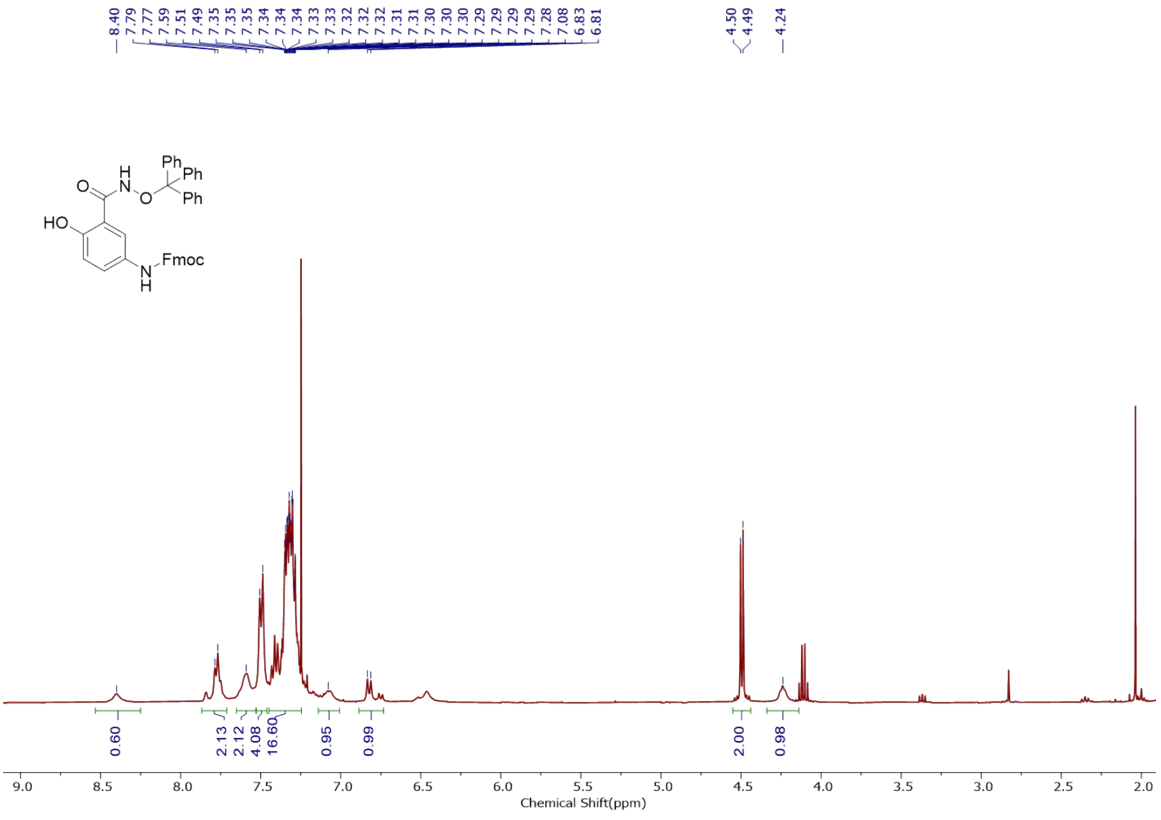


Figure S51. ^{1}H-NMR (400 MHz) spectrum of 3b recorded in $CDCl_3$

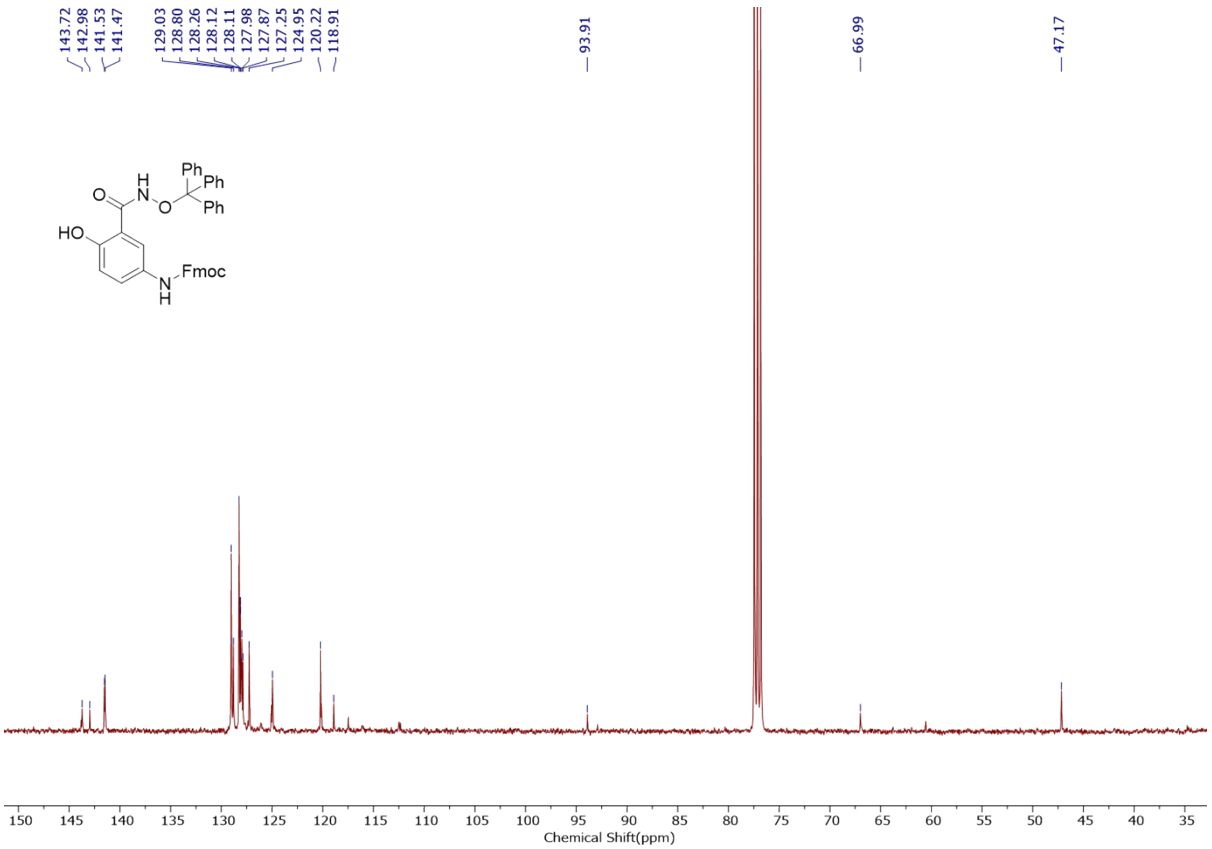


Figure S52. ^{13}C-NMR (101 MHz) spectrum of 3b recorded in $CDCl_3$

XXVII. References

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