Supporting Information for

The Role of Olefin Geometry in the Activity of Hydrocarbon Stapled Peptides Targeting Eukaryotic Translation Initiation Factor 4E (eIF4E)

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A. General Materials and Methods

General chemistry methods. RP-HPLC was performed using binary gradients of solvents A and B, where A is 0.1% HCO_2H in water and B is 0.1% HCO_2H in acetonitrile or 0.1% HCO_2H in methanol. Analytical RP-HPLC was performed using an Agilent 1260 Infinity HPLC equipped with a ZORBAX Eclipse SB-C18 column (4.6 × 150 mm; 5 μm) at a flow rate of 1 mL/min, with detection at 214 and 254 nm. Preparative RP-HPLC was performed using an Agilent 1260 Infinity HPLC equipped with a PrepHT SB-C18 column (21.2 × 150 mm; 5 μm) at a flow rate of 18.6 mL/min, with detection at 214 and 254 nm. In all cases, fractions were analyzed off-line using an Agilent Q-TOF HPLC-MS. All peptides were purified to >95% homogeneity. Peptide stock concentrations were determined using amino acid analysis.

General assay and biology methods. SPR was performed using a SensiQ Pioneer instrument and a HisCap chip (three-dimensional hydrogel surface) with 3 channels in series. CD spectra were recorded on a Jasco I-1500 CD-Spectropolarimeter. IC_{50} values were determined using PPI cat-ELCCA as reported; at least quadruplicates were performed for each peptide.[1] Chemiluminescence data was collected on a BioTek Cytation3. Gels were imaged on a ProteinSimple Fluorchem M Gel Imager. MDA-MB-231 and H1299 cells were a kind gift from Dr. Nouri Neamati. HCT116 cells were a kind gift from Dr. Judy Sebolt-Leopold. MDA-MB-231 cells were grown in RPMI-1640 media supplemented with 10% FBS (Atlanta Biologicals), 2 mM glutamine and 1% penicillin-streptomycin. H1299 cells were grown in RPMI-1640 media supplemented with 10% FBS and 2 mM glutamine. HCT116 cells were grown in McCoy's 5A media supplemented with 10% FBS and 2 mM glutamine. All cell lines were authenticated by STR profiling.

Data and statistical analysis. All data was analyzed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, www.graphpad.com). Two-sided t-tests were performed using Prism; equal variance between samples being compared was established. Graphs show mean ± standard deviation as described in the figure legends.

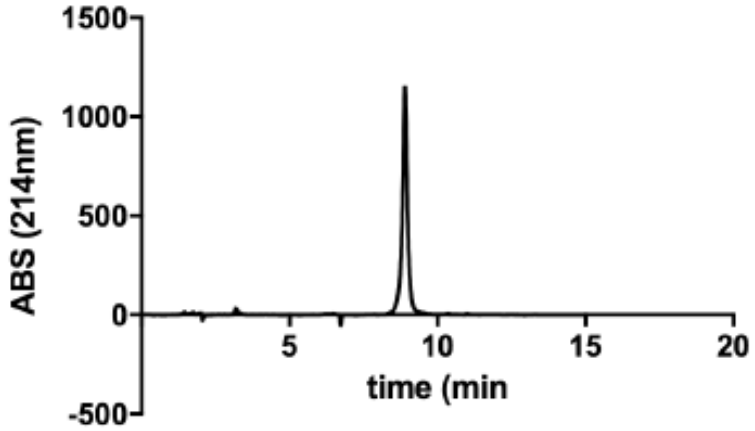
Materials. Fmoc-protected amino acids and Rink amide MBHA resin were purchased from P3 Biosystems and used as received. Fmoc-(*S*)-2-(4-pentenyl)glycine-OH was purchased from Ark Pharm and used as received. eIF4E (9742), 4E-BP1 (9644) and eIF4G (2858) antibodies were purchased from Cell Signaling Technology.

B. Peptide Synthesis

mHCS-4E-BP1 was synthesized on a 0.2-mmol scale in a 20-mL fritted syringe using MBHA Rink amide resin (0.2 – 0.4 mmol/g loading). In brief, the resin was swelled for 20 min at 25 °C in 1:1 solution of DMF and DCM. FMOC groups were removed following the addition of a 20% piperidine/DMF solution (10 mL) and gentle agitation for a total of 20 min at 25°C. After each FMOC deprotection and amino acid couplings, the resin was thoroughly washed with NMP, CH_2Cl_2, and DMF. Amino acid couplings were performed by addition of amino acid (1 mmol) pre-activated with HBTU (0.9 mmol), *N*,*N*-diisopropylethylamine (2 mmol) in NMP (5 mL), and agitated for 2–3 h at 25 °C. The peptide was stapled on resin by bubbling nitrogen gas in a DCE solution of Grubbs I catalyst at 6 mM for at least two hours.[2] The reaction was monitored through 10–20 mg resin test cleavages via LC–MS. The procedure was repeated until the reaction went to complete conversion, usually after three replicates. After stapling, the *N*-terminus was acetylated, and the peptide was

cleaved from the resin using TFA/thioanisole/water/triisopropylsilane (90:4:4:2) for 4 h at 25 °C. The resulting solution was added to glacial ether (~200 mL) for peptide precipitation. The precipitates were then collected, dissolved, and purified via RP-HPLC. Fractions containing the desired peptide were confirmed by LC-MS, lyophilized, re-dissolved in 1:1 acetic acid/water, and lyophilized again. The peptide was dissolved in 33% DMF/water and peptide stock concentration was determined via amino acid analysis.

mHCS-4E-BP1. HRMS (ESI+) calcd for 2048.08, found 2049.10 [M+H].



C. Circular Dichroism (CD) Spectroscopy

Peptides were dissolved in buffer (5 mM sodium phosphate buffer, pH 7.4) at concentrations between 25–100 μM. Data was collected between 180–260 nm with a step resolution of 0.1-nm and a speed of 50 nm/sec. Five accumulations were taken with the response time set to 1 s, bandwidth to 5 nm, and pathlength of 0.1 cm. The α-helical content of each peptide was calculated by dividing the mean residue ellipticity $[\varphi]_{222obs}$ by the theoretical $[\varphi]_{222}$ for a helical acetylated peptide of equivalent length (see equations below).[3]

Equation 1: $\%_H = (([\theta]_{obs222} - [\theta]_C)/([\theta]_{\infty 222} - [\theta]_C)) * 100$

Equation 2: $[\theta]_C = 2220 - 53T$

Equation 3: $[\theta]_{\infty 222} = (-44000 + 250T)(1 - \frac{k}{N_p})$

$\%_H$ = percent α-helix content, $[\theta]_{obs222}$= observed molar ellipticity at 222 nm

$[\theta]_C$= Random coil molar ellipticity, $[\theta]_{\infty 222}$= Infinite molar α-helix molar ellipticity

T = temperature in degrees Celsius, k = finite length correction

N_p = Number of peptide units

D. Surface Plasmon Resonance (SPR)

SPR was performed using a SensiQ Pioneer instrument and a HisCap chip (three-dimensional hydrogel surface) with 3 channels in series. Channel 1 was activated with $NiCl_2$ and 10×His-eIF4E[4] was immobilized by injection into the running buffer (10 mM HEPES-NaOH, pH 7.5, 225 mM NaCl, 10 mM imidazole, 0.1% Tween-20, and 5% Glycerol). The concentration was adjusted to about 600 RU. 50 µL of peptide was injected at 50 µL/min over a range of 10 concentrations in duplicate and random order (188 nM–4.0 µM) at 25 °C. The chip was regenerated in between each cycle with an injection 500 mM EDTA followed by reactivation with $NiCl_2$ and reimmobilization of 10×His-eIF4E. The association rate (k_a), dissociation rate (k_d), and binding constant (K_d) were obtained by fitting to a 1:1 interaction model using global data analysis with the Qdat software, which employs the Levenberg-Marquardt non-linear regression algorithm. Triplicate analyses were performed.

E. Cellular Assays

The cap pull-down assay was carried out as previously described.[5,6] Briefly, cells were grown in 6-cm dishes and treated with peptides (dissolved in 33% DMF/H_2O) for 6 h. Cells were then lysed in cap pull-down buffer (50 mM HEPES-KOH (pH 7.5), 150 mM KCl, 1 mM EDTA, 2 mM DTT and 0.1% Tween 20) containing protease inhibitors. Cell lysate was centrifuged at 15,000 rpm for 25 min. The supernatant was subsequently incubated for 2 h at 4 °C with m^7GDP-agarose resin. Beads were washed 3× with the cap pull-down buffer, 1× with TBS and 1× with water. Proteins were eluted by boiling in 2× LDS sample buffer for 10 min at 70 °C, resolved on a 4–12% Bis-Tris gel, and transferred to PVDF membrane in Towbin's Buffer. The membrane was blocked in 5% milk for 1 h at 25 °C, and then incubated with a primary antibody (overnight at 4 °C) and secondary antibody (1 h at 25 °C). Proteins were visualized by autoradiography. In all cases, the eIF4E level was used for normalization. Biological replicates were performed. Figures were formatted in Adobe Illustrator.

F. Supplemental Figures

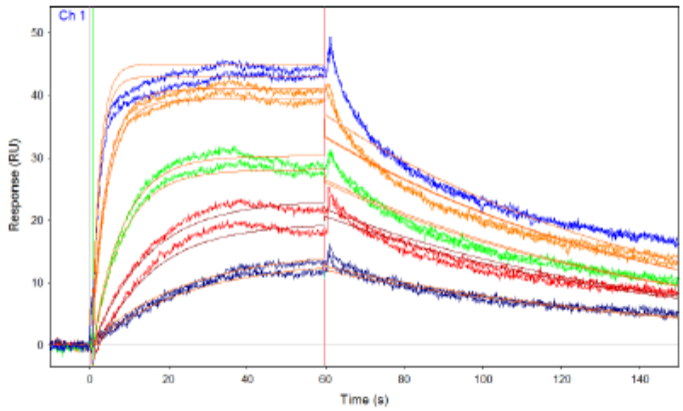


Figure S1. Representative sensorgram for **mHCS-4E-BP1** at 25 °C from duplicate runs at varying concentrations (62.5, 125, 250, 500 nM).

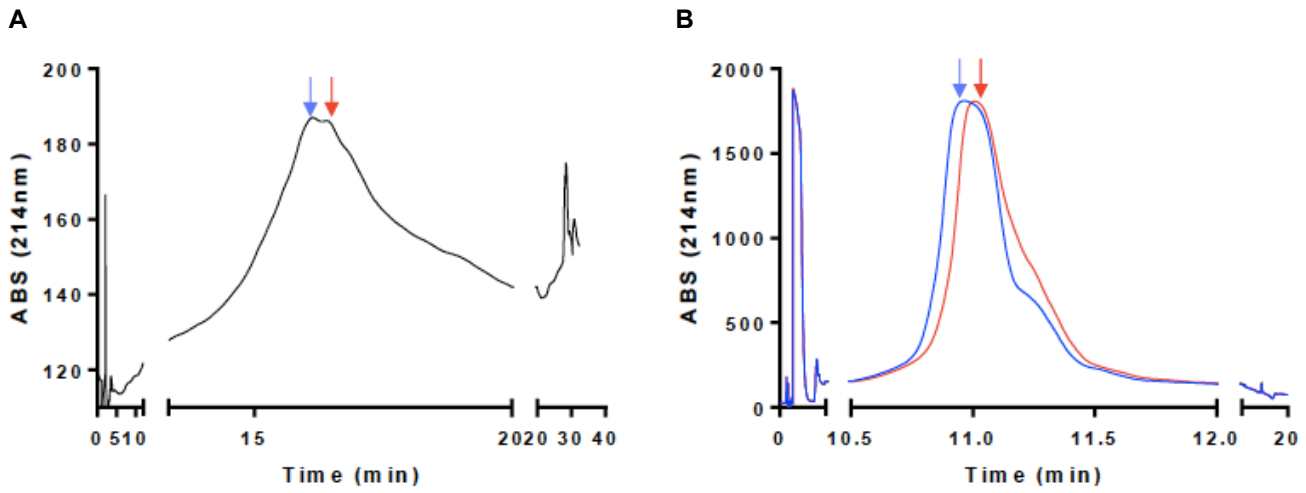


Figure S2. HPLC traces of **mHCS-4E-BP1** as (A) a mixture or (B) purified olefin isomers (blue = **mHCS-4E-BP1-i**; red = **mHCS-4E-BP1-ii**).

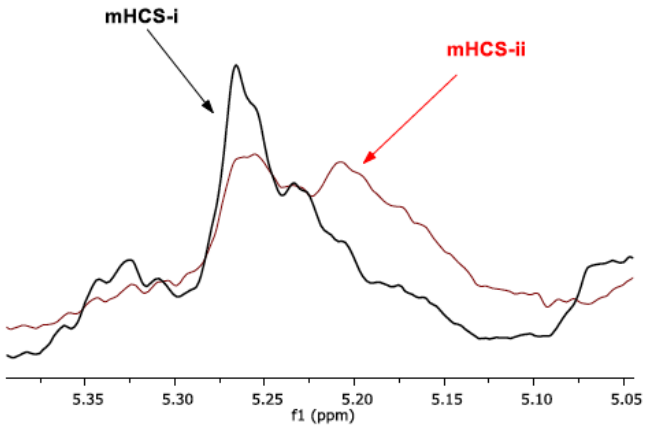


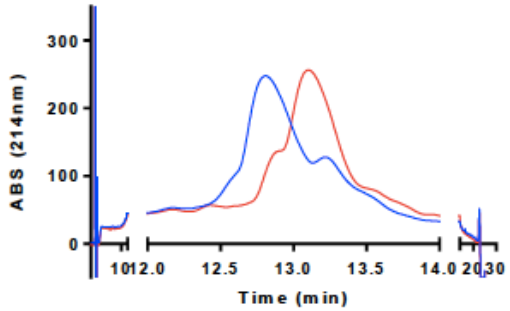
Figure S3. Overlaid ^{1}H NMR spectra of **mHCS-4E-BP1-i** (black) and **mHCS-4E-BP1-ii** (red) in D_2O using a Varian NMR 500 MHz instrument. Significant water contamination and poor solubility yielded unresolvable olefinic peaks for both isomers. Of note, experiments in DMSO to aid solubility were also attempted; however, many problems were encountered due to peptide oxidation (Met, Cys) in this solvent precluding analysis.

A

Peptide	Sequence
trunc-4E-BP1	AcNH-^{51}R I I Y D R K F L M E C R^{63}-CONH$_2$
trunc-mHCS-4E-BP1	AcNH-^{51}R I I Y D R # F L M # C R^{63}-CONH$_2$

= (*S*)-pentenylglycine

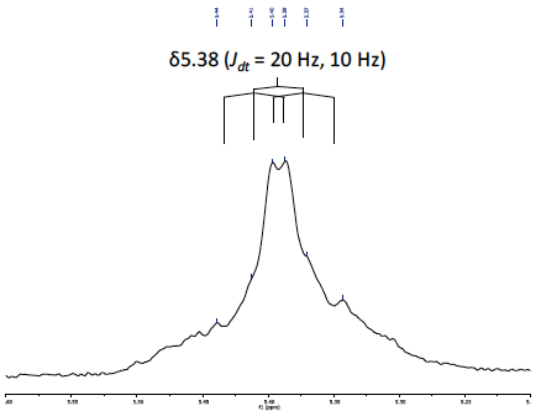
B



C

Peptide	Helicity (%)	IC_{50} (nM)	K_d (nM)
trunc-4E-BP1	20	60 - 84	17 ± 0.2
trunc-mHCS-4E-BP1-i	7	177 - 343	35 ± 5
trunc-mHCS-4E-BP1-ii	8	178 - 267	35 ± 6

D



E

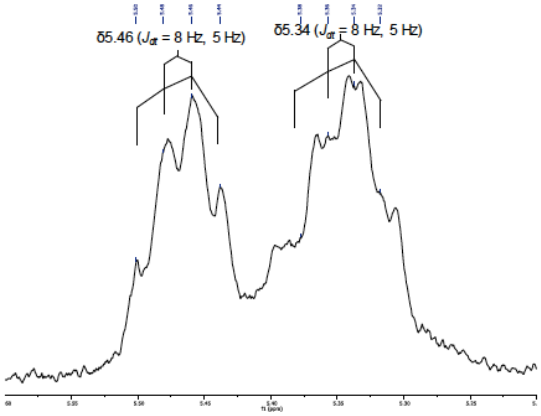


Figure S4. Truncated **mHCS-4E-BP1** (**trunc-mHCS-4E-BP1**). (A) Sequence. (B) HPLC traces of **trunc-mHCS-4E-BP1** isomers **i** (blue) and **ii** (red). (C) IC_{50} values presented as 95% confidence intervals for **trunc-4E-BP1**, **trunc-mHCS-4E-BP1-i** and **trunc-mHCS-4E-BP1-ii**. Measurements were made using PPI cat-ELCCA. (D) and (E) ^{1}H NMR spectra of olefin regions in **trunc-mHCS-4E-BP1-i** and **trunc-mHCS-4E-BP1-ii**. Spectra were collected in D_2O using a Varian NMR 500 MHz instrument.

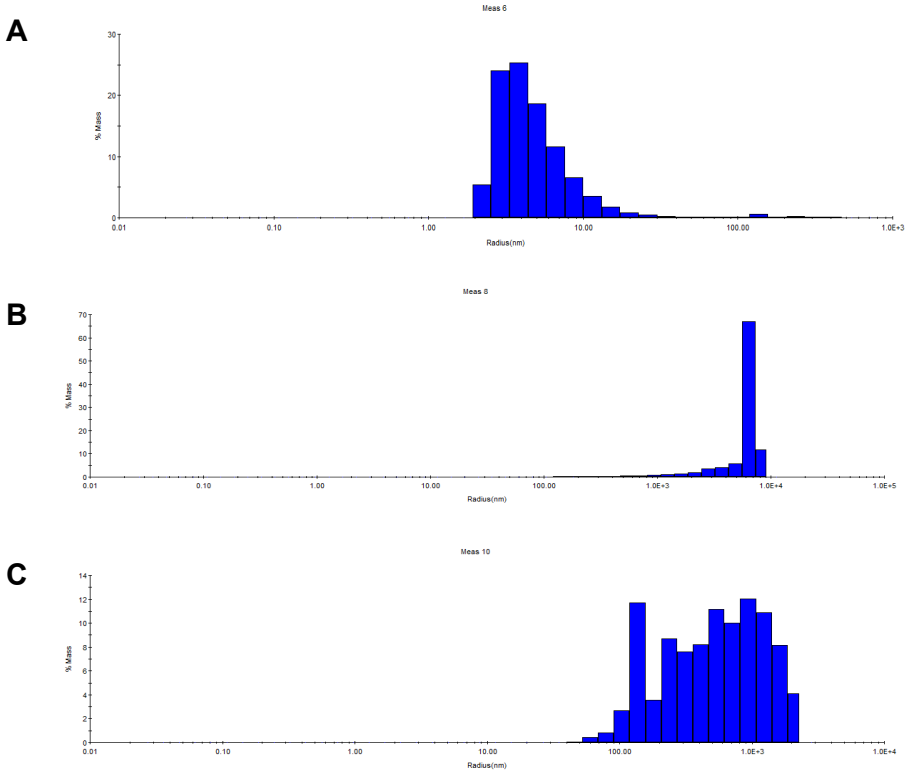


Figure S5. Dynamic light scattering (DLS) experiments with mHCS-4E-BP1. The following radii were measured at varying concentrations in water: (A) 30.5 µM, radius = 63 nm; (B) 61 µM, radius = 5900 nm; and (C) 122 µM, radius = 1300 nm.

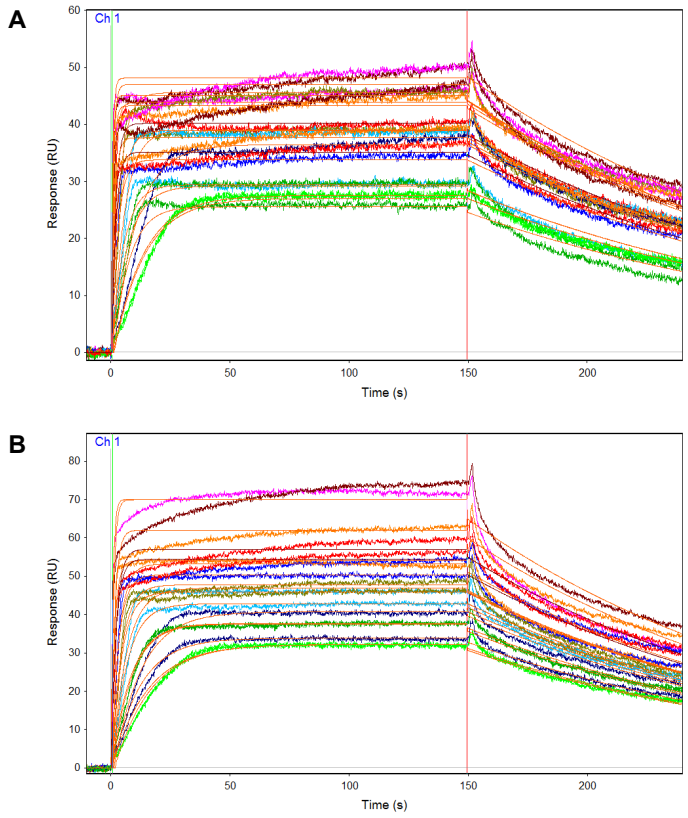


Figure S6. Representative sensorgrams for (A) **mHCS-4E-BP1-i** and (B) **mHCS-4E-BP1-ii** at 25 °C from duplicate runs at varying concentrations (62.5, 125, 250, 500 nM).

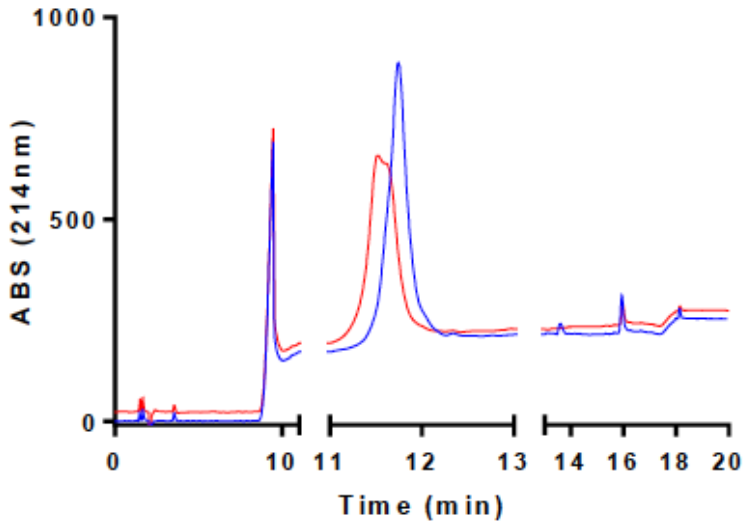


Figure S7. HPLC traces of **mHCS-4E-BP1** prepared using Grubbs I (red) or Grubbs II (blue) catalysts.

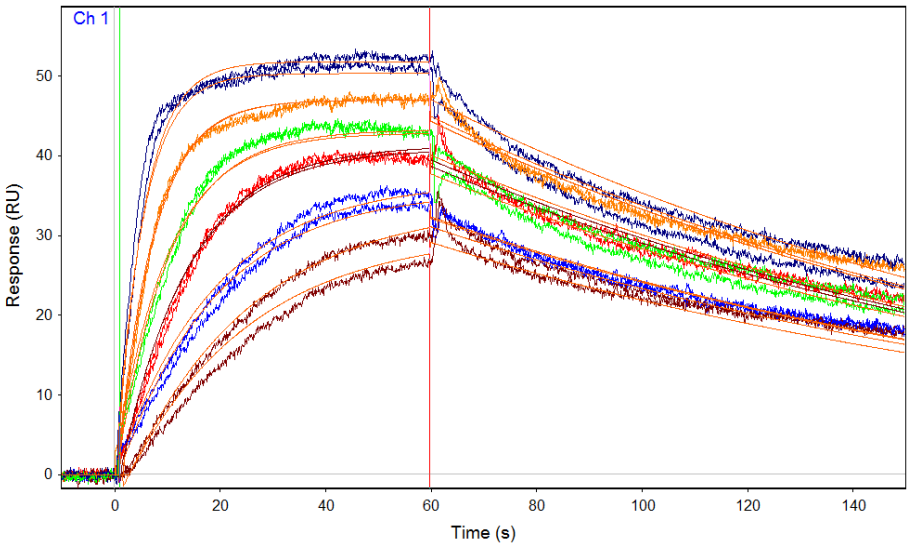


Figure S8. Representative sensorgram for **mHCS-4E-BP1** prepared using Grubbs II at 25 °C from duplicate runs at varying concentrations (62.5, 125, 250, 500 nM).

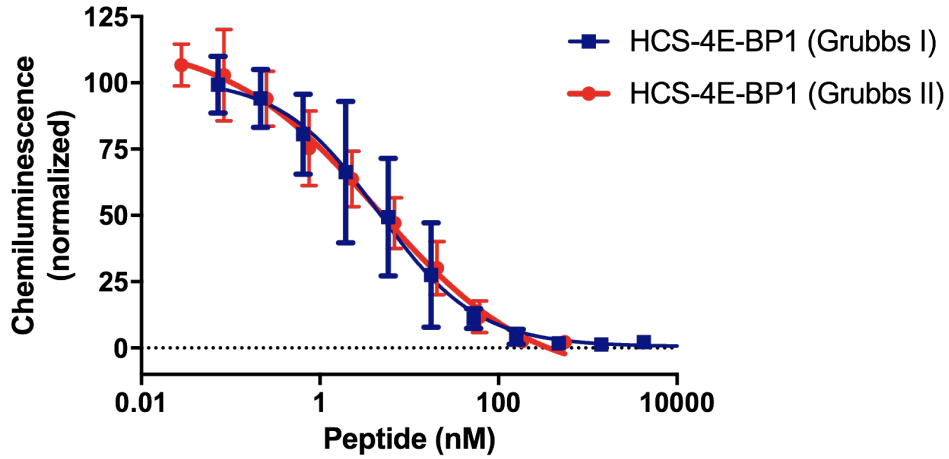


Figure S9. IC_{50} curves for **HCS-4E-BP1** prepared using either Grubbs I or II catalysts. Measured IC_{50} values as 95% confidence intervals were: 3.1–6.7 nM for Grubbs I and 2.7–7.2 nM for Grubbs II.

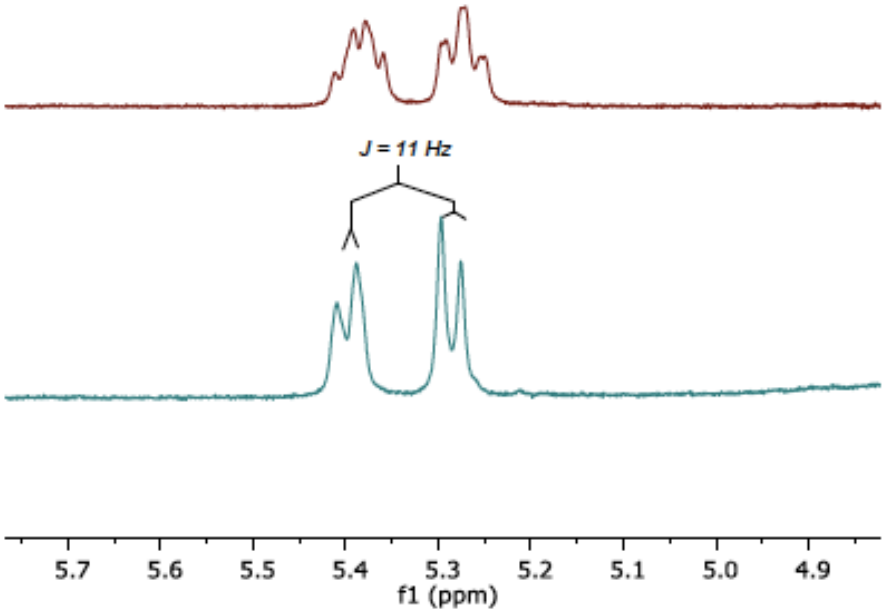


Figure S10. Stacked ^{1}H NMR of **sTIP–04** (red) and homo-decoupling of the protons at a chemical shift of 2.0–1.8 ppm (blue) during the acquisition. The resulting coupling constant of 11 Hz suggests that **sTIP–04** is a *cis* isomer.

G. References

1. J. M. Song, A. Menon, D. C. Mitchell, O. T. Johnson, A. L. Garner, *ACS Comb. Sci.* **2017**, *19*, 763–769.
2. Y. W. Kim, T. N. Grossman, G. L. Verdine, *Nat. Protoc.* **2011**, *6*, 761–771.
3. N. E. Shepherd, H. N. Hoang, G. Abbenante, D. P. Fairlie, *J. Am. Chem. Soc.* **2005**, *127*, 2974–2983.
4. E. E. Gallagher, J. M. Song, A. Menon, L. D. Mishra, A. Chmiel, A. L. Garner, *J. Med. Chem.* **2019**, *62*, 4967–4978.
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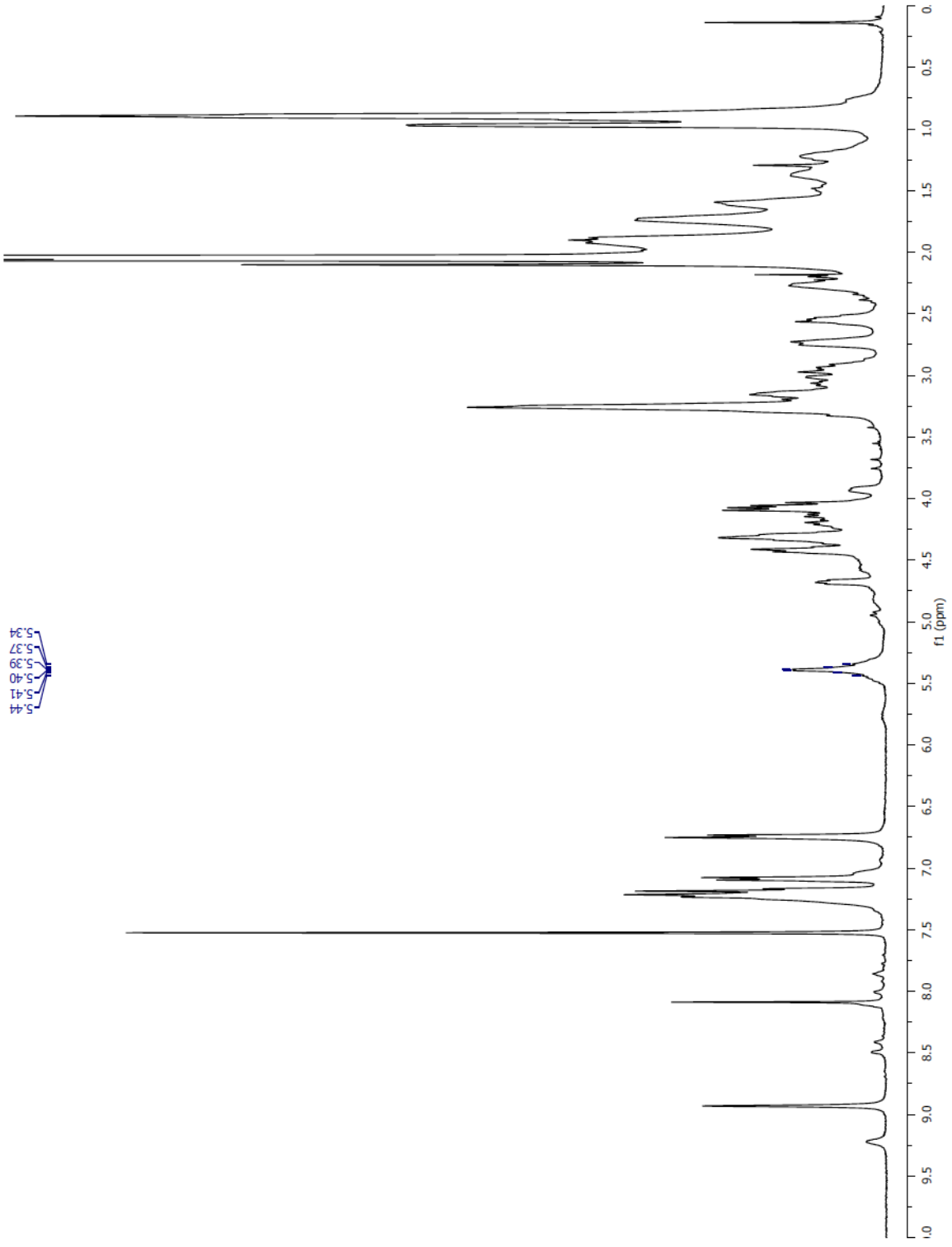
6. T. Kaur, A. Menon, A. L. Garner, *Eur. J. Med. Chem.* **2019**, *166*, 339–350.

H. Trunc-mHCS-4E-BP1-i and -ii NMR Spectra

Trunc-mHCS-4E-BP1-i (D_2O, 500 MHz):



Trunc-mHCS-4E-BP1-ii (D_2O, 500 MHz):