Supplementary Information (SI) for Organic & Biomolecular Chemistry.
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Supporting Information

A β-hairpin mimic built on a fluorinated isoxazoline-$\beta^{2,2}$-amino acid as a modulator of Tau protein aggregation

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I. General procedures for syntheses and physical characterizations

NMR spectra for small molecules were recorded on an ultra-field Bruker AVANCE 200 (^{1}H, 200 MHz, ^{13}C, 50 MHz, ^{19}F 188 MHz), and 300 (^{1}H, 300 MHz, ^{13}C, 75 MHz). NMR experiments for **β-FH1** and **β-FH2** were recorded on a 600MHz Bruker Avance-Neo, at COSPECT laboratory of the University of Milano. Chemical shifts δ are in ppm with the solvent resonance as the internal standard (^{1}H NMR, CD_3OH: δ = 3.31 ppm; ^{13}C NMR, CD_3OD: δ = 49.00 ppm). ^{19}F NMR chemical shifts were referenced to TFA as internal standard (in CD_3OD: δ = - 77.77 ppm). The following abbreviations are used: singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quintuplet (qt), multiplet (m), broad multiplet (brm), and broad singlet (brs), broad doublet (brd).

The following spectra were performed using a water suppression sequence by excitation esculping: homonuclear 2D [^{1}H-^{1}H] TOCSY, 2D [^{1}H-^{1}H] NOESY and 2D [^{1}H-^{1}H] ROESY were acquired using 16 for scans per t1 increments, a spectral width (sw) of 7200 Hz along both dimensions, 2.0 s relaxation delay and 2048 x 512 complex points in t2 and t1 respectively. In particular, 2D [^{1}H-^{1}H] TOCSY spectra were acquired using a mixing time of 80 ms; 2D [^{1}H-^{1}H] NOESY experiments were recorded with a mixing time of 200 ms; 2D [^{1}H-^{1}H] ROESY spectra were measured with a mixing time of 200 ms.

CD experiments were performed on a Jasco J-820 spectropolarimeter with a 0.1 cm quartz cuvette. The spectra were recorded from 190 to 250 nm with a 0.2 nm step and 2 s collection time per step, taking four averages and using a sensitivity of 100 mdeg and a scanning speed of 50 nm/min. The spectrum of the solvent was subtracted to eliminate interference from cell, solvent, and optical equipment. The CD spectra were plotted as mean residue ellipticity θ (degree*cm^{2}*dmol^{-1}) versus wavelength λ (nm). Noise-reduction was obtained using a Fourier-transform filter program.

ATR-FTIR spectroscopy measurements were made on a Perkin Elmer Spotlight 400 FT-IR spectrophotometer equipped with a diamond crystal attenuated total reflectance (ATR) accessory. All the sample were analysed at room temperature in the solid state. A total of 32 scans were performed for all measurements with a resolution of 4 cm^{-1} in the 4000-650 cm^{-1} spectral region. Transmittance has been recorded and transformed into absorbance [A = 2–log(T)]. Data processing was performed using solver in excel software (Microsoft). Deconvolution of the spectra was done in the spectral range of 1600–1700 cm^{-1}. The deconvoluted spectra were fitted with Gaussian band profiles. The positions and number of the components used as an input file for the curve-fitting function were obtained from the deconvoluted spectra. The quality of the fitting was estimated by the standard deviation and error squared.

HRMS were obtained using a TOF LCT Premier apparatus (water) with an electrospray ionization source.

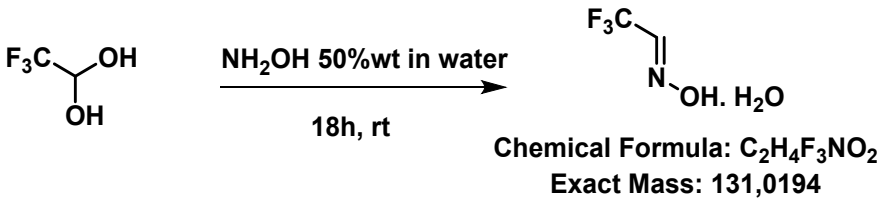
The purity of peptides and peptidomimetics determined by **RP-HPLC-QUAD** were made on an Agilent HPLC 1260 Infinity 600 bars at 215.8 nm. **Column A**: Cortecs T3 (Waters) – 2.7 µm – 2.1 x 50 mm. **Mobile phase**: ACN/H_2O + 0.1% FA. All the LC-MS spectra are presented with their **TIC** (**shown in black**) alongside the corresponding **UV spectra at 215.8 nm** (**shown in blue**).

Purification

Semi-preparative RP-HPLC purifications were performed on Agilent 1260 Infinity II 400 bars. **Column 1**: Pursuit C18 (10 mm x 250 mm-5µm), mobile phase: ACN/H_2O + 0.1% FA.

II. Solution-Phase Synthesis and Characterization

(*E*)-2,2,2-trifluoroacetaldehyde oxime (1)



Chemical Formula: $C_2H_4F_3NO_2$
Exact Mass: 131,0194

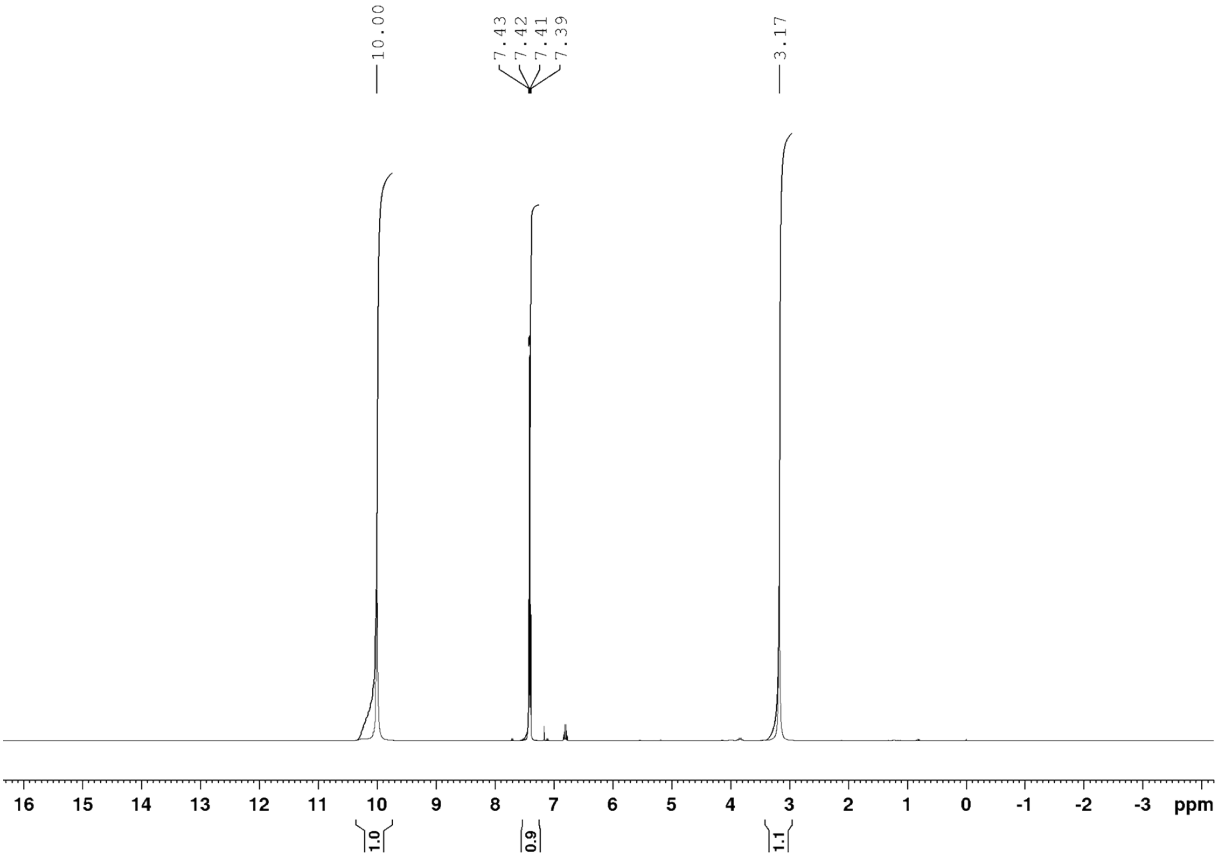
2,2,2-trifluoroethane-1,1-diol 10 (10.0 g, 86 mmol, 1 eq.) was added to an aqueous solution of hydroxylamine (50 % wt. in water) (6.8 mL, 103 mmol, 1.2 eq.). The reaction mixture was stirred 18 h at rt. The crude product was purified by a fractional distillation (bp: 80°C). Oxime was obtained as a colourless liquid (7.0 g, 62% yield).

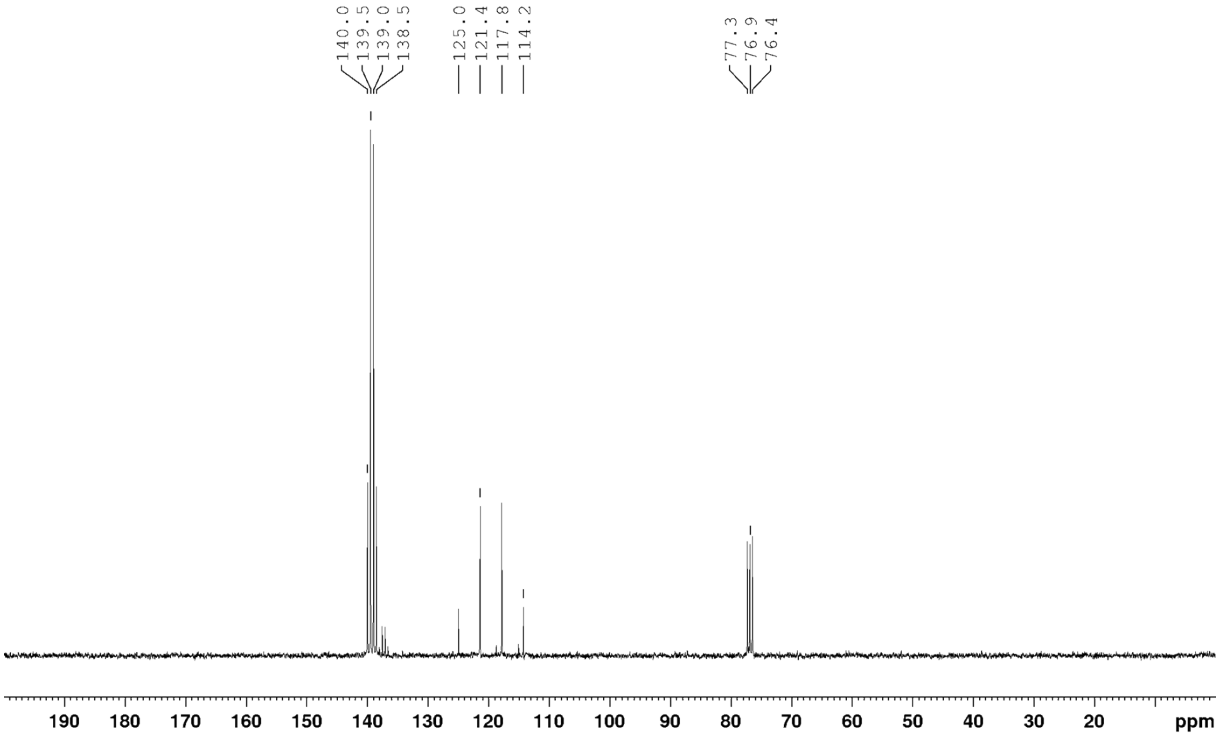
^{1}H NMR (300 MHz, $CDCl_3$): δ 10.00 (s, 1H, OH), 7.41 (q, *J* = 4.2 Hz, 1H, CH), 3.17 (s, 1H, H_2O).

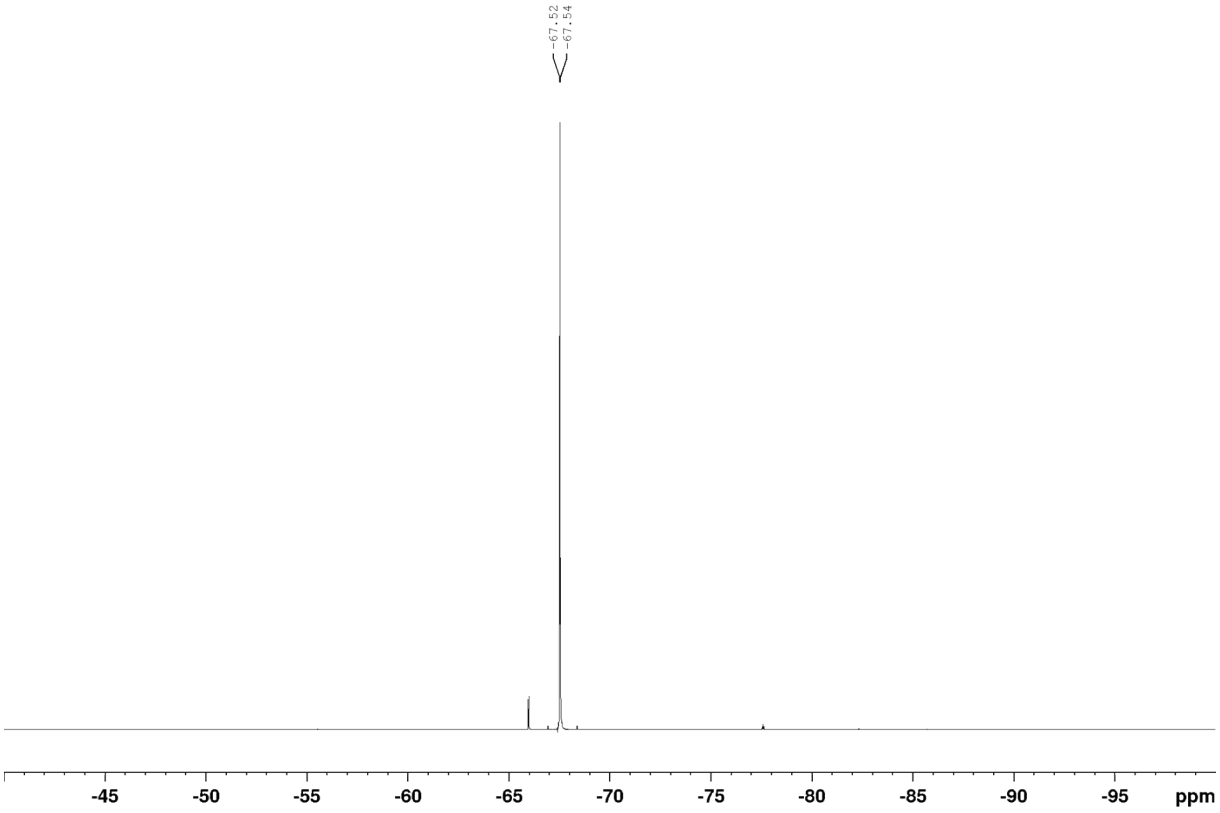
^{13}C NMR (75 MHz, $CDCl_3$): δ 139.2 (q, *J* = 37 Hz, CH), 119.6 (q, *J* = 270 Hz, CF_3).

^{19}F NMR (188 MHz, $CDCl_3$): δ -67.5 (d, *J* = 4.2 Hz, 3F, CF_3).

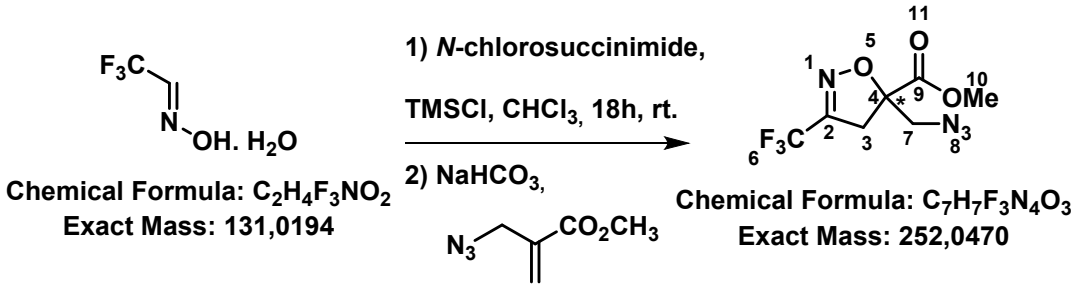
The analytical data are in accordance with the literature: *Beilstein J. Org. Chem.* 2013, 9, 2387–2394.




140.0
139.5
139.0
138.5
125.0
121.4
117.8
114.2
77.3
76.9
76.4
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

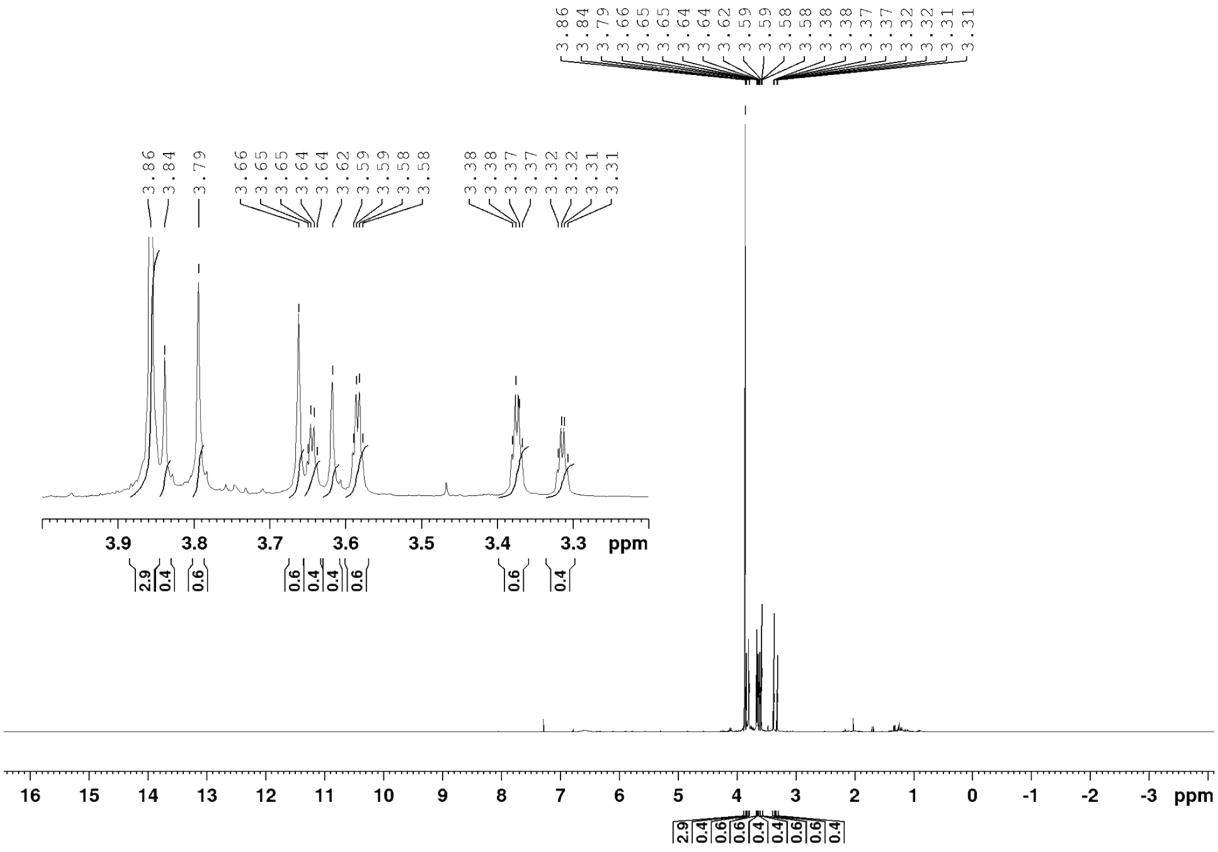

-67.52
-67.54
-45 -50 -55 -60 -65 -70 -75 -80 -85 -90 -95 ppm

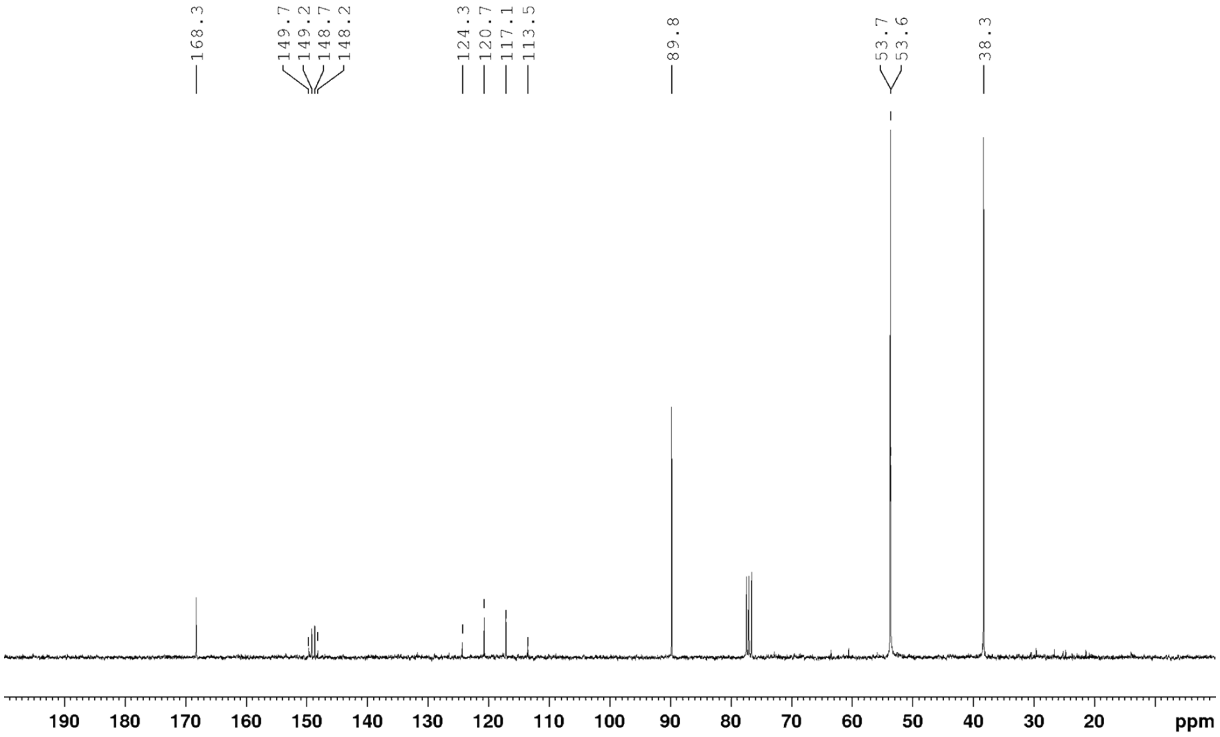
Methyl 5-(azidomethyl)-3-(trifluoromethyl)-4,5-dihydroisoxazole-5-carboxylate (2)

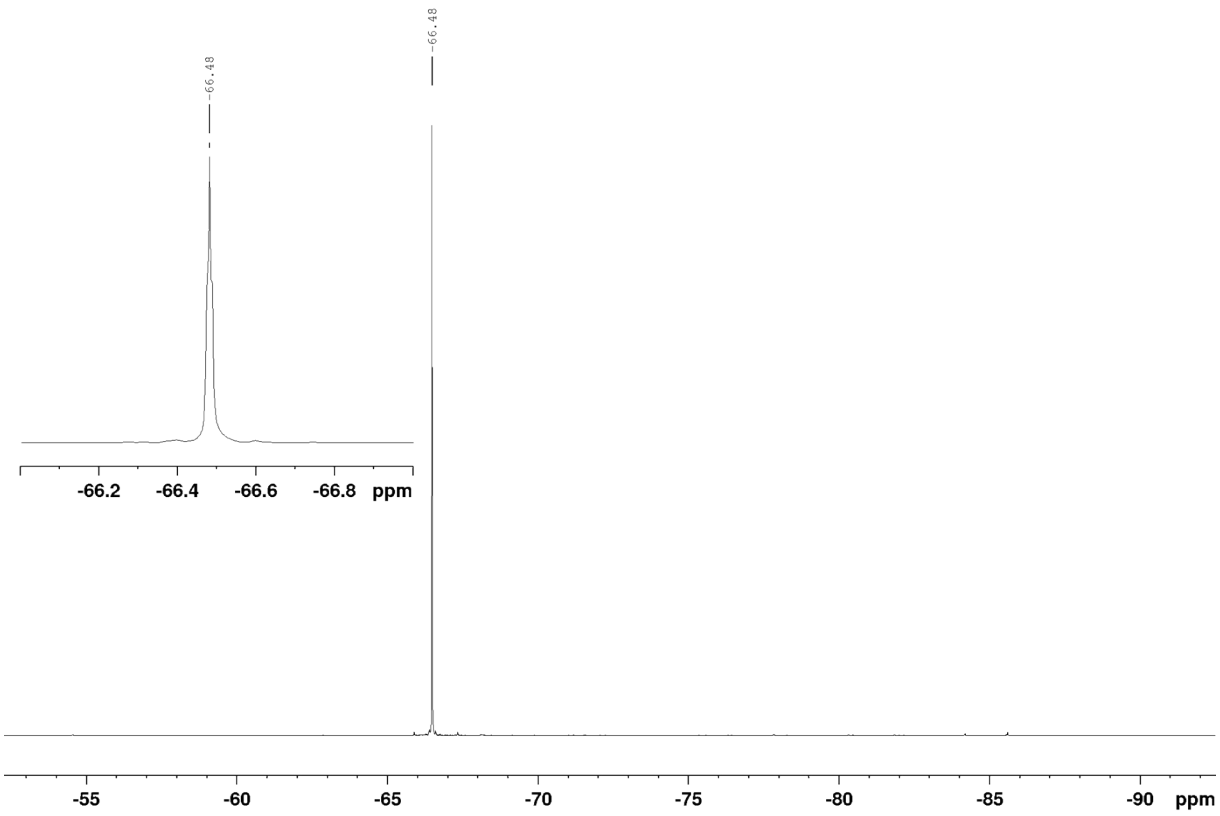


N-chlorosuccinimide (1.4 g, 10.7 mmol, 1.3 eq.) and trimethylchlorosilane (1 drop) were added to 2,2,2-trifluoroacetaldehyde oxime hemihydrate (1.0 g, 8.2 mmol, 1 eq.) in $CHCl_3$ (20 mL) at rt. The reaction mixture was stirred overnight. After obtaining the chloroxime, $NaHCO_3$ (1.0 g, 12.3 mmol, 1.5 eq.) and the corresponding methyl 2-(azidomethyl) acrylate (1.27 g, 9.0 mmol, 1.1 eq.) were added, and the resulting mixture was stirred overnight, then filtered and evaporated in vacuo. The crude was purified by flash chromatography. Dihydroisoxazole was obtained as a colourless oil (0.86 g, 45% yield).

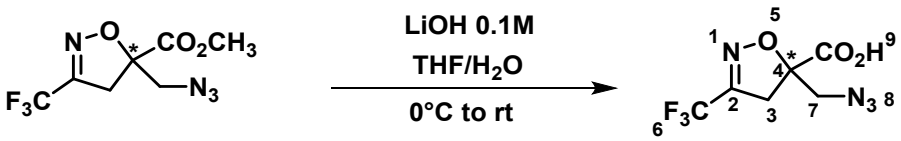
HRMS (ESI): calculated for $C_7H_7F_3N_4O_3$ [$(M+Na)^+$]: m/z 275.0362, found: m/z 275.0360.
^{1}H NMR (300 MHz, $CDCl_3$): δ 3.86 (s, 3H, H10), 3.82 (d, *J* = 13.3 Hz, 1H, H7'), 3.64 (d, *J* = 13.3 Hz, 1H, H7''), 3.61 (dq, *J* = 18 Hz, $J_{F\text{-}H}$ = 1.2 Hz, 1H, H3'), 3.34 (dq, *J* = 18 Hz, $J_{F\text{-}H}$ = 1.2 Hz, 1H, H3'').
^{13}C NMR (75 MHz, $CDCl_3$): δ 168.3 (C9), 148.9 (q, *J* = 38 Hz, C2), 118.9 (q, *J* = 271 Hz, C6), 89.8 (C4), 53.7 (C7), 53.6 (C10), 38.3 (C3).
^{19}F NMR (188 MHz, $CDCl_3$): δ -66.5 (s, 3F, CF_3).




168.3
149.7
149.2
148.7
148.2
124.3
120.7
117.1
113.5
89.8
53.7
53.6
38.3
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm


-66.48
-66.48
-66.2 -66.4 -66.6 -66.8 ppm
-55 -60 -65 -70 -75 -80 -85 -90 ppm

5-(azidomethyl)-3-(trifluoromethyl)-4,5-dihydroisoxazole-5-carboxylic acid (3)



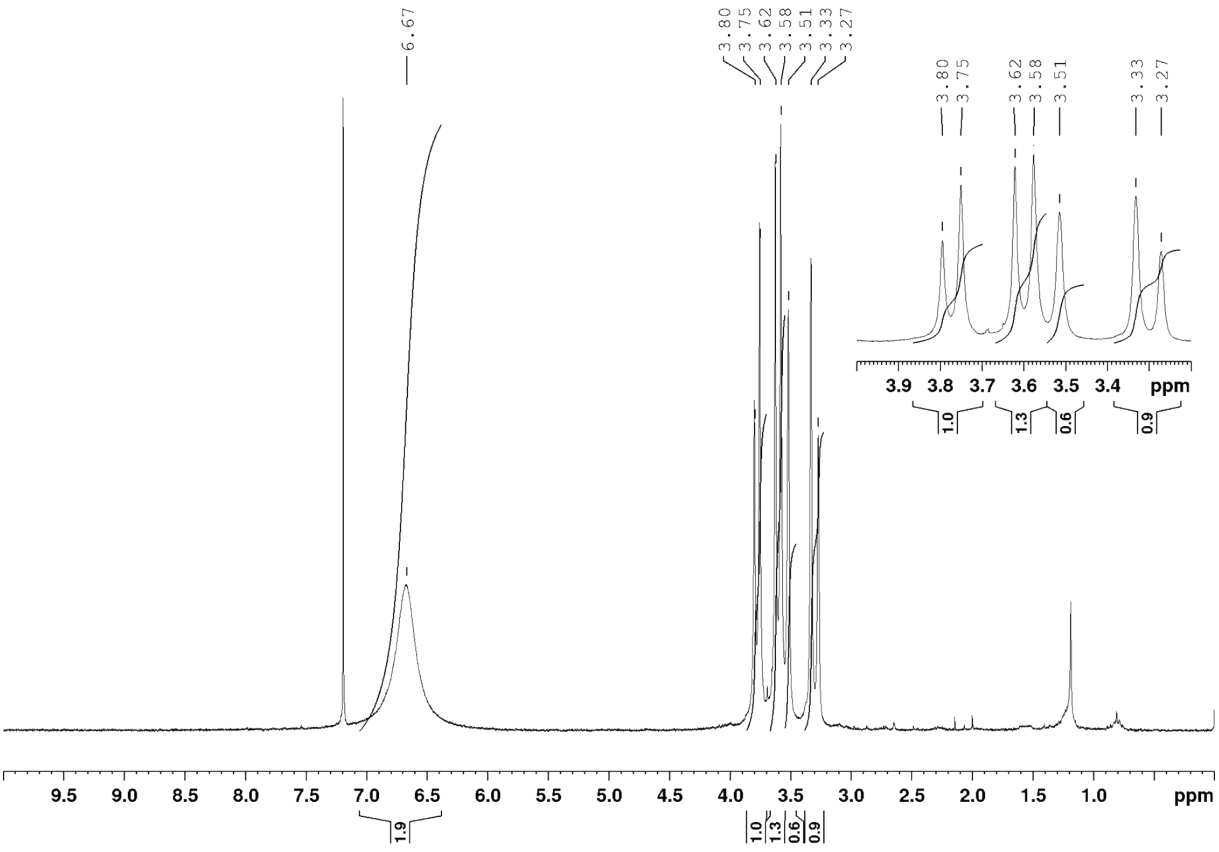
Chemical Formula: $C_6H_5F_3N_4O_3$
Exact Mass: 238,0314

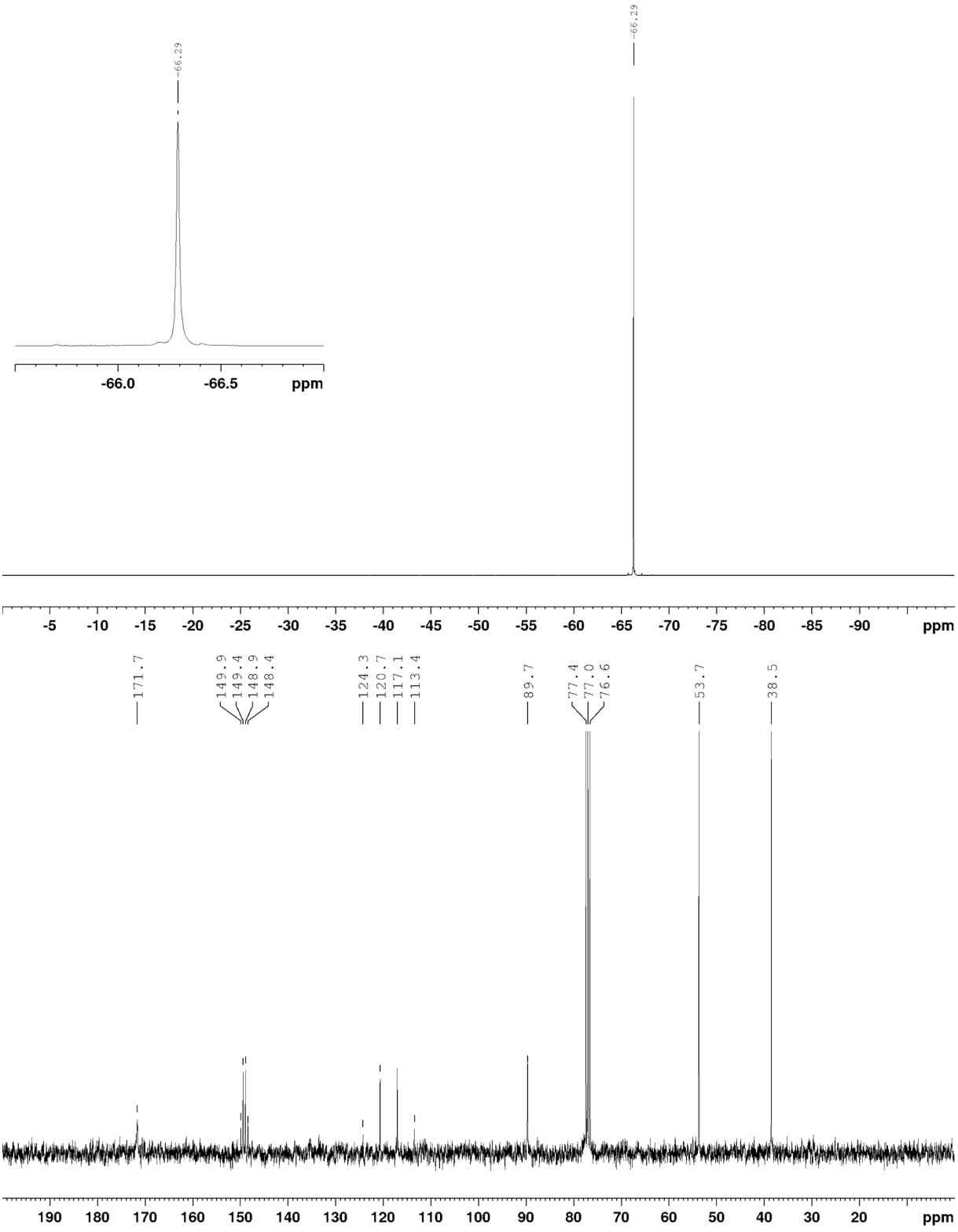
In a round-bottom flask equipped with a magnetic stirrer, the methyl ester derivative compound (1 eq.) was suspended in THF (1M). Afterwards, LiOH 0,1 M in H_2O (1.5 eq.) was added, and the reaction was let to stir for about 30 min at room temperature. At the end of the reaction, checked by TLC (hexane/EtOAc = 7:3), the solvent was removed under reduced pressure and then the aqueous solution was acidified with 10% HCl (checking the pH with litmus paper till pH 5) and extracted with ethyl acetate (3 x 15 mL). The combined organic phases were then dried over Na_2SO_4, and the solvent removed under reduced pressure, affording the acid as a white solid in quantitative yield.

HRMS (ESI): calculated for $C_6H_5F_3N_4NaO_3$ [$(M+Na)^+$]: m/z 261.0206, found: m/z 261.0210.
^{1}H NMR (300 MHz, $CDCl_3$): δ 6.67 (brs, 1H, H9, CO_2H), 3.77 (d, *J* = 13 Hz, 1H, H7), 3.60 (d, *J* = 13 Hz, 1H, H7), 3.55 (d, *J* = 18 Hz, 1H, H3), 3.30 (d, *J* = 18 Hz, 1H, H3).

^{13}C NMR (75 MHz, $CDCl_3$): δ 171.6 (C9), 149.2 (q, *J* = 38 Hz, C2), 118.9 (q, *J* = 273 Hz, C6), 89.7 (C4), 53.7 (C7), 38.5 (C3).

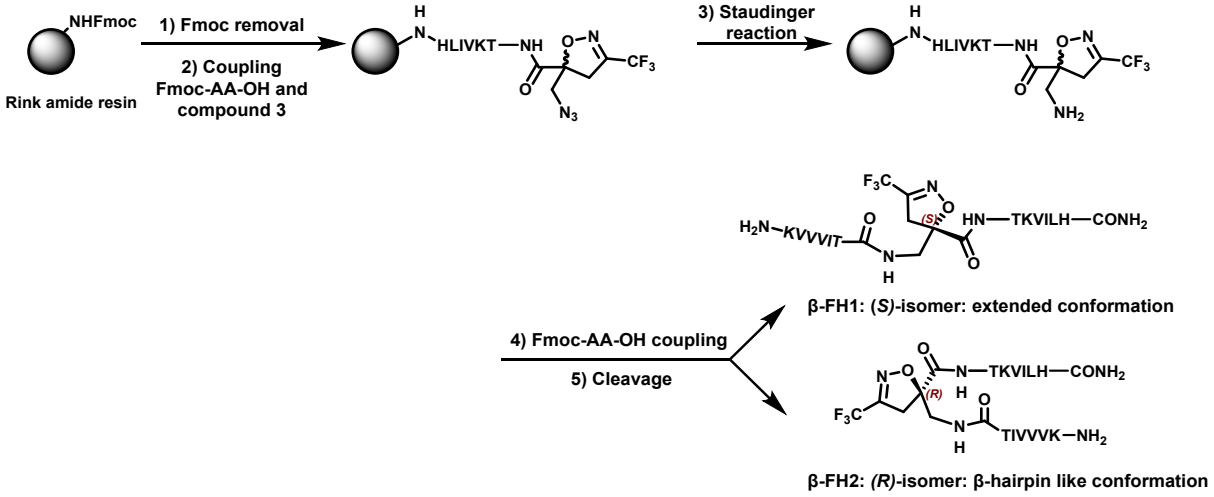
^{19}F NMR (188 MHz, $CDCl_3$): δ -66.29 (s, 3F, CF_3).




-66.29
-66.29
-66.0
-66.5
ppm
-5 -10 -15 -20 -25 -30 -35 -40 -45 -50 -55 -60 -65 -70 -75 -80 -85 -90 ppm
171.7
149.9
149.4
148.9
148.4
124.3
120.7
117.1
113.4
89.7
77.4
77.0
76.6
53.7
38.5
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

III. Solid phase general procedures

All reactions were carried out in plastic syringe tubes equipped with filters, agitated on an automated shaker using Rink Amide MBHA resin (100-200 mesh, 0.2 mmol scale, loading: 0.578 mmol/g). Removal of Fmoc group before each coupling was performed in 20% (v/v) piperidine/DMF for 15 min twice. Capping steps were performed by treating the resin with the mixture of acetic anhydride (0.25 M) and NMM (0.25 M) in DMF solution for 15 min. After each reaction, the resin was washed successively with DMF (3 × 10 mL), MeOH (3 × 10 mL) and DCM (3 × 10 mL). Prior to use, the Rink Amide resin was swollen in DMF for 1 h.



Scheme S1: Solid phase synthesis of peptide **β-FH1** and **β-FH2. 1) Fmoc removal:** 20 % Piperidine in DMF (v/v), rt, 30 min. **2) Fmoc-AA-OH coupling**: Fmoc-AA-OH (5 eq.), HCTU (5 eq.), NMM 20 % in DMF, rt, 30 min. **Compound 3 coupling:** DIC (2 eq.) Oxyma (2 eq.), **3** (2 eq.), DMF, rt, 16 h. **3) Staudinger reaction**: $P(Me)_3$ in toluene 1 M (7 eq.) and H_2O (49 eq.) in THF or DMF, rt, 16 h **4) Fmoc-AA-OH coupling:** Fmoc-AA-OH (5 eq.), HCTU (5 eq.), NMM 20 % in DMF, rt, 30 min. **5) Cleavage**: TFA/H_2O/TIPS/Phenol; 90/5/2.5/2.5, rt, 2h.

The **loading** was performed by suspending the resin in DMF/DCM (6/2 mL, 0.2 mmol scale) in the presence of collidine (17 eq.). In parallel, Fmoc-AA-OH (5 eq.) DIC (5 eq.) and Oxyma (5 eq.) were dissolved in DCM/DMF (33% v/v, 8 mL) and preactivated under magnetic stirring for 2 min. The activated amino acid solution was then added to the resin-containing reactor, and the mixture was agitated for 16 h at room temperature. Every following **coupling** using Fmoc-protected amino acid (Fmoc-AA-OH) was carried out twice to get satisfactory yields using Fmoc-AA-OH/HCTU (5.0/5.0 eq.) in a solution of NMM in DMF (20% v/v, 8 mL) for 30 min. The coupling of **3** to the sequence was processed using **3** (2 eq.) DIC (2 eq.) Oxyma (2 eq.), in DCM/DMF 33% (v/v) (8 mL), under mechanical stirring for 24h at room temperature. **Staudinger** reaction was performed under stirring with a solution of trimethylphosphine $P(Me)_3$ (1 M in Toluene, 7 eq.) and H_2O (49 eq.) in DMF (2 mL), at room

temperature for 16 h. The coupling of **3** and the Staudinger reaction was monitored by RP-HPLC-MS at 215.8 nm. After all amino acids of the sequence had been coupled to the resin and the Fmoc group was removed from the protected peptidomimetic attached to the resin, the peptidyl-resin was cleaved by mechanical stirring for 2 hours at room temperature using an acidic cocktail consisting of TFA/H_2O/TIPS/phenol in a 90:5:2.5:2.5 ratio.

Finally, purification of the crude compound by semi-preparative RP-HPLC using a 10–50% ACN/H_2O gradient over 20 minutes allowed the isolation of the diastereoisomers **β-FH1** and **β-FH2**.

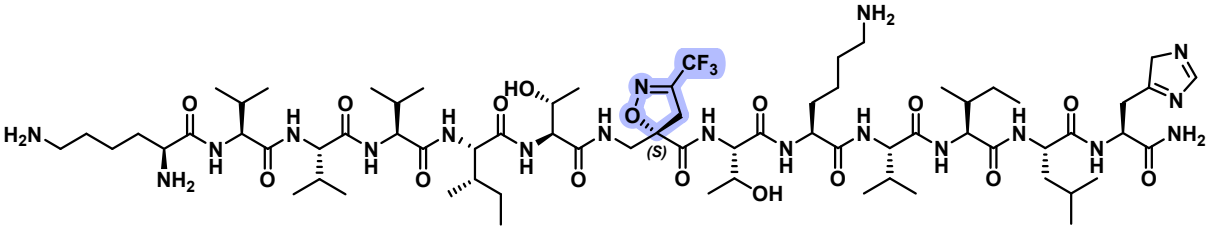
IV. General procedure for Pronase stability assays

A. By RP-HPLC-MS

The degradation of peptides and peptidomimetics by RP-HPLC-MS was performed according to the following procedure. The assay buffer was prepared with 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) BriJ 35, and adjusted to pH = 7.5. The peptide or peptidomimetic was dissolved in 200 µL of assay buffer to reach a concentration of 400 µM and stirred in a thermomixer at 37°C and 500 rpm for 5 min. Pronase E, a mixture of more than 10 proteases produced by Streptomyces griseus K-1, was dissolved in 200 µL of assay buffer to reach a concentration of 5 µg/mL and stirred in a thermomixer at 37°C for 5 min. Then, 30 µL of the peptide solution were transferred to an HPLC vial containing 30 µL of buffer and 6 µL of 10% TFA to be used as a control. Finally, 170 µL of the peptide solution was added to 170 µL of the Pronase solution in an Eppendorf tube and stirred in the thermomixer at 500 rpm and 37°C, resulting in final concentrations of 2.5 µg/mL for Pronase and 200 µM for the peptide. The reaction progress was monitored by RP-HPLC-MS at various time points: 0 min, 10 min, 30 min, 1 h, and 2 h. At the different time points transfer 60 µL of the reaction solution to an HPLC vial containing 6 µL of 10% TFA to stop proteolysis activity. The procedure was repeated for every time point and analysis of each of these vials were performed by RP-HPLC-MS, by **TIC** and **UV at 215.8 nm**, Mobile phase Gradient: 1-50% ACN in H_2O containing 0.1% FA in 6 min.

V. Characterization and stability assessment of β-FH1 and β-FH2

Peptidomimetic β-FH1: (*S*)-*N*-((2*S*,5*S*,8*S*,11*S*,14*S*,17*S*,18*S*)-2-((4*H*-imidazol-5-yl)methyl)-1-amino-14-(4-aminobutyl)-8-((*R*)-*sec*-butyl)-18-hydroxy-5-isobutyl-11-isopropyl-1,4,7,10,13,16-hexaoxo-3,6,9,12,15-pentaazanonadecan-17-yl)-5-((4*S*,7*S*,10*S*,13*S*,16*S*,19*S*)-19,23-diamino-7-((*S*)-*sec*-butyl)-4-((*S*)-1-hydroxyethyl)-10,13,16-triisopropyl-3,6,9,12,15,18-hexaoxo-2,5,8,11,14,17-hexaazatricosyl)-3-(trifluoromethyl)-4,5-dihydroisoxazole-5-carboxamide



Peptidomimetic β-FH1 was synthesized according to SPPS procedure. Compound was purified by semi-preparative RP-HPLC (Column 1); (Gradients of 1-50% ACN in H_2O containing 0.1% FA in 15 min, isolated yield after purification: 12 %, 11.7 µmol, 18 mg).

Molecular weight: 1541.9269 g/mol;

HRMS: Calcd. for $[C_{70}H_{122}F_3N_{19}O_{16} + H]^+$: m/z 1542.9342, found: m/z 1542.9333 $[M + H]^+$; Calcd. for $[C_{70}H_{122}F_3N_{19}O_{16} + 2\ Na]^{2+}$: m/z 782.9617, found: m/z 782.9612 $[M + 2\ Na]^{2+}$.

HPLC purity (215.8 nm): RP-HPLC-QUAD (column A); (Gradients of 1-50% ACN in H_2O containing 0.1% FA in 6 min); Rt = 5.471 min, 100 %.

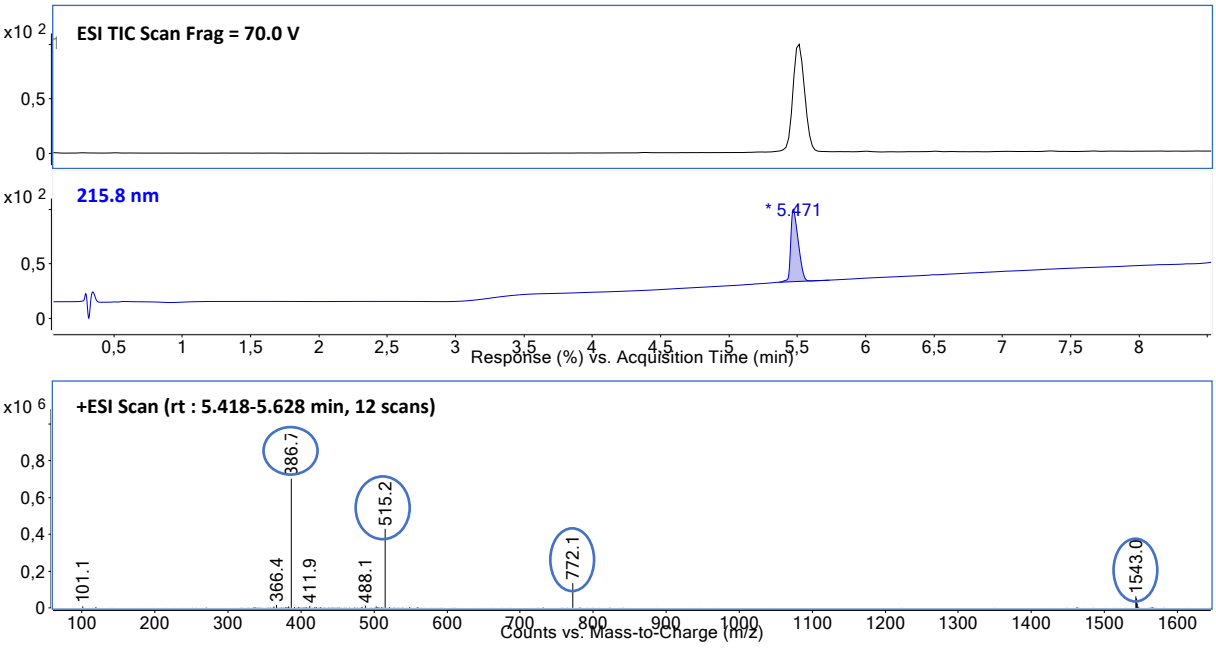
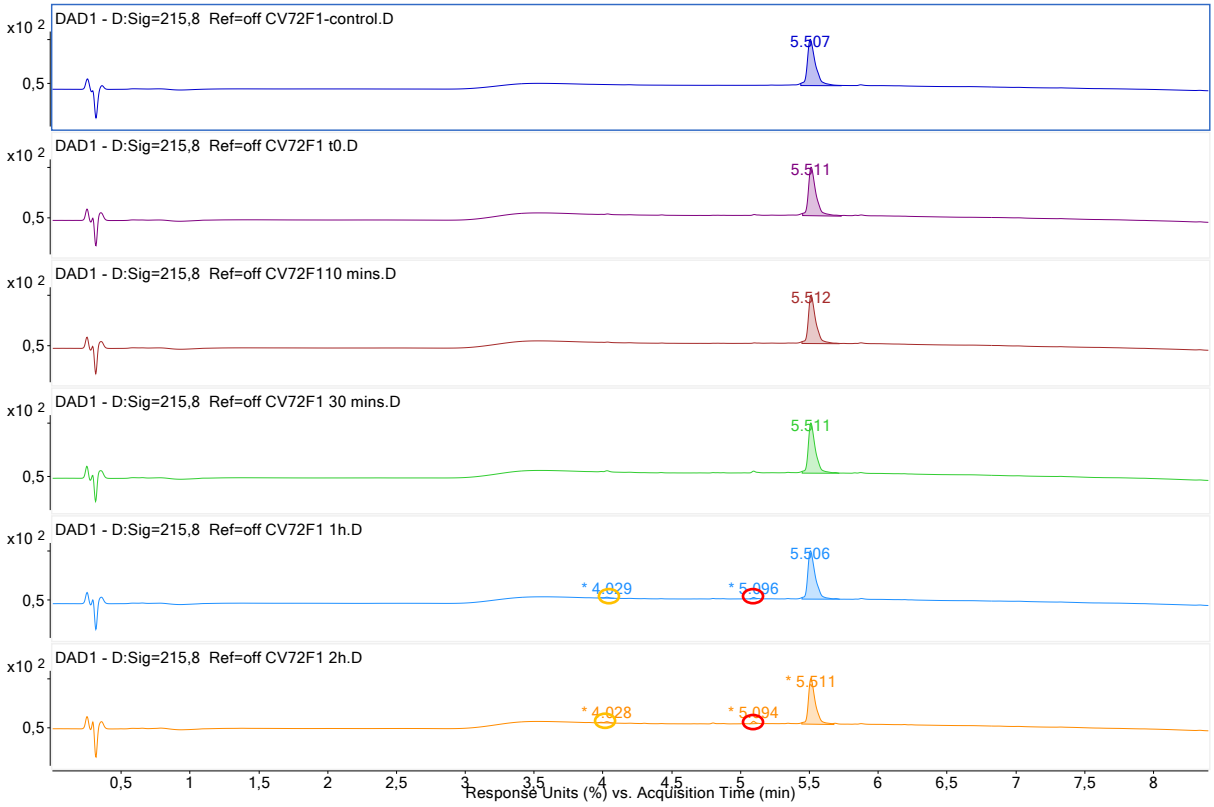
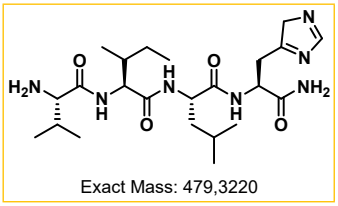
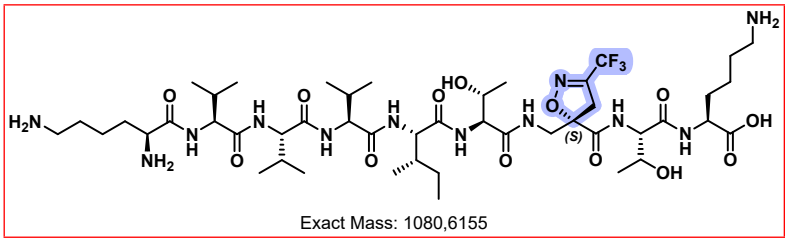


Figure S1. LC–MS/UV characterization of **β-FH1**. Overlay of TIC (total ion chromatogram, ESI⁺) and UV (λ = 215.8 nm) showing a single major peak at Rt = 5.471 min. The peak-apex MS matches $[M+H]^+$ (calc. m/z 1542.9, found m/z 1543.0). UV purity ≥ 95%.



Identified Structures of Cleaved Peptide Residues:





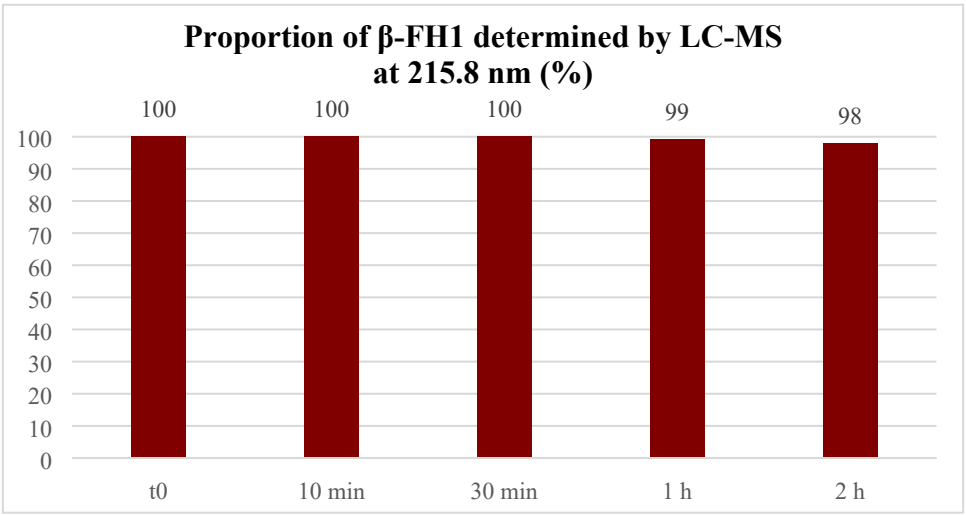
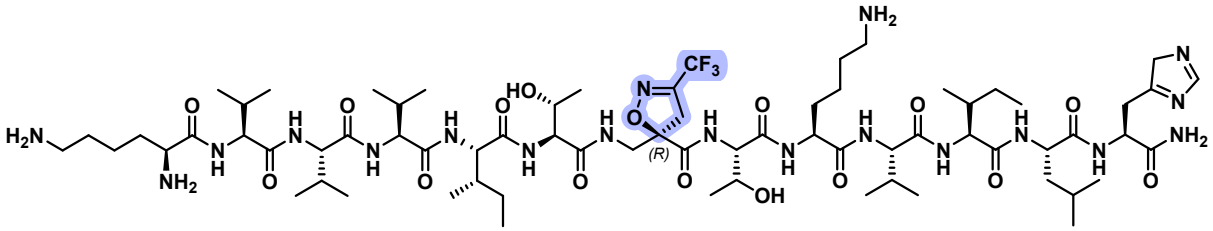


Figure S2. Pronase stability assay and cleavage mapping by RP-HPLC-MS (TIC, and UV at 215.8 nm). UV chromatograms (215.8 nm) of **β-FH1** recorded at the indicated time points after incubation with Pronase E. Reverse-phase separation on column 1 using a 1–50% ACN in H_2O + 0.1% formic acid linear gradient over 6 min.

Peptidomimetic β-FH2: (*R*)-*N*-((2*S*,5*S*,8*S*,11*S*,14*S*,17*S*,18*S*)-2-((4*H*-imidazol-5-yl)methyl)-1-amino-14-(4-aminobutyl)-8-((*R*)-*sec*-butyl)-18-hydroxy-5-isobutyl-11-isopropyl-1,4,7,10,13,16-hexaoxo-3,6,9,12,15-pentaazanonadecan-17-yl)-5-((4*S*,7*S*,10*S*,13*S*,16*S*,19*S*)-19,23-diamino-7-((*S*)-*sec*-butyl)-4-((*R*)-1-hydroxyethyl)-10,13,16-triisopropyl-3,6,9,12,15,18-hexaoxo-2,5,8,11,14,17-hexaazatricosyl)-3-(trifluoromethyl)-4,5-dihydroisoxazole-5-carboxamide

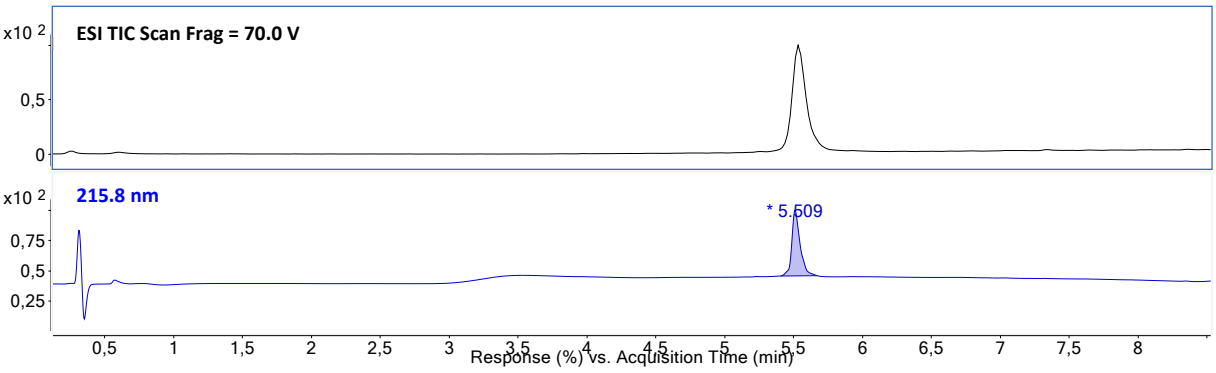


Peptidomimetic β-FH2 was synthesized according to SPPS procedure. Compound was purified by semi-preparative RP-HPLC (Column 1); (Gradients of 1-50% ACN in H_2O containing 0.1% FA in 15 min, isolated yield after purification: 10 %, 10.376 µmol, 16 mg).

Molecular weight: 1541.9269 g/mol;

HRMS: Calcd. for $[C_{70}H_{122}F_3N_{19}O_{16} + H]^+$: m/z 1542.9342, found: m/z 1542.9333 $[M + H]^+$; Calcd. for $[C_{70}H_{122}F_3N_{19}O_{16} + Na]^+$: m/z 1564.9161, found: m/z 1564.9158 $[M + Na]^+$.

HPLC purity (215.8 nm): RP-HPLC-QUAD (column A); (Gradients of 1-50% ACN in H_2O containing 0.1% FA in 6 min); Rt = 5.509 min, 100 %.



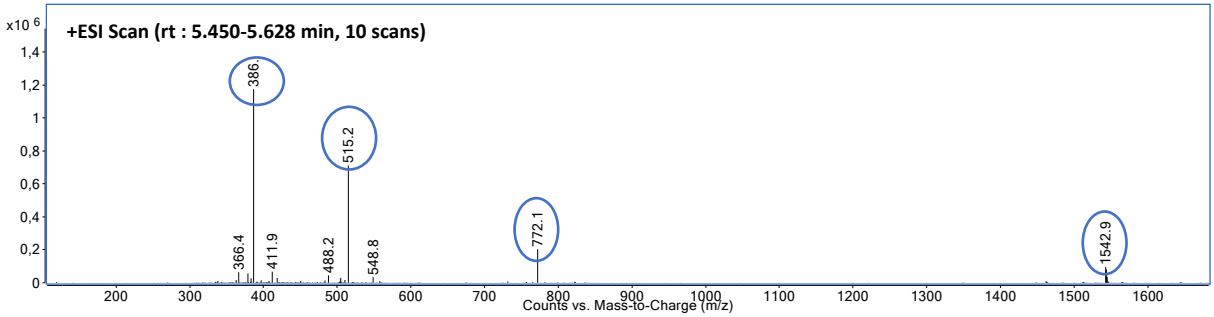
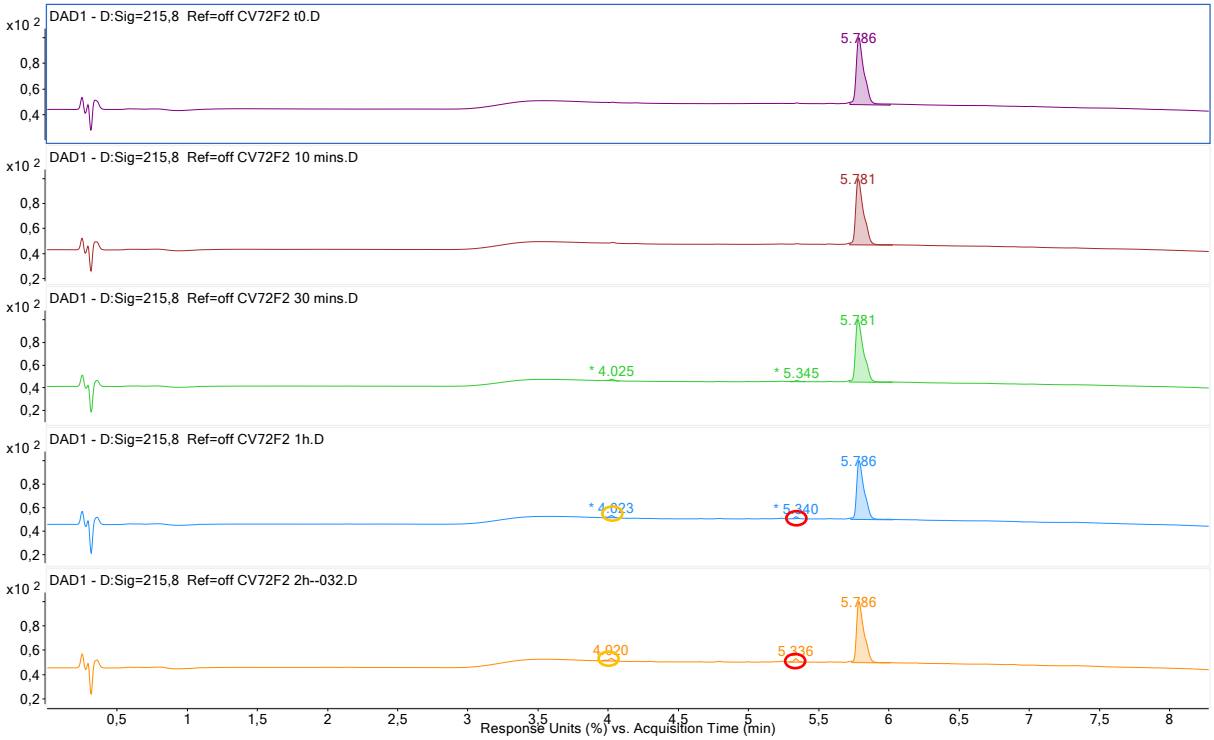
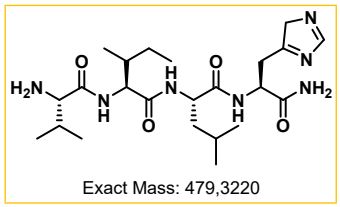
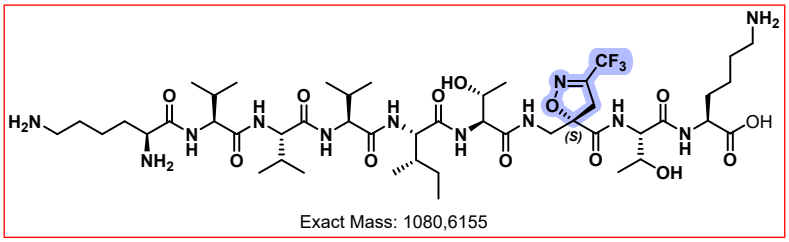


Figure S3. LC–MS/UV characterization of **β-FH2**. Overlay of TIC (total ion chromatogram, ESI⁺) and UV (λ = 215.8 nm) showing a single major peak at Rt = 5.471 min. The peak-apex MS matches $[M+H]^+$ (calc. m/z 1542.9, found m/z 1543.0). UV purity ≥ 95%.



Identified Structures of Cleaved Peptide Residues:





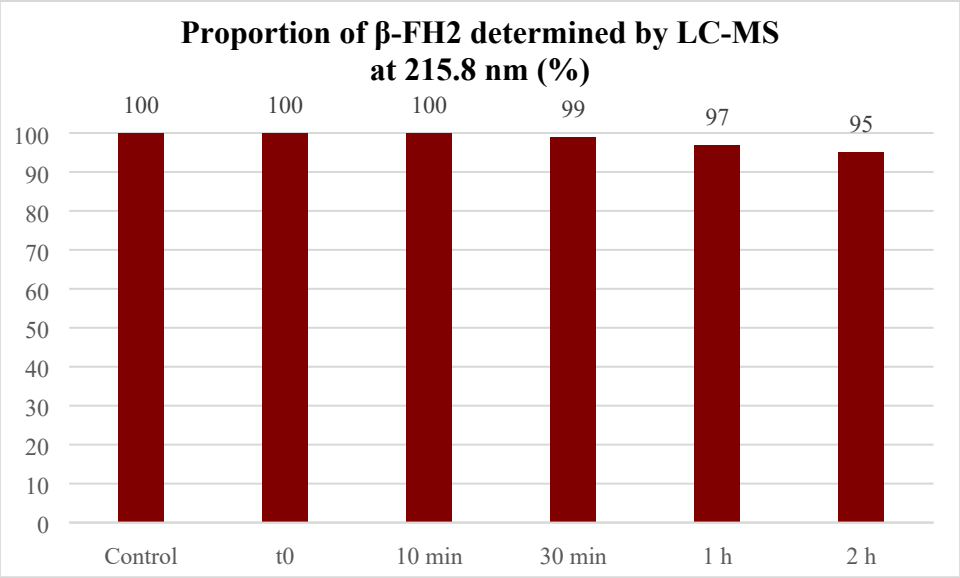


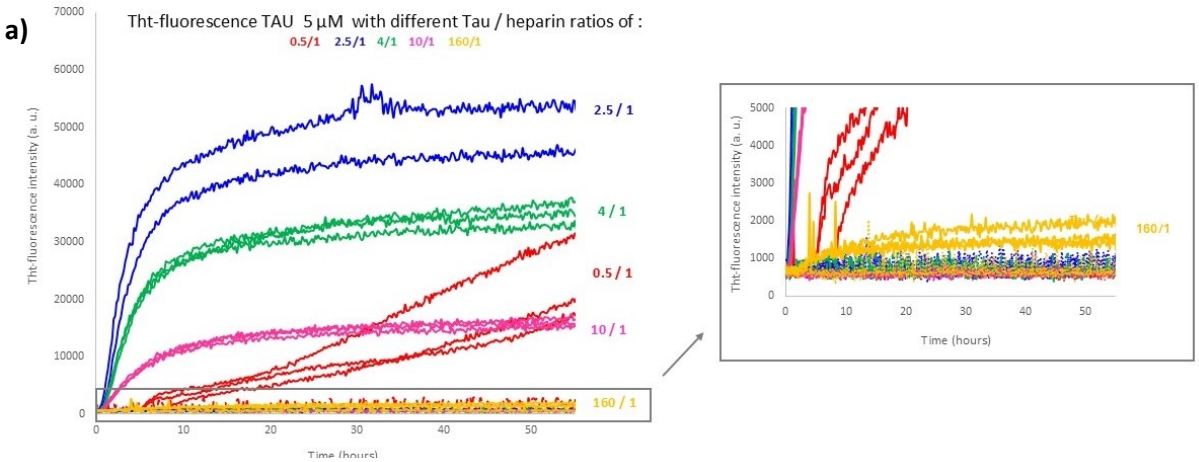
Figure S4. Pronase stability assay and cleavage mapping by RP-HPLC-MS (TIC, and UV at 215.8 nm). UV chromatograms (215.8 nm) of **β-FH2** recorded at the indicated time points after incubation with Pronase E. Reverse-phase separation on column 1 using a 1–50% ACN in H_2O + 0.1% formic acid linear gradient over 6 min.

VI. Production and purification of recombinant Wt-Tau441 and Tau441ΔK280

Human Tau (Wt-Tau441) and the ΔK280 deletion variant (Tau441ΔK280) were overproduced in *Escherichia coli* Bl21(DE3) and purified following published protocols.[1] In brief, transformed cells were grown at 37°C until reaching an Abs_{600nm} of 0.6–0.8. Then protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 5 h. Cells were harvested and resuspended in lysis buffer (50 mM sodium phosphate pH 7, 20 mM NaCl, 1 mM EGTA, 0.5 mM DTT and anti-proteases) before disruption by sonication. The lysate was cleared by centrifugation, and the supernatant was heated to 75 °C for 10 min then centrifuged again. Proteins were purified by cation-exchange chromatography (HiTrap SP FF column, Cytiva) followed by size exclusion chromatography (Superdex 75 column, Cytiva) in 20 mM Hepes pH 7.2, 100 mM KCl, 0.5 mM DTT. Proteins were concentrated using Amicon® Ultra unit and their concentrations were estimated by measurement of their absorbance at 280 nm. Finally, they were snap frozen in liquid nitrogen and stored at -70°C until use.

VII. Fluorescence-Detected ThT Binding Assay (Wt-Tau441 and TauΔK280)

Due to a modified Tau expression and purification protocol compared to our previous studies, aggregation conditions were re-optimized for ThT fluorescence assays (**Figure S5**). The Tau/heparin ratio was increased from 1:160 (used with our previous protein batches) to 1:10 to achieve consistent aggregation kinetics. Furthermore, heparin addition was delayed by one hour, as immediate aggregation upon heparin addition resulted in an extremely short lag phase, preventing accurate kinetic curve acquisition.[2]



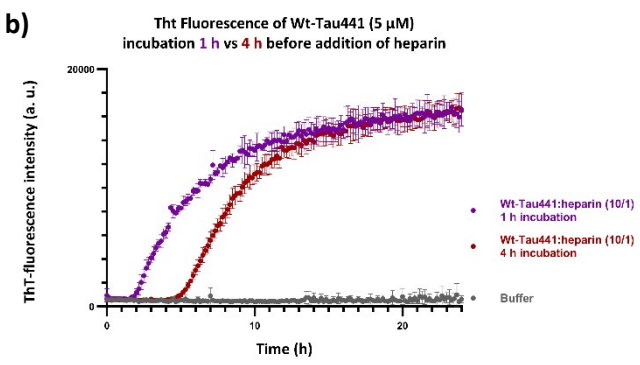


Figure S5. Optimization of Wt-Tau441 (5 µM) fibrillization kinetics. **a)** Effect of varying heparin concentrations: red curves (Tau/Heparin 5 µM/10 µM, ratio 0.5:1), blue (5 µM/2 µM, ratio 2.5:1), green (5 µM/1.25 µM, ratio 4:1), pink (5 µM/0.5 µM, ratio 10:1) and yellow (5µM/0.03125 µM, ratio 160:1). **b)** Impact of delayed heparin addition: fibrillization when heparin (Tau/Heparin, ratio 10:1) is added after 1 h of incubation (pink curves) and after 4 h of incubation (purple curves). The control curves without Tau are represented in dashed lines.

Wt-Tau441 and TauΔK280 were produced and purified as reported in the previous paragraph. ThT fluorescence was measured to evaluate the development of Tau fibrils over time using a fluorescence plate reader (Vantastar, BMG Labtech, Germany) at 440/480 nm excitation/emission wavelengths with 384-wells flat-bottom black plates (final volume in the wells of 40 µL). Stock Tau solutions were diluted to 20 µM in NaPi 25 mM, NaCl 25 mM, EDTA 2.5 mM, pH 6.8, and 2 mM stock solutions of compounds to test were dissolved in milliQ water. Experiments were initiated by adding the Tau solution (final Tau concentration: 5 µM) to each well containing Thioflavin-T (25 µM) in a buffer composed of 25 mM NaPi, 25 mM NaCl, 2.5 mM EDTA, pH 6.8, with or without the compounds to test at various concentrations. Tested compound/Wt-Tau ratios included 5/1 (25 µM), 2/1 (10 µM), and 1/1 for **β-FH1**, **β-FH2**, and **MB** (only 0.1/1 for MB). With TauΔK280, only the ratios 5/1 and 2/1 were tested. Heparin BC (Heparin sodium salt H-3149, average MW 18 kDa from Sigma-Aldrich, final concentration 0.5 µM) was added 60 min later, and the ThT fluorescence intensity of each sample (performed in triplicate) was recorded for a total of 24 h under continuous agitation (double orbital shaking, 300 rpm) at 37 °C on plates sealed with an optical transparent film. The fluorescence assays were performed at least twice in triplicate (n = 6) on different days, with the same batch of peptide. The ability of compounds to inhibit Tau aggregation was assessed considering the increase of the experimental fluorescence intensity (F) between 1 h and 24 h and is expressed relative to the same value of Tau control as a percentage: $\Delta F_{Tau + compound} / \Delta F_{Tau} \times 100$ (mean ± SD, n = 6). Effect of compounds on Wt-Tau441 and TauΔK280 fibrillization in the presence of the tested compounds are represented in **Table S1 and Table S2**, respectively.

Table S1. Table summarizing the effects of compounds on Wt-Tau441 fibrillization assessed by ThT-fluorescence spectroscopy at 5/1, 2/1, and 1/1 ratios of compound/Wt-Tau441 (+ 0.1/1 for **MB**).

Compound	**Compound/Tau441-ΔK280 ratio**	**$\Delta F_{Tau+compound}$ relative to $\Delta F_{Tau441-\Delta K280}$ (%)**
β-FH1	(5/1)[a] (2/1)[a] (1/1)[a]	SA[b], ND[c] SA[b], ND[c] SA[b], ND[c]
β-FH2	(5/1) (2/1) (1/1)	3.2±1.2% 44.2±8.0%* only 5 replicates 76.5±11.4%
MB	(5/1) (2/1) (1/1) (0.1/1)	6.0±0.6% 21.3±2.1% 32.2±2.6% 87.2±3.3%

The inhibitory effect was assessed by the increase in fluorescence intensity (ΔF) between 1 h and 24 h, expressed as a percentage relative to the Tau control: $\Delta F_{Tau + compound} / \Delta F_{Tau} \times 100$ (mean ± SD, n = 6).[a] Compound **β-FH1** self-aggregates at 25 µM, 10 µM, and 5 µM. [b] SA = Self-aggregation. [c] ND = value Not Determined.

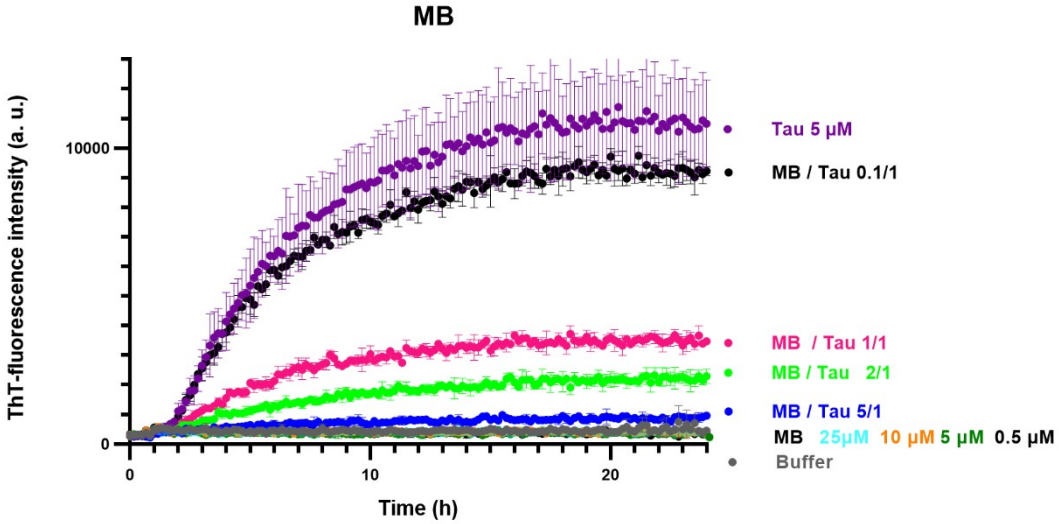


Figure S6. Curves of ThT fluorescence assays over time in the absence (purple curve) and in the presence of **MB** at compound/Wt-Tau441 ratios of 5/1 (blue), 2/1 (green), 1/1 (pink), 0.1/1 (black), for Wt-Tau441 fibrillization (5 µM) represented as a mean (triplicate±SD) and expressed as arbitrary units (a.u). The control curves of the compound are represented in light blue (25 µM), orange (10 µM), green (5 µM), black (0.5 µM) and the buffer in grey.

Table S2. Table summarizing the effects of compounds on Tau441-ΔK280 fibrillization assessed by ThT-fluorescence spectroscopy at 5/1, and 2/1 ratios of compound/Tau441-ΔK280.

Compound	**Compound/Tau441-ΔK280 ratio**	**$\Delta F_{Tau+compound}$ relative to $\Delta F_{Tau441-\Delta K280}$ (%)**
β-FH1	(5/1)[a] (2/1)[a]	SA[b], ND[c] SA[b], ND[c]
β-FH2	(5/1) (2/1)	1.0±0.8% 13.8±6.6%
MB	(5/1) (2/1)	1.3±0.8% 5.7±1.6%

The inhibitory effect was assessed by the increase in fluorescence intensity (ΔF) between 1 h and 24 h, expressed as a percentage relative to the Tau control: $\Delta F_{Tau + compound} / \Delta F_{Tau} \times 100$ (mean ± SD, n = 6).[a] Compound **β-FH1** self-aggregates at 25 µM and 5 µM. [b] SA = Self-aggregation. [c] ND = value Not Determined.

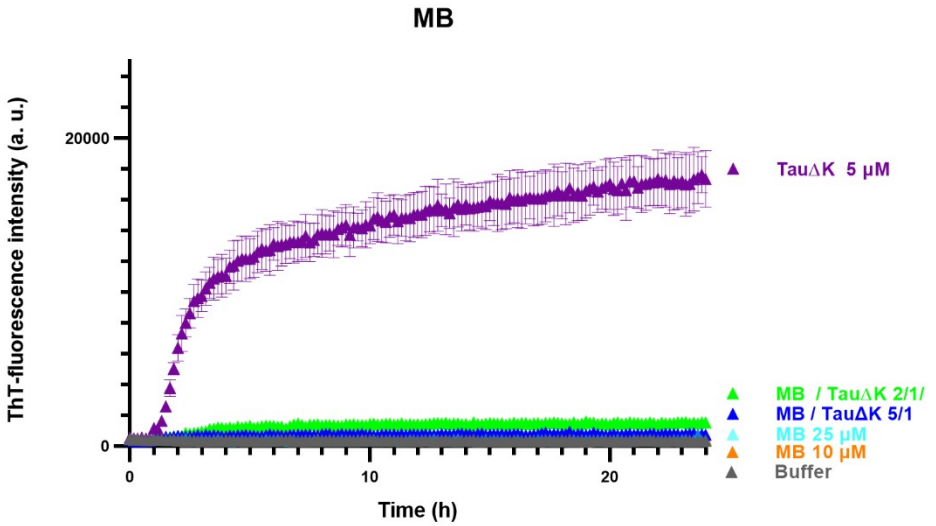


Figure S7. Curves of ThT fluorescence assays over time in the absence (purple curve) and in the presence of **MB** at compound/Wt-Tau441 ratios of 5/1 (blue), 2/1 (green), for Tau441-ΔK280 fibrillization (5 µM) represented as a mean (triplicate±SD) and expressed as arbitrary units (a.u). The control curves of the compound are represented in light blue (25 µM), orange (10 µM) and the buffer in grey.

VIII. Transmission Electron Microscopy for Wt-Tau441 and Tau441-ΔK280

Reactions were performed using the same aggregation assay conditions as for the ThT experiment (37 °C, continuous double-orbital shaking at 300 rpm, 24 h incubation; ThT measurements in triplicate). Tau (5 µM) was incubated with test compounds at a 5/1 compound/Tau molar ratio (25 µM compound). To avoid any interference of ThT with TEM imaging, two sets of reactions were run in parallel and incubated under identical conditions: one set contained ThT (25 µM) for aggregation monitoring, and the second set was ThT-free and used exclusively for TEM imaging. After incubation, aliquots from ThT-free reactions were deposited on grids and processed for negative-stain TEM as described below.

Samples were deposited onto copper grids coated with Formevar and a carbon film (ECF400-Cu, 400 mesh, Science Services, Munich, Germany). The grids were pre-treated with ambient air in a glow

discharge (Pelco easiGlow). The sample (5 µL) was applied onto the grids, and after a sedimentation time of 1-2 minutes, the excess suspension was removed with filter paper. Grids were then negatively stained with 2% (w/v) phosphotungstic acid (PTA) (5 µL) for 30-60 seconds, and excess stain was removed with filter paper. The prepared grids were analyzed with a microscope (JEOL JEM-1400), a cold field emission electron gun, and an applied acceleration voltage of 80/120 kV. A CMOS camera with direct detection (Gatan RIO9, 3kx3k) was used for digital recording. The images were processed using the image-processing system GMS3 (DigitalMicrograph version 3.x) and the image editing software ImageJ.

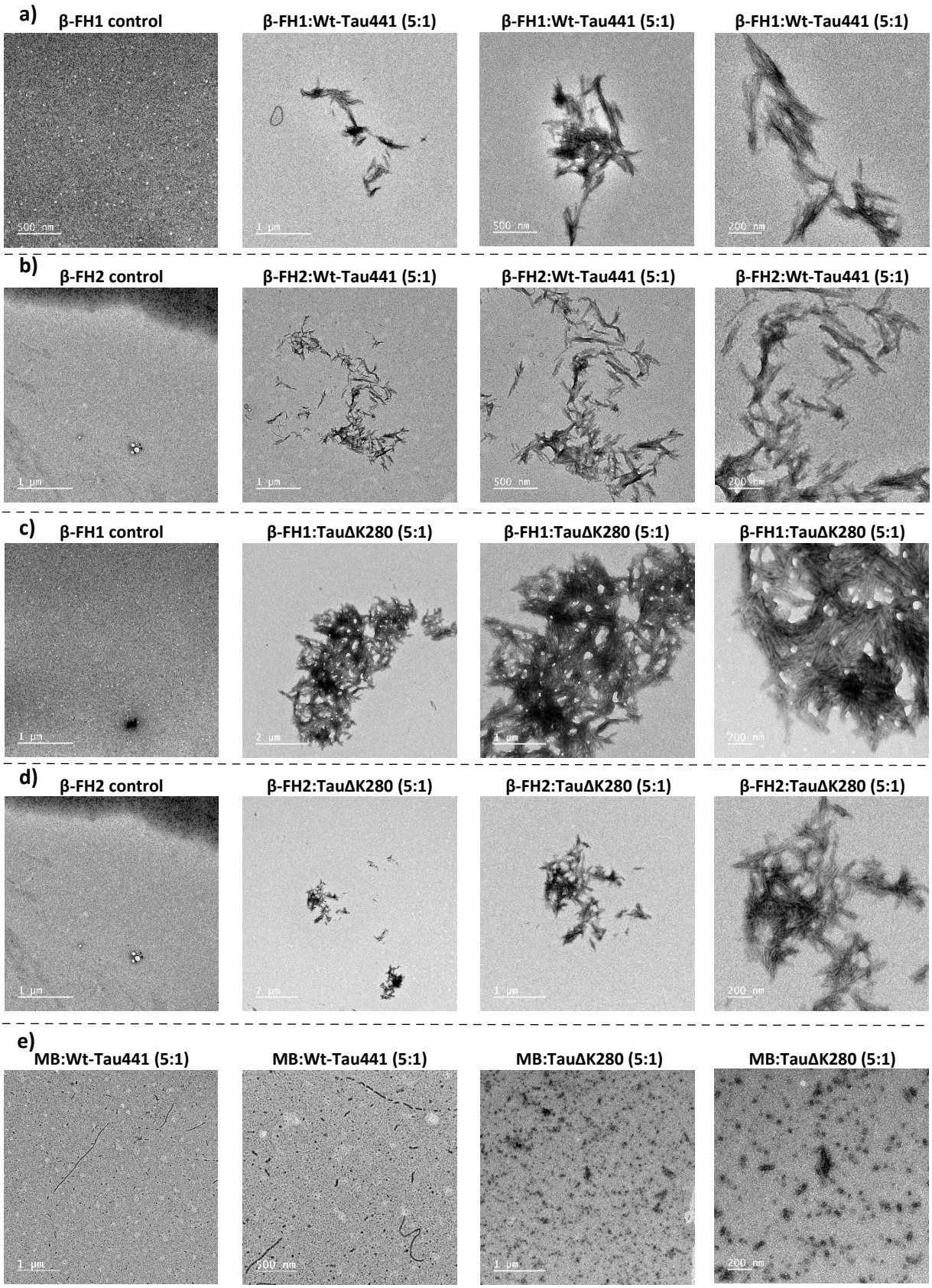
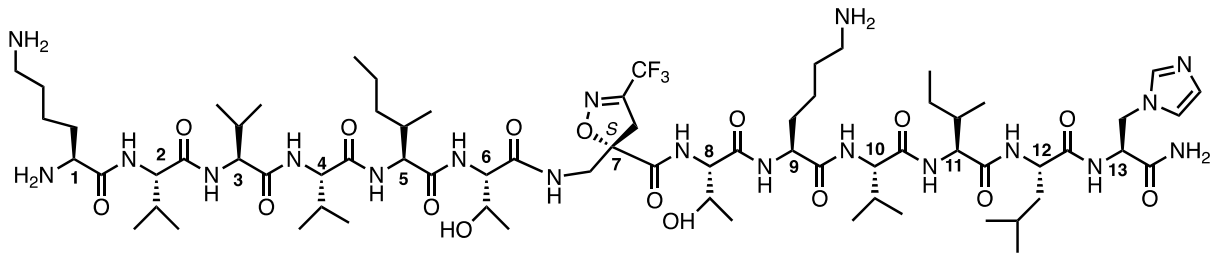


Figure S8. Transmission electron micrographs of compounds alone (control) and in presence of Wt-Tau441 *or* Tau441-ΔK280. **a) β-FH1** in presence of Wt-Tau441 **b) β-FH1** in presence of Tau441-ΔK280 **c) β-FH2** in presence of Wt-Tau441 **d) β-FH2** in presence of Tau441-ΔK280 **e) MB** in presence of Wt-Tau441 and Tau441-ΔK280. Samples were negatively stained with 2% (w/v) phosphotungstic acid (PTA), pH ~7.0, and imaged using a transmission electron microscope at 120 kV. Scale bars = [2 µm], [1 µm], [500 nm], [200 nm].

IX. Conformational characterization of peptidomimetics β-FH1 and β-FH2

A. NMR characterization and conformational analyses of peptidomimetics β-FH1 and β-FH2



β-FH1

Table S3. ^{1}H (black), ^{13}C (blue) resonances and ROEs spectroscopic data for **β-FH1** (CD_3OH, 4.0 mg in 750 µL, T = 298K)

Residue	NH (*J*, Hz)	H_α	H_β	H_γ and others	ROEs (200 ms)
Lys-1	-	4.01 (52.7)	1.88 (31.1)	H_ε: 2.95 (39.3), H_γ: 1.48 (21.4), H_δ: 1.69 (26.9)	H_α: $H_{\beta-Lys1}$(w)
Val-2	8.49 (*J* 8.0)	4.38 (59.2)	2.06 (30.8 region)	0.97 (w)	NH: $H_{\alpha-Lys1}$ (s), $H_{\beta-Val2}$(w)
Val-3	8.41 (*J* 9.2)	4.32 (58.8)	2.03 (30.8 region)	0.93	NH: $H_{\alpha-Val2}$ (s), $H_{\beta-Val3}$(m)
Val-4	8.19 (*J* 9.0)	4.36[a]	2.015 (30.8 region)	0.92	NH: $H_{\alpha-Val3}$ (s), $H_{\beta-Val4}$(w)
Ile-5	8.39 (*J* 8.3)	4.37 (57.8)	1.87 (36.8)	H_γ: 1.19/1.54 (24.7), Me: 0.93	NH: $H_{\alpha-Val4}$ (s)[b], $H_{\beta-Ile5}$ (w)
Thr-6	7.88 (*J* 7.2)	4.36 [a]	4.11 (67.2)	Me: 1.17 (d, *J* 6.3); (18.9)	NH: $H_{\alpha-Ile5}$ (s) Me: $H_{\beta-Thr6}$ (w)
S-Isox-7	8.28 (t, *J* 6.5)		3.83 (brs) (43.2)	3.55, 3.43 (AB system, *J* 18.1) (38.1)	NH: $H_{\alpha-Thr6}$ (s), $H_{\beta-Isox}$ (w)
Thr-8	7.84 (*J* 6.4)	4.36[a]	4.16 (67.2)	Me: 1.21 (d, *J* 6.1); (18.9)	NH: $H_{\alpha-Thr8}$ (s), $H_{\beta-Thr8}$ (vw) Me: $H_{\beta-Thr8}$ (w)
Lys-9	8.53 (*J* 7.4)	4.49 (53.4)	1.87 (31.1)	H_ε: 2.94 (39.3), H_γ: 1.51 (22.2), H_γ: 1.70 (31.4)	NH: $H_{\alpha-Thr8}$ (s), $H_{\gamma-Lys9}$(w) H_α: $H_{\beta-Val10}$ (w)
Val-10	8.13 (*J* 7.9)	4.36[a]	2.014 (30.8 region)	0.92	NH: $H_{\alpha-Lys9}$ (m), $H_{\beta-Val11}$(vw)
Ile-11	8.27 Overl.	4.26 (57.8)	1.86 (36.8)	H_γ: 1.53, 1.23 (24.7), Me: 0.91	NH: $H_{\alpha-Val11}$(m) H_α: $H_{\beta-Ile12}$(w)
Leu-12	8.31 (*J* 7.8)[c]	4.45 (52.1)	1.64/1.52 (40.2)	H_γ: 1.52 Me: 0.92 region	NH: $H_{\alpha-Ile12}$ (s)[b], $H_{\beta-Leu12}$ (w)
His-13	8.31 (*J* 7.8) [c]	4.67 (52.2)	3.25 (dd, *J* 15.3, 5.9), 3.10 (dd, *J* 15.3, 7.7) (27.1)	H_{2ring}: 8.72 (133.6) H_{4ring}: 7.60 (117.2) NH_2: 7.23, 6.93	NH: $H_{\alpha-Leu12}$ (s)[b] $H4_{ring}$: $H_{\alpha-His-13}$ (m) H_α: NH_{His} (vw)

[a] ^{13}C chemical shifts associated to 4.36 signals: 58.7, 59.5; [b]Tentatively assigned due to the overlapped H_α signals of His-14/ Leu-13; [c]Overlapped signals: the *J value is no higher than 7.8 Hz*

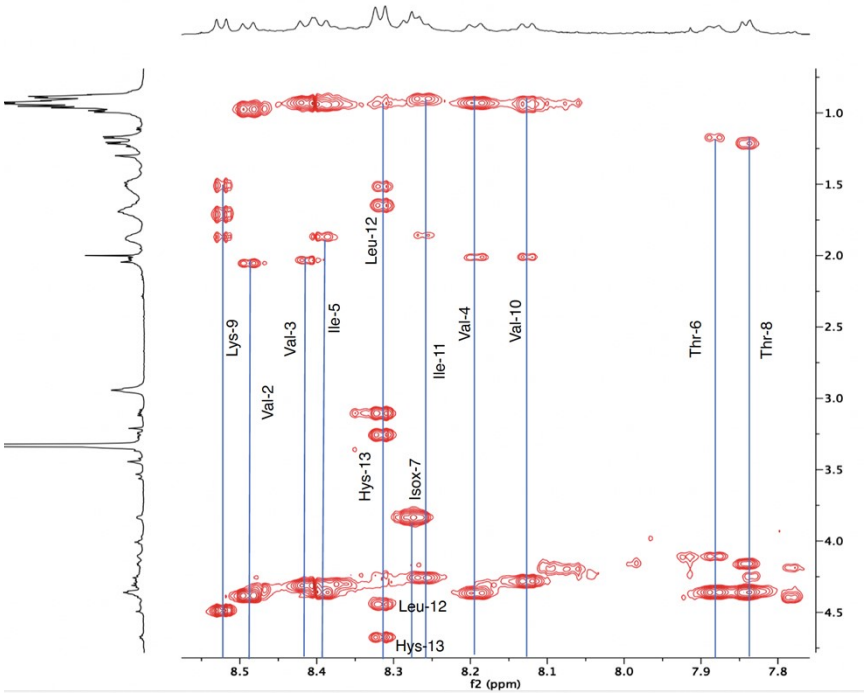


Figure S9. TOCSY experiment of **β-FH1** (CD_3OH, 4.0 mg in 750 μL, T = 313 K, 80 ms): zoom of NH/CH region.

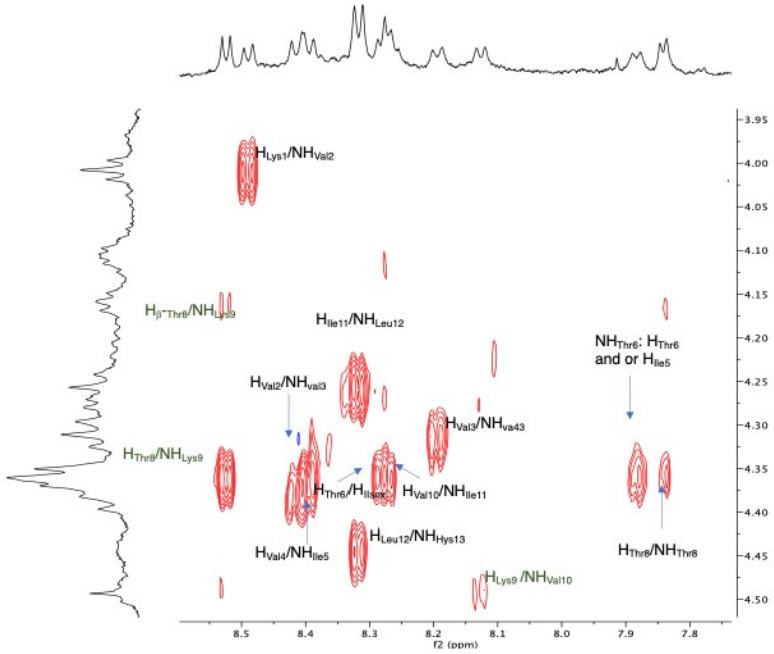


Figure S10. ROESY experiment of **β-FH1** (CD_3OH, 4.0 mg in 750 μL, T = 298 K, 200 ms): zoom of NH/CH_α region

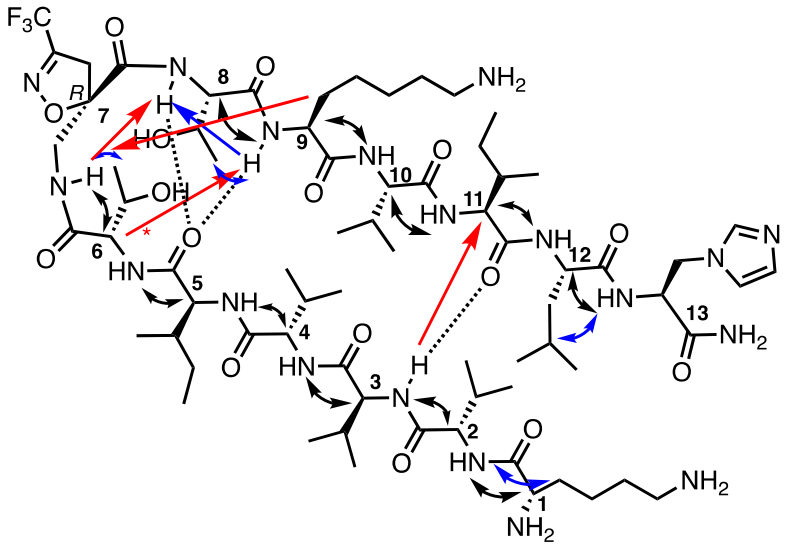


Table S4. ^{1}H (black), ^{13}C (blue) resonances and NOE for **β-FH2** (CD_3OH, 4.1 mg in 750 µL, T = 313 K)[a]

Residue	NH	H_α	H_β	H_δ and others	NOEs (200 ms)
Lys-1	-	4.13	1.92 (30.3)	H_ε: 2.97 (38.9), H_γ: 1.52 (21.9), H_δ: 1.73 (26.4)	
Val-2	8.49	4.39 (58.5)	2.09 (30.2 region)	Me: 1.00, 0.97	NH: $H_{\alpha-Lys1}$ (s) H_β: NH_{-Val2} (w)
Val-3	8.29	4.47	2.04 (30.2 region)	Me: 0.95, 0.92	NH: $H_{\alpha\text{-}Val2}$ (m), $H_{\alpha\text{-}Ile11}$ (m) H_β: NH_{-Val3} (vw)
Val-4	8.36	4.36	2.03 (30.2 region)	Me: 0.91 region	NH: $H_{\alpha\text{-}Val3}$ (s) H_β: NH_{-Val4} (w)
Ile-5	8.374	4.55	1.91 (35.7)	H_γ: 1.17/1.55 (24.3), Me: 0.94	NH: $H_{\alpha\text{-}Val4}$ (s)
Thr-6	8.13	4.36 (58.5)	4.16 (66.5)	Me:1.16 (d, *J* 5.0); (18.3)	NH: $H_{\alpha\text{-}Ile5}$ (s), Me_{Thr6}(vw) H_α: NH_{Lys9*} Me: NH_{Isox} (w)
***R*-Isox-7**	8.17		3.98, 3.76 (42.6)	3.61, 3.47 (AB system, *J* 17.9) (38.1)	NH: H_α(s)/Me(w)$_{Thr6}$, $H_{\beta\text{-}Lys9}$ (w), NH_{Thr8} (w)
Thr-8	7.75	4.21 (59.5)	4.32 (65.6)	Me: 1.18 (d, *J* 5.1); (18.3)	NH: NH_{Lys9} (w), NH_{Isox7} (w), H_α(m)/H_β(m)/Me(s)$_{Thr8}$ Me: NH_{Lys9} (m)
Lys-9	8.29	4.52	1.90 (30.1)	H_ε: 2.95 (38.9), H_γ: 1.53/1.46 (21.9), H_δ: 1.68 (26.2)	NH: H_α (s)/Me(m)$_{-Thr8}$, $H_{\beta\text{-}Lys9}$ (m), NH_{Thr8} (w)
Val-10	8.19	4.25 (59.3)	2.06 (30.2 region)	Me: 0.99, 0.93	NH: $H_{\alpha\text{-}Leu9}$(s) H_β: NH_{-Val10} (w)
Ile-11	8.24	4.32	1.85 (35.8)	H_γ: 1.16/1.59 (24.3), Me: 0.88, 0.86	NH: $H_{\alpha-Val0}$ (s) H_α: NH_{-Val3} (m) H_β: NH_{-Ile11} (w) H_γ (1.16): $H_{\alpha-Ile11}$
Leu-12	8.35	4.53	1.69 (39.7)	H_γ: 1.53, Me: 0.90 region	NH: $H_{\alpha\text{-}Ile11}$ H_β: NH_{Leu12}
His-13	8.33	4.70	3.32 (overl.) 3.18 (dd, *J* 15.6, 8.2) (26.1)	H_{2ring}: 8.79 (132.8) H_{4ring}: 7.39 (116.4) NH_2: 7.57, 7.17	NH: H_α(s)/H_γ(s)$_{-Leu12}$ H_α(s)/H_β(w)$_{-His}$, H_{4ring}: H_α/H_β(w)$_{His}$, Me (0.89, w; 0.94, vw) NH_2 (7.57): $H_{\alpha-His}$ (m)

					NH_2 (7.17): $H_{\beta\text{-}His}$ (w)

*Tentatively assigned

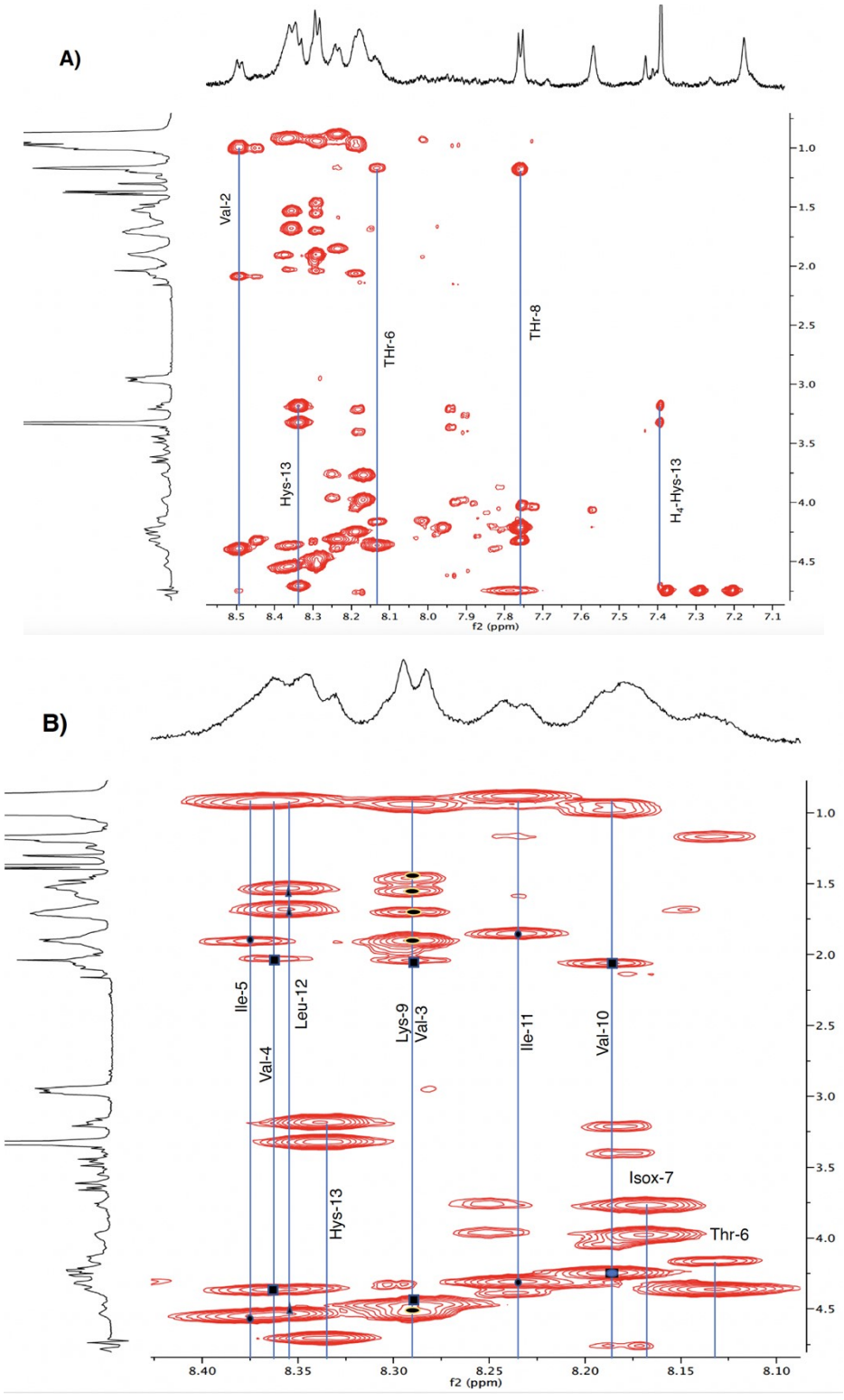


Figure S11. TOCSY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K): **A)** NH/CH region; **B)** zoom of NH/CH region.

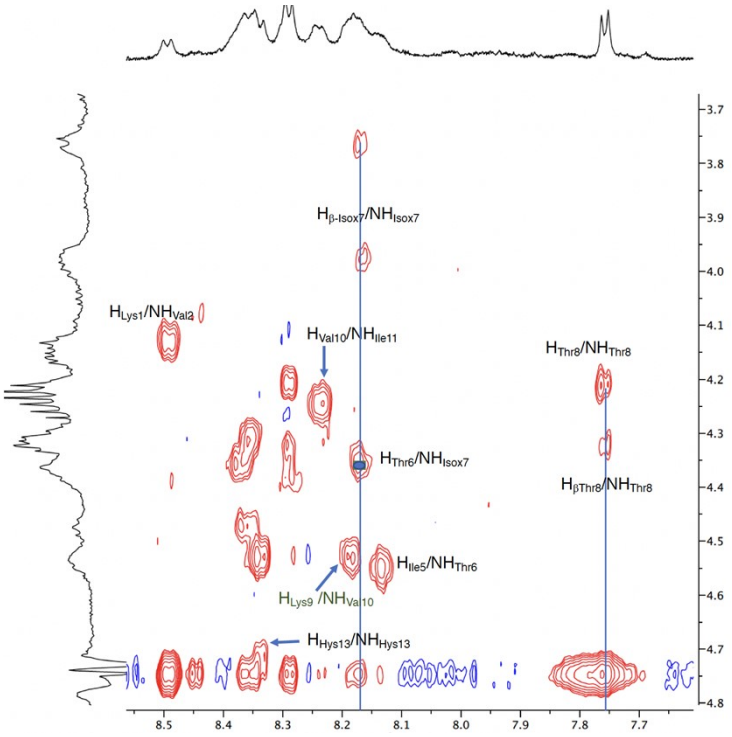


Figure S12. NOESY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K, 200 ms): zoom of NH/CH_α region.

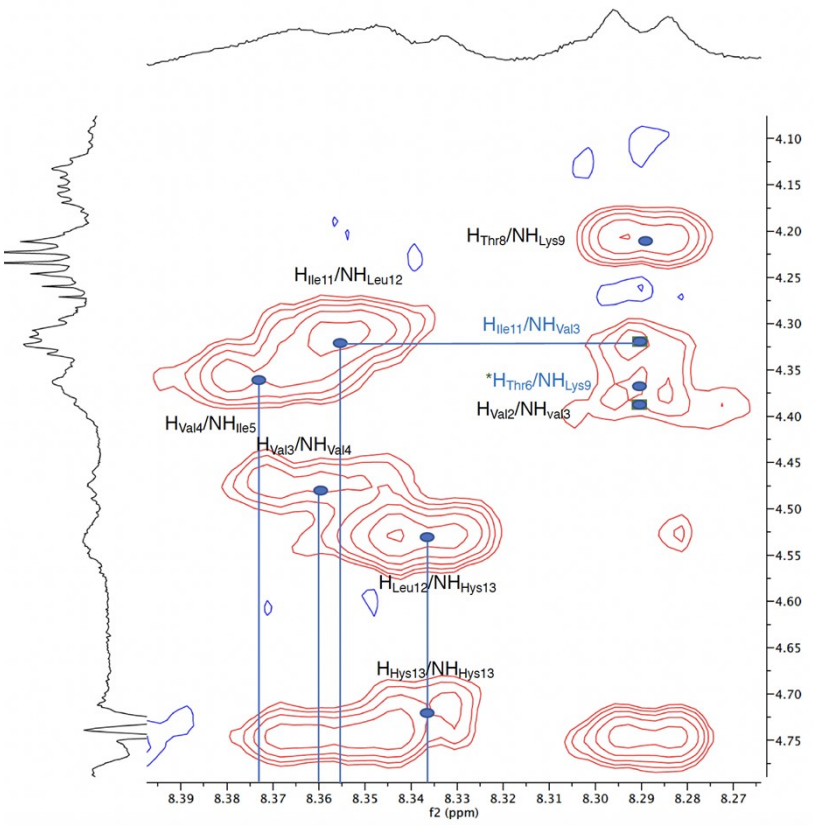


Figure S13. NOESY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K, 200 ms): Zoom of NH (8.39-8.27 δ region)/CH_α region (Black: CH_α/NH (*i, i+1*) NOEs; Blu: intra-strand NOEs); *Tentatively assigned due to the overlapped signals.

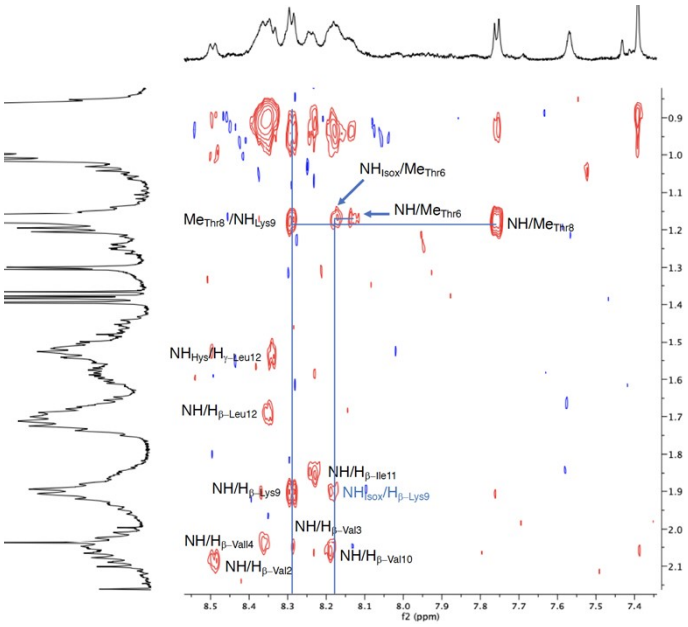


Figure S14. NOESY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K, 200 ms): NH/CH (high field region; Blue: intra-strand NOE).

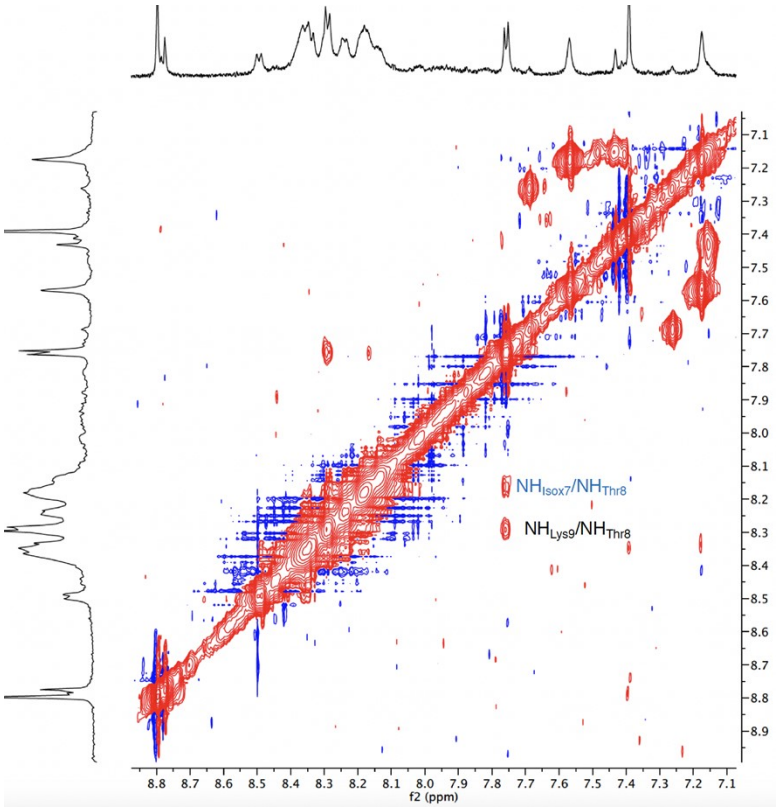


Figure S15. NOESY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K, 200 ms): NH/NH region; Black: NH/NH (i, i+1) NOE; Blue: intra-strand NOE).

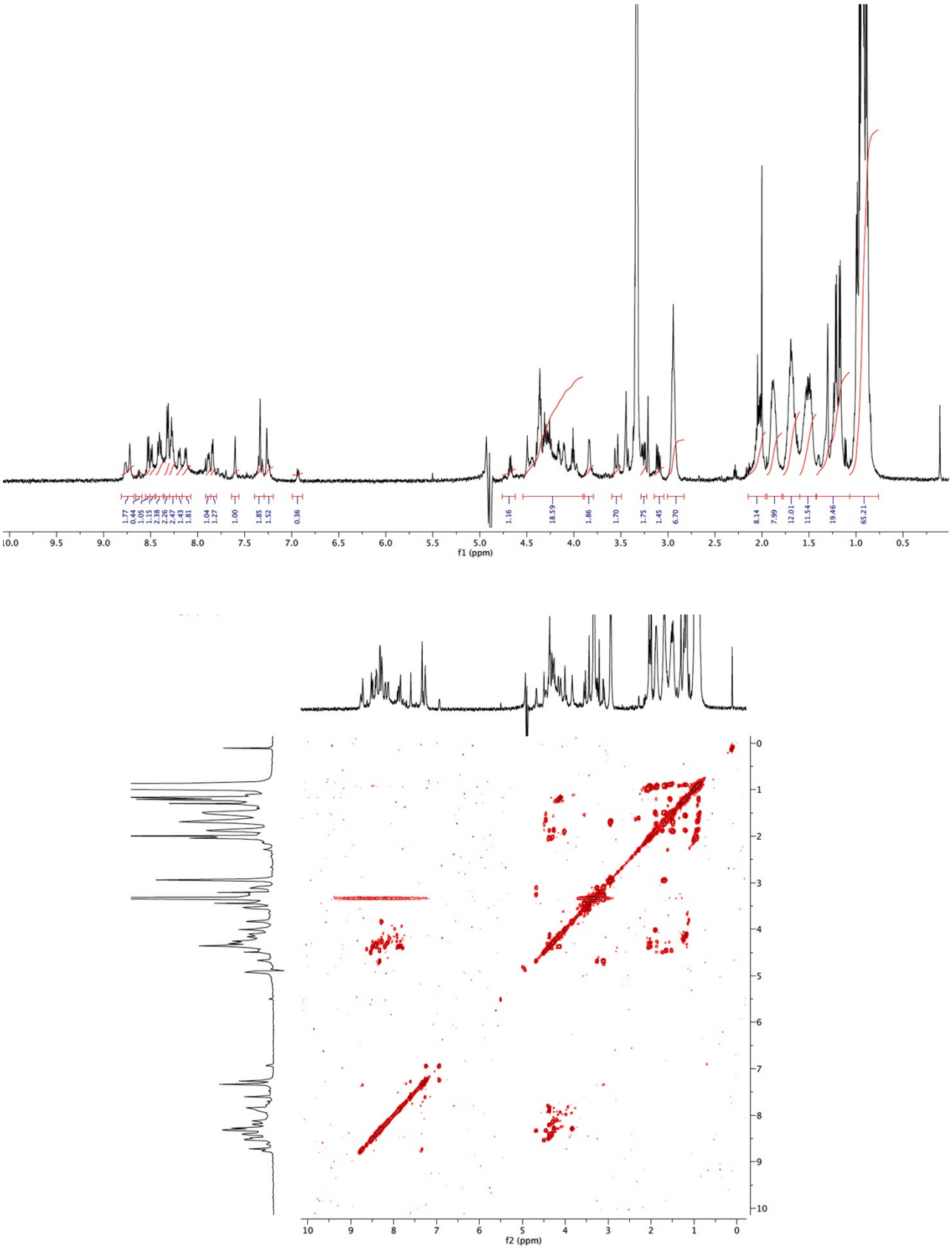
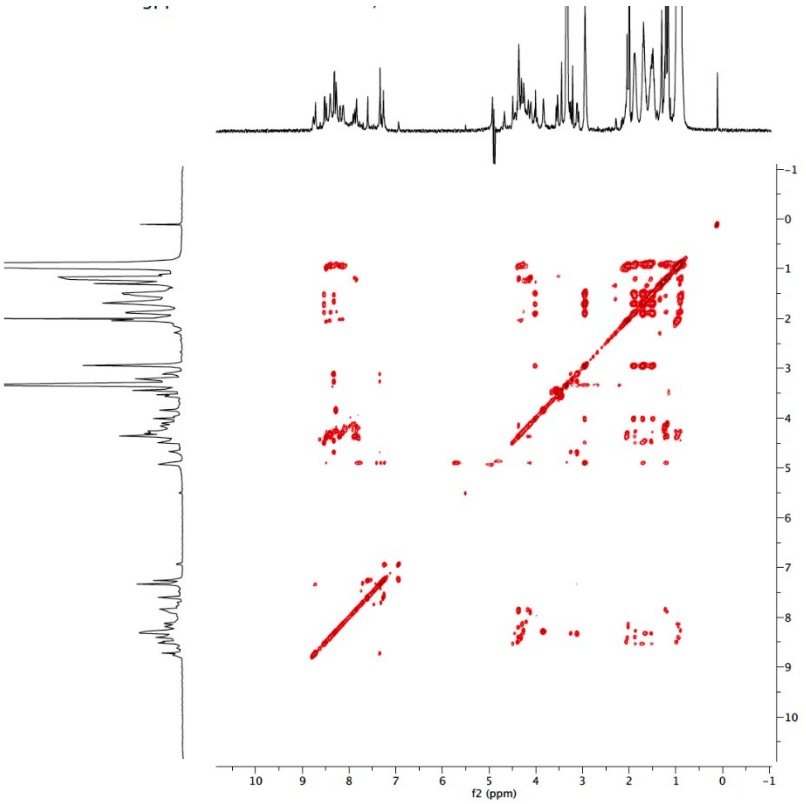


Figure S16. ^{1}H NMR experiment of **β-FH1** (CD_3OH, 4.0 mg in 750 μL, T = 298 K)

Figure S17. COSY experiment of **β-FH1** (CD_3OH, 4.0 mg in 750 μL, T = 298 K)



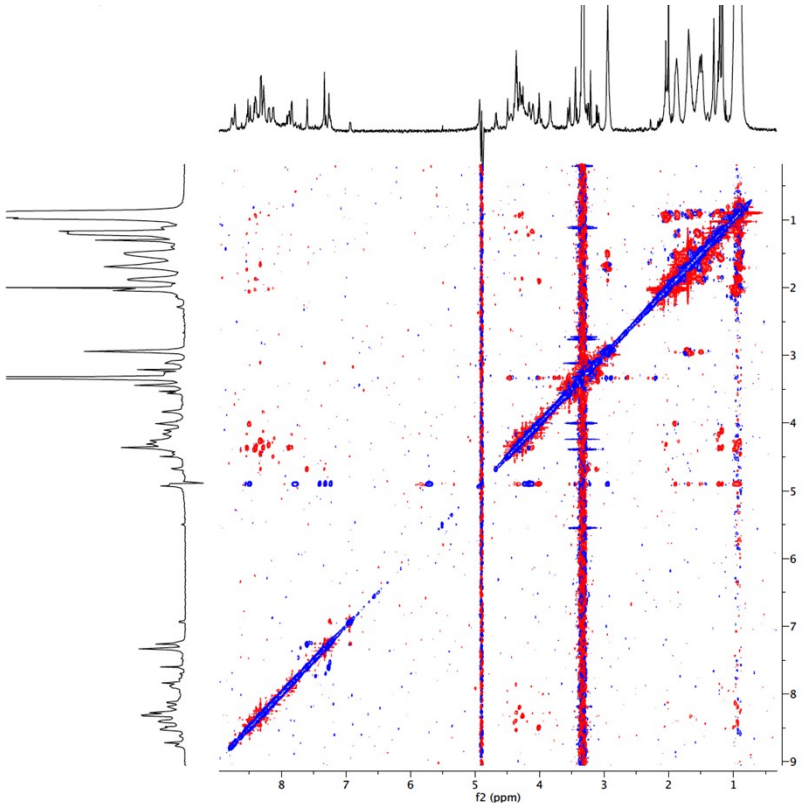


Figure S18. TOCSY experiment of **β-FH1** (CD_3OH, 4.0 mg in 750 μL, T = 298 K)

Figure S19. ROESY experiment of **β-FH1** (CD_3OH, 4.0 mg in 750 μL, T = 298 K)

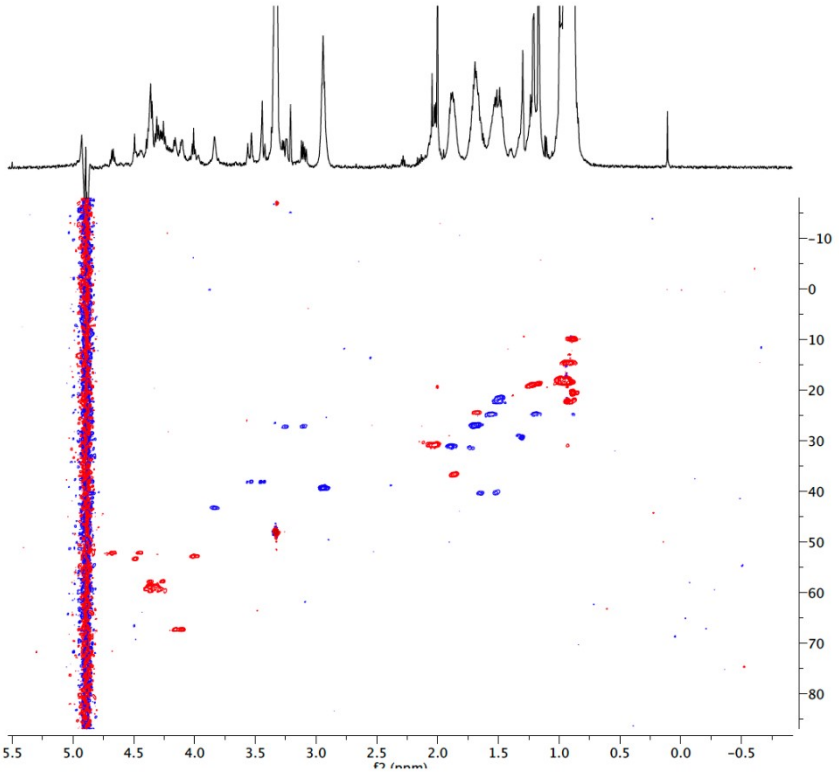


Figure S20. HSQC experiment of **β-FH1** (CD_3OH, 4.0 mg in 750 μL, T = 298 K): zoom of H-high field region

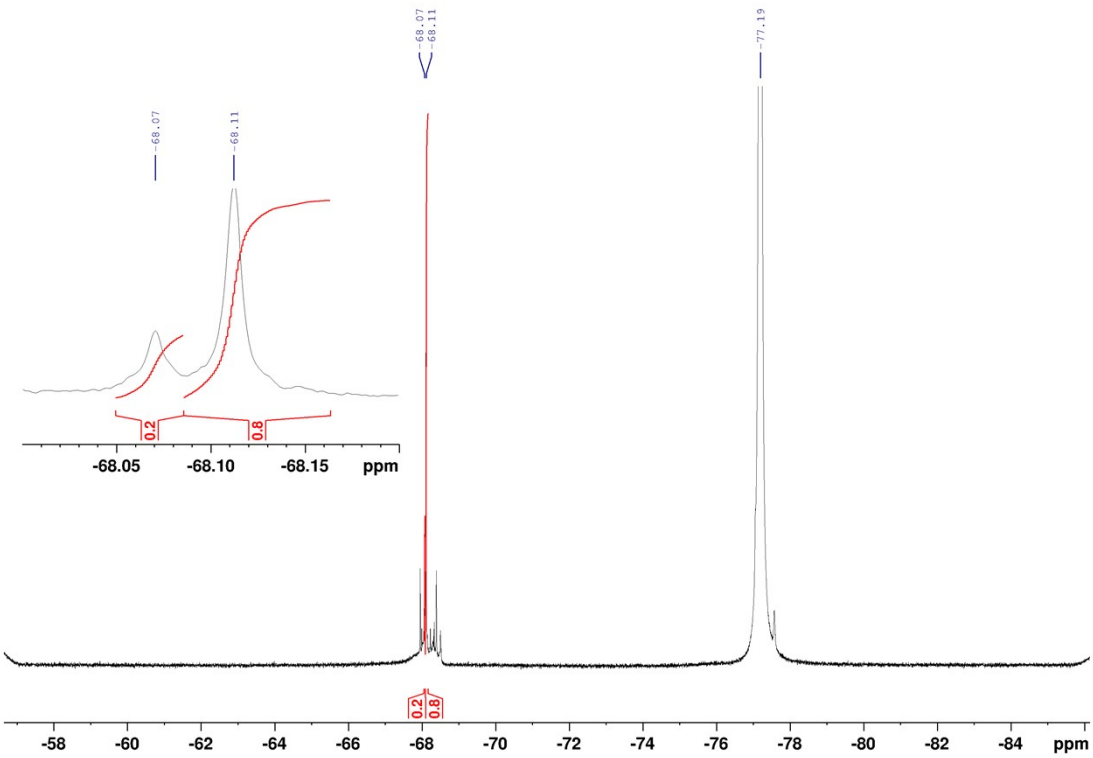


Figure S21a. ^{19}F NMR experiment of **β-FH1** (CD_3OH, 3.715 mg in 600 μL, T = 278 K).

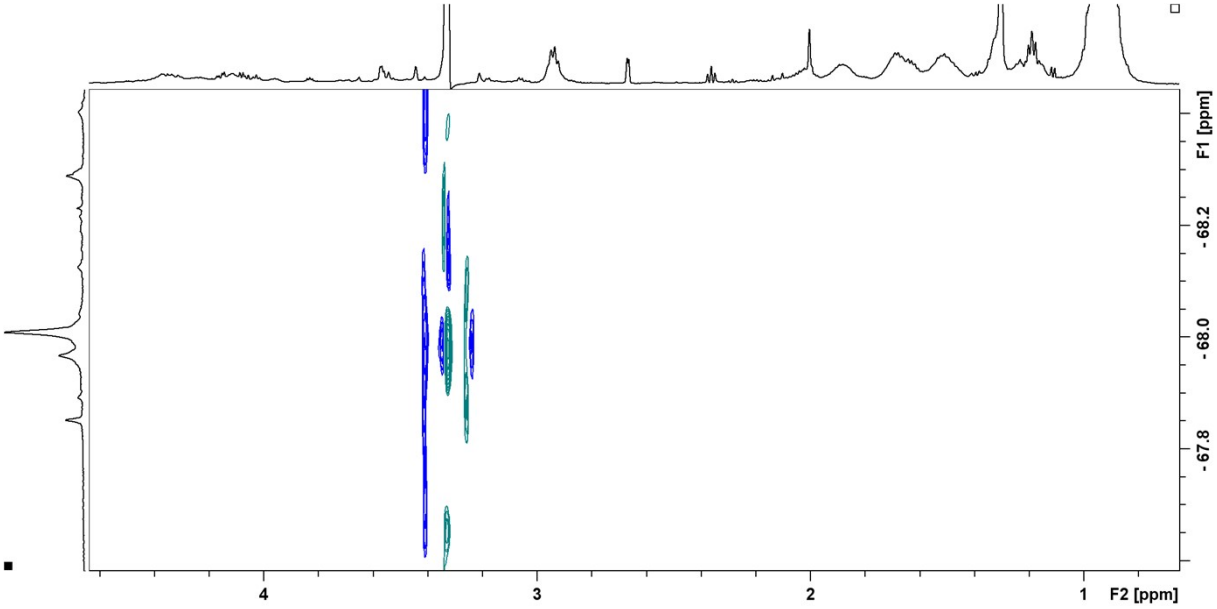


Figure S22.b. ^{19}F-^{1}H HOESY experiment of **β-FH1** (CD_3OH, 3.715 mg in 600 μL, T = 298 K).

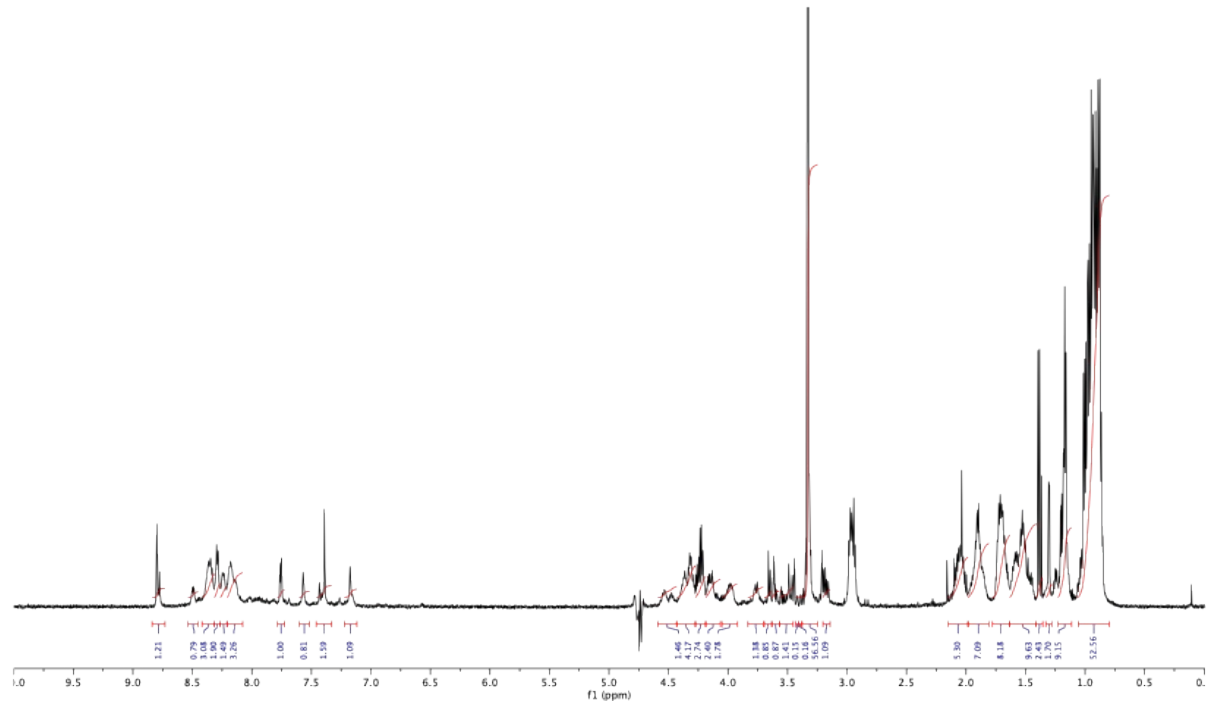


Figure S23. ^{1}H NMR experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K)

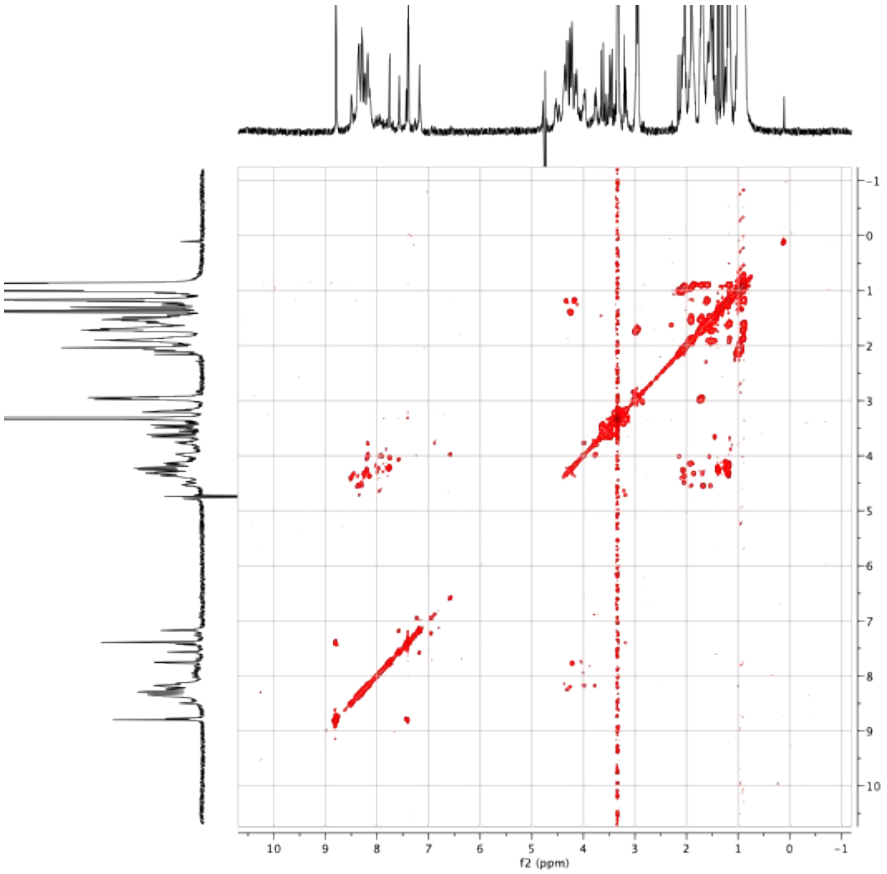


Figure S24. COSY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 µL, T = 313 K)

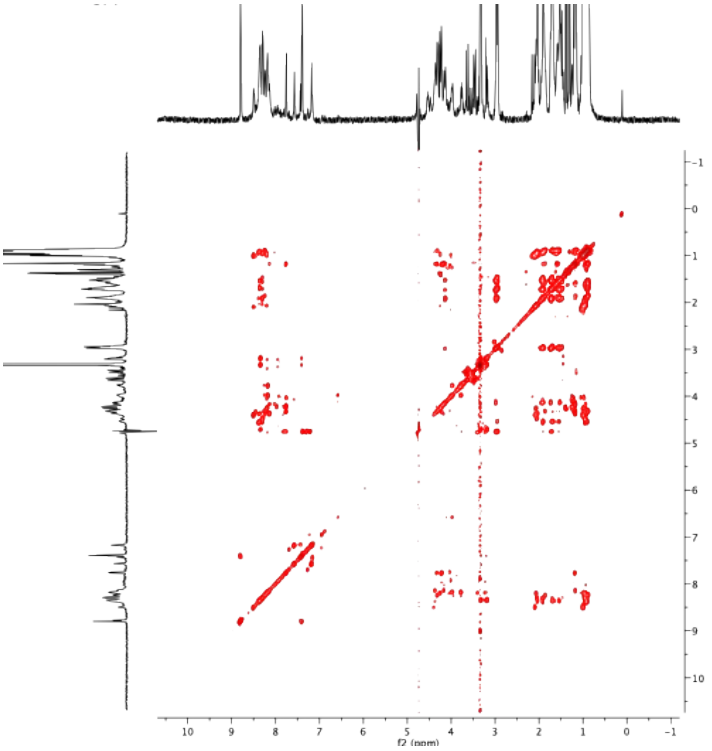


Figure S25. TOCSY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 µL, T = 313 K)

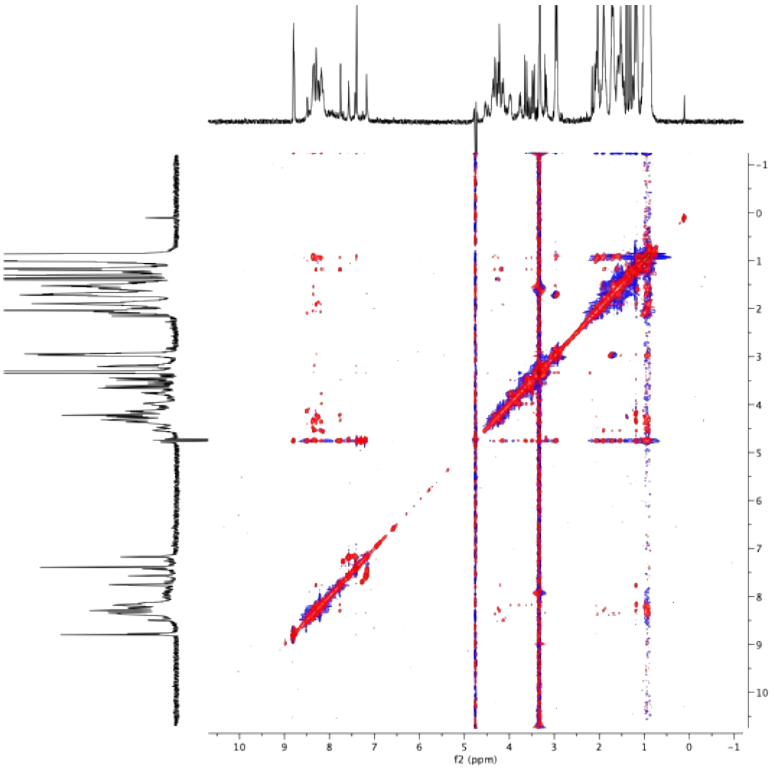


Figure S26. NOESY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K, 200 ms)

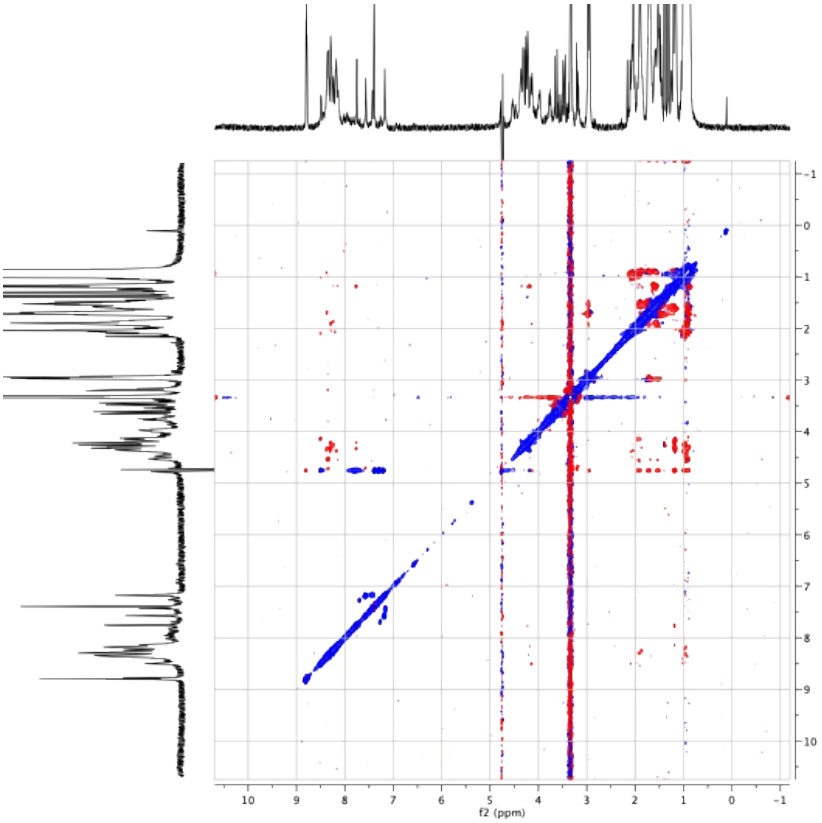


Figure S27. ROESY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K, 200 ms)

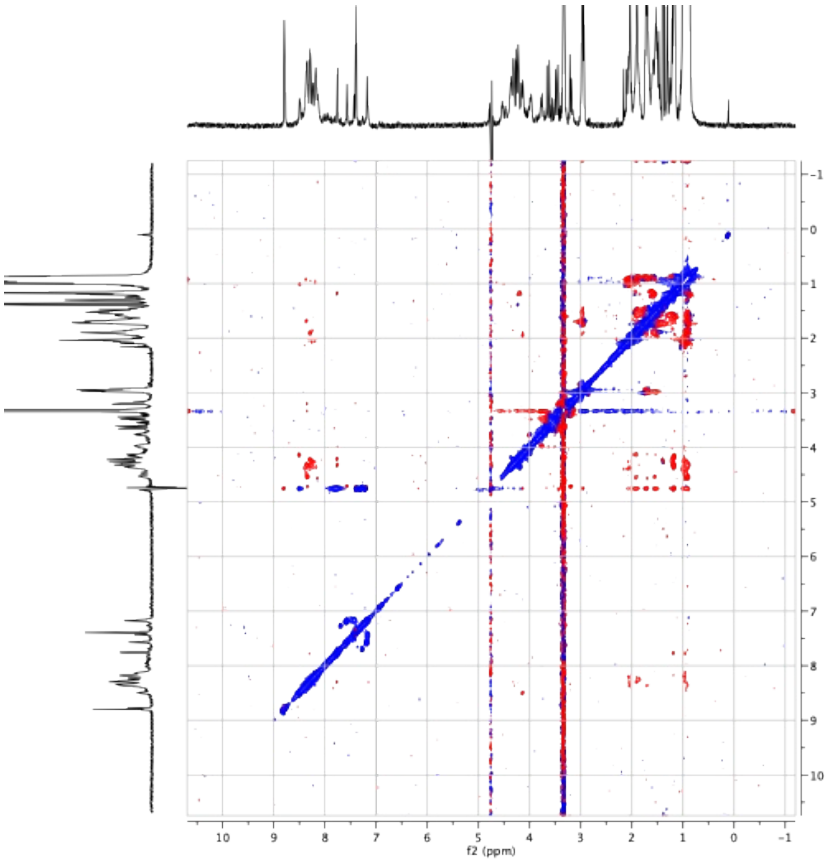


Figure S28. ROESY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K, 150 ms)

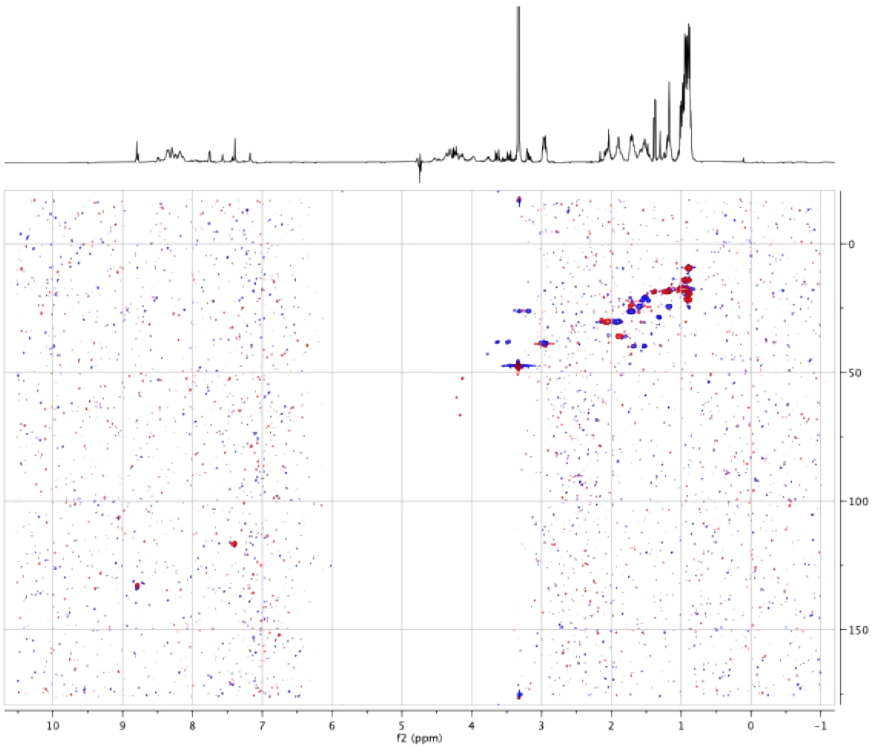


Figure S29. HSQC experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 313 K)

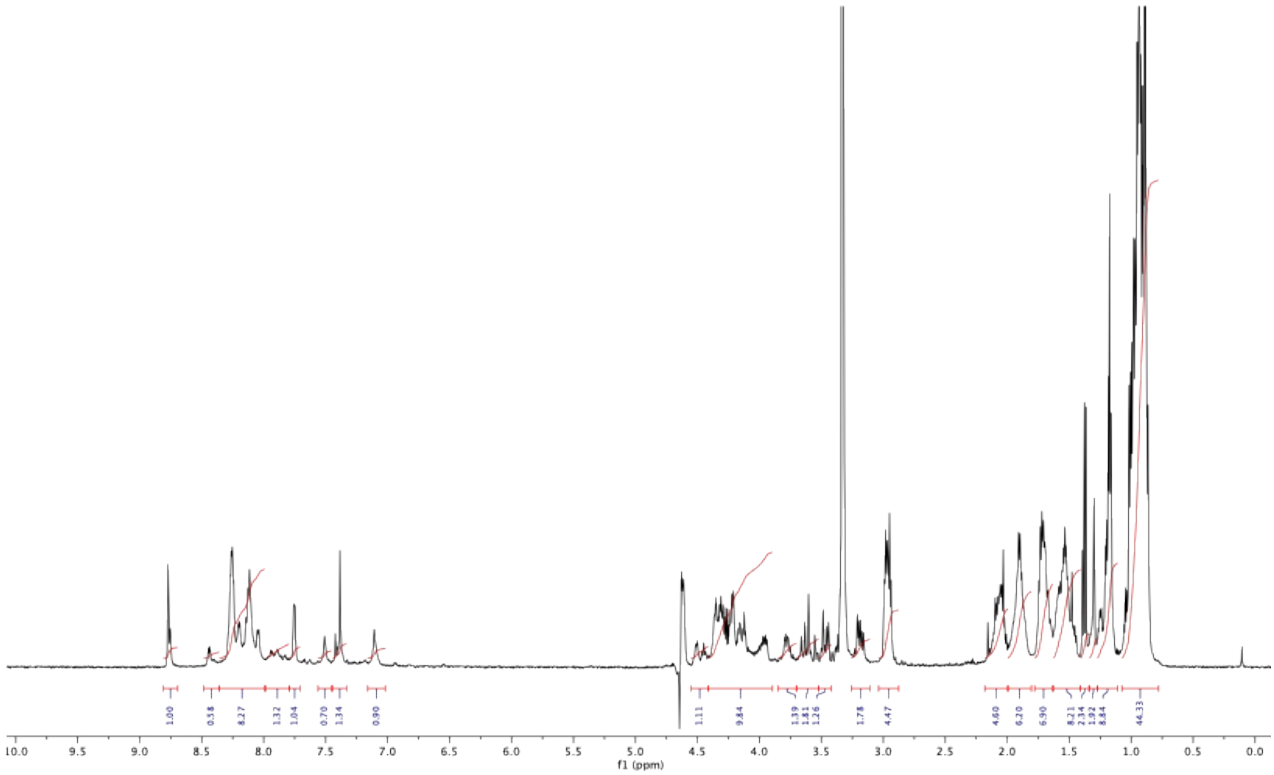


Figure S30. ^{1}H NMR experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 323 K)

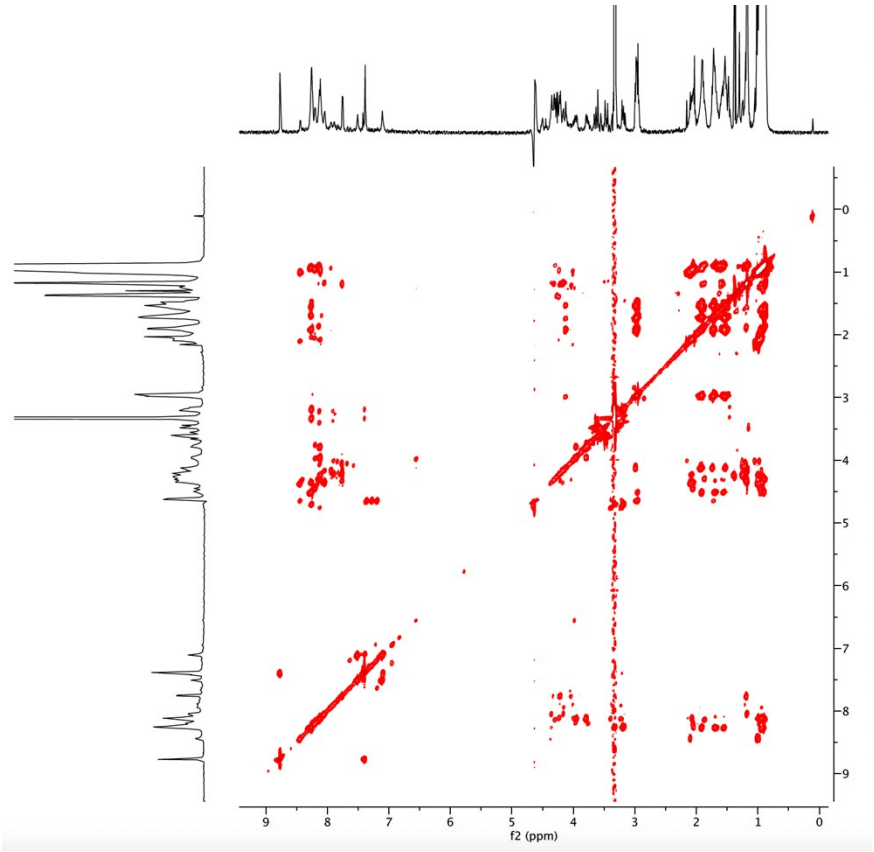


Figure S31. TOXY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 323 K)

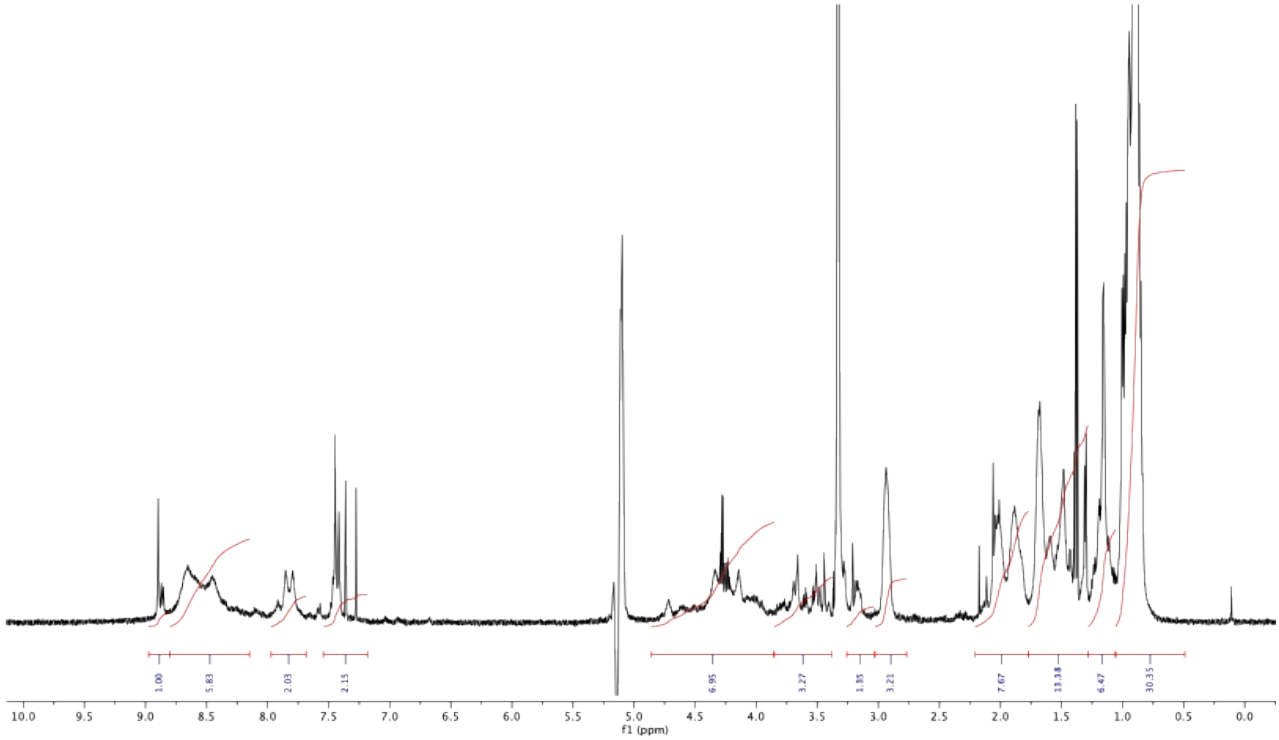


Figure S32. ^{1}H NMR experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 273 K)

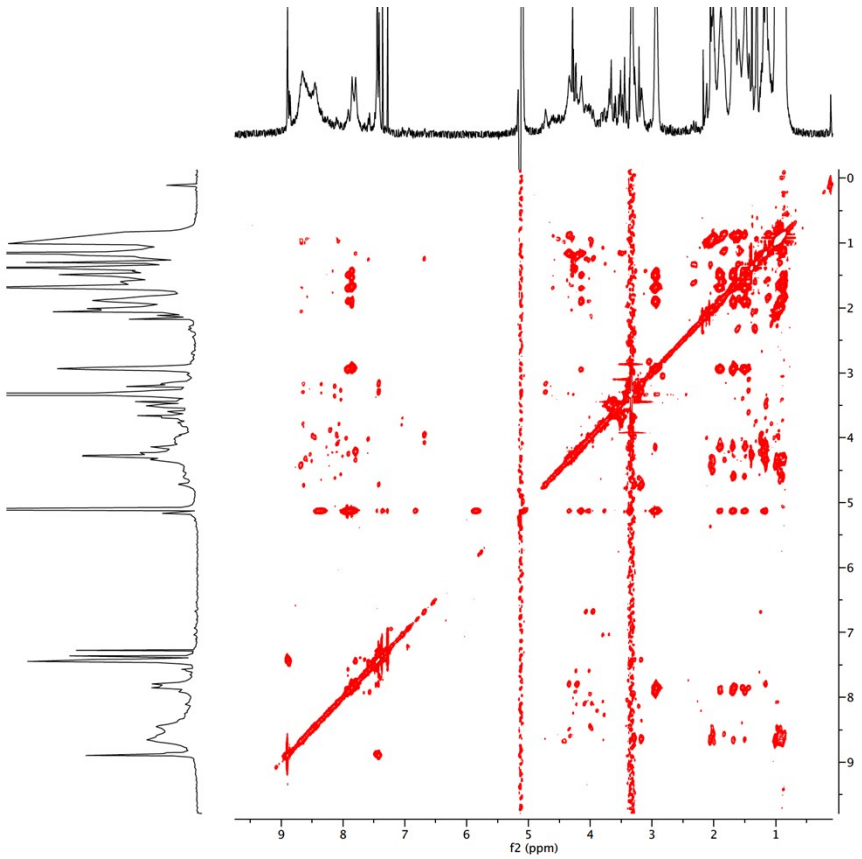


Figure S33. TOCSY experiment of **β-FH2** (CD_3OH, 4.1 mg in 750 μL, T = 273 K)

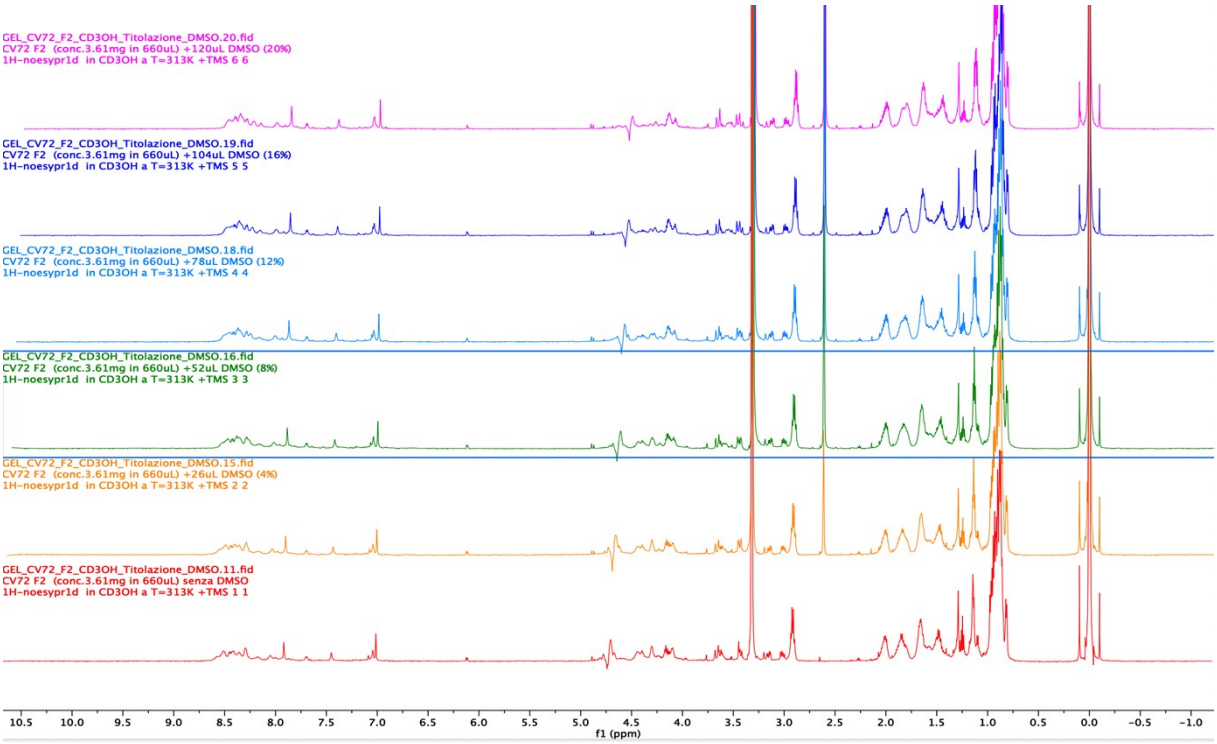


Figure S34. DMSO-d_6 (0, 4, 8, 12, 16, 20%) titration of experiment of **β-FH2** (CD_3OH, 3.61 mg in 660 μL, T = 313 K)

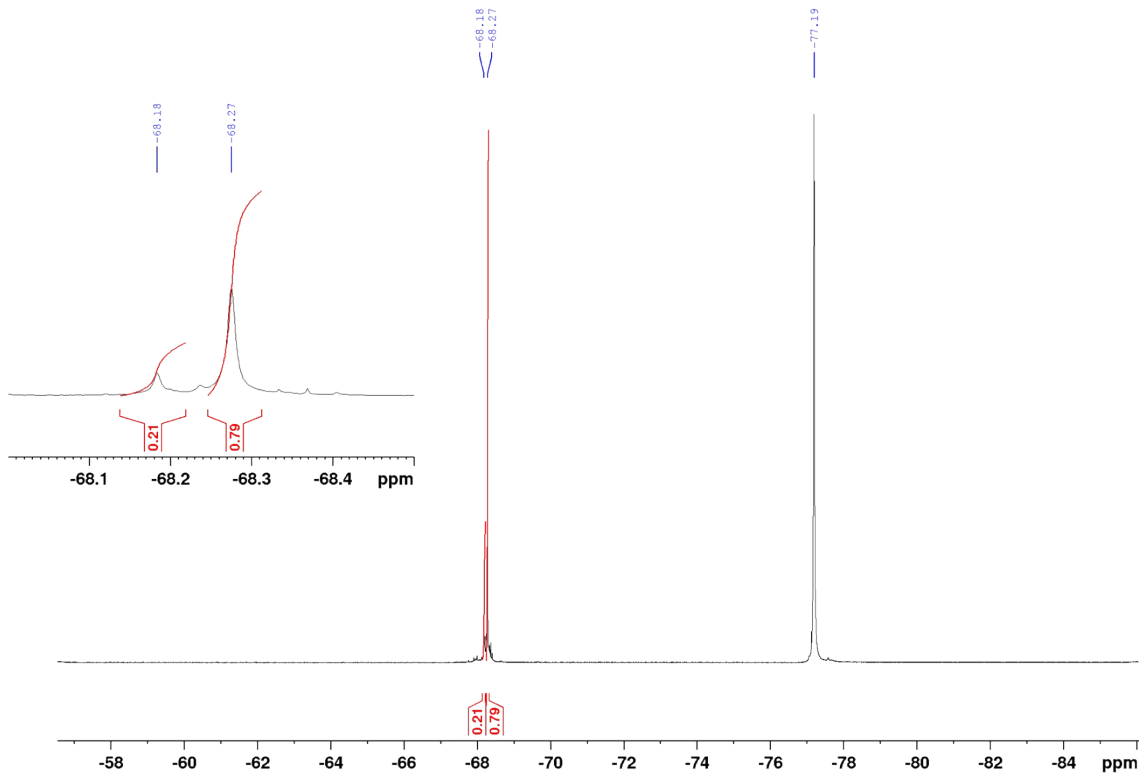


Figure S35. ^{19}F NMR experiment of **β-FH2** (CD_3OH, 3.51 mg in 660 μL, T = 278 K).

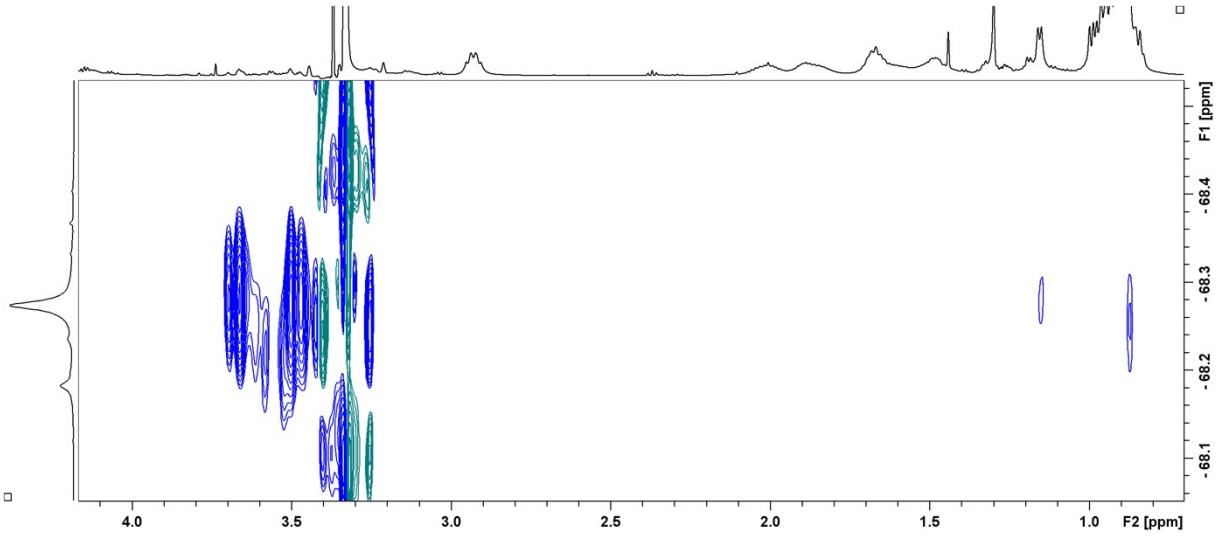


Figure S36. ^{19}F-^{1}H HOESY NMR experiment of **β-FH2** (CD_3OH, 3.51 mg in 660 µL, T = 278 K).

X. Cellular biological assays

A. Live-cell imaging and Fluorescence Decay after Photoactivation (FDAP)

FDAP experiments were performed essentially as previously described.[3] Briefly, cells expressing Tau441-ΔK280 were plated on 35-mm glass-bottom culture dishes (MatTek, USA), transfected, and neuronally differentiated by medium exchange to serum-reduced DMEM containing 100 ng/mL 7S mouse NGF. After 3 days, the medium was exchanged to serum-reduced DMEM without phenol red with NGF, and the respective compounds were added at 25 µM. After 20 h, live cell imaging was performed using a laser scanning microscope (Nikon Eclipse Ti2-E(Nikon, Japan)) equipped with a LU-N4 laser unit with 488-nm and 405-nm lasers and a Fluor 60×ultraviolet-corrected objective lens (NA 1.4) enclosed in an incubation chamber at 37°C and 5% CO_2. Photoactivation was performed with a 405-nm laser using the microscope software (NIS-Elements version AR 5.02.03 (Nikon, Japan)). A series of consecutive images was acquired at a frequency of 1 frame/s, and 112 images were collected per activated cell at a resolution of 256×256 pixels. Effective diffusion constants were determined by fitting the fluorescence decay data from the photoactivation experiments using a one-dimensional diffusion model function for FDAP.[4] A refined reaction-diffusion model was used to estimate the association rate k^*_{on} and the dissociation rate k_{off} constant of Tau binding to microtubules.[5]

B. Determination of Cell Viability via MTT Assay

PC12 cells were cultured in 96-well plates with 5 × 10^3 cells/well in serum-reduced medium supplemented with 100 ng/ml 7S mouse NGF. The cells were incubated for 48 h to induce neuronal differentiation. Concentration-response profiling for **β-FH1** and **β-FH2** was performed in triplicates at five concentrations from 0.2 to 100 µM. **β-FH1** or **β-FH2** was added to each well 20 h prior to measurement. Control wells remained untreated. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the wells at a final concentration of 1 mg/ml. The cells

were incubated for a further 2 h in the cell culture incubator before the reaction was stopped by adding 50 µl of lysis buffer (20% w/v sodium dodecyl sulfate in 1:1 v/v *N*,*N*-dimethylformamide/water, pH 4.7). After overnight incubation at 37 °C, the optical density of the formazan product was determined at 570 nm. The MTT conversion measurements were normalized to the optical density of the negative controls.

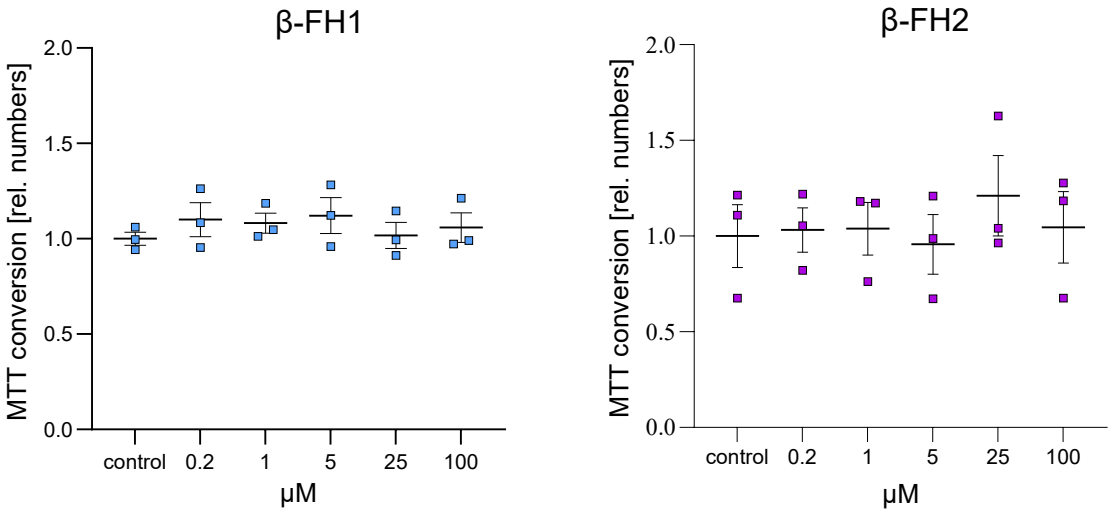


Figure S37: MTT assays of **β-FH1** and **β-FH2** to measure metabolic activity as an indicator of cell viability. Neuronally differentiated PC12 cells were exposed to the respective compound for 20 hours. Each compound and concentration was measured in triplicate on three independent assay plates; mean±SEM of the three plates is shown. Concentrations up to 100 µM did not impair cell viability.

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