Supporting Information

Covalent Targeting of Ras G12C by Rationally Designed Peptidomimetics

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Table S1. Computational alanine scanning for determination of hot-spot residues in the Ras-Sos interaction (PDB 1NVW and 1BKD)[1].

Sos αH Helix (929-944): FFGIYLTNILKTEEGN

Residue	Δ ΔG (kcal/mol) from 1NVW	Δ ΔG (kcal/mol) from 1BKD
F 929	**1.64**	**1.45**
F 930	0.03	0.05
G 931	--	--
I 932	0.07	0.05
Y 933	0.01	--
L 934	0.65	0.65
T 935	**1.11**	**1.59**
R 936	0.28	0.83
I 937	--	--
L 938	0.63	0.69
K 939	0.27	0.44
T 940	-0.13	0.05
E 941	--	--
E 942	**1.10**	0.34
G 943	--	--
N 944	**2.35**	**2.63**

Table S2. PDB codes with accessible cysteines within 8 Å of a high-affinity helix at the PPI

PDB Codes				
1D2Z	1GH6	1JEQ	1N1J	1Q7L
1TUE	1UKV	1VRA	1X3Z	1Y8Q
1ZOQ	2A1J	2DCU	2H6F	2PBI
2QSF	2VE7	2WAX	2XA0	2Y9M
2Y9Y	2ZIV	3A1G	3AA0	3AXJ
3AYH	3BLH	3C98	3DSS	3DXE
3FGR	3IO8	3IF8	3MMY	3MQP
3MV2	3O3M	3RGF	3RO2	3S4W
3SF4	3TIX	3ZHE	4AXG	4C59
4C9B	4CC9	4CCG	4CZZ	4DBP
4DCJ	4E17	4F9C	4GEH	4GG2
4GIZ	4HST	4JPS	4KVM	4NFU

Peptide Characterization

Table S3. Mass spectroscopic characterization of αHBS, α_3βHBS, and control peptides.

Compound	Sequence	L938 Modifications	Calculated $[M+H]^+$	Observed $[M+H]^+$
HBS_{SOS}	XFEG*IYRLELLKAEEAN-NH_2	N/A	1988.0	1988.9
UNC_{SOS}	Ac-FEGIYRLELLKAEEAN-NH_2	N/A	1936.0	1936.4
1	XFEG*IYRLELKKAEEAN-NH_2	L938K	2003.1	2003.6
2a	XFEG*IYRLEL(K)KAEEAN-NH_2	L938K + Acrylamide	2057.1	2057.1
2b	XFEG*IYRLEL(K)KAEEAN-NH_2	L938K + β-Ala + Acrylamide	2128.7	2128.7
3a	XFEG*IYRLEL(K)KAEEAN-NH_2	L938K + VSA1	2164.1	2164.7
3b	XFEG*IYRLEL(K)KAEEAN-NH_2	L938K + VSA2	2178.1	2178.8
4a	XFEG*IYRLEL(K)KAEEAN-NH_2	L938K + VSO1	2135.0	2135.8
4b	XFEG*IYRLEL(K)KAEEAN-NH_2	L938K + VSO2	2149.1	2149.8
α_3βHBS$_{SOS}$	ZFEG*I_βYRLE_βLLKA_βEEAN-NH_2	N/A	2043.1	2043.8
α_3βUNC$_{SOS}$	Ac-FEGI_βYRLE_βLLKA_βEEAN-NH_2	N/A	1977.1	1977.8
α_3βMUT$_{SOS}$	ZAEG*I_βYRLE_βLLKA_βEAAA-NH_2	N/A	1866.1	1866.2
5	ZFEG*I_βYRLE_βLKKA_βEEAN-NH_2	L938K	2059.1	2059.4
6	ZFEG*I_βYRLE_βLK(K)A_βEEAN-NH_2	L938K + VSA2	2233.2	2234.0
7	ZFEG*I_βYRLE_βLK(K)A_βEEAN-NH_2	L938K + EBA	2235.2	2235.4
8	ZAEG*I_βYRLE_βLK(K)A_βEAAA-NH_2	L938K + VSA2	2057.1	2057.6
Flu-αHBS	XFEG*IYRLELLKAEEANK(Flu)-NH_2	N/A	2519.2	2519.8
Flu-α_3βHBS	ZFEG*I_βYRLE_βLLKA_βEEANK(Flu)-NH_2	N/A	2560.2	2560.4

Residues in blue font depict β^3-residues; G* denotes bridged *N*-allylglycine residue; X = 4-pentenoic acid; Z = 5-hexenoic acid residue; Flu is 5-carboxyfluorescein linked via an amide bond to Lys side chain.

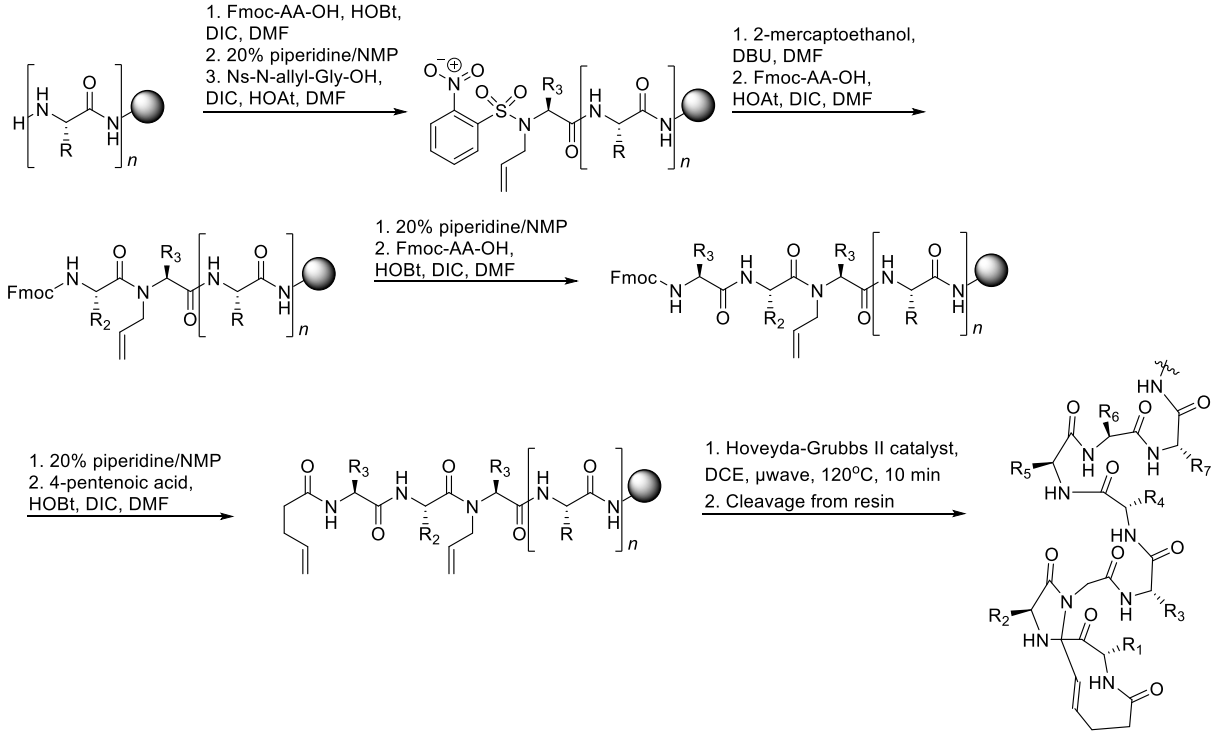
Supporting Methods

General Information

All reagents were obtained from commercial suppliers and used without further purification unless otherwise noted. All solvents were purchased anhydrous and used without further purification. Fmoc α-amino acids, Fmoc β^3-amino acids, and peptide synthesis reagents were purchased from Novabiochem and Chem-Impex International. Hoveyda-Grubbs 2nd Generation catalyst and molecular biology grade salts and buffers were obtained from Sigma. HisPur Ni-NTA resin was acquired from Thermofisher Scientific. Peptides were synthesized manually or on a Gyros Technologies Prelude X batch peptide synthesizer. Completed peptides were purified using reverse-phase high-performance liquid chromatography (RP-HPLC) on C_{18} columns with a Thermofisher Scientific Ultimate 3000 HPLC with a diode array detector. Purity checks were conducted with an Agilent 1260 Infinity series RP-HPLC with a diode array detector on a C_{18} analytical column. High-resolution mass spectroscopy was performed on an Agilent 1260 Infinity High-Performance Liquid Chromatography (HPLC) and a Bruker UltrafleXtreme MALDI-TOF (matrix-assisted laser assisted desorption/ionization time-of-flight).

Peptide Synthesis and Characterization

Synthesis of linear and HBS peptides



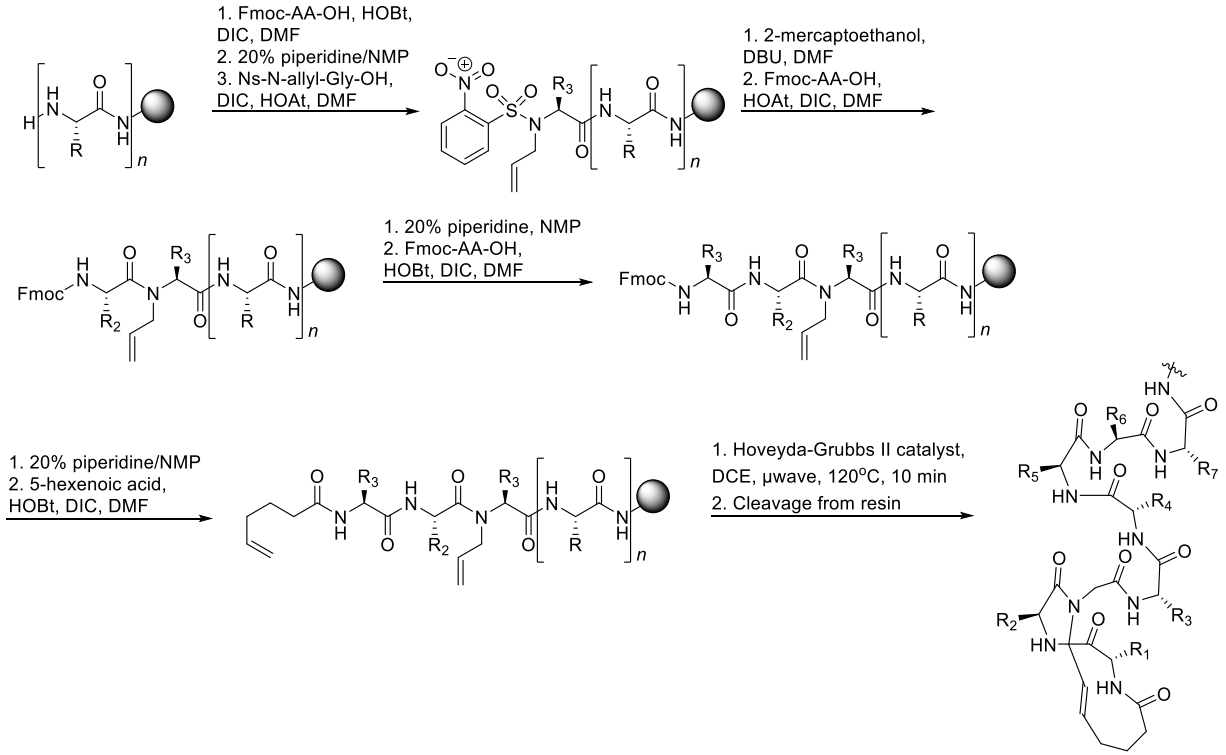
Scheme S1. General synthetic scheme of linear and HBS peptides.

HBS synthesis was conducted as reported in previous studies.[1] The compounds are synthesized with standard Fmoc solid-phase peptide synthesis on Knorr Rink Amide resin (Novabiochem). Unless otherwise specified, amino acids were coupled using Fmoc-AA-OH (5 eq.), 1-hydroxybenzotriazole (HOBt, 5 eq.), and diisopropylcarbodiimide (DIC, 5 eq.) in dimethylformamide (DMF) up to the *i*+3rd residue of the putative helix. Nosyl-*N*-allyl-Gly-OH (G*, 3 eq.) can be readily synthesized in solution[2] and is coupled overnight with DIC (3 eq.) and 1-hydroxy-7-azabenzotriazole (HOAt, 3 eq.). The 2-nitrobenzenesulfonamide (Nosyl or Ns) protecting group is removed with three 30 minute and one 60 minute sessions using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 10 eq.) and 2-mercaptoethanol (10 eq.) in DMF. The resulting secondary amine is coupled onto the next amino acid (Fmoc-AA-OH, 10 eq.) overnight with DIC (10 eq.) and HOAt (10 eq.). The peptide is further

elongated and finished with the placement of 4-pentenoic acid at the N-terminus of the peptide via normal coupling conditions (5 eq. DIC, 5 eq. HOBt). The resin is dried overnight by placement in a dessicator or under vacuum. After flushed with nitrogen, ring-closing metathesis (RCM) is performed with the ruthenium-based Hoveyda-Grubbs 2nd Generation catalyst (20 mol%) in 1,2-dichloroethane (DCE) under microwave irradiation at 120°C for 10 minutes as previously described[1-6]. Resin was washed with DMF (3x), dichloromethane (DCM, 3x), and methanol (3x) before and after each coupling. Coupling reactions were also monitored by either ninhydrin-based Kaiser or chloranil tests. Upon completion, peptides were cleaved from resin using a mixture of trifluoroacetic acid (TFA)/thioanisole/anisole/1,2-ethanedithiol (90%/5%/3%/2% v/v) or another suitable cleavage cocktail depending on the amino acid sequence. The cleavage solution is filtered with subsequent removal of solvent by rotary evaporation. Then, the peptide is precipitated, washed (3x) with cold diethyl ether, filtered, and dissolved in a mixture of water:acetonitrile. The peptide is finally purified via RP-HPLC on a 5-95% acetonitrile gradient in water with 0.1% TFA on a C_{18} column and characterized by MALDI-TOF spectroscopy.

The unconstrained peptides were synthesized with a similar protocol as the HBS without the addition of the *N*-allylglycine and 4-pentenoic acid. Instead, the completed peptide undergoes a final Fmoc deprotection and protected with an acetyl group at the N-terminus using a solution of 0.5 M acetic anhydride and 5% diisopropylethylamine (DIEA) in NMP for 30 minutes prior to cleavage from resin.

Synthesis of $\alpha_3\beta$HBS peptides



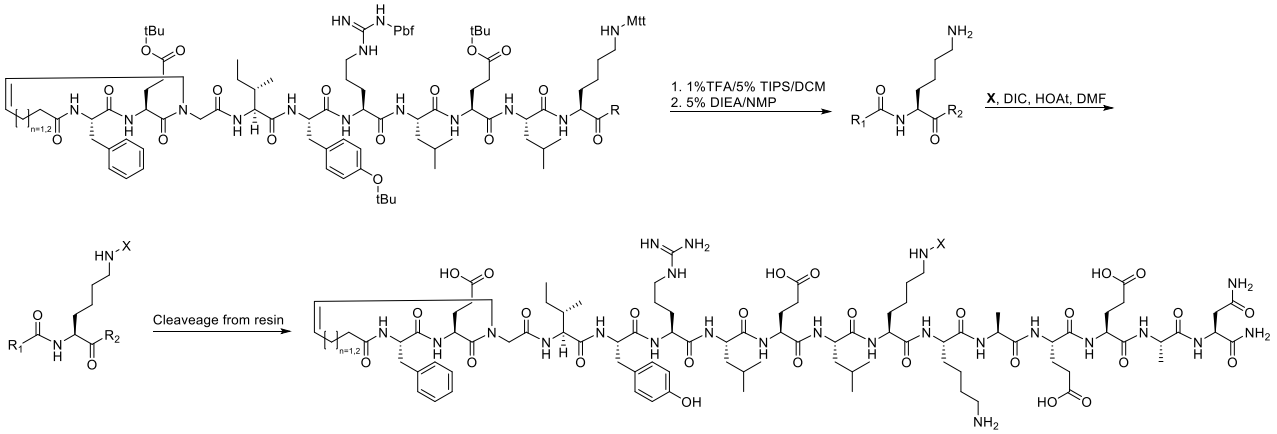
Scheme S2. General synthetic scheme of linear and $\alpha_3\beta$HBS peptides.

$\alpha_3\beta$HBS synthesis was performed under a similar protocol to that of αHBS peptides. Briefly, the peptides are coupled via standard Fmoc solid-phase peptide synthesis up to the *i*+5th residue of the putative helix. β^3-residues are coupled manually with the appropriate Fmoc-β^3-AA-OH (3 eq.), DIC (3 eq.), and HOAt (3 eq.) in DMF overnight at room temperature. Nosyl-*N*-allylglycine (G*) is coupled and subsequently deprotected as previously described. The peptide is further elongated and finished with the installment of 5-hexenoic acid at the N-terminus of the peptide under normal coupling conditions. As previously described, the HBS macrocycle is completed with RCM and thoroughly washed before proceeding to the next appropriate step. The peptides are cleaved from resin

before being purified via RP-HPLC on a acetonitrile/water gradient (0.1% TFA v/v) on a C_{18} column and characterized by MALDI-TOF spectroscopy.

The unconstrained version of the $\alpha_3\beta$ peptides were synthesized with a similar protocol to the HBS peptides without the addition of the *N*-allylglycine and 5-hexenoic acid. Instead, the completed peptide undergoes a final Fmoc deprotection and protected with an acetyl group at the N-terminus using a solution of 0.5 M acetic anhydride and 5% DIEA (v/v) in NMP for 30 minutes prior to cleavage from resin.

Synthesis of covalent peptidomimetics



Scheme S3. General synthetic scheme of covalent HBS and $\alpha_3\beta$HBS peptidomimetics.

During peptide synthesis, amino acids bearing orthogonal protecting groups were incorporated at appropriate positions within the sequence. For covalent peptide designs peptides, Fmoc-Lys(Mtt)-OH was primarily used. Upon completion of the sequence (linear) or post-RCM (HBS), they are subjected to selective deprotection of the 4-methyltrityl group with a solution of TFA/triisopropylsilane (TIPS)/DCM (1%/5%/94%) 6x for 10 minutes each. The peptides are then washed with DIEA (2x) to neutralize the resulting trifluoroacetate salt. The Michael acceptor is installed by coupling the electrophile (5 eq.) with DIC (5 eq.) and HOAt (10 eq.) in DMF for 4-6 hours at room temperature.

For acrylamide-based covalent peptidomimetics, acrylic acid (5 eq.) is coupled onto the deprotected amine of the sequence with DIC (5 eq.) and HOAt (10 eq.) in DMF overnight at room temperature. The peptides were cleaved from resin, precipitated, filtered, and purified according to the aforementioned protocol.

For vinyl sulfonamide-based covalent peptidomimetics, **VSA1-2** (5 eq.) are coupled onto the deprotected amine of the sequence with DIC (5 eq.) and HOAt (10 eq.) in DMF overnight at room temperature. The peptides were cleaved from resin, precipitated, filtered, and purified according to the aforementioned protocol.

For vinyl sulfone-based covalent peptidomimetics, **VSO1-2** (5 eq.) are coupled onto the deprotected amine of the sequence with DIC (5 eq.) and HOAt (10 eq.) in DMF overnight at room temperature. The peptides were cleaved from resin, precipitated, filtered, and purified according to the aforementioned protocol.

Synthesis of fluorescein-labeled peptides

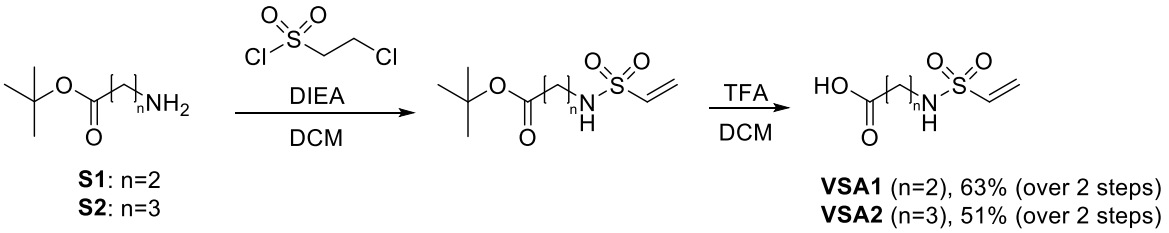
The indicated peptide sequences were synthesized as previously described with a C-terminal Mtt-protected Lys residue. Upon Mtt deprotection, the resin was treated with fluorescein isothiocyanate (5/6-FITC, 3 eq.) and DIEA (3 eq.) in DMF overnight. The fluorescein-conjugated peptides were then cleaved from resin, purified via reverse-phase HPLC, and characterized as described previously.

Synthetic Procedures and Characterization

General Information

All reagents were obtained from commercial suppliers and used without further purification unless otherwise noted. All solvents were purchased anhydrous and used without further purification. Unless otherwise specified, all reactions were conducted under dry conditions. Silica gel flash chromatography was performed manually using self-packed columns under air pressure. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy data were collected at 298 K on a Bruker AVANCE 400 MHz spectrometer with chemical shifts reported in parts per million relative to $CDCl_3$ (^{1}H NMR; 7.26 ppm, ^{13}C NMR; 77.36 ppm) and d_6-DMSO (^{1}H NMR; 2.54 ppm, ^{13}C NMR; 40.45 ppm). High-resolution mass spectroscopy was performed on an Agilent 1260 Infinity High-Performance Liquid Chromatography (HPLC) and a Bruker UltrafleXtreme MALDI-TOF.

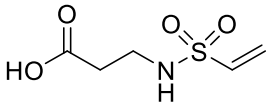
General Procedure A. Vinyl sulfonamide-based covalent warhead synthesis



Scheme S4. General synthetic scheme of vinyl sulfonamide-based covalent warheads.

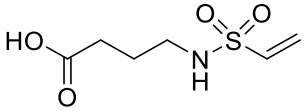
Precursors **S1** or **S2** (5.5 mmol, 1.1 eq.) and triethylamine (3.07 mL, 22 mmol, 4.4 eq.) were dissolved in 10 mL DCM and cooled to 0°C in an ice-water bath. 2-chloroethanesulfonyl chloride (522.5 µL, 5 mmol) was dissolved in 10 mL DCM and added dropwise via dropping funnel to the stirred solution over the course of one hour. The resulting solution was stirred for an additional 2 hours at 0°C before being allowed to warm to room temperature and stirred overnight. The reaction was concentrated *in vacuo* and purified via flash column chromatography (5% MeOH/DCM). The relevant fractions were collected, concentrated *in vacuo* to produce a yellow oil, and proceeded to the next step without further purification or characterization. The residue was then dissolved in a solution of 1:1 trifluoroacetic acid and DCM (20 mL total) and stirred for 4 hours at room temperature. The solution was concentrated *in vacuo* and dried overnight for final quantification and characterization.

3-(vinylsulfonamido)propanoic acid (VSA1)



General procedure A was followed with β-alanine tert-butyl ester hydrochloride salt (**S1**, 5.5 mmol, 1.1 eq.) as the starting material to afford **VSA1** (622 mg, 63% over two steps). ^{1}H NMR (400 MHz, *d6*-DMSO) δ 12.18 (b, 1H, -COOH), 7.31 (b, 1H, -NH-SO2), 6.68 (dd, 1H, -CH2=, 16 Hz, 12 Hz), 5.95-6.04 (m, 2H, =CH2), 2.99-3.04 (m, 2H, -CH2-NH), 2.41 (t, 2H, O2C-CH2-, 8 Hz). ^{13}C NMR (400 MHz, *d6*-DMSO) δ 172.2, 136.5, 125.5, 38.2, 34.3. MS (ESI) calculated for $C_5H_8NO_4S$ $[M+H]^+$: 180.0, found: 180.2.

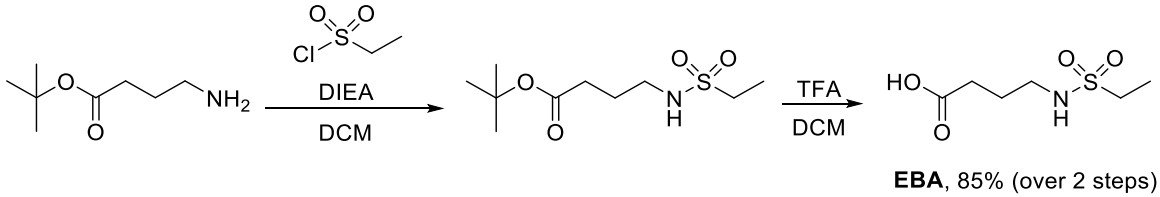
4-(vinylsulfonamido)butanoic acid (VSA2)



General procedure A was followed with 4-aminobutanoate tert-butyl ester hydrochloride salt (**S2**, 5.5 mmol, 1.1 eq.) as the starting material to afford **VSA2** (541 mg, 51% over two steps). ^{1}H NMR (400 MHz, *d6*-DMSO) δ 12.01 (b, 1H, -COOH), 7.26 (b, 1H, -NH-SO2), 6.68 (dd, 1H, -CH=, 16 Hz, 12 Hz), 5.94-6.02 (m, 2H, =CH2), 2.80-2.85 (m, 2H, -CH2-NH), 2.25 (t, 2H, HOOC-CH2-, 6 Hz), 1.68 (m, 2H, CH2-CH2-CH2).^{13}C NMR (400

MHz, *d6*-DMSO) δ 173.8, 136.7, 125.2, 41.5, 30.5, 24.6. MS (ESI) calculated for $C_6H_{11}NO_4S$ $[M+H]^+$: 194.0, found: 194.1.

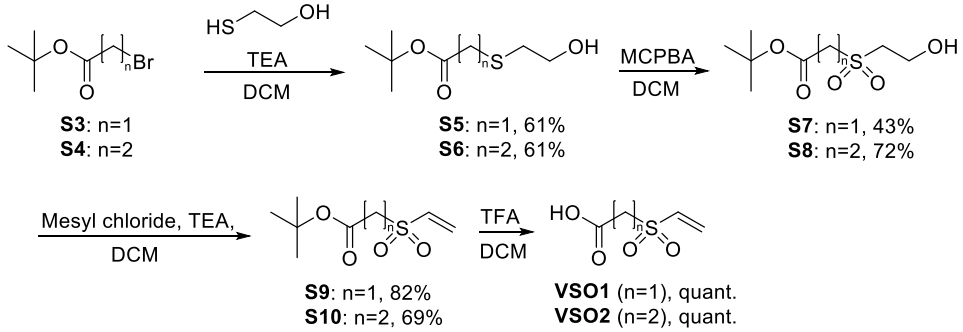
4-(ethylsulfonamido)butanoic acid (EBA)



Scheme S5. Synthetic scheme of **EBA**.

4-aminobutanoate tert-butyl ester hydrochloride salt (5.5 mmol, 1.1 eq.) and triethylamine (1.46 mL, 10.5 mmol, 2.1 eq.) were dissolved in 10 mL DCM and cooled to 0°C in an ice-water bath. Ethanesulfonyl chloride (473.8 µL, 5 mmol) was dissolved in 10 mL DCM and added dropwise via dropping funnel to the stirred solution over the course of one hour. The resulting solution was stirred for an additional 2 hours at 0°C before being allowed to warm to room temperature and stirred overnight. The reaction was concentrated *in vacuo* and purified via flash column chromatography (5% MeOH/DCM). The relevant fractions were collected, concentrated *in vacuo* to produce a yellow oil, and proceeded to the next step without further purification or characterization. The residue was then dissolved in a solution of 1:1 trifluoroacetic acid and DCM (20 mL total) and stirred for 4 hours at room temperature. The solution was concentrated *in vacuo* and dried overnight to afford **EBA** (842.3 mg, 85% over two steps). ^{1}H NMR (400 MHz, *d6*-DMSO) δ 12.06 (b, 1H, -OH), 7.02 (b, 1H, -NH-SO2), 2.89-2.99 (m, 4H, -CH2-NH, O2S-CH2-), 2.26 (t, 2H, HOOC-CH2-, 7.2 Hz), 1.63-1.67 (m, 2H, CH2-CH2-CH2), 1.17 (t, 3H, -CH3, 7.6 Hz). ^{13}C NMR (400 MHz, *d6*-DMSO) δ 173.9, 45.0, 41.5, 30.5, 25.0, 8.0. MS (ESI) calculated for $C_6H_{11}NO_4S$ $[M+H]^+$: 196.1, found: 196.2.

General Procedure B. Vinyl sulfone-based covalent warhead synthesis



Scheme S6. General synthetic scheme of vinyl sulfone-based covalent warheads.

Step 1

Precursors **S3** (10.25 mmol) or **S4** (9.566 mmol) and triethylamine (2.1 eq.) were dissolved in 20 mL DCM and cooled to 0°C in an ice-water bath. 2-mercaptoethanol (1.1 eq.) was added dropwise to the cooled solution. The reaction was then allowed to stir overnight at room temperature and monitored by thin-layer chromatography (TLC). The mixture was filtered and concentrated *in vacuo*. The residue was purified via flash column chromatography (1:1 hexane:ethyl acetate) to afford compounds **S5** (1.21 g, 61%) and **S6** (1.2 g, 61%) as a yellow oil. **S5**: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 3.76 (t, 2H, -CH2-OH, 6Hz), 3.18 (s, 2H, -CH2-S), 2.82 (t, 2H, S-CH2-, 4Hz), 2.52 (b, 1H, -OH), 1.47 (s, 9H, -CH3). MS (ESI) calculated for $C_8H_{16}O_3S$ $[M+H]$: 193.1, found: 192.9. **S6**: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 3.75 (t, 2H, -CH2-OH, 6Hz), 2.73-2.79 (m, 4H, O2C-CH2-, S-CH2-), 2.53 (t, 2H, -CH2-S, 6Hz), 2.25 (s, 1H, -OH), 1.46 (s, 9H, -CH3). MS (ESI) calculated for $C_9H_{17}O_3S$ $[M+H]^+$: 207.1, found: 207.2.

Step 2

Meta-chloroperoxybenzoic acid (3 eq.) was dissolved in 10 mL DCM and cooled to 0°C in an ice-water bath. **S5** (6.3 mmol) or **S6** (5.8 mmol) was dissolved in 10 mL of DCM and added to the cooled solution. The resulting mixture was stirred at 0°C for one hour until the formation of a white precipitate. The reaction was then allowed to warm to room temperature and stirred for an additional 16 hours. The reaction was filtered and concentrated *in vacuo*. The residue was purified via flash column chromatography (1:1 hexane:EtOAc) to afford compounds **S7** (600 mg, 43%) and **S8** (721 mg, 72%) as a white solid. **S7**: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 4.16 (t, 2H, -CH2-OH, 6 Hz), 4.05 (s, 2H, -CH2-SO2), 3.51 (t, 2H, O2S-CH2-, 4 Hz), 1.52 (s, 9H, -CH3). MS (ESI) calculated for $C_8H_{16}O_5S$ [M+H]: 225.1, found: 225.0. **S8**: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 4.13 (t, 2H, -CH2-OH, 6 Hz), 3.41 (t, 2H, -CH2-SO2, 4 Hz), 3.25 (t, O2S-CH2-, 4 Hz), 2.81 (t, 2H, O2C-CH2-, 8 Hz), 1.46 (s, 9H, -CH3). MS (ESI) calculated for $C_9H_{17}O_5S$ $[M+H]^+$: 239.1, found: 239.4.

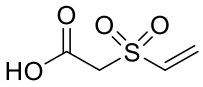
Step 3

S**7** (2.68 mmol) or **S8** (4.2 mmol) was dissolved in 25 mL of DCM and cooled to 0°C in an ice-water bath. A solution of triethylamine (3 eq.) and mesyl chloride (1.5 eq.) in 10 mL of DCM was added slowly to the cooled reaction mixture. The reaction was then allowed to warm to room temperature and stirred overnight. The mixture was concentrated *in vacuo* and purified via flash column chromatography (1:1 hexane:EtOAc) to afford compounds **S9** (455 mg, 82%) and **S10** (634 mg, 69%). **S9**: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 6.88 (dd, 1H, -CH=, 16 Hz, 8 Hz), 6.49 (d, 1H, =CH2, 16 Hz), 6.21 (d, 1H, =CH2, 8 Hz), 3.92 (s, 2H, O2C-CH2-), 1.50 (s, 9H, -CH3). MS (ESI) calculated for $C_8H_{14}O_4S$ [M+H]: 207.1, found: 207.5. **S10**: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 6.63 (dd, 1H, -CH=, 16 Hz, 8 Hz), 6.44 (d, 1H, =CH2, 16 Hz), 6.17 (d, 1H, =CH2, 8 Hz), 3.28 (t, 2H, -CH2-SO2, 8 Hz), 2.70 (t, 2H, O2C-CH2-, 6 Hz), 1.44 (s, 9H, -CH3). MS (ESI) calculated for $C_9H_{16}O_4S$ $[M+H]^+$: 221.1, found: 221.3.

Step 4

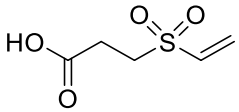
S**9** (2.19 mmol) or **S10** (2.88 mmol) was dissolved in a solution of 1:1 trifluoroacetic acid and DCM (20 mL total) and stirred for 4 hours at room temperature. The solution was concentrated *in vacuo* and dried overnight to produce **VSO1** (329 mg, quant.) and **VSO2** (531 mg, quant.) as an off-white solid for final quantification and characterization.

2-(vinylsulfonyl)acetic acid (VSO1)



General procedure B was followed with t-butyl bromoacetate (**S3**, 10.25 mmol) as the starting material in step 1 to afford **VSO1**. ^{1}H NMR (400 MHz, *d6*-DMSO) δ 13.35 (b, 1H, -COOH), 7.00-7.07 (dd, 1H, -CH=, 16 Hz, 12 Hz), 6.26 (m, 2H, =CH2), 4.29 (s, 2H, -CH2-S). ^{13}C NMR (400 MHz, *d6*-DMSO) δ 163.9, 136.9, 129.6, 58.3. MS (ESI) calculated for $C_4H_6O_4S$ $[M+H]^+$: 151.0, found: 151.2.

3-(vinylsulfonyl)propanoic acid (VSO2)



General procedure B was followed with tert-butyl 3-bromopropionate (**S4**, 9.57 mmol) as the starting material in step 1 to afford **VSO2**. ^{1}H NMR (400 MHz, *d6*-DMSO) δ 12.47 (b, 1H, -COOH), 6.865 (dd, 1H, -CH2=, 16 Hz, 12 Hz), 6.14 (m, 2H, =CH2), 3.23 (t, 2H, -CH2-S, 6 Hz), 2.47 (t, 2H, -CH2-CH2, 8 Hz). ^{13}C NMR (400 MHz, *d6*-DMSO) δ 171.2, 136.2, 130.0, 48.5, 27.0. MS (ESI) calculated for $C_5H_8O_4S$ $[M+H]^+$: 165.0, found: 165.2.

Biochemical Procedures and Characterization

Protein Purification

His_6-H-Ras (residues 1-166), His_6-H-Ras(G12C) (residues 1-166), and His_6-Sos^{Cat} (residues 550-1050) in pProEx HTb expression vector and Glutathione S-transferase (GST)-tagged Ras binding domain (RBD) of Raf were expressed in *Escherichia coli* (BL21). Cells were grown at 37°C to an absorbance of 0.7 at 600 nm. Protein expression was induced with 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 hours at 30°C or overnight at 18°C. The bacterial cells were pelleted via centrifugation at 4500 rpm for 25 minutes. Bacterial pellets were resuspended in a lysis buffer containing 20 mM Tris pH 7.4, 300 mM NaCl, 2 mM 2-mercaptoethanol, and a cOmplete, EDTA-free protease inhibitor cocktail (Roche). For Ras purification, the lysis, elution and dialysis buffers were also supplemented with 2.5 mM $MgCl_2$. The resuspended pellets were sonicated using a Branson Cell Disrupter 200. Clarified lysates were formed upon centrifugation at 13,000 rpm for 20 minutes at 4°C and incubated with charged Ni-NTA resin (Invitrogen) at 4°C for 1 hour. The resin beads were washed five times with resuspension buffer containing 5 mM imidazole. The His_6-tagged proteins were eluted via gravity flow with buffer containing 200 mM imidazole in buffer containing 20 mM Tris pH 7.4, and 300 mM NaCl. Eluted proteins were dialyzed twice against buffer containing 20 mM Tris pH 7.4, 300 mM NaCl, and 2.5 mM $MgCl_2$ for H-Ras and 20 mM Tris pH 7.4, 300 mM NaCl for Sos^{Cat}. The eluted proteins were concentrated with 3000 (H-Ras) and 10,000 (Sos^{Cat}) Da molecular weight cutoff Amicon centrifugal columns (Millipore) in dialysis buffer containing 10% glycerol (v/v) before snap-frozen in liquid N_2 and stored at -80°C until further use.

2D NMR Spectroscopy

Experiments were performed on a Bruker AVANCE 600 MHz spectrometer equipped with a ZXI probe and 3D gradient control. Samples were prepared by dissolving 1 mg of peptide in 400 μL of 10% acetonitrile-*d*3 in deionized water titrated to pH 4.0 with trifluoroacetic acid. The 2D TOCSY spectra were employed to discern the chemical shifts of the amide protons and side chains. Solvent suppression was achieved with a 3919 Watergate pulse sequence. All 2D spectra were recorded at 25°C by collecting 10240 complete data points in the *t*2 domain by averaging 40 scans and 512 increments in the *t*1 domain with the States-TPPI mode. All TOCSY experiments are performed with a mixing time of 80 ms, and NOESY with the mixing time of 200 ms. The data were processed and analyzed using the Bruker TOPSPIN program. The original free induction decays (FIDs) were zero-filled to give the final matrix of 5120 by 5120 real data points. A 90° sine-square window function was applied in both dimensions.

Structure Calculations

The solution structure of the peptide was computed using a Monte Carlo-based conformational search and energy minimization protocol (Macromodel). The force field was applied to a helical starting conformation. The 20 lowest energy structures from different starting conformations show minimal overall deviation. The NOE restraints were categorized into three groups: strong (2.5 Å upper limit), medium (4.0 Å upper limit), and weak (5.5 Å upper limit). The $^3J_{NH\text{-}CHa}$ coupling constants for all residues except G3 (due to the lack of an amide hydrogen) were used to calculate φ angles by applying the Pardi parameterized Karplus equation.

Circular Dichroism

CD experiments were conducted on an AVIV Stopped-Flow Circular Dichroism 202SF spectrometer equipped with a temperature controller using 1 mm length cells and a scan speed of 1.0 nm/min at 298 K. The generated spectra were averaged over 10 scans with baseline subtraction. The samples were prepared in a buffer containing 1 mM Tris and 15 mM NaCl to a final peptide concentration of 50 μM.

Proteolytic Stability - Trypsin

Solutions were prepared containing 300 μM peptide, 73 μM tryptophan, and 1 ng/μL trypsin in 100 mM Tris pH 8.3 buffer and incubated at room temperature. At specific timepoints (15, 30, 45, 60, 90 min, 2 h, 4 h, 6 h, 9 h, 12 h, 24 h, 48 h, and 96 h), 11 μL trypsin solution was removed and immediately quenched in 20 μL of 2% TFA

(aqueous) solution. Quenched solutions were immediate frozen at -20°C. Immediately after thawing, the quenched samples were analyzed on reverse-phase analytical HPLC on a C_{18} column and compared undegraded peptide to the internal standard (tryptophan).[6] Generated curves for all proteolytic stability assays are the averaged data for biological triplicates.

Proteolytic Stability - Serum

Peptides were dissolved in 20 mM Tris pH 8.3, 300 mM NaCl to a concentration of 400 μM. Peptide solutions and 50% human serum in RPMI-1640 media were incubated separately in a 37°C water bath for 15 minutes. The peptide solutions (450 μL) were added to 50% human serum solution (450 μL, final serum concentration of 25%) and incubated at 37°C for 24 hours. At 0, 2, 5, 10, and 24 h, three 50 μL aliquots were removed and immediately quenched with 100 μL of 96% ethanol-containing tryptophan (83 μM final). Quenched samples were cooled to 4°C for 30 minutes and subsequently centrifuged (14,000 rpm for 5 min). The supernatant (110 μL) was removed, diluted with water (55 μL), and frozen at -80°C. Immediately after thawing, quenched samples were run on reverse-phase analytical HPLC on a C_{18} column and compared undegraded peptide to the internal standard (tryptophan).

Proteolytic Stability – Proteinase K

The procedure for proteinase K-based degradation assays was identical to that used for trypsin, except solutions were prepared to a final concentration of 1 ng/μL of proteinase K.

Fluorescence Polarization Binding Assay

The relative binding affinities of peptides for N-terminal His_6-tagged H-Ras (1-166) were determined using direct fluorescence polarization assays with fluorescein-tagged HBS peptides (Flu-αHBS, Flu-$\alpha_3\beta$HBS). These experiments were conducted with a DTX 880 Multimode Detector (Beckman) at 25°C with excitation and emission wavelengths set to 485 and 535 nm, respectively. The addition of increasing concentrations (0-500 μM) of His_6-H-Ras to a 15 nM solution of fluorescein-labeled HBS peptides in 20 mM Tris, 300 mM NaCl, 1 mM $MgCl_2$, 0.1% pluronic F68, pH = 7.4 was carried out in black, U-bottom 96-well plates (Brand). The generated binding affinity (K_D) values for each peptide are from biological duplicate studies and were determined via fitting to a sigmoidal dose-response nonlinear regression model on Graphpad Prism 6.

$$K_{D1} = (R_T*(1-F_{SB}) + L_{ST}*F_{SB}^2)/F_{SB}-L_{ST} \text{ (SJ 1)}$$

where:

R_T = Total concentration of H-Ras (1-166)

L_{ST} = Total concentration of fluorescence peptide

F_{SB} = Fraction of bound fluorescence peptide

Heteronuclear Single Quantum Spectroscopy (HSQC)

The purification procedure is nearly identical to the previously described protocol with a couple exceptions. The *E. coli* (BL21) cells containing the His_6-H-Ras construct were grown at 37°C in minimal M9 media supplemented with $^{15}NH_4Cl$ as the sole nitrogen source.[5] Protein expression was induced with 1 mM IPTG at O.D. 1.0 overnight at 16°C. Protein purification and subsequent concentration were performed as described above. The His_6-tag was cleaved off upon incubation with recombinant His_6-tagged Tobacco Etch Virus (TEV) protease (Invitrogen) overnight at 4°C according to the manufacturer's protocol. The resulting protein mixture was loaded onto a charged Ni-NTA column (Invitrogen), and the tag-less protein was collected in the flow-through fraction. Uniformly ^{15}N-labeled H-Ras underwent buffer exchange into PBS, concentrated with an Amicon Ultra centrifugal filter (Millipore), and supplemented with 10% D_2O prior to analysis. Data collection was conducted on a 900 MHz Bruker 4-channel NMR system equipped with a cryoprobe at 30°C and analyzed by the BioSpin software (Bruker). For peptide titration experiments, 0.2, 0.5, 1, 2, 5, and 10 equivalents of $\alpha_3\beta$HBS were dissolved in d6-DMSO and incubated with ^{15}N-H-Ras (21 μM).

Intact Protein Mass Spectrometry

H-Ras (1-166) G12C sequence: H-Ras in black, His_6-TEV in red, available cysteines in bold

Molecular Weight (Da): 22270.92

MSYYHHHHHH DYDIPTTENL YFQGAMGS MTEYKLVVVG A**C**GVGKSALT IQLIQNHFVD EYDPTIEDSY RKQVVIDGET **C**LLDILDTAG QEEYSAMRDQ YMRTGEGFL**C** VFAINNTKSF EDIHQYREQI KRVKDSDDVP MVLVGNK**C**DL AARTVESRQA QDLARSYGIP YIETSAKTRQ GVEDAFYTLV REIRQH

Experiments were carried out as described unless otherwise noted. His_6-tagged recombinant H-Ras G12C (1-166) was reacted with covalent peptides (50 eq.) in 1X phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, KH_2PO_4, pH 7.55) and 10 mM ethylenediaminetetraacetic acid (EDTA) for 24 hours at room temperature and protected from light. All tested covalent peptidomimetics were initially dissolved in dimethylsulfoxide (DMSO) as concentrated stocks. After the specified reaction time, the unlabeled protein or protein-inhibitor solution was quenched with 2% (v/v) acetic acid, precipitated with the addition of acetonitrile (2:1 v/v final), vortexed, centrifuged, and left at room temperature for 20 minutes. The solution is then centrifuged at 10,000 rpm for 10 minutes before the subsequent removal of supernatant. The residue is washed with water, centrifuged at the same conditions, and repeated once more. After the final round of centrifugation, the pellet is dissolved in a mixture of methanol:chloroform (40:60). 2 µL of this solution is dissolved in an equivalent volume of Super-DHB matrix solution (9:1 gentisic acid:2-hydroxy-5-methoxybenzoic acid). 2 µL of the resultant mixture is then plated onto the MALDI plate. The sample is measured in Linear/Positive mode with a detection threshold of 20-50 kDa. All labeling reactions were analyzed via intact protein MS with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectroscopy (Bruker UltrafleXtreme). For specificity control labeling experiments, the procedure remained the same excepting the removal of EDTA (when indicated) and substitution of the H-Ras G12C mutant with either wild-type H-Ras (1-166) or bovine serum albumin (BSA, Bio-Rad).

In-solution Tryptic Digest Mass Spectrometry

As previously described, His_6-tagged recombinant H-Ras (G12C (1-166) was labeled with the desired covalent peptide (50 eq.) in 1X PBS, supplemented with 10 mM EDTA (v/v), for 24 hours at room temperature and protected from light. The reaction mixture was resolved with one-dimensional SDS-PAGE and stained with Coomassie Blue. The desired protein band was digested using a published in-gel trypsin digestion protocol. [3] In brief, the individual protein gel bands were excised, destained, and dehydrated through treatments of 100 mM NH_4HCO_3/acetonitrile (1:1 v/v) and an additional volume of neat acetonitrile. The dehydrated gel pieces are digested in a solution of trypsin (13 ng/µL in 10 mM NH_4HCO_3/acetonitrile (9:1 v/v)) overnight at 37°C. The digested protein fragments are subjected to MALDI-TOF MS without the need for further extraction.

Nucleotide Association Assay

This experiment was conducted in a similar manner as the nucleotide exchange assay and utilizes the same plate preparation and plate reader. H-Ras G12C was loaded with mant-GDP and purified by size-exclusion chromatography (see above). 10 µL of the prepared protein (1 µM final) in reaction buffer (25 mM Tris, pH 7.5, 50 mM NaCl, and 5 mM EDTA) was added to each well. The first well was reserved for the negative control with only mant-GDP (1 µM final) and protein present. The second well contained mant-GDP, unlabeled GDP (200 µM final), and protein and served as the positive control. Samples were measured in duplicates for each experiment. For the remaining wells, the final mix consisted of protein, mant-GDP, and peptide (titrated in a 2-fold dilution series, 10 points). Fluorescence intensities were collected on a Flexstation 3 Multi-Mode Microplate Reader (360 nm excitation, 440 nm emission, Molecular Devices). The generated curves visualize the averaged data from technical triplicates of two biological replicates. A sigmoidal curve fit was employed to determine the IC_{50} in Graphpad Prism 6 after normalization using the data from the first two wells.

Nucleotide Exchange Assay

This procedure was adapted from a published protocol.[4] The recombinantly expressed H-Ras G12C protein (10 μM) was labeled according to the protocol for intact protein MS overnight and protected from the light. The resulting mixture was incubated with 200 μM mant-GDP (Jena Biosciences) in the presence of 5 mM EDTA. $MgCl_2$ was added to a final concentration of 20 mM after 1 hour of gentle shaking at room temperature. The protein was purified via gravity flow through a NAP-5 column to remove free nucleotide and unreacted peptide. Concentrations of the collected protein was determined by absorbance at 280 nm with a NanoDrop 2000 spectrophotometer. For the exchange assay, 10 μL of the prepared protein (1 μM final) in reaction buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM $MgCl_2$) was added to wells in a black, U-shape 96-well plate (Brand) already filled with reaction buffer. The reaction was initiated upon the final addition of 5 μL Sos (1 μM final) or buffer. The fluorescence was observed for 100 minutes at 10-second intervals on a Flexstation 3 Multi-Mode Microplate Reader (360 nm excitation, 440 nm emission, Molecular Devices). The gathered data was normalized to t = 0 s, converted to a percentage, and plotted as a curve. Dissociation rates (k_{off}) and IC_{50} values were determined using first-order exponential decay fit with Graphpad Prism 6.

$$Y = (Y_0 - \text{Plateau})*e^{-kt} + \text{Plateau}$$

where,

t = time (seconds)

k = rate constant reciprocal to the X-axis units

Flow Cytometry

The indicated cell lines were seeded at 1 x 10^5 cells/well in clear polystyrene 24-well plates (Corning) and incubated overnight. The initial growth media is replaced with serum-free media and incubated for 2 hours at 37°C. Upon aspiration, the cells are incubated in serum-free media containing 0.4% DMSO (v/v) for one hour. The cells were then treated with 1 μM (final) of fluorescein-conjugated peptides in serum-free media for another hour while protected from light. All compounds are dissolved as concentrated stocks in DMSO. Each well was aspirated and treated with 1X trypsin (0.25% trypsin, 2.21 mM EDTA, Corning Cellgro) for 10 minutes at 37°C. After trypsinization, the resulting solution was mixed with cold serum-free media and collected. The samples are centrifuged at 500 rpm for 5 minutes at 4°C. The supernatant was removed, and the cell pellets were resuspended with cold PBS before placed on ice. Each sample was treated with 10% trypan blue (v/v) immediately before analysis by flow cytometry on a Becton Dickinson Accuri flow cytometer. The presented data consists of the median fluorescence intensities for at least 10,000 cells/sample and processed using FlowJo (Tree Star Inc.).

Live Cell Fluorescence Microscopy

The indicated cell lines were seeded at 5 x 10^5 cells/well in poly-D-lysine coated 35 mm plates (MatTek) and incubated overnight. The growth media is aspirated and washed with serum-free media. The cells are then incubated with 1 μM (final) of fluorescein-conjugated peptides dissolved in serum-free media (0.4% DMSO v/v) for 4 hours at 37°C while protected from light. All compounds are dissolved as concentrated stocks in DMSO. After the specified incubation time, each plate was aspirated and treated with Hoechst dye solution (Hoechst 33342, ThermoFisher Scientific) for 10 minutes to stain cell nuclei. The Hoechst dye stock solution (10 mg/mL) was diluted 1:2000 in PBS to form the working mixture. The dye solution was removed, and the plate was gently washed 3x with PBS. DMEM (high glucose, HEPES, no phenol red, 20% FBS) was added to each plate and used as the imaging solution. Fluorescence images were acquired on an Eclipse Ti Fluorescence Microscope (Nikon) equipped with NIS-Elements imaging software and using a 40x objective lens.

Cell Culture

T24 and HeLa cells were maintained in Dulbecco's modified eagle medium (DMEM, Corning Cellgro) supplemented with 10% fetal bovine serum (FBS, Innovative Research), 1X penicillin/streptomycin (EMD Millipore), and 10 mM HEPES buffer (Sigma-Aldrich). H358 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 1X penicillin/streptomycin, 10 mM HEPES buffer, and 1X sodium pyruvate (Sigma-Aldrich). All cells were kept in a humidified incubator at 37°C and 5% CO_2.

Cell Viability Assay

Cell viability was monitored using the MTT assay (Sigma-Aldrich, M2128), which measures cell proliferation rate and metabolic activity of mitochondria. This colorimetric assay depends on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2, 5-dyphenyltetrazolium bromide (MTT) by cellular dehydrogenase enzymes to its insoluble formazan precipitate, resulting in a purple color. Cells were plated in clear 96 well clear plates at 2000 cells/well and allowed to affix overnight at 37ºC. Each well is aspirated and washed with serum-free media before introducing peptide inhibitors dissolved in serum-free media with 1% DMSO to desired concentrations (90 µL/well). The cells are incubated in the presence of peptide for 4 hours at 37ºC under serum-free conditions. FBS was then added to each well (10% v/v final), and the plate was incubated for an additional 72 hours. MTT reagent solution is composed of Thiazolyl Blue Tetrazolium Bromide dissolved in Dulbecco's Phosphate Buffered Saline (DPBS), pH 7.4 to a concentration of 5 mg/mL and subsequently sterile-filtered into a light-protected container. After the specified incubation times, MTT reagent was added to each well (0.45 mg/mL/well final). The cells are incubated at 37ºC for an additional 4 hours. Upon careful removal of the supernatant, 150 µL DMSO (solubilization solution) is added to each well and mixed to ensure complete solubilization and release of the insoluble purple formazan precipitate into solution. Absorbance values are recorded with a Synergy HT Multi-Detection Microplate Reader (BioTek) at 570 nm. Each plate included a positive death control (cells with 10% DMSO), a negative control (cells with 0.05% DMSO), and a blank (no cells with 0.05% DMSO).

ERK Activation Assay

H358 or HeLa cells were initially grown to confluence, serum-starved for 4 hours, and treated with the indicated peptides dissolved in serum-free media at specified concentrations for 20 hours. The cells were then lysed in buffer containing 25 mM Tris-HCl (pH 7.4), 120 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 mg/mL pepstatin, 50 mM NaF, 1% aprotinin, 10 mg/mL leupeptin, 1 mM Na_3VO_4, 10 mM benzamidine, 10 mg/mL soybean trypsin inhibitor, 1% NP-40, and 0.25% sodium doexycholic acid. The lysates were subjected to SDS-PAGE and Western Blot analysis. Levels of ERK2 and phosphorylated ERK were detected with anti-ERK2 (Upstate Biotechnology, 1:1000) and anti-phospho-ERK1/2 (Cell Signaling, 1:1000) antibodies, respectively. Anti-vinculin antibody was used for the loading control. The gels were visualized with the Odyssey Infrared Imaging System (LiCor) and quantified on ImageJ (NIH).

Supplementary Figures

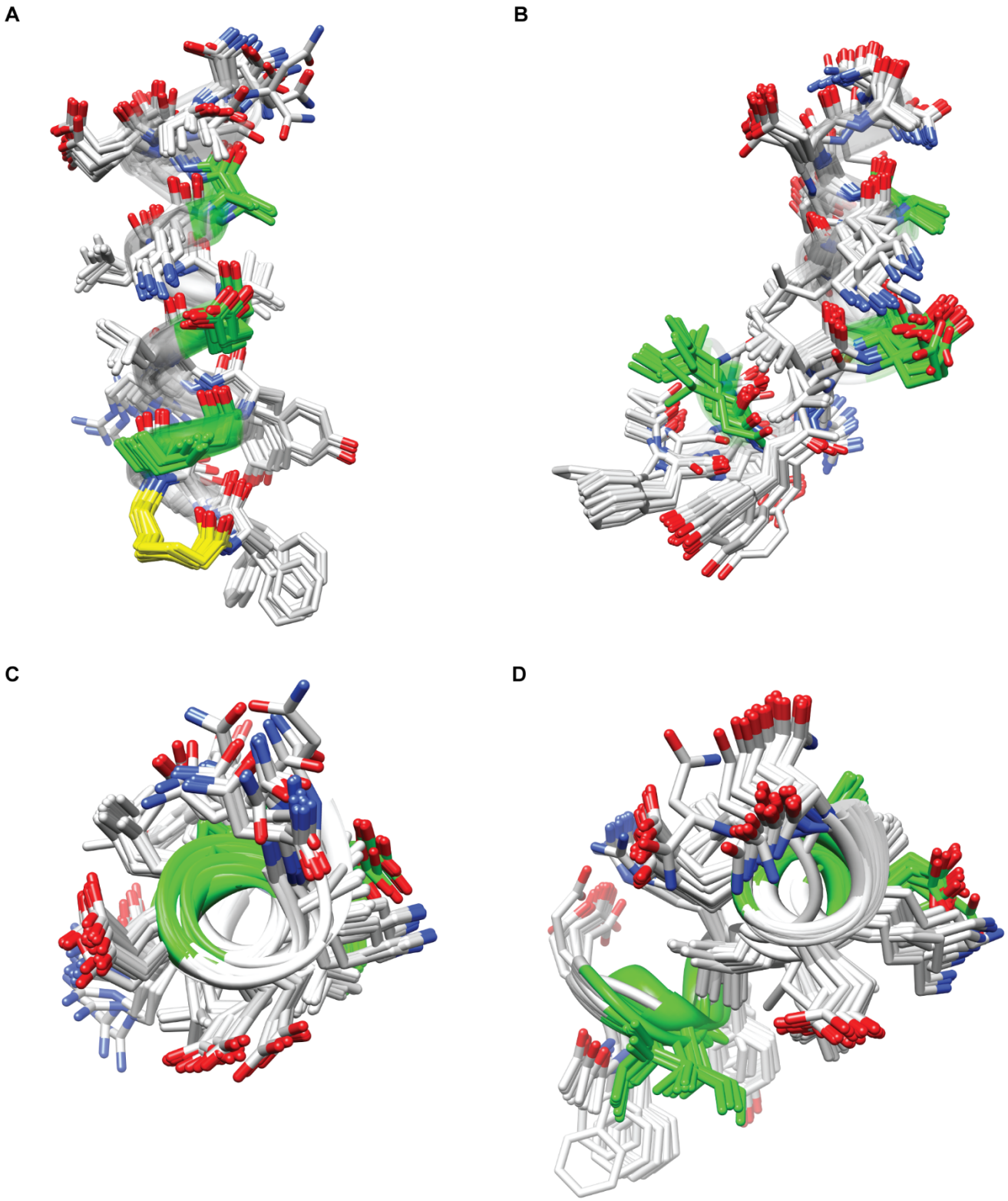


Figure S1. Ensemble of the 20 lowest energy solution structures of (A) **α$_3$βHBS$_{SOS}$** and (B) **α$_3$βUNC$_{SOS}$** in 10% d3-acetonitrile in H_2O (pH = 4.0) at 298 K.

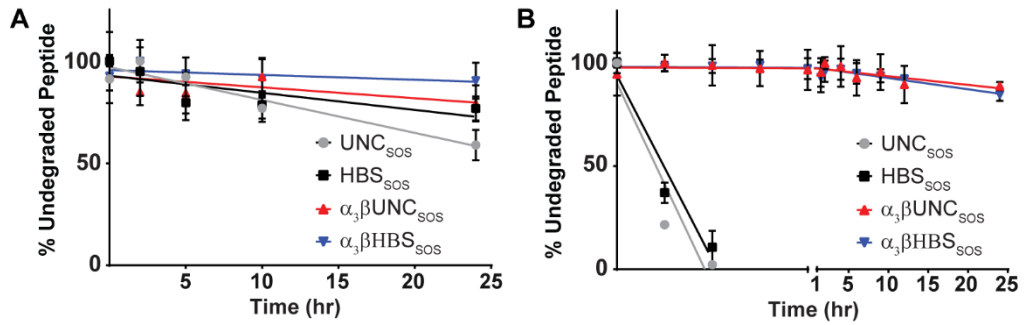


Figure S2. Measurement of proteolytic degradation of indicated peptides by (A) 25% serum or (B) proteinase K over 24 hours. Error bars are mean ± SD of biological triplicates.

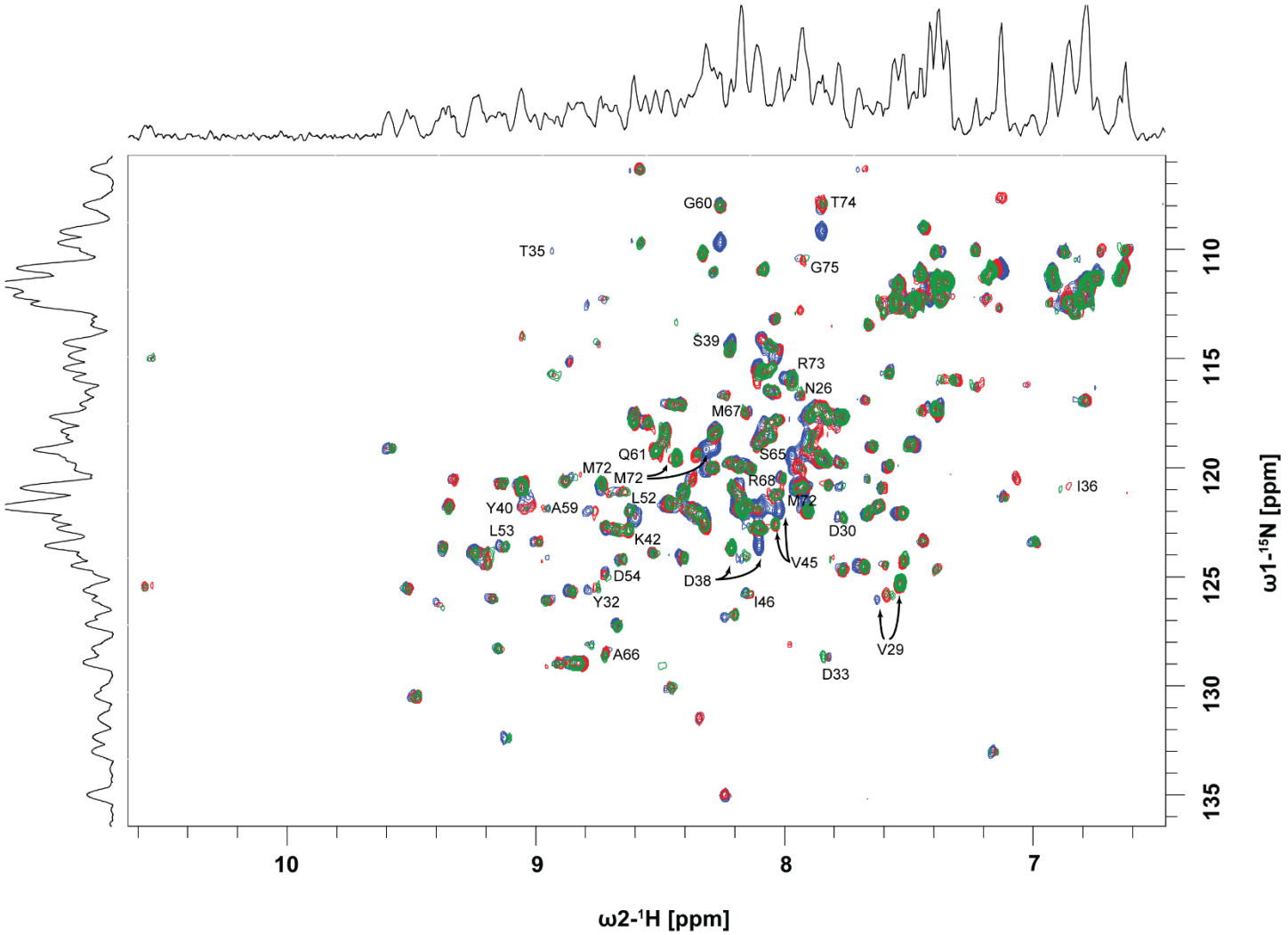


Figure S3. ^{1}H-^{15}N HSQC overlaid spectrum of H-Ras only (blue), H-Ras:**$\alpha_3\beta$HBS$_{SOS}$** (1:1, red), and H-Ras:**$\alpha_3\beta$HBS$_{SOS}$** (1:5, green).

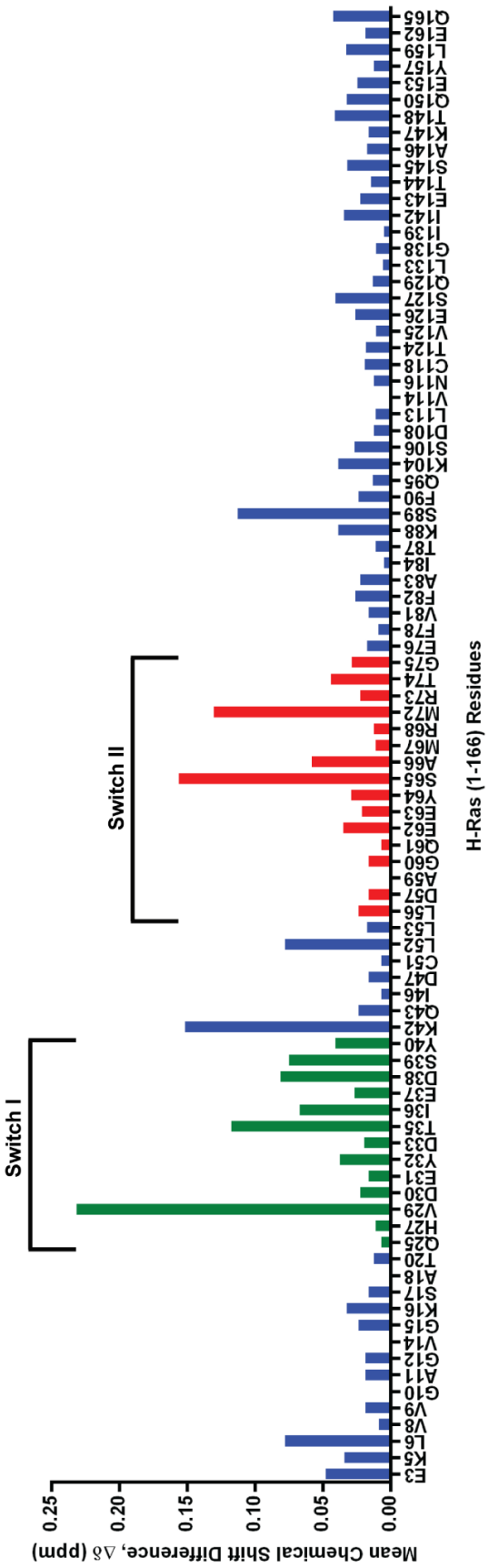


Figure S4. Mean chemical shift difference ($\Delta\delta_{NH}$) plot indicating resonance shifts upon addition of increasing amounts of **$\alpha_3\beta$HBS$_{SOS}$**.

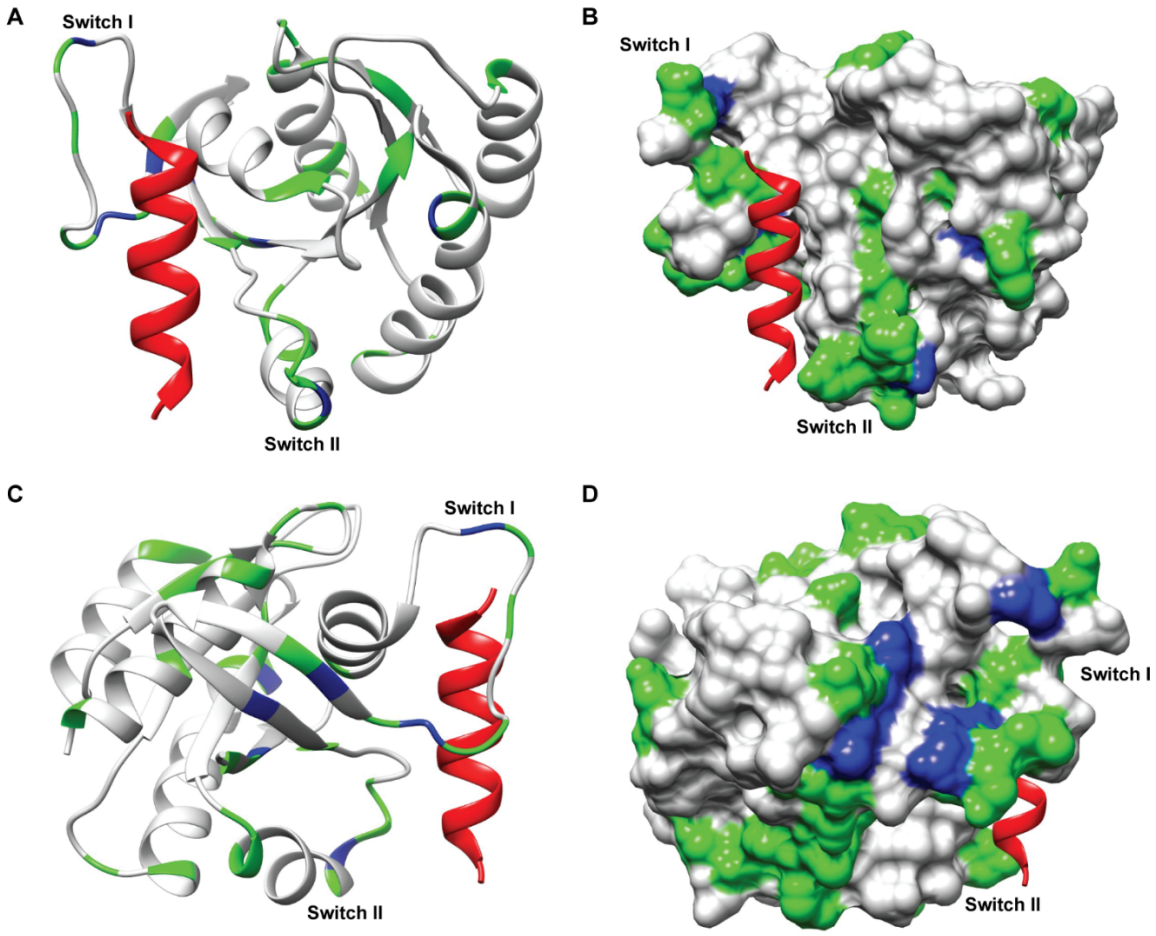


Figure S5. (A) Ribbon and (B) surface view of the ^{15}N-labeled H-Ras-**$\alpha_3\beta$HBS$_{Sos}$** interaction upon titrating increasing amounts of **$\alpha_3\beta$HBS$_{Sos}$**. The Switch I and II regions of Ras have been highlighted along with the αH helix of Sos (red). The (C) ribbon and (D) surface view of the ^{15}N-labeled H-Ras-**$\alpha_3\beta$HBS$_{Sos}$** interaction rotated 180°. Residues are colored according to the degree of mean chemical shift change: minimal (< 0.02, light gray), moderate (0.02 – 0.075, green), and significant (> 0.075, blue).

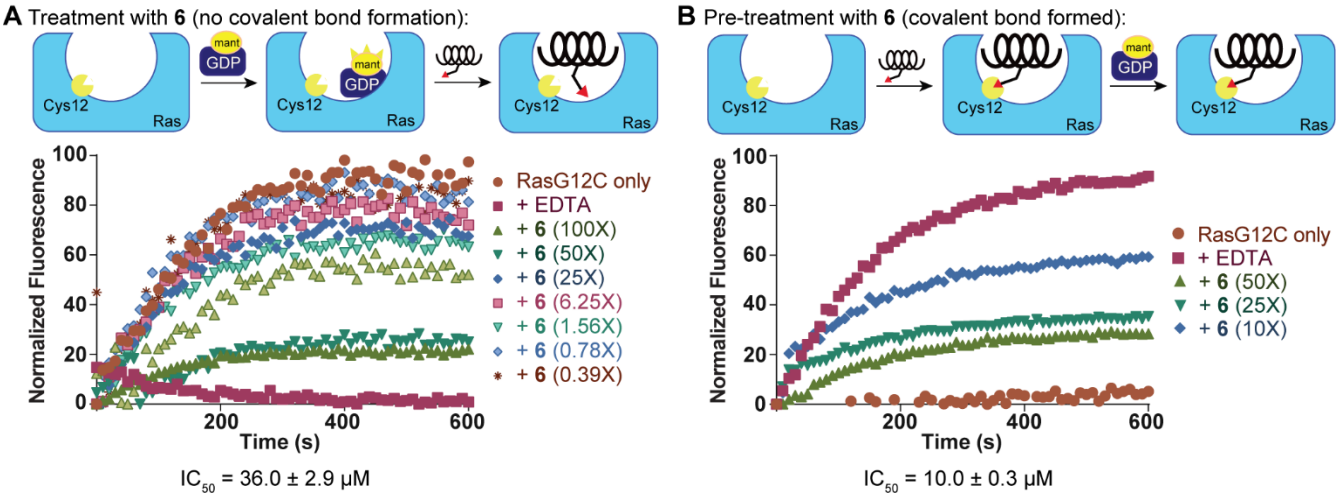


Figure S6. (A) Nucleotide association assay with H-Ras G12C (1 μM) with fluorescent mant-GDP serial dilutions of peptide **6**. (B) Nucleotide association assay with H-Ras G12C (1 μM) pre-treated and labeled with peptide **6** for 24 hours prior to exposure to fluorescent nucleotide. Error bars are mean ± SD of biological duplicates.

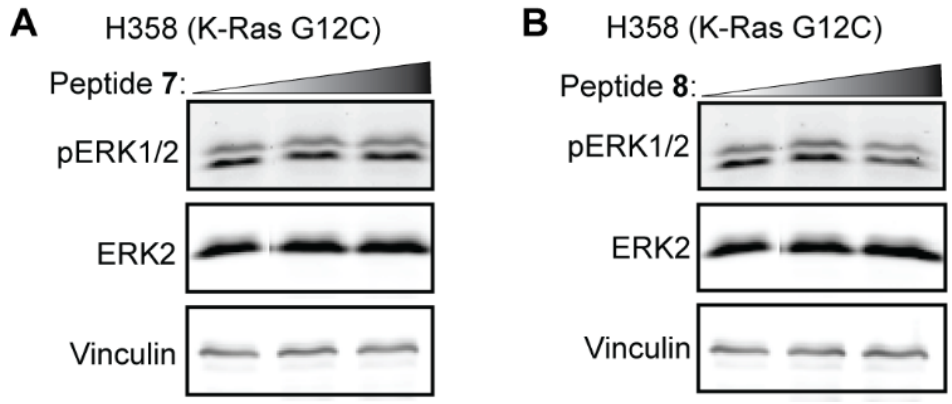


Figure S7. Representative Western blots probing for the downstream effector ERK post-treatment with increasing concentrations of (A) **7** (Ala mutant $\alpha_3\beta$HBS with the vinyl sulfonamide warhead, n=2) in H358 cells (DMSO, 50 μM, and 100 μM), and (B) **8** ($\alpha_3\beta$HBS with the deactivated warhead) in H358 cells (DMSO, 50 μM, and 100 μM).

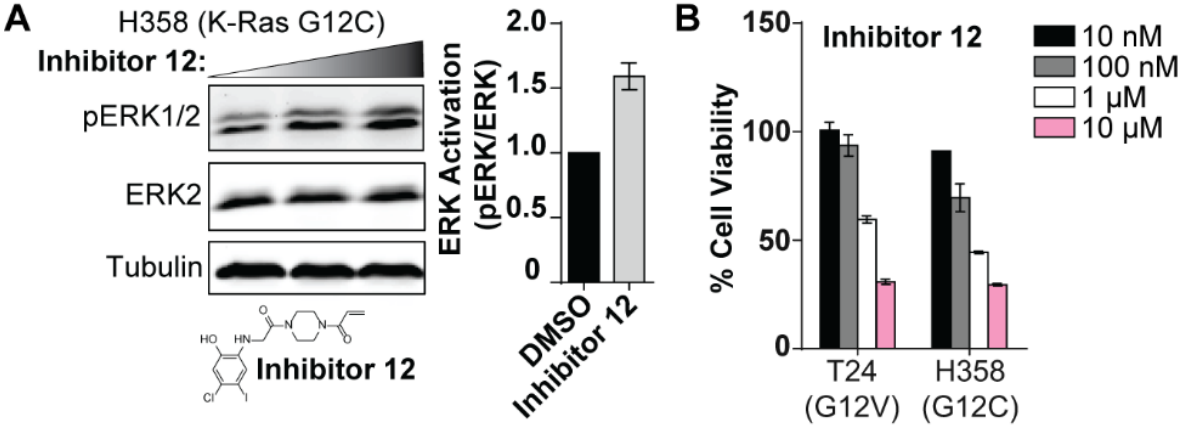


Figure S8. (A) Left: Representative Western blots probing the downstream effector ERK post-treatment with increasing concentrations of the K-Ras G12C allosteric **Inhibitor 12** in H358 cells (DMSO, 25 μM, and 50 μM). Right: Representative bar graphs displaying overall ERK activation at 50 μM of each compound relative to the DMSO control. (B) Comparison of cell viability between T24 (H-Ras G12V) and H358 (K-Ras G12C) cells treated with Inhibitor 12. Error bars are mean ± SD of biological duplicates.

2D NMR Chemical Shifts and Spectra

Table S4. NOE distance restraints used for structure determination of **$\alpha_3\beta$HBS$_{SOS}$**. Distances of 2.0 ± 0.5 Å, 3.0 ± 1.0 Å, and 4.0 ± 0.5 Å were incorporated for strong, medium, and weak NOE intensities, respectively. β^3-amino acid residues are denoted with blue letters.

Residue-Atom 1	Residue-Atom 2	Chemical Shift of Atom 1 (ppm)	Chemical Shift of Atom 2 (ppm)	NOE Intensity
F1Aromatic(o)	F1β	7.229	2.892	Weak
F1	Z0α	8.137	2.254	Weak
F1	Z0β	8.137	1.516	Weak
F1	F1β	8.137	2.892	Weak
F1	E2	8.137	8.454	Weak
E2	Z0β	8.454	1.516	DID NOT USE
E2	F1α	8.454	4.511	Medium
E2	E2α	8.454	4.007	Medium
I$_\beta$4	G3α	7.903	3.665	Weak
I$_\beta$4	Y5	7.903	8.079	Weak
Y5Aromatic(o)	Y5Aromatic(m)	7.006	6.712	Strong
Y5Aromatic(m)	Y5β	6.712	2.927	Weak
Y5Aromatic(o)	Y5β	7.006	2.927	Strong
Y5	E2β	8.079	4.007	DID NOT USE
Y5	I$_\beta$4CH2	8.079	2.434	Weak
Y5	I$_\beta$4CH2	8.079	2.270	Medium
Y5	Y5Aromatic(o)	8.079	7.006	Weak
Y5	Y5α	8.079	4.289	Medium
Y5	Y5β	8.079	2.927	Weak
Y5	R6	8.079	7.844	Weak
Y5	I$_\beta$4δ	8.079	0.767	Weak
R6	E2α	7.844	4.182	Weak
R6	Y5α	7.844	4.289	Weak
R6	Y5β	7.844	2.927	Weak
R6	R6α	7.844	4.019	Medium
R6	R6β	7.844	1.624	Weak
R6	R6γ	7.844	1.599	Medium
R6	L7	7.844	7.691	Weak
R6	L7δ	7.844	0.080	DID NOT USE
L7	I$_\beta$4α	7.691	3.960	Weak
L7	R6β	7.691	1.624	Weak
L7	L7α	7.691	4.078	Strong
L7	L7β	7.691	1.495	Medium
L7	L7δ	7.691	0.802	Weak
L7	E$_\beta$8	7.691	7.738	Weak*
E$_\beta$8	I$_\beta$4α	7.738	3.960	Weak
E$_\beta$8	L7β	7.738	1.495	Medium
E$_\beta$8	E$_\beta$8α	7.738	4.078	Strong
E$_\beta$8	E$_\beta$8β	7.738	1.765	Medium
E$_\beta$8	E$_\beta$8CH2	7.738	2.409	Medium
E$_\square$8	E$_\beta$8CH2	7.738	2.374	Medium
E$_\beta$8	L9	7.738	7.943	Weak
L9	Y5α	7.943	8.079	Weak*

L9	R6α	7.943	4.019	Weak*
L9	E_{β}8CH2	7.943	2.409	Weak
L9	E_{β}8CH2	7.943	2.374	Strong
L9	L9α	7.943	4.054	Strong
L9	L9β	7.943	1.459	Strong
L9	L9γ	7.943	1.247	Weak
L9	L9δ	7.943	0.791	Strong
L9	L10	7.943	7.910	Weak*
L10	R6α	7.910	4.019	Weak
L10	L7α	7.910	4.078	Weak*
L10	L9α	7.910	4.054	DID NOT USE
L10	L10α	7.910	4.172	Weak
L10	L10β	7.910	1.577	Strong
L10	L10δ	7.910	0.767	Medium
L10	K11	7.910	7.762	Medium
K11	L7α	7.762	4.078	Weak*
K11	L7δ	7.762	0.802	Weak
K11	E_{β}8α	7.762	4.078	Weak*
K11	L10α	7.762	4.172	Weak
K11	K11δ	7.762	1.566	Weak
K11	α12	7.762	7.644	Weak
A_{β}12	E_{β}8α	7.644	4.078	Weak*
A_{β}12	L9α	7.644	4.054	Weak
A_{β}12	L10α	7.644	4.172	Weak
A_{β}12	K11α	7.644	4.007	Medium
A_{β}12	A_{β}12α	7.644	4.148	Weak
A_{β}12	A_{β}12β	7.644	1.084	Weak
A_{β}12	A_{β}12CH2	7.644	2.423	Weak
A_{β}12	A_{β}12CH2	7.644	2.352	Weak
E13	L10α	8.208	4.172	Weak*
E13	A_{β}12	8.208	7.644	Weak
E13	A_{β}12CH2	8.208	2.423	Strong
E13	A_{β}12CH2	8.208	2.352	DID NOT USE
E13	E13α	8.208	4.101	Medium
E13	E13β	8.208	1.953	Medium
E13	E14	8.208	8.290	Strong
E14	L10α	8.290	4.172	Weak*
E14	K11α	8.290	4.007	Weak
E14	A_{β}12β	8.290	1.084	DID NOT USE
E14	E13α	8.290	4.101	Medium
E14	E14α	8.290	4.183	Medium
E14	E14β	8.290	2.035	Weak
E14	E14γ	8.290	2.397	Medium
E14	A15	8.290	7.996	Weak
A15	A_{β}12CH2	7.996	2.352	DID NOT USE
A15	E14β	7.996	1.941	Weak
A15	A15α	7.996	4.183	Medium
A15	A15β	7.996	1.319	Medium
A15	N16	7.996	8.067	Weak*
N16	A15α	8.067	4.183	Weak

N16	A15β	8.067	1.319	Weak
N16	TerminalNH2	8.067	7.000	Weak
N16Amide	N16Amide	7.457	6.766	Strong
TerminalNH2	TerminalNH2	7.000	7.273	Strong

*Denotes NOE cross-peaks that could not be cleanly assigned due to overlapping signals and were therefore labeled as weak intensities.

Table S5. Calculated dihedral angles, ϕ, derived from $^3J_{NH\text{-}C\alpha H}$ coupling constants for **α₃βHBS$_{SOS}$**.

Residue	ϕ
F1	-88.0 ± 15°
E2	-67.9 ± 15°
I_β4	-82.9 ± 15°
Y5	-73.5 ± 15°
R6	-100.3 ± 15°
L7	-98.8 ± 15°
E_β8	-85.4 ± 15°
L9	-104.5 ± 15°
L10	-104.5 ± 15°
K11	-96.8 ± 15°
A_β12	-93.6 ± 15°
E13	-82.9 ± 15°
E14	-93.6 ± 15°
A15	-104.5 ± 15°
N16	-55.9 ± 15°

$^3J_{NH\text{-}C\alpha H}$ values were either obtained directly from ^{1}H NMR spectrum or calculated from TOCSY or NOESY data using Protocol #1.[7] ϕ angles were calculated using the Karplus Equation $J_{NH\text{-}C\alpha H} = 6.4\cos^2(\phi - 60°) - 1.4\cos(\phi - 60°) + 1.9$.

Table S6. NOE distance restraints used for structure determination of **α₃βUNC$_{SOS}$**. Distances of 2.0 ± 0.5 Å, 3.0 ± 1.0 Å, and 4.0 ± 0.5 Å were incorporated for strong, medium, and weak NOE intensities, respectively. β^3-amino acid residues are denoted with blue letters. A total of 107 NOE constraints were used for the structure calculation (60 intraresidue, 31 sequential, and 16 medium to long-range).

Residue-Atom 1	Residue-Atom 2	Chemical Shift of Atom 1 (ppm)	Chemical Shift of Atom 2 (ppm)	NOE Intensity
F1	Ac	8.146	1.893	Strong
F1	F1α	8.146	4.451	Weak
F1	F1β	8.146	2.996	Weak
F1	F1β	8.146	2.902	Weak
F1	E2	8.146	8.299	Weak
F1Aromatic(o)	F1α	7.164	4.451	Weak
F1Aromatic(o)	F1β	7.164	2.996	Weak
F1Aromatic(o)	F1Aromatic(m)	7.164	7.258	Weak
F1Aromatic(m)	F1Aromatic(p)	7.258	7.352	Strong
E2	F1α	8.299	4.451	Medium
E2	E2α	8.299	4.123	Medium
E2	E2β	8.299	1.845	Weak
E2	E2γ	8.299	2.291	Weak
E2	G3	8.299	7.750	Weak
G3	G3α	7.750	3.630	Weak

G3	G3α	7.750	3.747	Strong
G3	$I_{\beta}4$	7.750	7.348	Medium
$I_{\beta}4$	F1α	7.348	4.451	Weak
$I_{\beta}4$	G3α	7.348	3.630	Weak
$I_{\beta}4$	G3α	7.348	3.747	Weak
$I_{\beta}4$	$I_{\beta}4\alpha$	7.348	3.958	Weak
$I_{\beta}4$	$I_{\beta}4\beta$	7.348	1.411	Weak
$I_{\beta}4$	$I_{\beta}4\delta$	7.348	0.742	Weak
$I_{\beta}4$	$I_{\beta}4$CH2	7.348	2.280	Weak
$I_{\beta}4$	Y5	7.348	7.923	Weak
Y5	E2α	7.923	4.123	Weak*
Y5	$I_{\beta}4$CH2	7.923	2.280	Strong
Y5	Y5α	7.923	4.287	Medium
Y5	Y5β	7.923	2.890	Strong
Y5	Y5Aromatic	7.923	7.000	Weak
Y5Aromatic	$I_{\beta}4\beta$	7.000	1.411	DID NOT USE
Y5Aromatic	$I_{\beta}4\gamma'$	6.718	0.789	DID NOT USE
Y5Aromatic(o)	Y5	7.000	7.923	Weak
Y5Aromatic(o)	Y5α	7.000	4.287	Weak
Y5Aromatic(m)	Y5β	6.718	2.890	Weak
Y5Aromatic(o)	Y5β	7.000	2.890	Strong
Y5Aromatic(o)	Y5Aromatic(m)	7.000	6.718	Strong
R6	E2α	7.994	4.123	Weak*
R6	$I_{\beta}4$CH2	7.994	2.280	Weak
R6	Y5α	7.994	4.287	Strong
R6	R6α	7.994	4.005	Medium
R6	R6β	7.994	1.517	Weak
R6	R6γ	7.994	1.669	Strong
R6	R6δ	7.994	3.078	Medium
R6	L7	7.994	7.712	Medium
R6guanidine	Y5β	7.090	2.890	Medium
L7	$I_{\beta}4\alpha$	7.712	3.958	Weak*
L7	R6α	7.712	4.005	Strong
L7	L7α	7.712	4.088	Strong
L7	L7β	7.712	1.658	Medium
L7	L7β	7.712	1.505	Strong
L7	L7δ	7.712	0.770	Weak
$E_{\beta}8$	Y5α	7.736	4.287	Weak
$E_{\beta}8$	$E_{\beta}8\gamma$	7.736	2.280	Weak
$E_{\beta}8$	$E_{\beta}8\gamma$	7.736	2.222	Weak
$E_{\beta}8$	$E_{\beta}8$CH2	7.736	2.350	Strong
L9	R6α	7.958	4.005	Weak*
L9	$E_{\beta}8$	7.958	7.736	Weak
L9	$E_{\beta}8$CH2	7.958	2.350	Strong
L9	L9α	7.958	4.017	Strong
L9	L9β	7.958	1.434	Strong
L9	L9β	7.958	1.564	Strong
L10	R6α	7.912	4.005	Weak*
L10	L7α	7.912	4.088	Weak*
L10	L9α	7.912	4.017	Strong

L10	L9β	7.912	1.434	Weak
L10	L10α	7.912	4.146	Medium
L10	L10β	7.912	1.575	Strong
L10	K11	7.912	7.829	Strong
K11	L7α	7.289	4.088	Weak*
K11	$E_{\beta}8\alpha$	7.289	4.080	Weak*
K11	L10α	7.289	4.146	DID NOT USE
K11	K11α	7.289	4.005	Medium
K11	K11β	7.289	1.646	Medium
K11	K11γ	7.289	1.352	Weak
K11	$A_{\beta}12$	7.289	7.630	Medium
$A_{\beta}12$	L9α	7.630	4.017	Weak*
$A_{\beta}12$	K11α	7.630	4.005	Medium
$A_{\beta}12$	K11β	7.630	1.646	Weak
$A_{\beta}12$	$A_{\beta}12\alpha$	7.630	4.135	Medium
$A_{\beta}12$	$A_{\beta}12\beta$	7.630	1.082	Medium
$A_{\beta}12$	$A_{\beta}12CH2$	7.630	2.421	Weak
$A_{\beta}12$	$A_{\beta}12CH2$	7.630	2.315	Weak
$A_{\beta}12$	E13	7.630	8.205	Weak
E13	L10α	8.205	4.146	Weak*
E13	$A_{\beta}12\beta$	8.205	1.082	Weak
E13	$A_{\beta}12CH2$	8.205	2.421	Strong
E13	E13α	8.205	4.088	Medium
E13	E13β	8.205	1.951	Weak
E13	E13β	8.205	2.021	Weak
E13	E13γ	8.205	2.374	Medium
E13	E14	8.205	8.287	Medium
E14	L10α	8.287	4.146	Weak*
E14	E13α	8.287	4.088	Medium
E14	E14α	8.287	4.181	Medium
E14	E14β	8.287	2.033	Weak
E14	E14β	8.287	1.939	Medium
E14	E14γ	8.287	2.408	Weak
E14	A15	8.287	8.001	Weak
A15	K11α	8.001	4.005	Weak*
A15	$A_{\beta}12\alpha$	8.001	4.135	Weak*
A15	N16	8.001	8.001	Medium
A15	A15α	8.001	4.170	Medium
A15	A15β	8.001	1.305	Medium
N16	A15α	8.076	4.170	Weak
N16	N16α	8.076	4.545	Weak
N16	$TerminalNH_2$	8.076	7.270	Weak
N16Amide	N16Amide	7.457	6.765	Strong
N16Amide	$TerminalNH_2$	7.457	7.270	Weak
$TerminalNH_2$	$TerminalNH_2$	7.270	6.990	Strong

*Denotes NOE cross-peaks that could not be cleanly assigned due to overlapping signals and were therefore labeled as weak intensities.

Table S7. Calculated dihedral angles, ϕ, derived from $^3J_{NH\text{-}C\alpha H}$ coupling constants for **α₃βUNC$_{SOS}$**.

Residue	ϕ
F1	-79.0 ± 15°
E2	-79.0 ± 15°
G3	-73.6 ± 15°
I_β4	-101.7 ± 15°
Y5	-80.6 ± 15°
R6	-90.9 ± 15°
L7	-104.7 ± 15°
L9	-75.9 ± 15°
L10	-85.5 ± 15°
K11	-79.0 ± 15°
A_β12	-101.7 ± 15°
E13	-69.7 ± 15°
E14	-83.8 ± 15°
A15	-80.6 ± 15°
N16	-83.8 ± 15°

$^3J_{NH\text{-}C\alpha H}$ values were either obtained directly from ^{1}H NMR spectrum or calculated from TOCSY or NOESY data using Protocol #1.[7] ϕ angles were calculated using the Karplus Equation $J_{NH\text{-}C\alpha H} = 6.4\cos^2(\phi - 60°) - 1.4\cos(\phi - 60°) + 1.9$. A $^3J_{NH\text{-}C\alpha H}$ value for E_β8 could not be determined.

Table S8. ^{1}H NMR assignments and chemical shifts (δ, ppm) for α₃βHBS$_{SOS}$ (293 K) in 10% d3-acetonitrile in H_2O with TFA (pH = 4.0). **α₃βHBS$_{SOS}$** has two conformations, one major and an alternate minor one. The table below has chemical shifts for the major conformation.

Residue	NH	Hα	Hβ	Hγ	Hδ	Hε/*CH_2**	Other
Z0	N/A	2.254	1.516	ND	5.12	5.27	ND
F1	8.149	4.511	2.892	N/A	N/A	N/A	7.229 (o)
E2	8.454	4.007	1.988	N/A	N/A	N/A	N/A
G3	N/A	3.665 3.870	N/A	N/A	N/A	N/A	N/A
I_β4	7.91	3.96	ND	ND	0.767	*2.434* *2.270*	N/A
Y5	8.079	4.289	2.927	N/A	N/A	N/A	7.006 (o) 6.712 (m)
R6	7.844	4.019	1.624 1.425	1.599	3.068	N/A	7.103 (guanidine)
L7	7.691	4.078	1.495	ND	0.8024	N/A	N/A
E_β8	7.738	4.078	1.765 1.648	2.211 2.258	N/A	*2.409* *2.374*	N/A
L9	7.938	4.054	1.459	1.247	0.791	N/A	N/A
L10	7.91	4.172	1.577	ND	0.767	N/A	N/A
K11	7.762	4.007	1.683	1.307	1.5655	2.869	7.413 (amine)
A_β12	7.644	4.148	1.084	N/A	N/A	*2.423* *2.352*	N/A
E13	8.208	4.101	1.953	2.352	N/A	N/A	N/A
E14	8.29	4.183	1.941 2.035	2.397	N/A	N/A	N/A
A15	7.996	4.183	1.319	N/A	N/A	N/A	N/A

N16	8.067	4.547	2.728 2.657	N/A	N/A	N/A	7.457 6.766
Terminal Amide	7.000 7.273	N/A	N/A	N/A	N/A	N/A	N/A

The β^3-amino acid residues are denoted with blue letters. Protons are labeled as α, β, γ, δ, or ε relative to the amide nitrogen instead of the carbonyl and the backbone methylene resonances are italicized. *Denotes the backbone methylene protons of the β^3-residues or the ε-side chain protons of lysine residues. ND denotes protons for which chemical shifts could not be determined. For aromatic protons, (o) denotes ortho protons, (m) denotes meta protons.

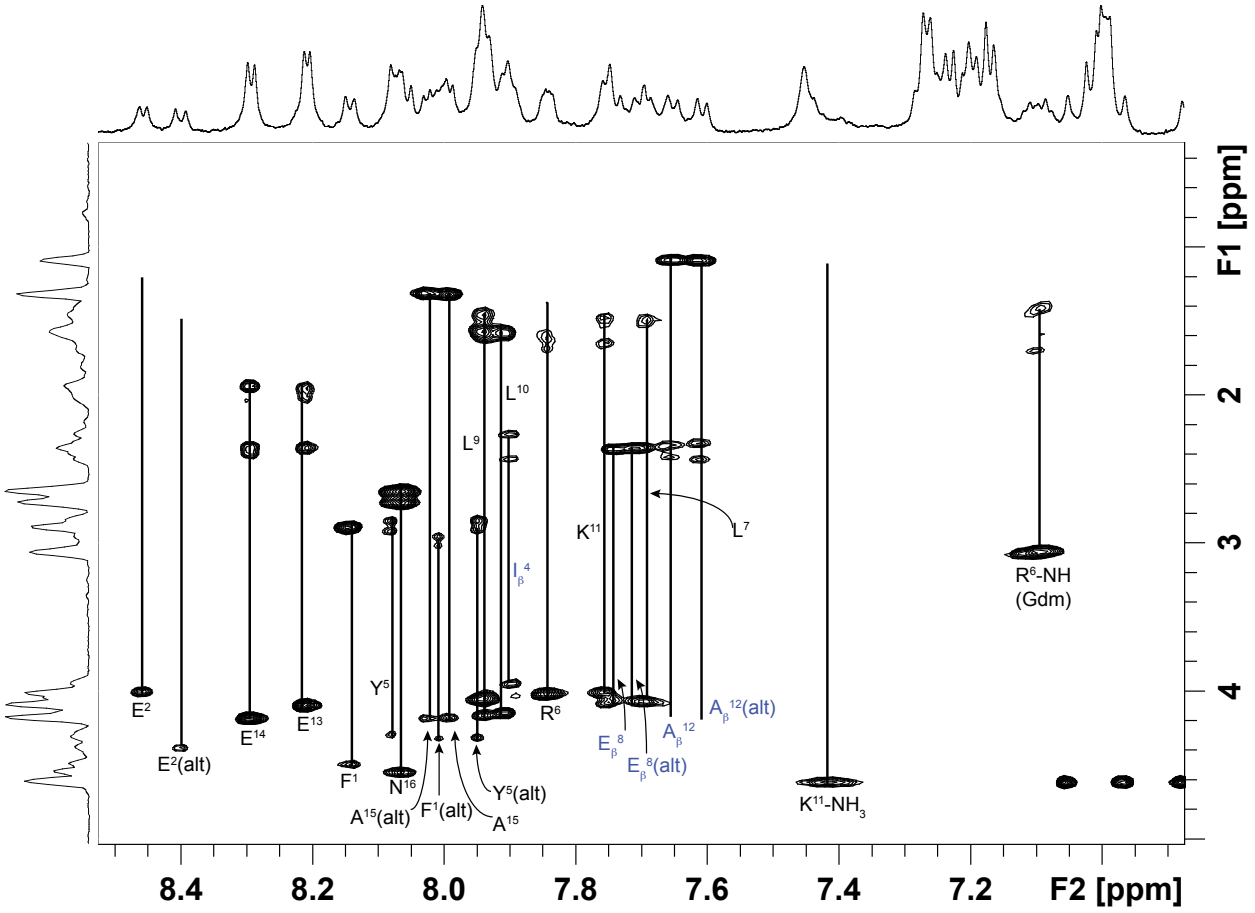


Figure S9. NH-Cα region of the TOCSY spectrum (600 MHz, 298 K) of **α₃βHBS$_{SOS}$** in 10% d3-acetonitrile in H_2O, pH = 4.0. β^3-amino acid residues are denoted with blue letters. (Alt) denotes minor conformer chemical shifts (only discernible for F1, E2, Y5, Eβ8, Aβ12, and A15).

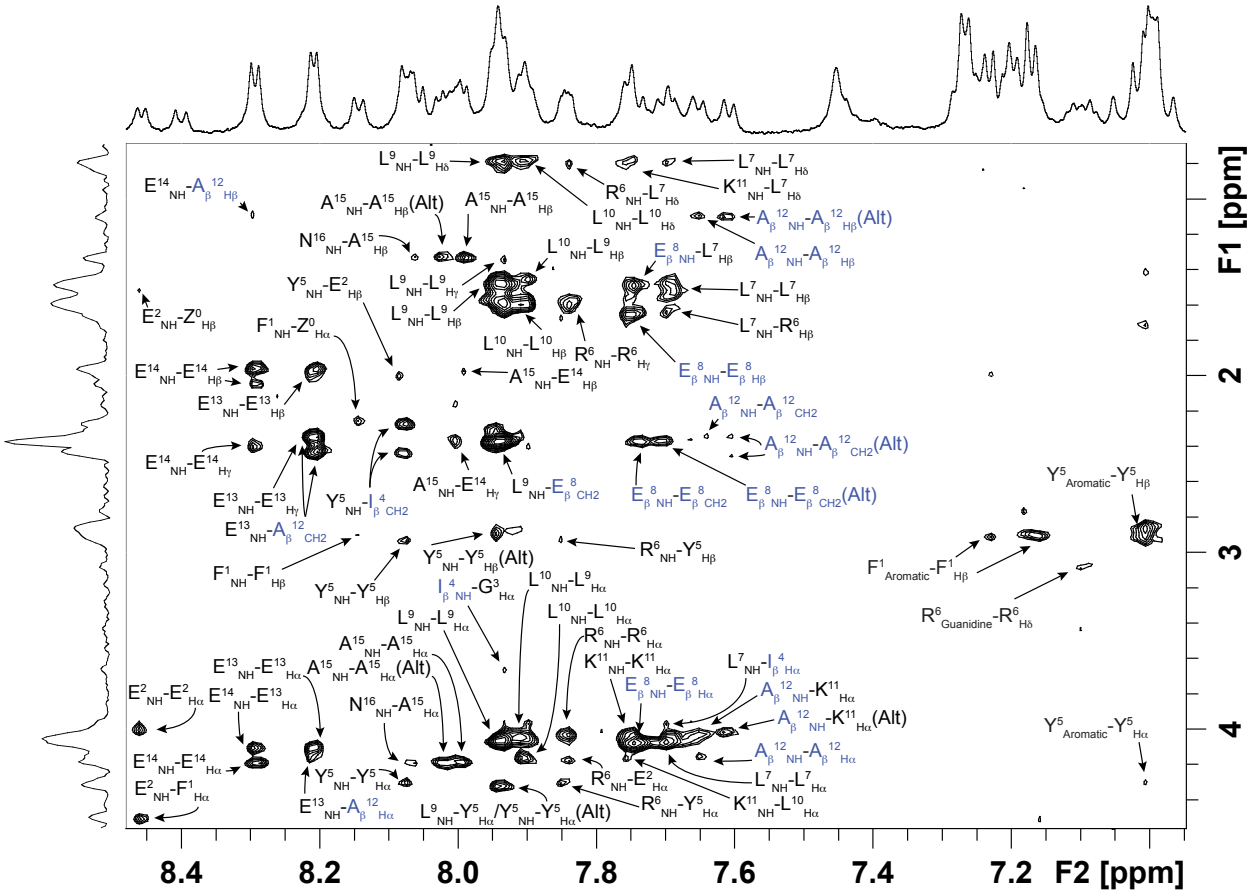


Figure S10. NH-Cα region of the NOESY spectrum (600 MHz, 298 K) of **α$_3$βHBS$_{SOS}$** in 10% d3-acetonitrile in H_2O, pH = 4.0. β^3-amino acid residues are denoted with blue letters. (Alt) denotes minor conformer chemical shifts (only discernible for F1, E2, Y5, Eβ8, Aβ12, and A15).

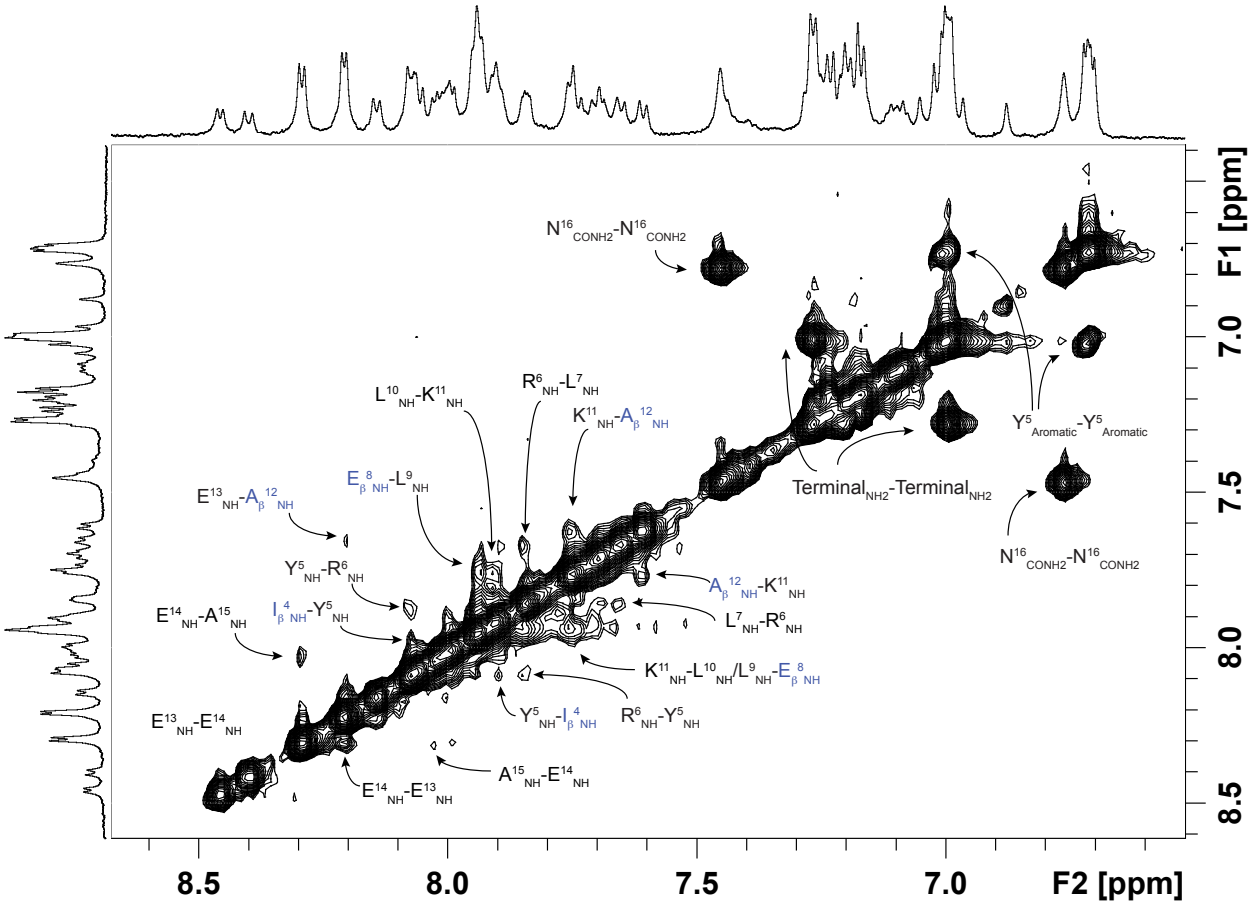


Figure S11. Amide region of the NOESY spectrum (600 MHz, 298 K) of **α₃βHBS$_{SOS}$** in 10% d3-acetonitrile in H_2O, pH = 4.0. β^3-amino acid residues are denoted with blue letters.

Table S9. ^{1}H NMR assignments and chemical shifts (δ, ppm) for **α₃βUNC$_{SOS}$** (293 K) in 10% d3-acetonitrile in H_2O with TFA (pH = 4.0).

Residue	NH	Hα	Hβ	Hγ	Hδ	Hε/*CH$_2$**	Other
Ac	N/A	1.893	N/A	N/A	N/A	N/A	N/A
F1	8.146	4.451	2.996 2.902	N/A	N/A	N/A	7.352(p) 7.258(m) 7.164 (o)
E2	8.299	4.123	1.845	2.291	N/A	N/A	N/A
G3	7.75	3.630 3.747	N/A	N/A	N/A	N/A	N/A
I_β4	7.348	3.958	1.411	0.789 (Me)	0.742	*2.327* *2.280*	N/A
Y5	7.923	4.287	2.89	N/A	N/A	N/A	7.000 (o) 6.718 (m)
R6	7.994	4.005	1.505 1.423	1.669	3.078	N/A	7.09
L7	7.712	4.088	1.505 1.658	ND	0.777	N/A	N/A

$E_{\beta}8$	7.736	4.08	1.751 1.634	2.280 2.222	N/A	*2.350*	N/A
L9	7.958	4.017	1.434 1.564	ND	0.777	N/A	N/A
L10	7.912	4.146	1.575	ND	0.765	N/A	N/A
K11	7.829	4.005	1.646	1.352	1.5518	2.867	7.395
$A_{\beta}12$	7.63	4.135	1.082	N/A	N/A	*2.421* *2.315*	N/A
E13	8.205	4.088	1.951 2.021	2.374	N/A	N/A	N/A
E14	8.287	4.181	2.033 1.939	2.408 2.362	N/A	N/A	N/A
A15	8.001	4.17	1.305	N/A	N/A	N/A	N/A
N16	8.076	4.545	2.714 2.644	N/A	N/A	N/A	7.457 6.765
Terminal Amide	7.270 6.999	N/A	N/A	N/A	N/A	N/A	N/A

The β^3-amino acid residues are denoted with blue letters. Protons are labeled as being α, β, γ, δ, or ε relative to the amide nitrogen instead of the carbonyl and the backbone methylene resonances are italicized. *Denotes the backbone methylene protons of the β^3-residues or the ε-side chain protons of lysine residues. ND denotes protons for which chemical shifts could not be determined. For aromatic protons, (o) denotes ortho protons, (m) denotes meta protons, (p) denotes the para proton.

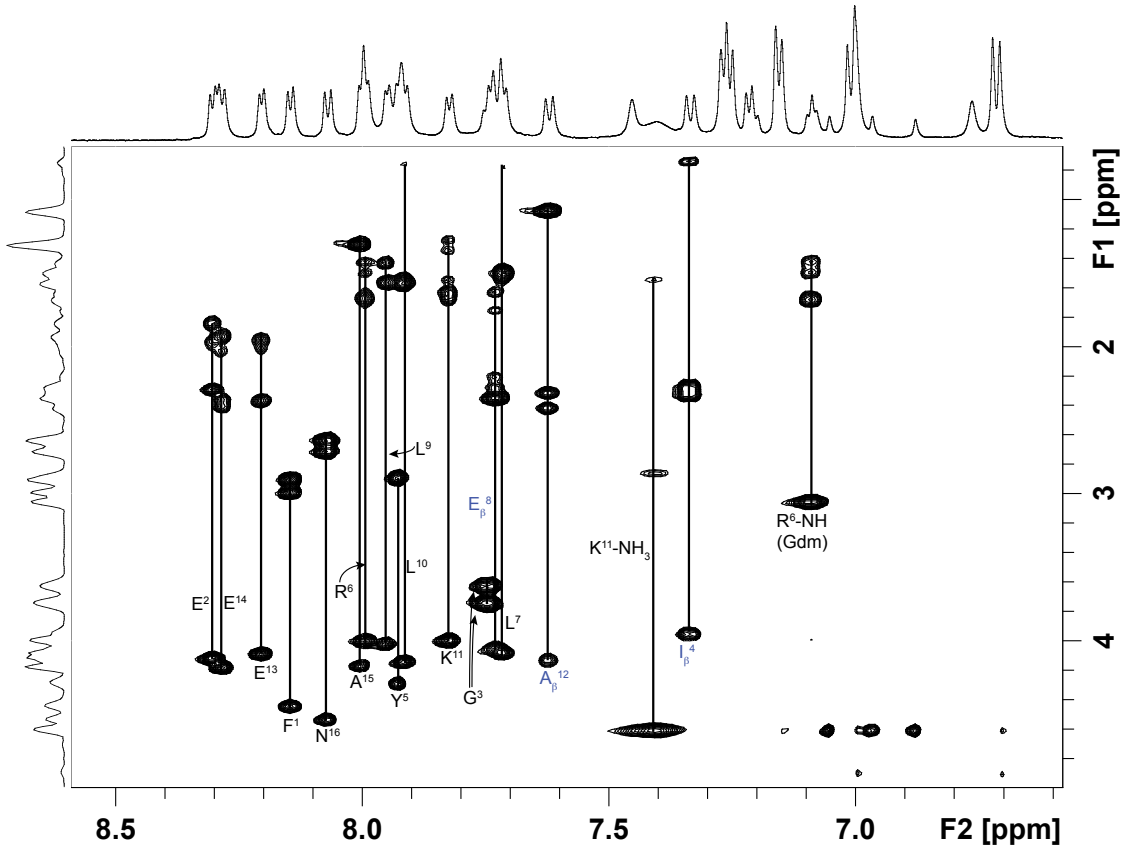


Figure S12. NH-Cα region of the TOCSY spectrum (600 MHz, 298 K) of **α$_3$βUNC$_{SOS}$** in 10% d3-acetonitrile in H_2O, pH = 4.0. β^3-amino acid residues are denoted with blue letters.

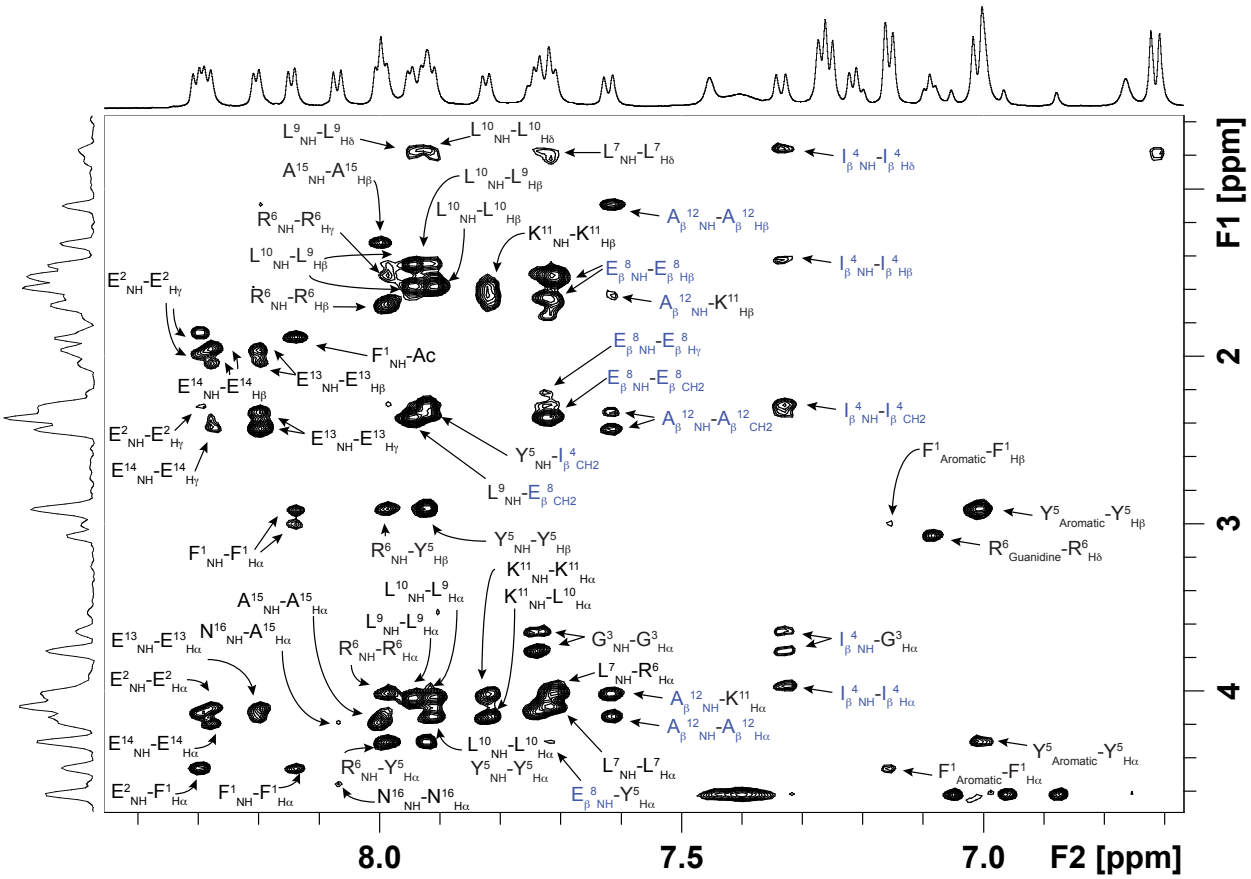


Figure S13. NH-Cα region of the NOESY spectrum (600 MHz, 298 K) of **α$_3$βUNC$_{SOS}$** in 10% d3-acetonitrile in H_2O, pH = 4.0. β^3-amino acid residues are denoted with blue letters.

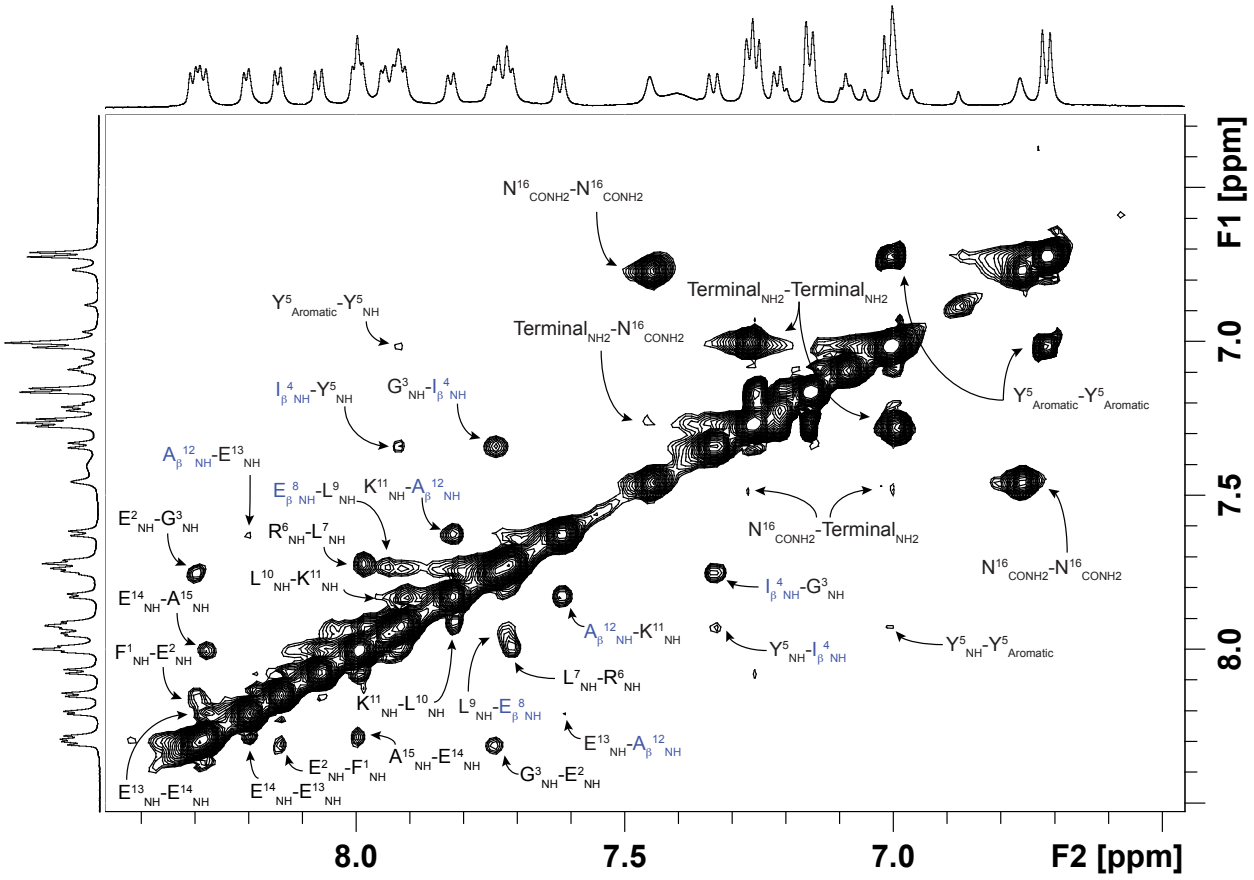


Figure S14. Amide region of the NOESY spectrum (600 MHz, 298 K) of **$\alpha_3\beta$UNC$_{SOS}$** in 10% d3-acetonitrile in H_2O, pH = 4.0. β^3-amino acid residues are denoted with blue letters.

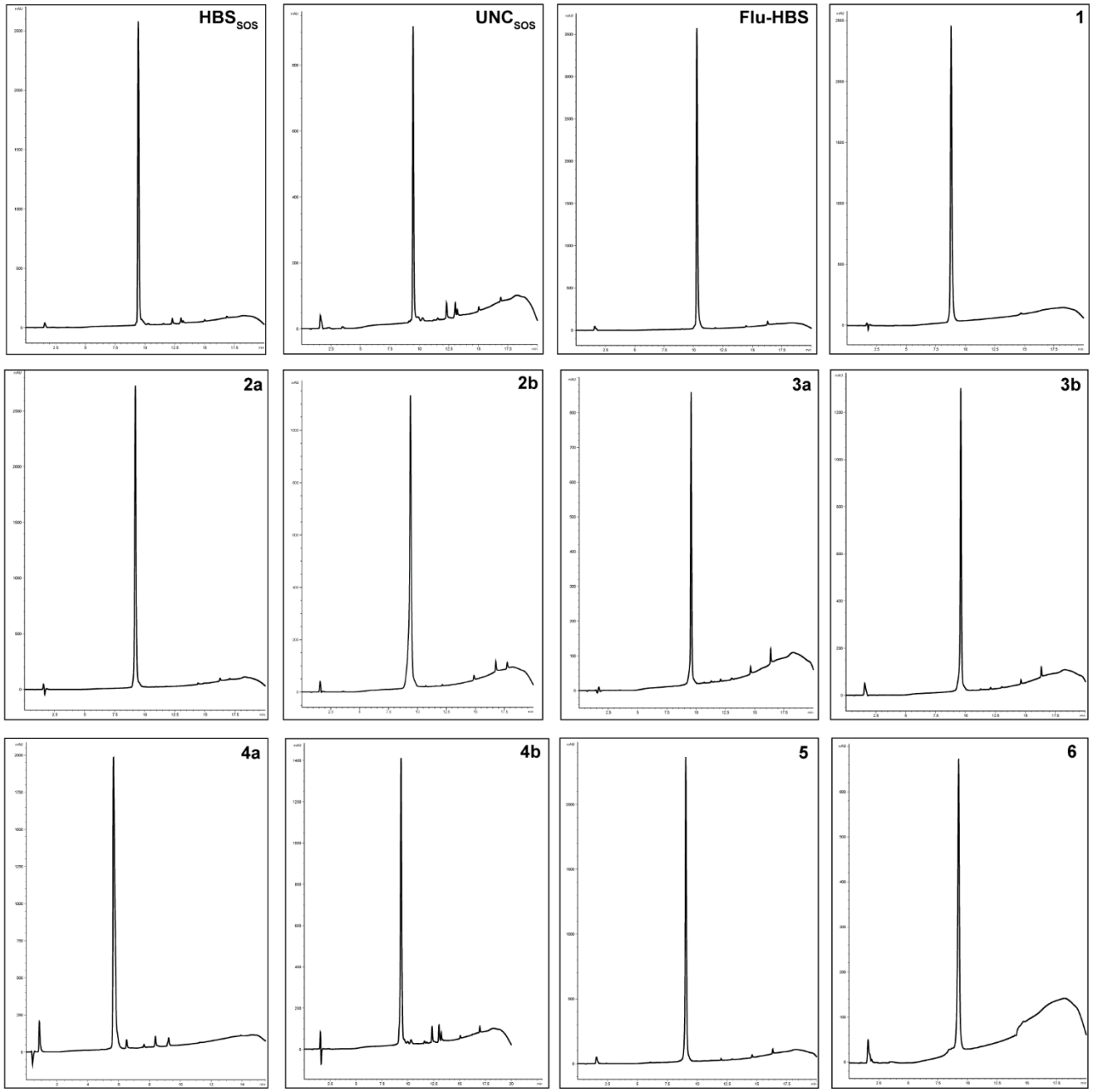


Figure S15. Analytical HPLC traces of αHBS peptides.

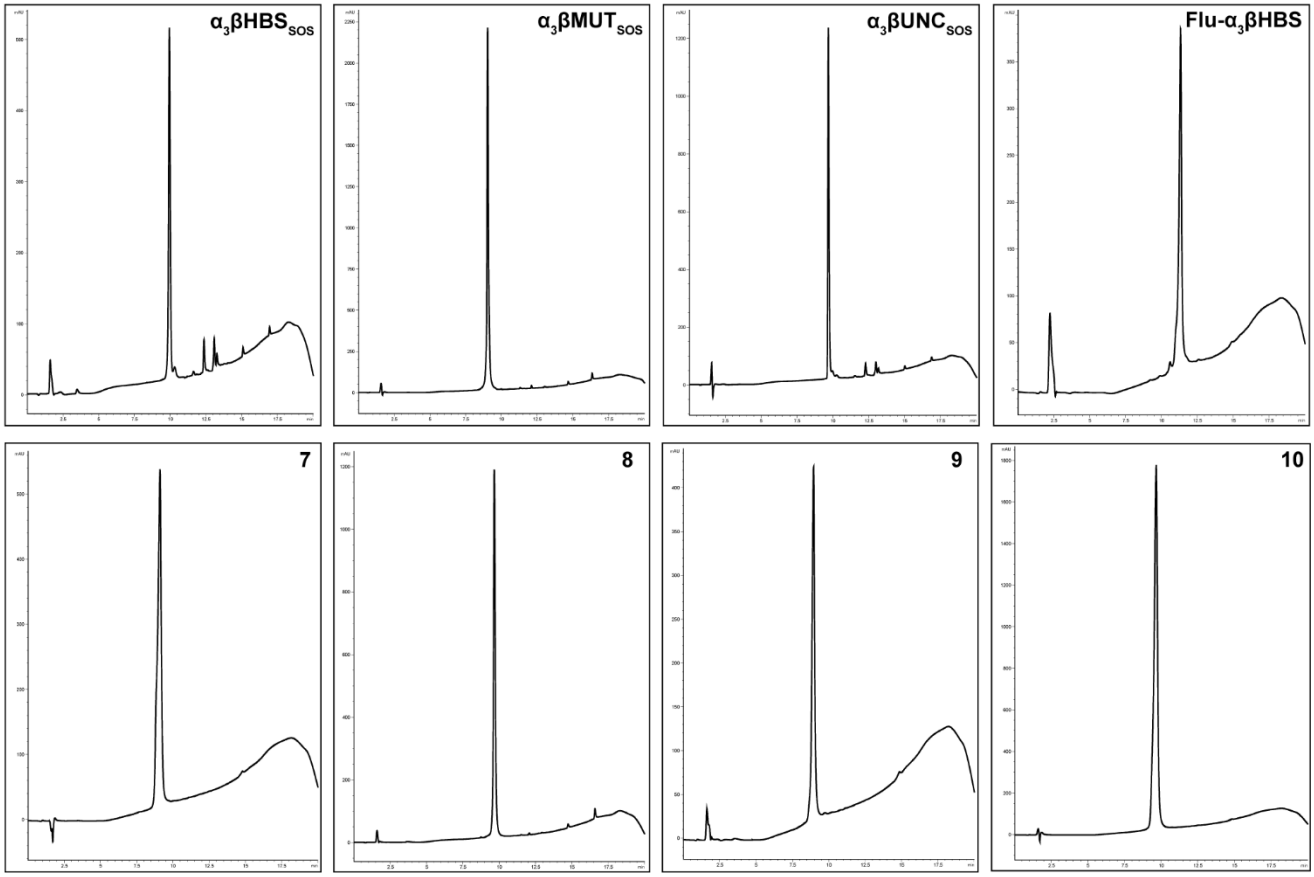


Figure S16. Analytical HPLC traces of $\alpha_3\beta$HBS peptides.

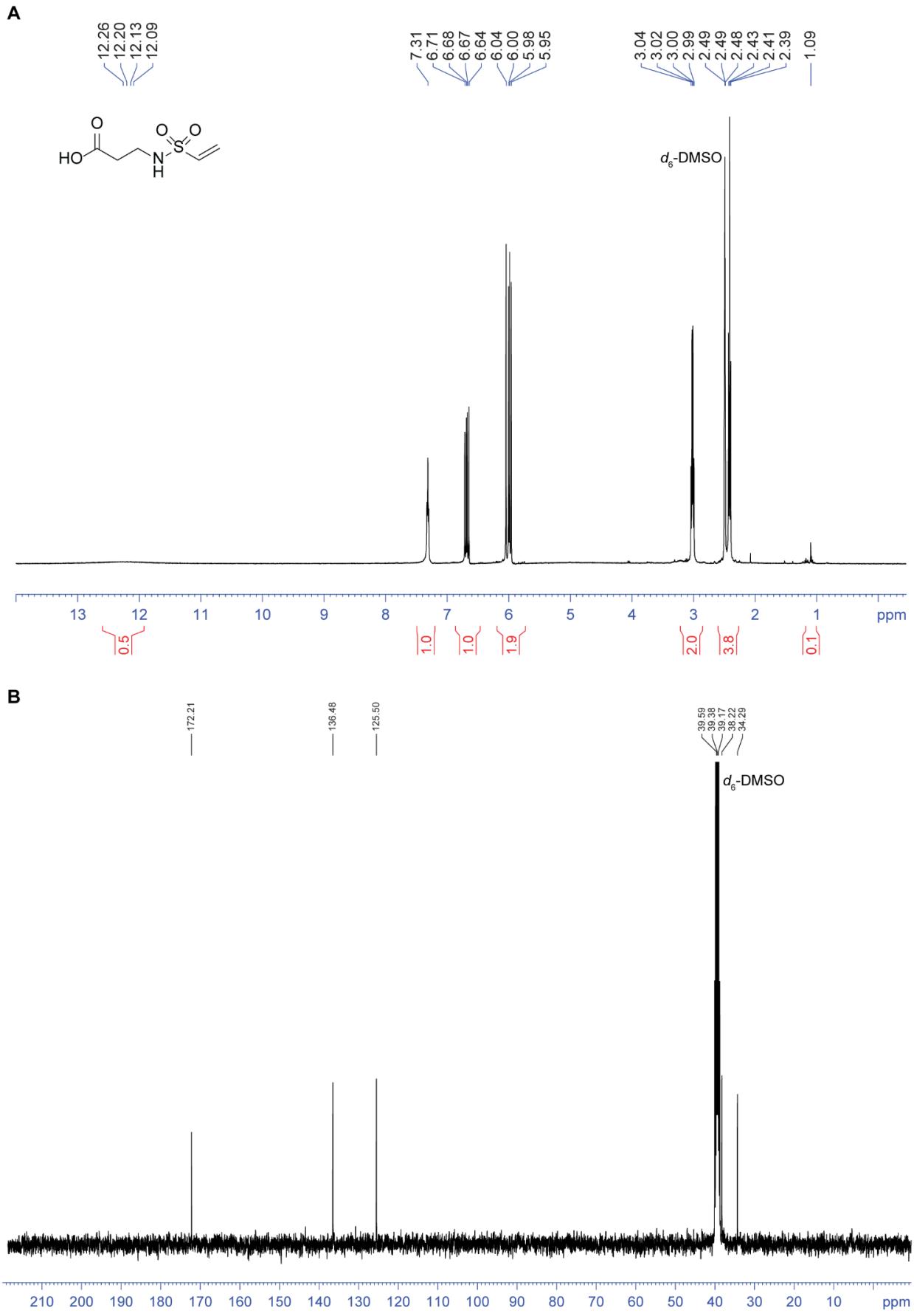


Figure S17. ^{1}H- and ^{13}C-NMR spectra of **VSA1**.

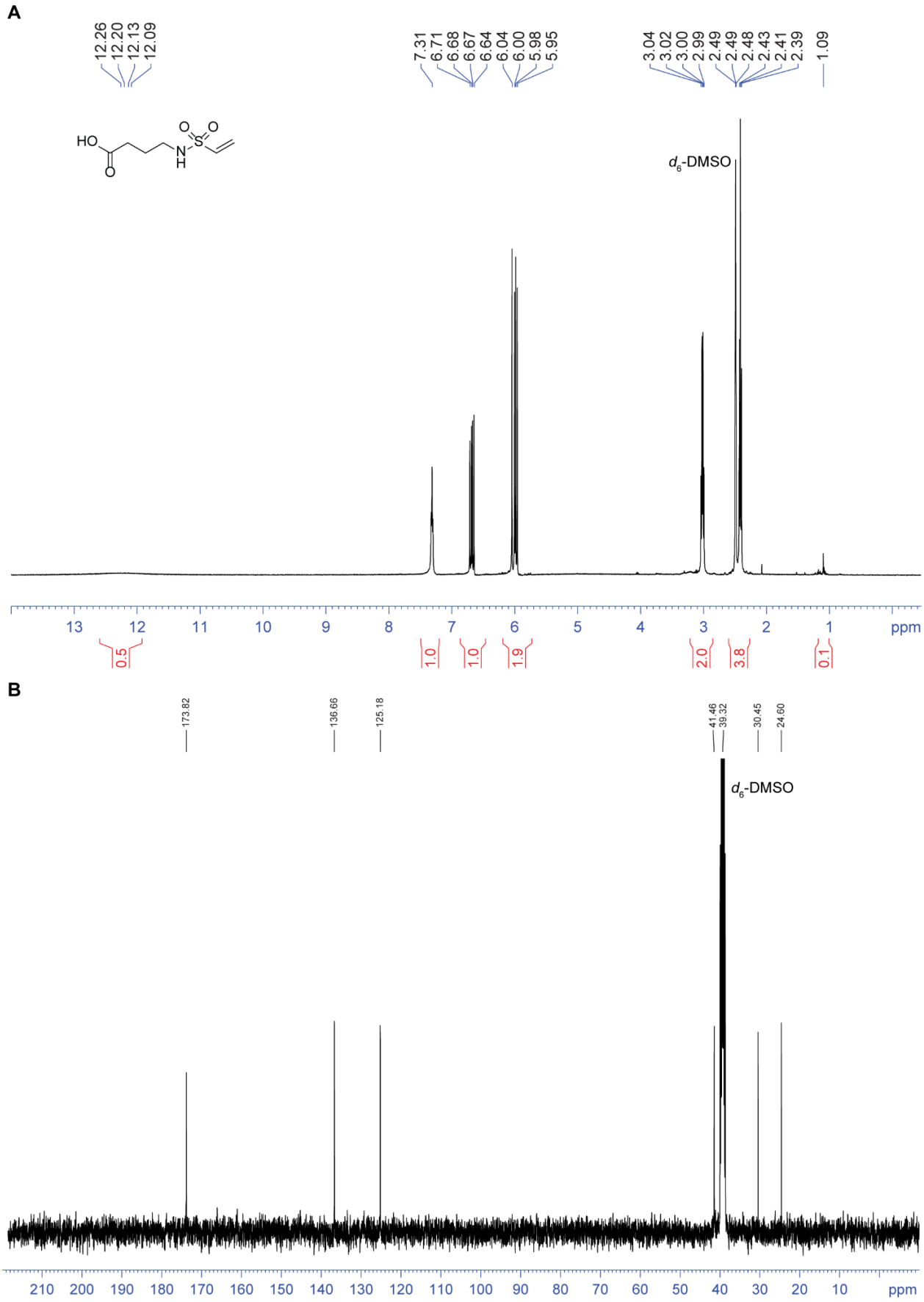


Figure S18. ^{1}H- and ^{13}C-NMR spectra of **VSA2**.

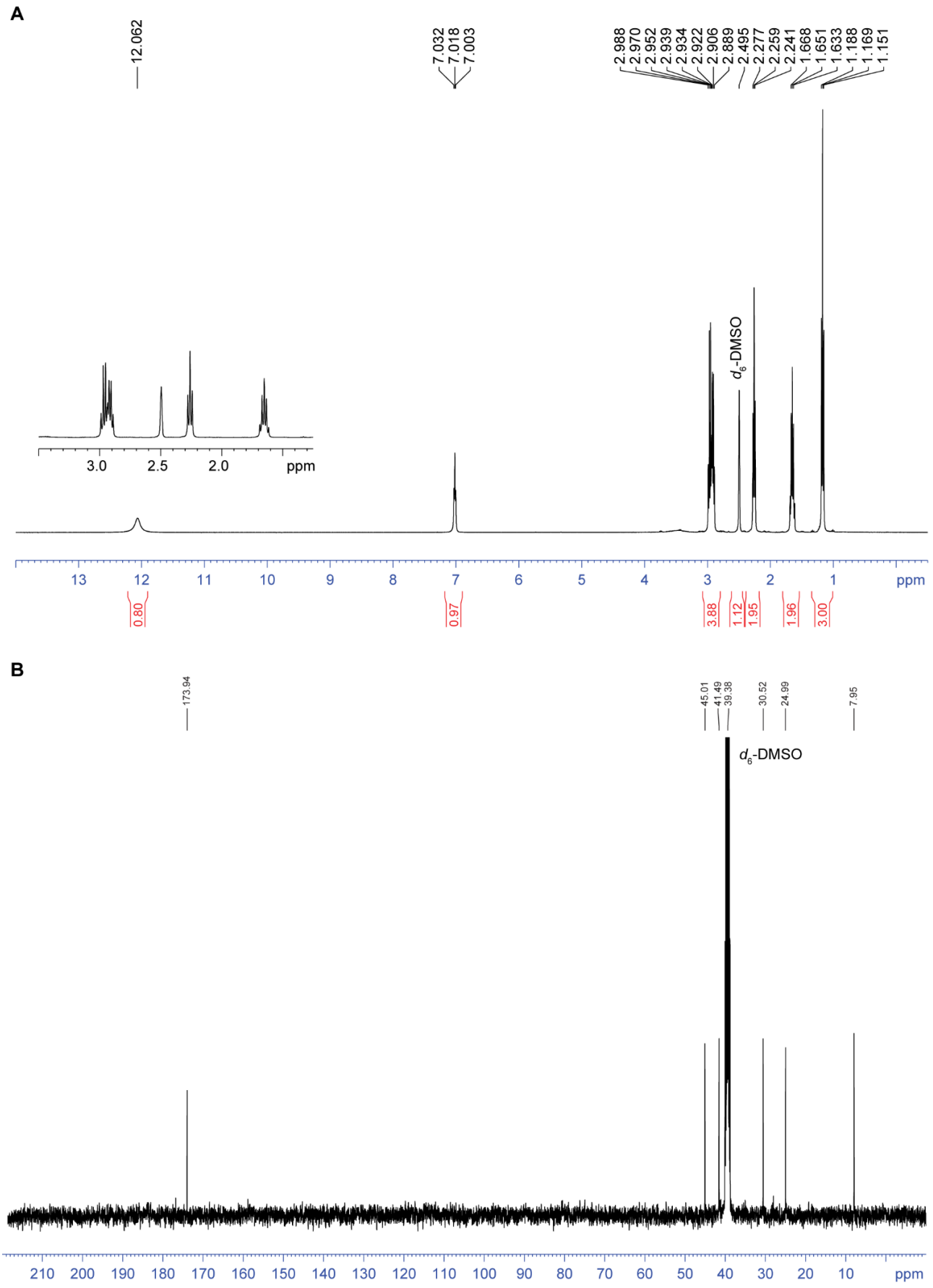


Figure S19. ^{1}H- and ^{13}C-NMR spectra of **EBA**.

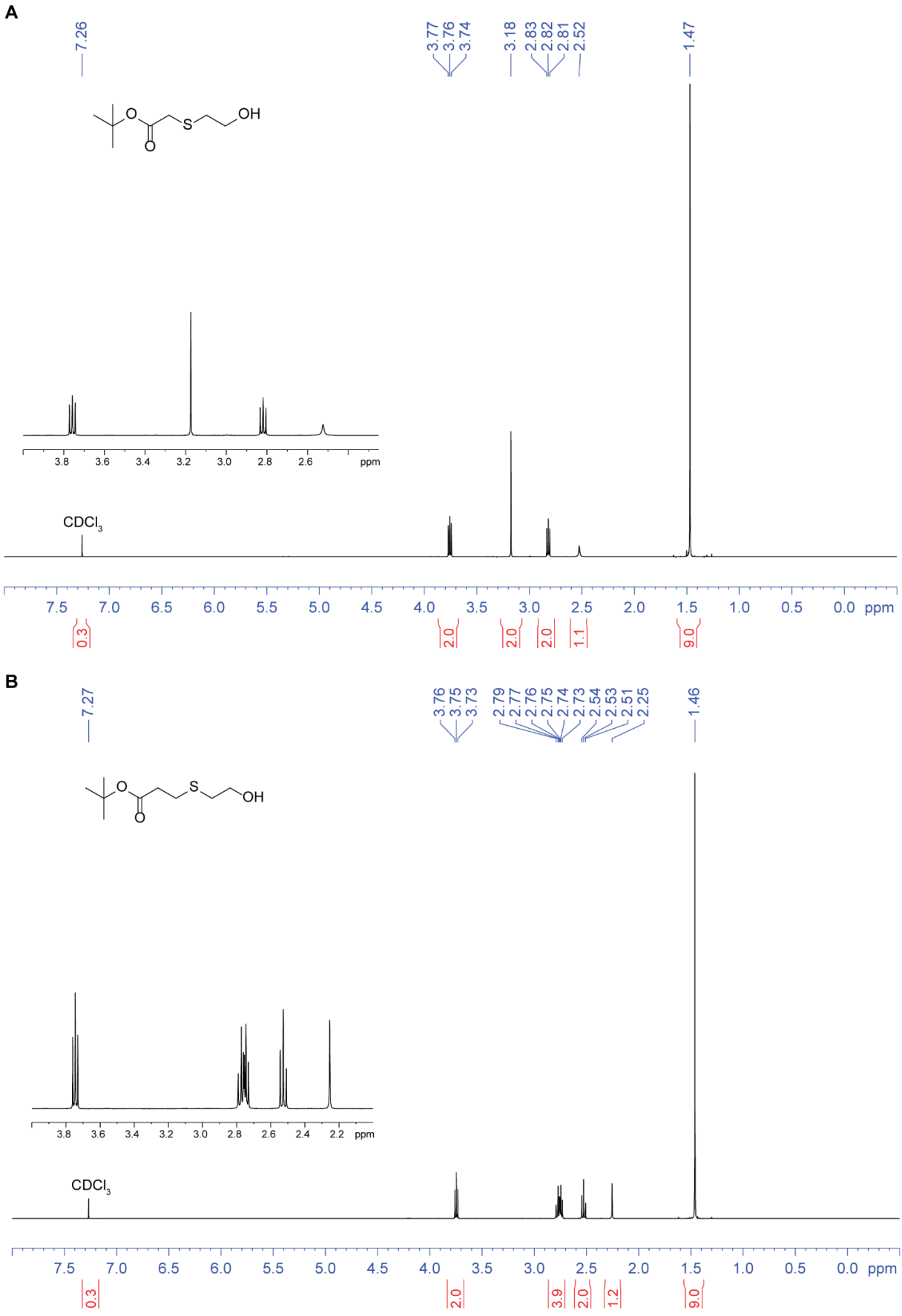


Figure S20. ^{1}H-NMR spectra of **S5** and **S6**.

Figure S21. [1]H-NMR spectra of **S7** and **S8**.

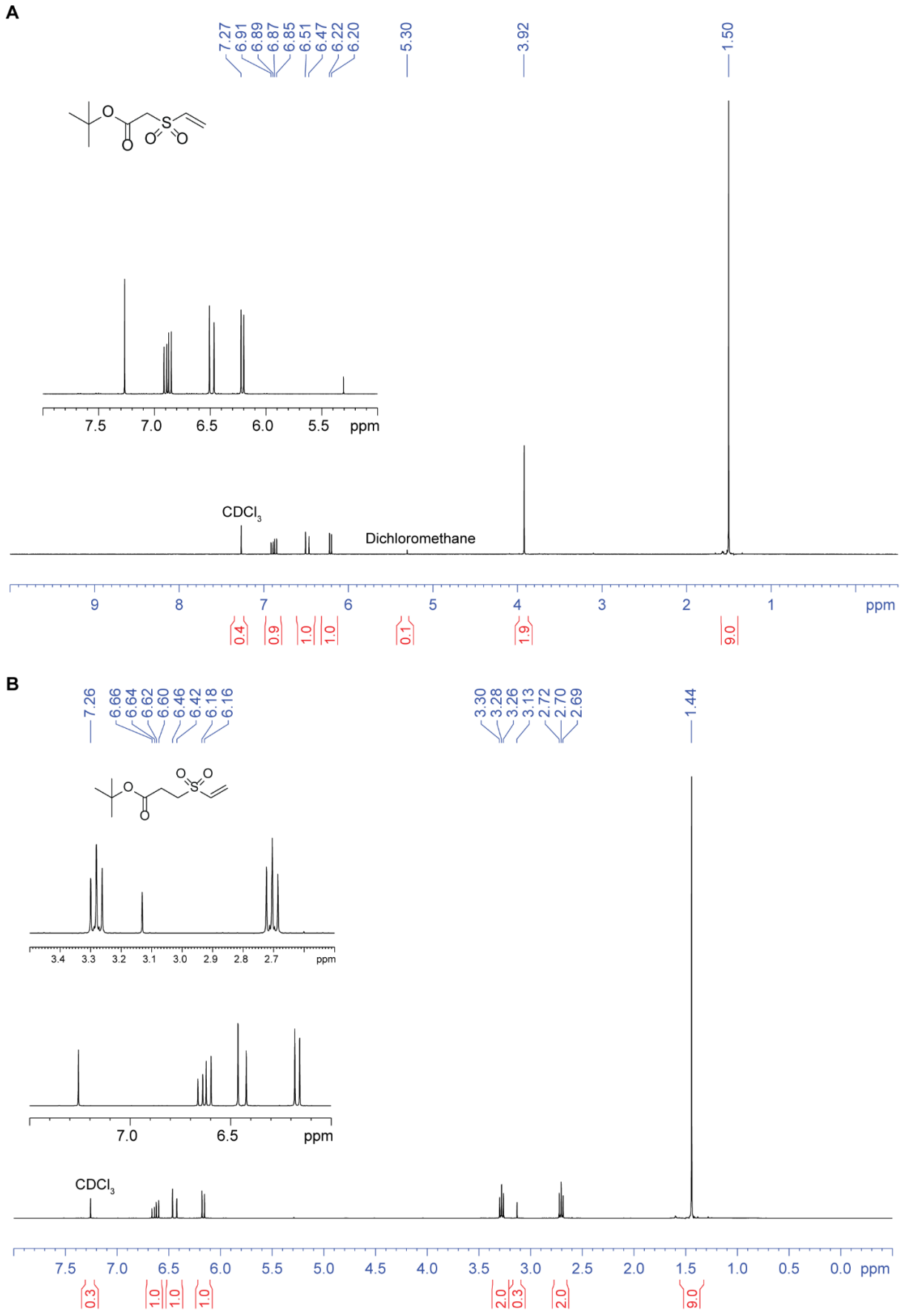


Figure S22. [1]H-NMR spectra of **S9** and **S10**.

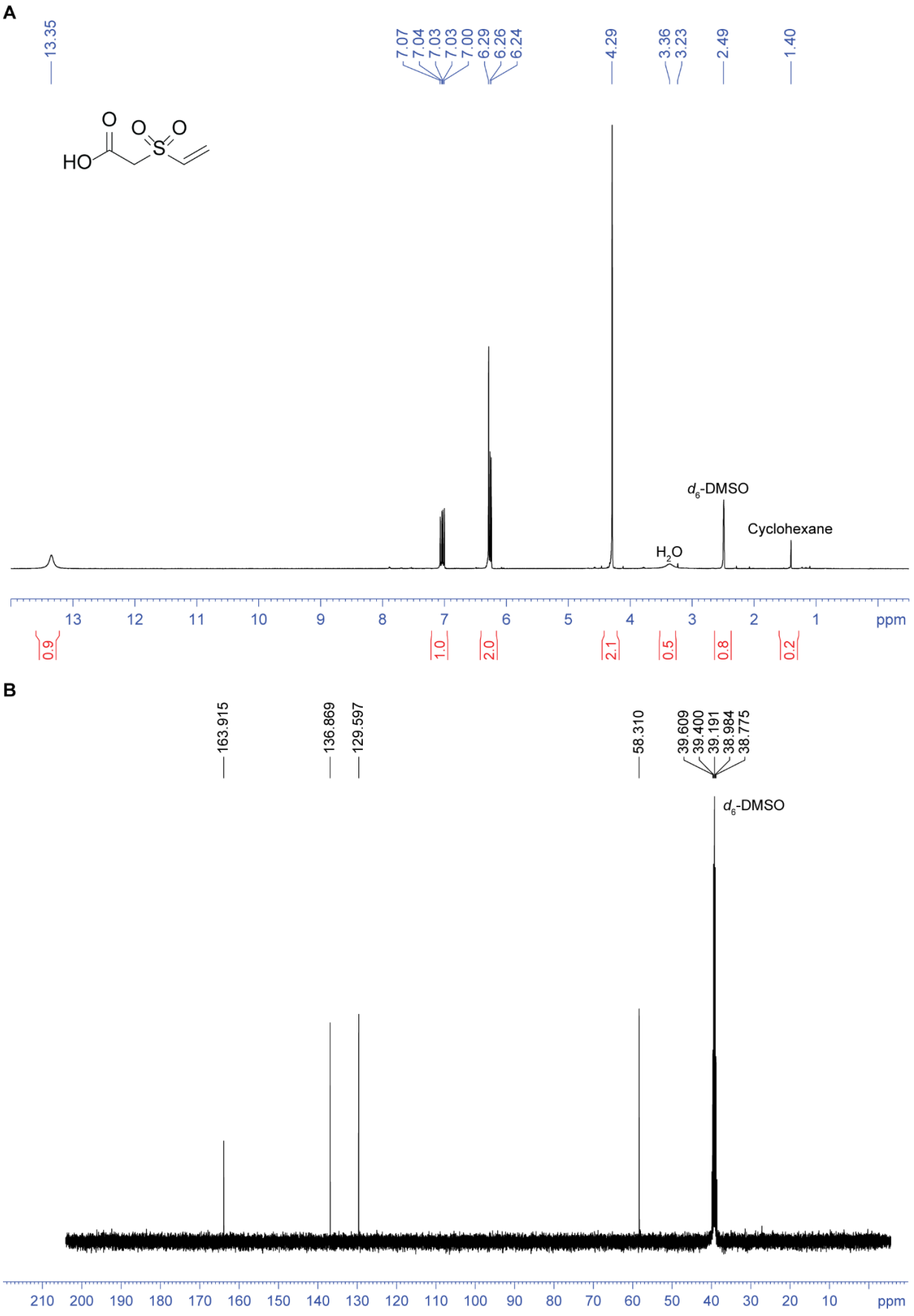


Figure S23. ^{1}H- and ^{13}C-NMR spectra of **VSO1**.

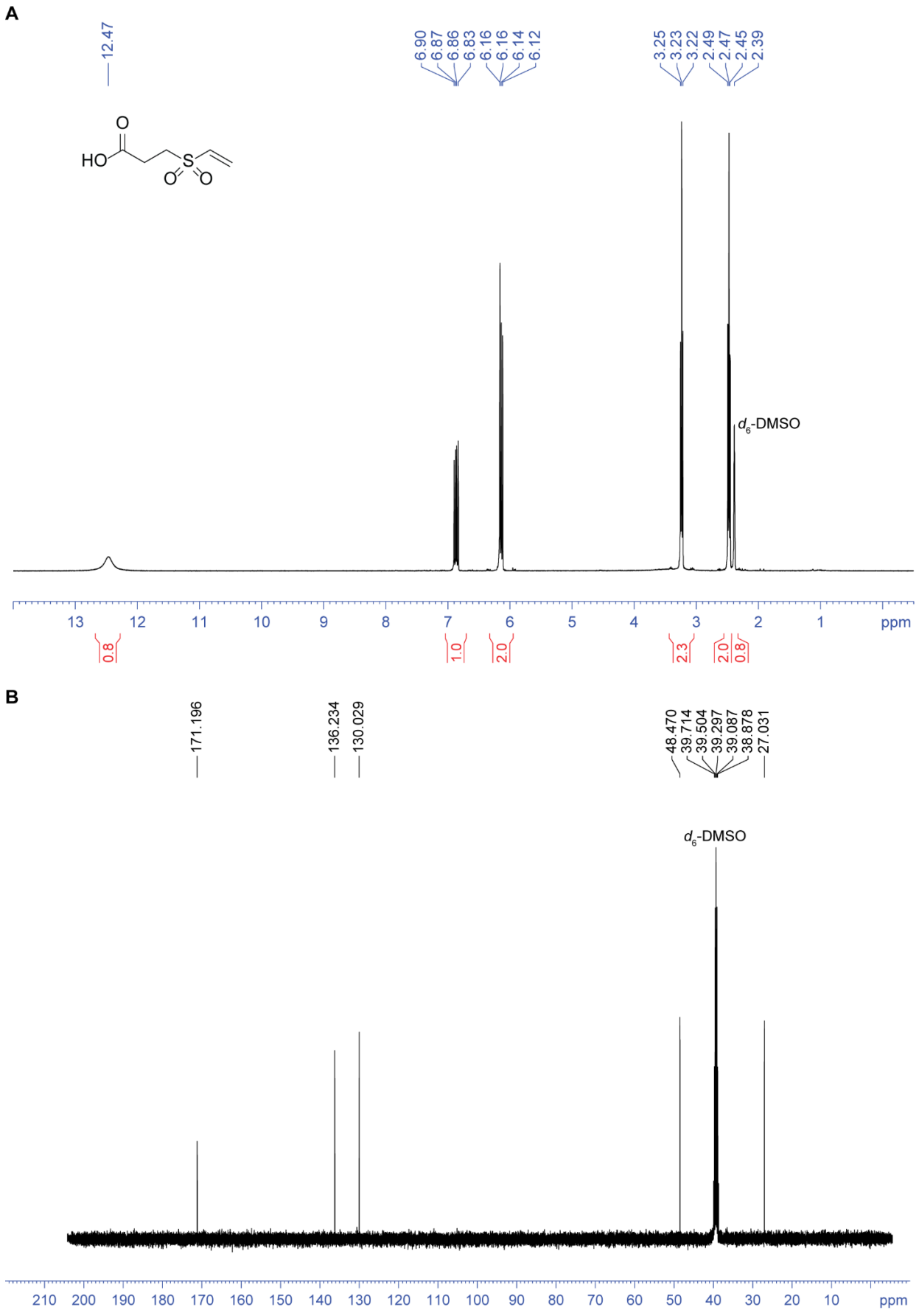


Figure S24. ^{1}H- and ^{13}C-NMR spectra of **VSO2**.

References

(1) Miller, S. E.; Thomson, P. F.; Arora, P. S., *Curr. Protoc. Chem. Biol.* **2014,** *6* (2), 101-16.
(2) Joy, S. T.; Arora, P. S., *Chem. Commun.* **2016,** *52* (33), 5738-41.
(3) Shevchenko, A.; Tomas, H.; Havlis, J.; Olsen, J. V.; Mann, M., *Nat. Protoc.* **2006,** *1* (6), 2856-60.
(4) Ostrem, J. M.; Peters, U.; Sos, M. L.; Wells, J. A.; Shokat, K. M., *Nature* **2013,** *503* (7477), 548-51.
(5) Ito, Y.; Yamasaki, K.; Iwahara, J.; Terada, T.; Kamiya, A.; Shirouzu, M.; Muto, Y.; Kawai, G.; Yokoyama, S.; Laue, E. D.; Walchli, M.; Shibata, T.; Nishimura, S.; Miyazawa, T., *Biochemistry* **1997,** *36* (30), 9109-19.
(6) Patgiri, A.; Joy, S. T.; Arora, P. S., *J. Am. Chem. Soc.* **2012,** *134* (28), 11495-502.
(7) Wang, Y. J.; Nip, A. M.; Wishart, D. S., *J. Biomolec. NMR* **1997,** *10* (4), 373-382.