Supporting information

SI_nhPHIP

Parahydrogen Hyperpolarized NMR Detection of Underivatized Short Oligopeptides

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1. Chemicals and materials

Pyridine, 3-methylpyridine, 3-fluoro-4-methylpyridine (3F4MePy), 4-methylpyridine, 3,5-dimethylpyridine, 4-fluoropyridine, 4-tertbutylpyridine, 3,5-difluoropyridine, 3-fluoropyridine, L-alanine, L-leucine, L-phenylalanine were purchased from common international chemical suppliers and used as supplied. Alanine di-, tri-, and pentamer were purchased from Sigma Aldrich. Papain inhibitor (GGYR), Leucine enkephalin (YGGFL), and Hexapeptide-11 (FVAPFP) were purchased from Cayman Chemical Company. The iridium catalyst complex precursor [Ir(Cl)(COD)(IMes)] (COD: cyclooctadiene, IMes: 1,3-bis(2,4,6-trimethylphenyl)-imidazole-2-ylidene) was prepared according to published methods.[1,2] 1-methyl-1,2,3-triazole (mtz) was synthesized based on a published procedure[3] at −21 ˚C and purified by vacuum distillation.

Urine sample handling (Figure S16) was approved by the Estonian Research Ethics Committee of the National Institute for Health Development of Estonia (Decision No 686). The urine sample used in this work was donated by a healthy adult. Urine was collected as morning first midstream urine and stored at -80 °C. Frozen sample was allowed to thaw over a room temperature water bath prior to sample preparation. Excess urine sample material was discarded after melting, in accordance with local regulations on handling human derived samples.

2. nhPHIP experiments

nhPHIP experiments were carried out similarly to a published procedure[10] with slight changes in cosubstrate and analyte concentrations. The active nhPHIP catalyst ($[Ir(H_2)(IMes)(cosubstrate)_3]^+$, was prepared *in situ* in a 5 mm intermediate pressure valved NMR tube. Ir-catalyst and cosubstrate were added into the tube, pressurized under 5 bar of H_2, shaken, and allowed to react for 2 h before adding oligopeptide solution. The tube was connected to the nhPHIP setup, repressurized under 5 bar of H_2 and inserted into the NMR spectrometer. NMR sample assembly is described fully in The Supporting Information of our previous publication.[10]

Hyperpolarized NMR was conducted by leading 50% enriched parahydrogen (pH_2) gas into the tube through a PEEK capillary under pulse program control. pH_2 was produced by catalytic conversion at liquid N_2 temperature. The conversion was conducted continuously in flow using iron(III) oxide (Sigma-Aldrich, product 371254-50G, 30-50 mesh) as hydrogen conversion catalyst. pH_2 bubbling was facilitated by a custom-built bubbling control box that switched hydrogen gas flow through the sample tube.[10]

NMR experiments were recorded at on an 800 MHz Bruker Avance III spectrometer equipped with a room temperature inverse probe or a cryogenically cooled probe. Hyperpolarized spectra were recorded at 10 °C and 25 °C sample temperature, using a SEPP pulse sequence[11] for 1D (64 scans unless stated otherwise) and zero-quantum COSY for 2D measurements (2 scans, 256 increments).[10]

To accelerate the formation of equilibrium *ax-eq* complex, NMR sample was heated at 37.5 or 50 °C water bath between 5 to 7.5 min as specified in the main text. After that time, the sample was transferred directly into the spectrometer, which was operating at 10 °C sample temperature. The sample was allowed to stabilize in the spectrometer for a few minutes before recording multiple nhPHIP 1D experiments to ensure sample stability.

2.2.Sample preparation

2.2.1. Sample preparation for alanine peptides under basic conditions

After dissolving an oligopeptide in water, NaOH was added to reach pH 11. Solution was lyophilized to remove excess water and re-dissolved in methanol-d_4 prior to the nhPHIP experiment. Ir-catalyst and cosubstrate were dissolved in methanol-d_4. The nhPHIP sample consisted of 1.2 mM iridium catalyst [Ir(Cl)(COD)(IMes)], 18-fold excess of 3F4MePy and 0.1 mM oligopeptide with a total sample volume of 600 µL.

2.2.2. Sample preparation for functional peptides

Three functional oligopeptides were chosen as examples that cover a broad range of applications. Papain inhibitor is a tetrapeptide (GGYR), which inhibits the peptidase activity of papain, a proteolytic enzyme. Leucine enkephalin (YGGFL) is an endogenous neuropeptide found in the brains of animals, including humans. Hexapeptide-11 (FVAPFP) is a signaling peptide used in cosmeceuticals for its wrinkle smoothing properties. Two sample preparation protocols were followed:

a) After dissolving an oligopeptide in water, NaOH was added to reach pH 7.4 – 7.9. Solution was lyophilized to remove excess water and re-dissolved in methanol-d_4 prior to the nhPHIP experiment. Ir-catalyst and cosubstrate were dissolved in methanol-d_4. The nhPHIP sample consisted of 1.2 mM iridium catalyst [Ir(Cl)(COD)(IMes)], 18-fold excess of 3F4MePy and 0.1 mM oligopeptide with a total sample volume of 600 µL;
b) Peptide was dissolved in methanol-d_4 as supplied. Ir-catalyst and cosubstrate were dissolved in methanol-d_4. The nhPHIP sample consisted of 1.2 mM iridium catalyst [Ir(Cl)(COD)(IMes)], 8-fold excess of 3F4MePy and 0.1 mM oligopeptide with a total sample volume of 600 µL.

2.2.3. Sample preparation for alanine peptides under neutral conditions

Oligopeptide, Ir-catalyst and cosubstrate were dissolved in methanol-d_4. The nhPHIP sample consisted of 1.2 mM [Ir(Cl)(COD)(IMes)], 18-fold excess of 3F4MePy and 0.1 mM oligopeptide with a total sample volume of 600 µL.

2.2.4. Sample preparation for ^{15}N-labelled alanine peptides

A[15N]-A-A-OMe and A-A[15N]-A-A-OMe, Ir-catalyst and cosubstrate were dissolved in methanol-d_4. The nhPHIP sample consisted of 1.2 mM [Ir(Cl)(COD)(IMes)], 8-fold excess of 3F4MePy and 0.7 mM oligopeptide with a total sample volume of 600 µL.

2.2.5. Sample preparation for A_2 and A_3 nhPHIP complex characterization by ^{1}H, COSY, NOESY, HSQC and HMBC

Oligopeptide, Ir-catalyst and cosubstrate were dissolved in methanol-d_3. The nhPHIP sample consisted of 1.2 mM [Ir(Cl)(COD)(IMes)], 18-fold excess of 3F4MePy and 1 mM oligopeptide with a total sample volume of 600 µL.

2.3.nhPHIP chemosensor system optimization

Ten cosubstrates (mtz, pyridine, and pyridine derivatives) were tested on a mixture of amino acids (Ala, Leu, Phe) under basic conditions. Mixture of Phe, Leu, and Ala with 1 mM concentration of

each analyte was prepared in *MilliQ* H_2O. The mixture was brought to pH 11 with addition of NaOH, lyophilized, and dissolved in methanol-d_4 prior the nhPHIP experiment.

The main factors which were considered in choosing an optimal cosubstrate were intensity of nhPHIP signals, signal line width, number of signals produced per analyte, stability of signal intensity in longer measurements, and Ir-catalyst activation time with the chosen cosubstrate. For example, 3,5-dimethylpyridine formed amino acid nhPHIP complexes, but their lifetimes were short and the intensity of nhPHIP signals dropped four-fold within 30 min; 3,5-difluoropyridine did not produce an active Ir-catalyst after 4 h reaction time; 4-fluoropyridine did not give any observable signals in the expected δ −23.5 and −28.5 regions; etc. Out of the tested cosubstrates, 3-fluoro-4-methylpyridine (3F4MePy) showed the most intensive signals for amino acid nhPHIP complexes at 25 ˚C of Ala, Leu and Phe. Since measurements at lower temperatures gave narrower lines and more intensive signals, fallowing experiments were carried out at 10 ˚C.

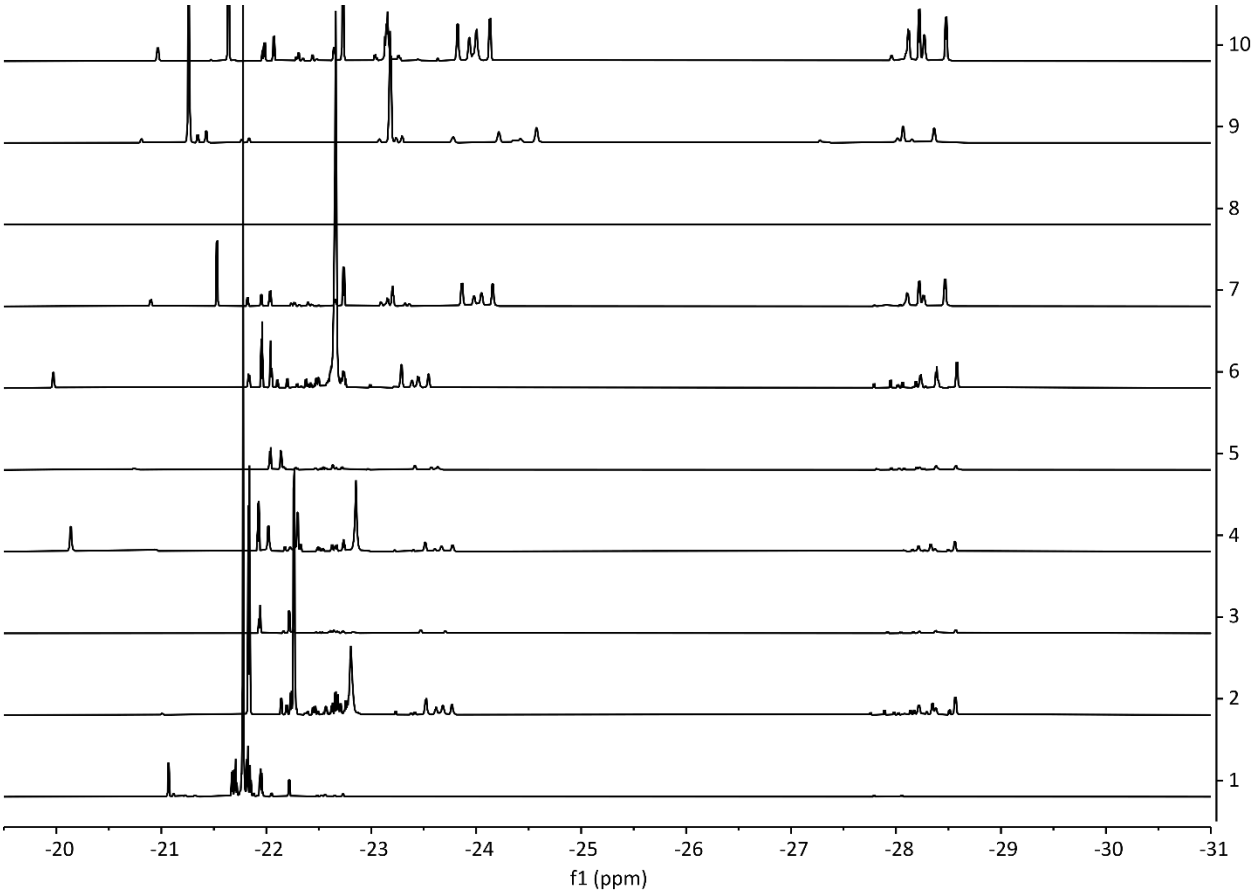


Figure S1. Screening of co-substrates on a mixture of Ala, Leu and Phe under basic conditions recorded at 298 K. 1 – mtz, 2 – pyridine, 3 – 3-methylpyridine, 4 – 4-methylpyridine, 5 – 3,5-dimethylpyridine, 6 – 4-tertbutylpyridine, 7 – 3-fluoropyridine, 8 – 4-fluoropyridine (no observable signals), 9 – 3,5-difluoropyridine, 10 – 3-fluoro-4-methylpyridine.

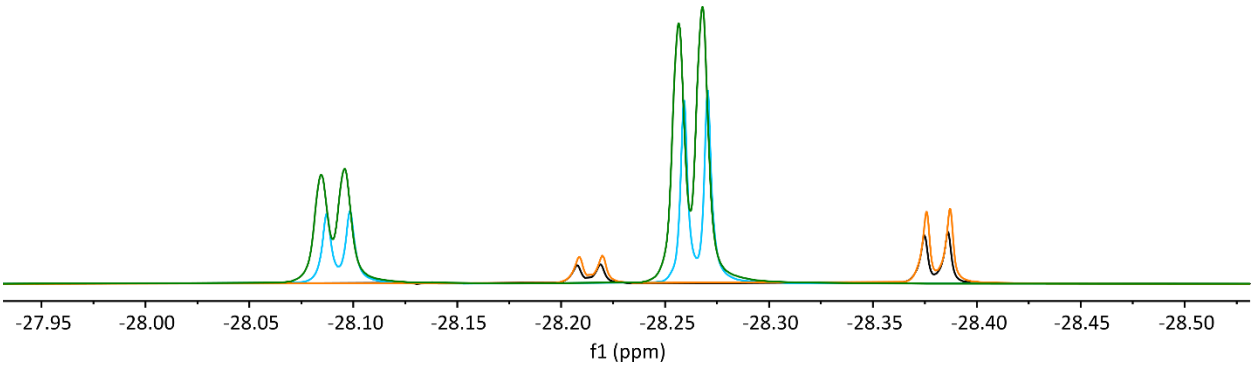


Figure S2. nhPHIP spectra of H_A *ax-eq* signals of L-alanine-nhPHIP complex with pyridine as cosubstrate before heating (black) and after heating at 50 ˚C for 7.5 min (orange), L-alanine-nhPHIP complex with 3F4MePy as cosubstrate before heating (blue) and after heating (green). Spectra recorded at 283 K. 3F4MePy improves noticeably the nhPHIP response.

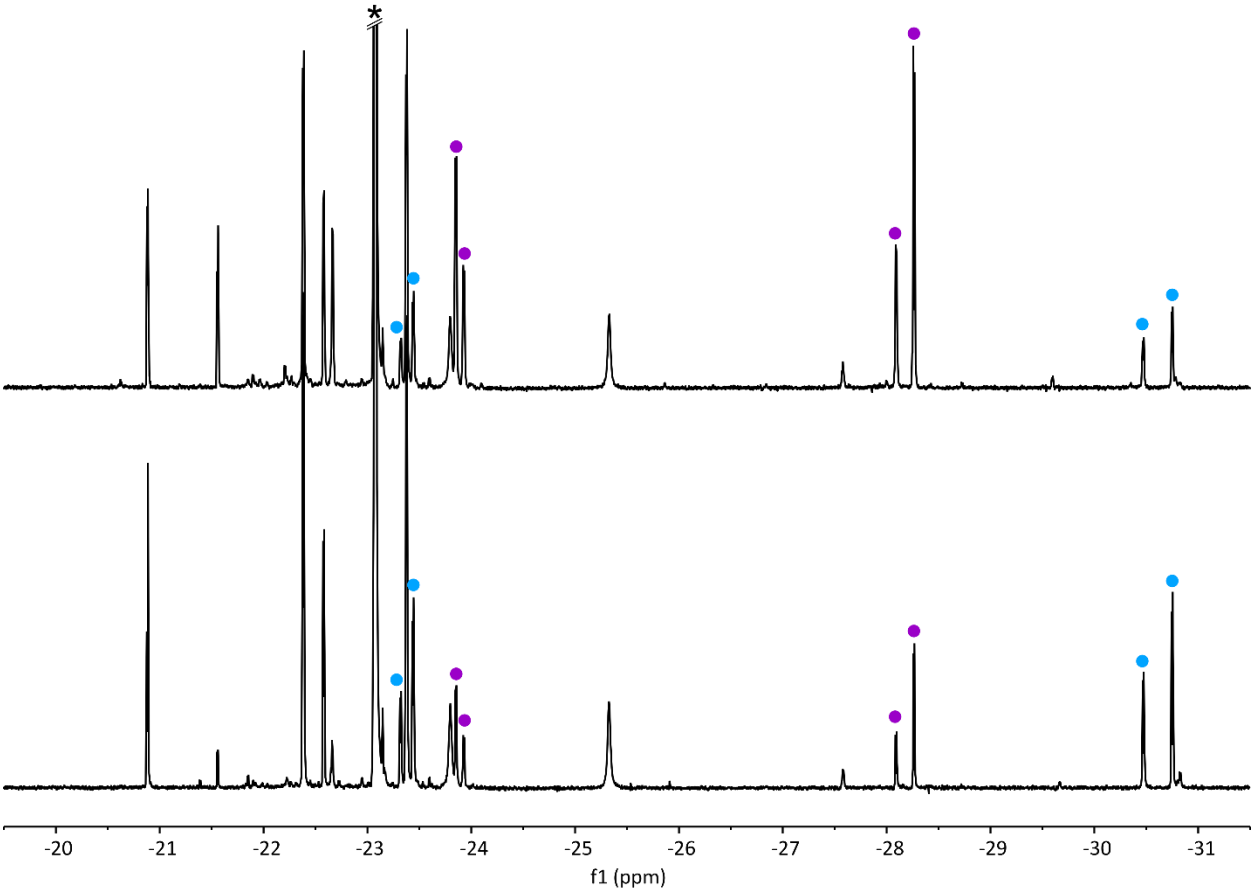


Figure S3. nhPHIP spectra of A_5 under basic conditions (bottom), A_5 under basic conditions after heating at 50 ˚C for 7.5 min (upper). Blue circles correspond to diastereomers of A_5 *ax-eq* complex. Spectra demonstrates the intensity increase of desired nhPHIP signals upon heating. Commercial A_5 contains traces of alanine monomer, undetectable by regular NMR, but observable with nhPHIP, indicated with violet circles. Asterisk (*) denotes the Ir-complex of 3F4MePy. Unassigned signals arise from iridium-complex of solvent and impurities. Sample contained a 1:18:0.08 ratio of catalyst : 3F4MePy : A_5, recorded at 10 °C.

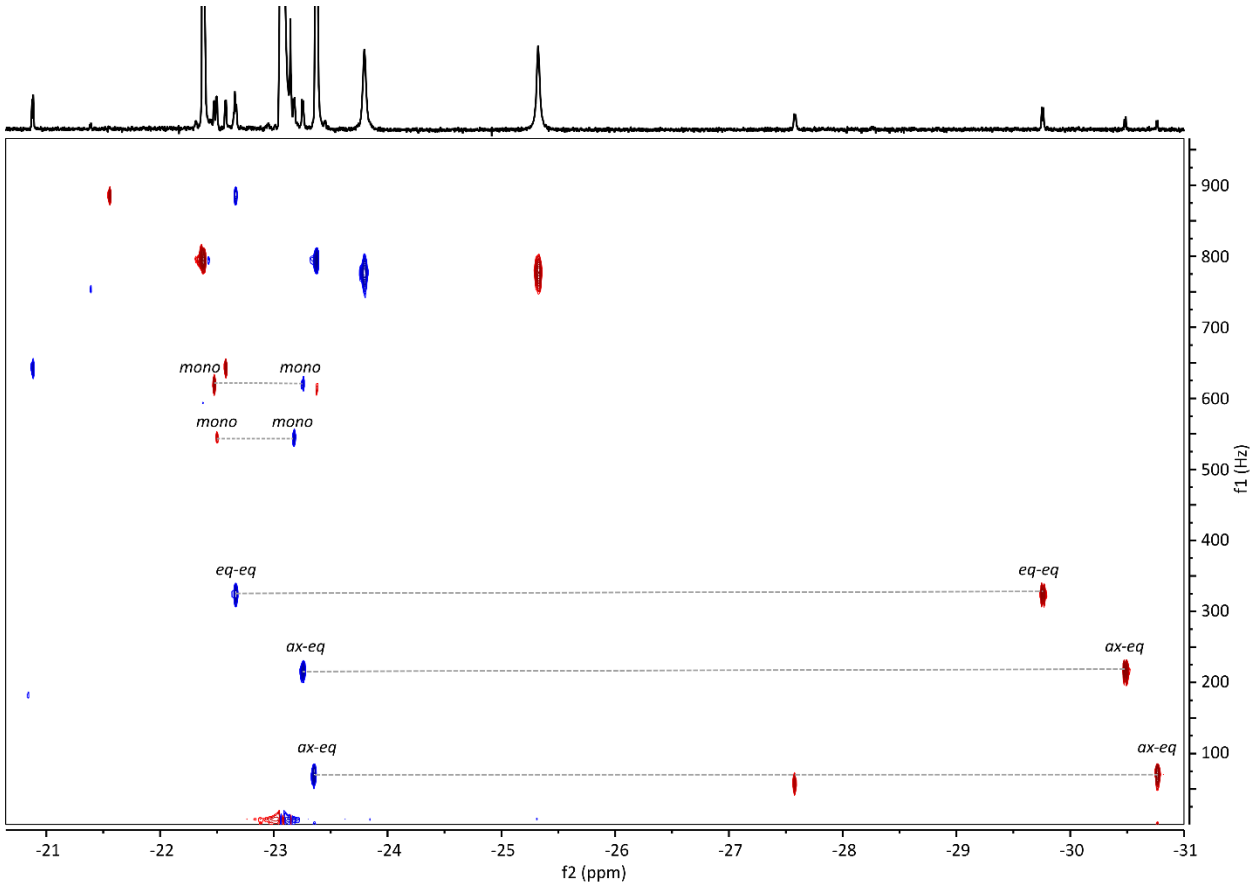


Figure S4. 2D nhPHIP zero quantum spectrum[10] of A_3 recorded at 283 K. Opposite phase (red and blue) H_A and H_X signals on the same zero-quantum frequency represent a single nhPHIP complex. Note that the hydride signal pairs for analyte complexes are folded in f_1 dimension since it was recorded in a 1000 Hz spectral width in f_1. Unassigned signals arise from iridium-complex of 3F4MePy, solvent, and impurities.

2.4.Elucidation of oligopeptide binding modes

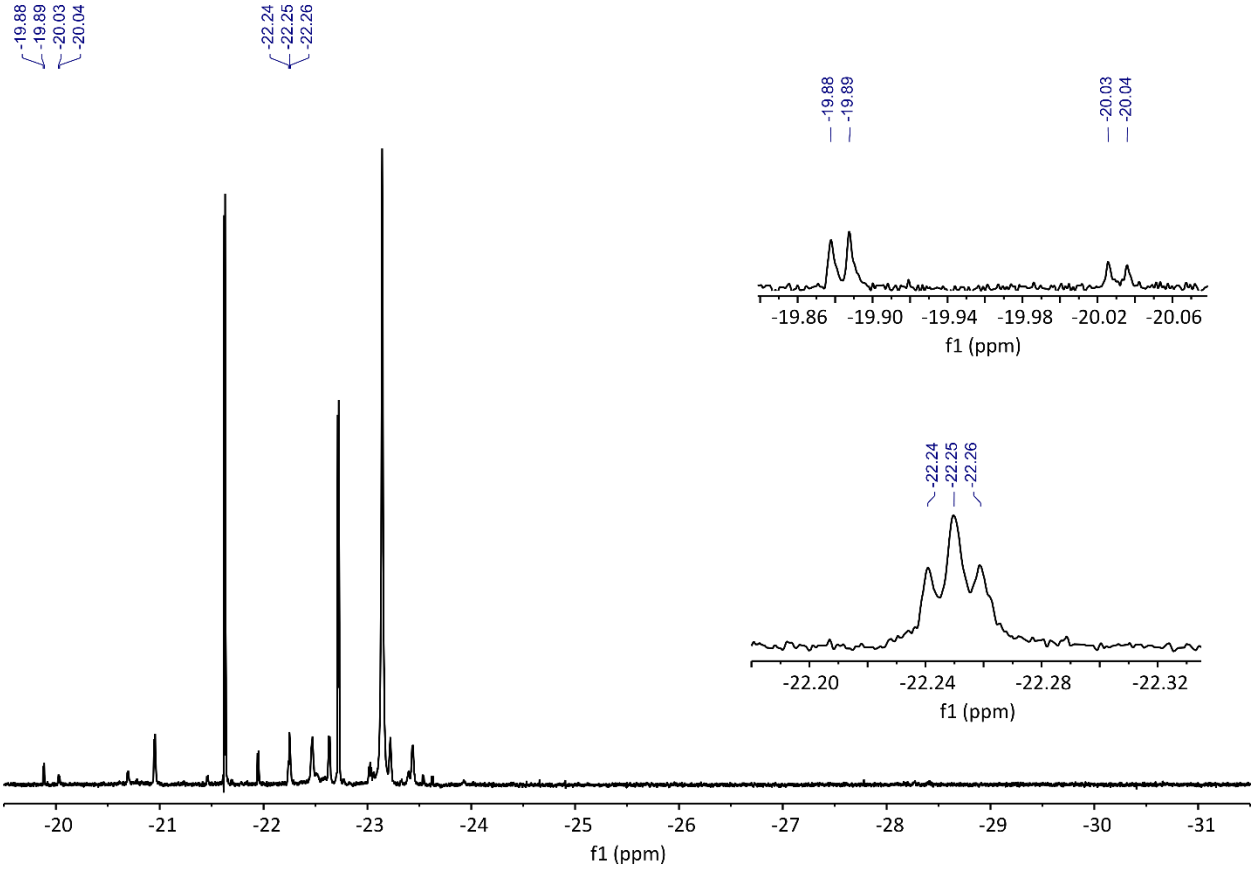


Figure S5. nhPHIP spectrum of sec-butylamine displays hydrides' chemical shifts characteristic for N-ligand binding. Recorded at 283 K. Unassigned signals arise from iridium-complex of 3F4MePy, solvent, and impurities.

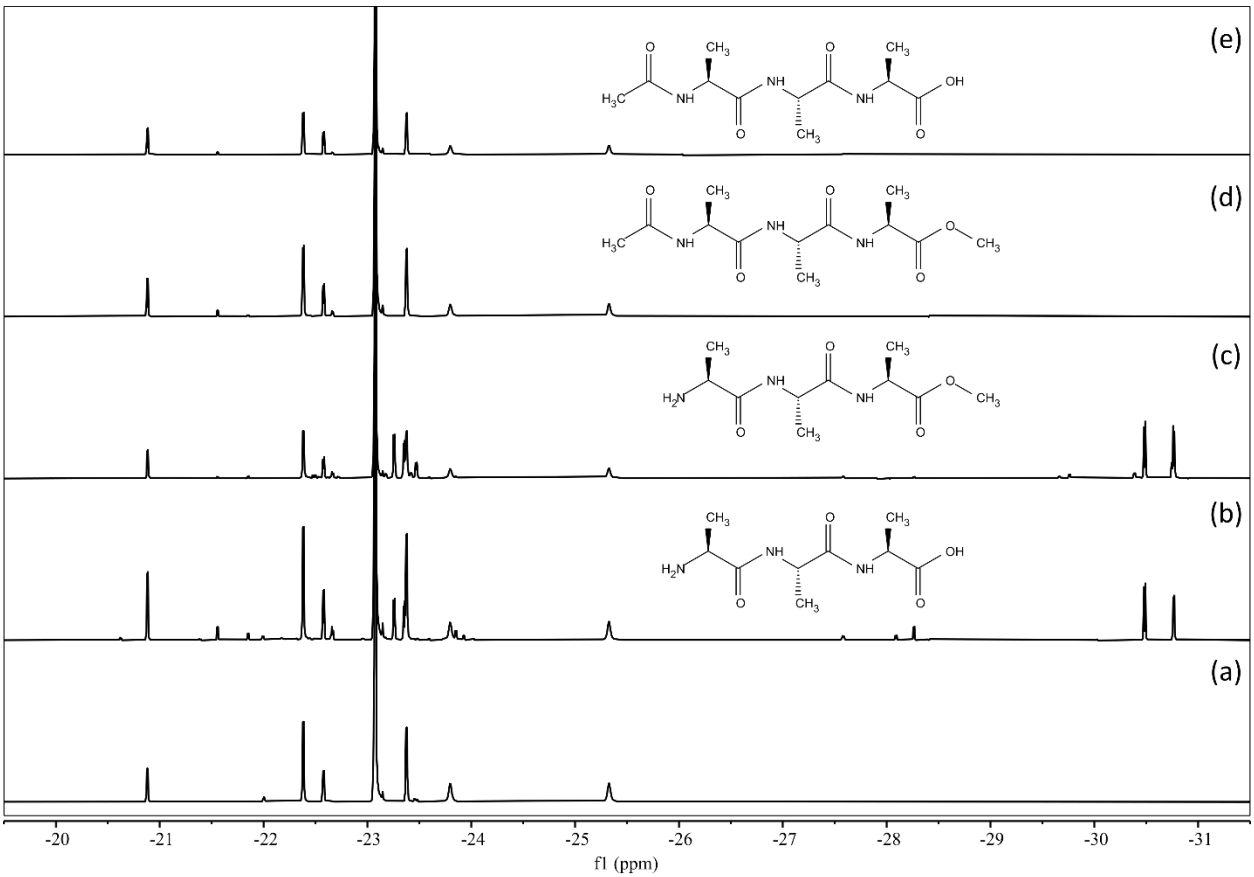


Figure S6. nhPHIP spectra of (a) Ir-catalyst and 3F4MePy, (b) A_3, (c) A-A-A-OMe, (d) Ac-A-A-A-OMe, and (e) Ac-A-A-A, recorded at 283 K. Under basic conditions only A_3 and A-A-A-OMe give rise to nhPHIP signals at −30.5 ppm region with similar chemical shifts (see Figure S7). This indicates that complexation to the Ir-catalyst happens through N-terminus and O-binding is likely provided by one of the peptide bond carbonyl groups. The C-terminus is not involved.

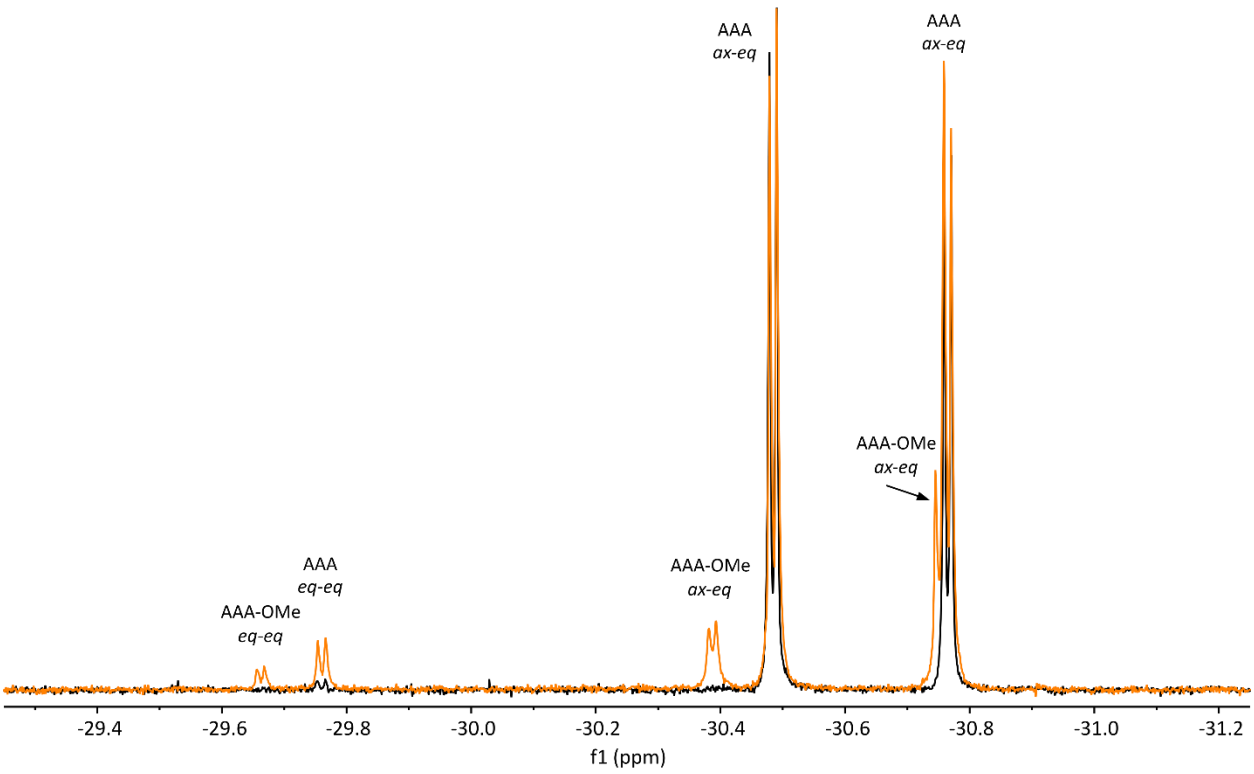


Figure S7. Superimposed nhPHIP H_A spectra of A_3 (black) and A-A-A-OMe (orange) under basic conditions, recorded at 283 K. Basic conditions do not influence chemical shifts, as explained in the main text and shown in Figure 2. However, methyl ester group in synthesized A-A-A-OMe is partly cleaved under basic conditions, which is why both A-A-A-OMe and A_3 complexes' nhPHIP signals are observable in the orange spectrum.

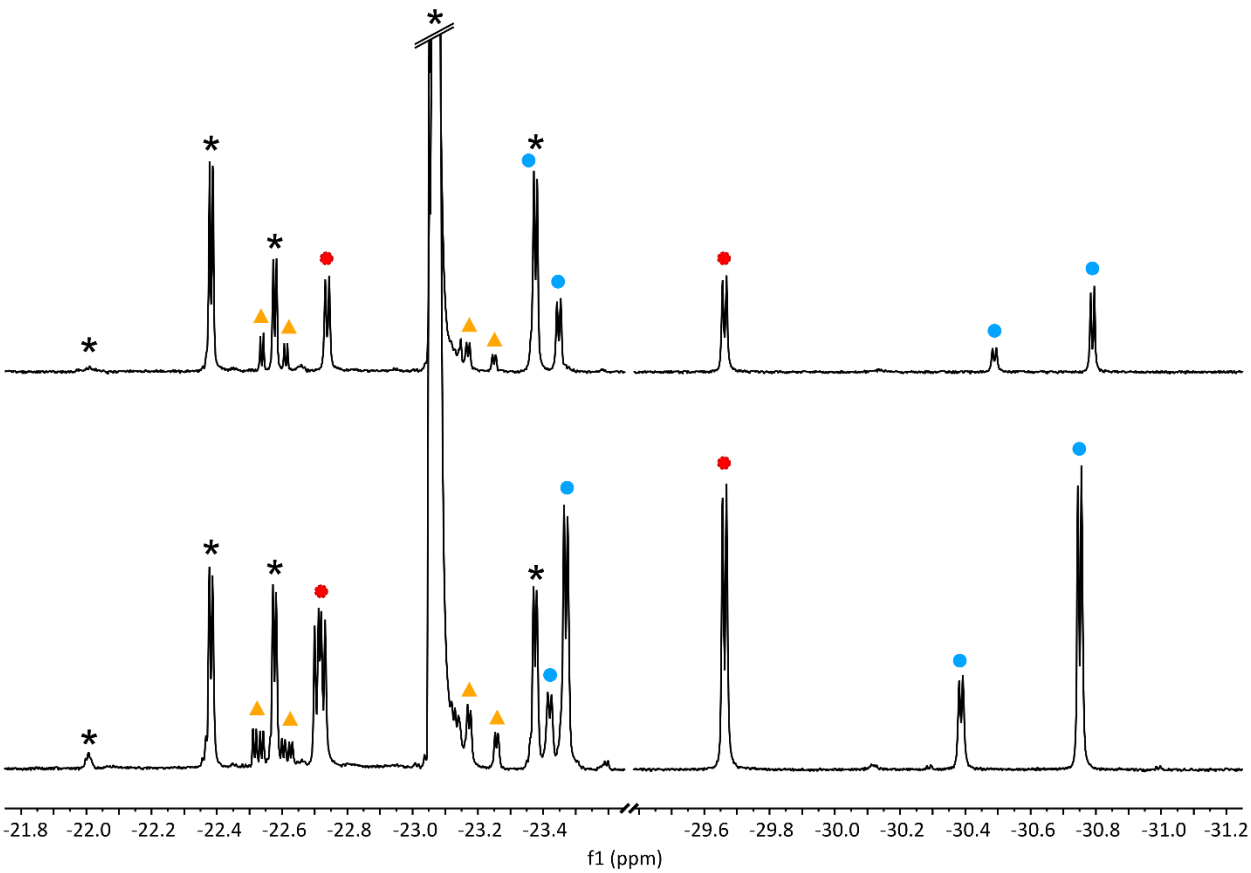


Figure S8. nhPHIP spectra of A[15N]-A-A-OMe (bottom) and A-A[15N]-A-A-OMe (upper) recorded at 283 K. Blue circles correspond to diastereomers of the *ax-eq* complex, red circles to the *eq-eq* complex, and yellow triangles to diastereomers of *mono* complex. Note that some signals overlap in 1D, but are resolved in 2D spectrum (Figure S9). Asterisk (*) denotes signals from the Ir-complex of 3F4MePy, solvent, and impurities. Samples contained a 1:8:0.6 ratio of catalyst : 3F4MePy : peptide. The lower cosubstrate (3F4MePy) loading produces a larger proportion of kinetic *mono* and *eq-eq* products. The lack of ^{15}N-coupling effects in the upper spectrum proves that the second Ala residue nitrogen is not involved in *eq* binding.

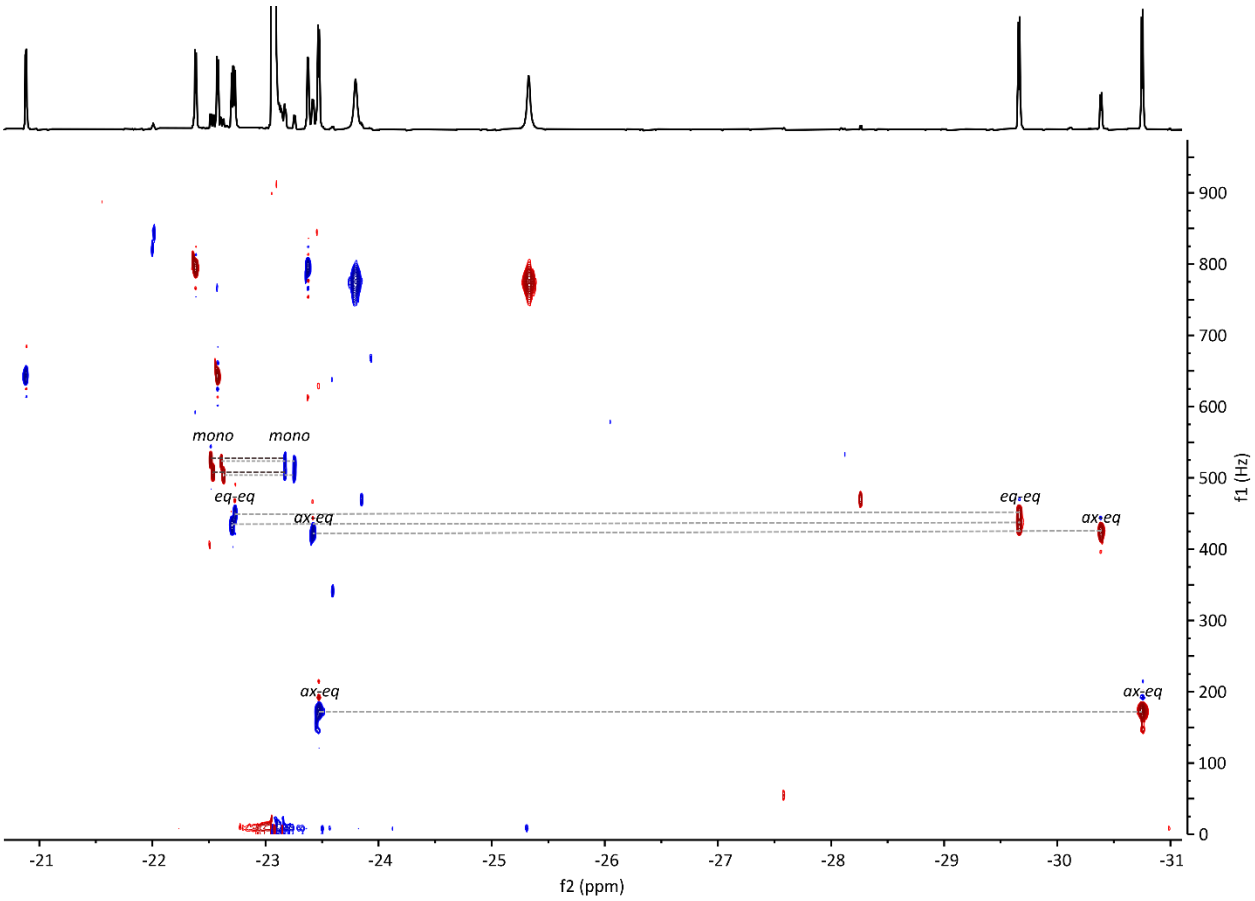


Figure S9. 2D nhPHIP zero quantum spectrum of A[15N]-A-A-OMe, recorded at 283 K. Opposite phase (red and blue) H_A and H_X signals on the same zero-quantum frequency represent a single nhPHIP complex. *J*-coupling from ^{15}N introduces an additional set of multiplets with distinct zero-quantum frequencies. Note that the hydride signal pairs for analyte complexes are folded in f_1 dimension since it was recorded in a 1000 Hz spectral width in f_1. Unassigned signals arise from iridium-complex of 3F4MePy, solvent, and impurities.

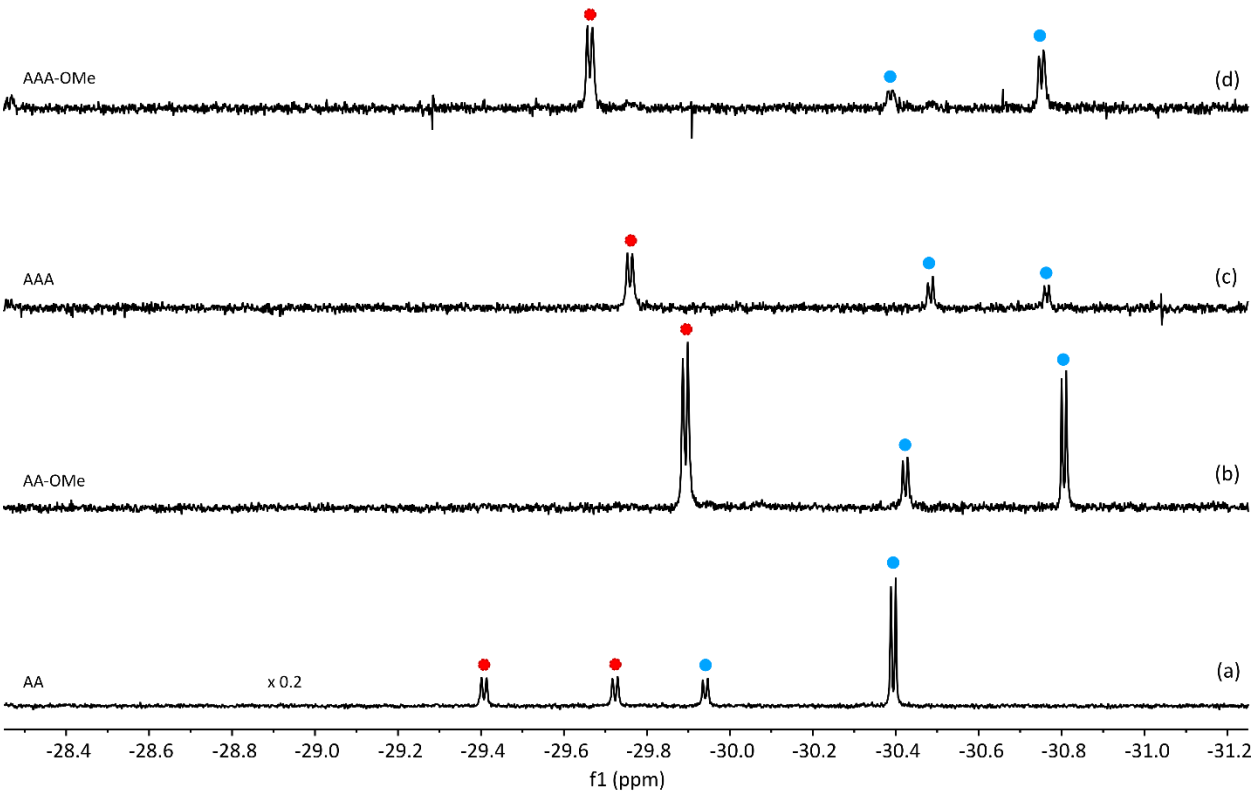


Figure S10. nhPHIP H_A spectra of (a) A_2, (b) A-A-OMe, (c) A_3, (d) A-A-A-OMe, recorded at 283 K. Blue circles correspond to diastereomers of *ax-eq* complex, red circles to *eq-eq* complex. DFT suggests the possibility of A_2 tail-to-tail dimerization, which causes chemical shift differences compared with A-A-OMe, A_3 and A-A-A-OMe.

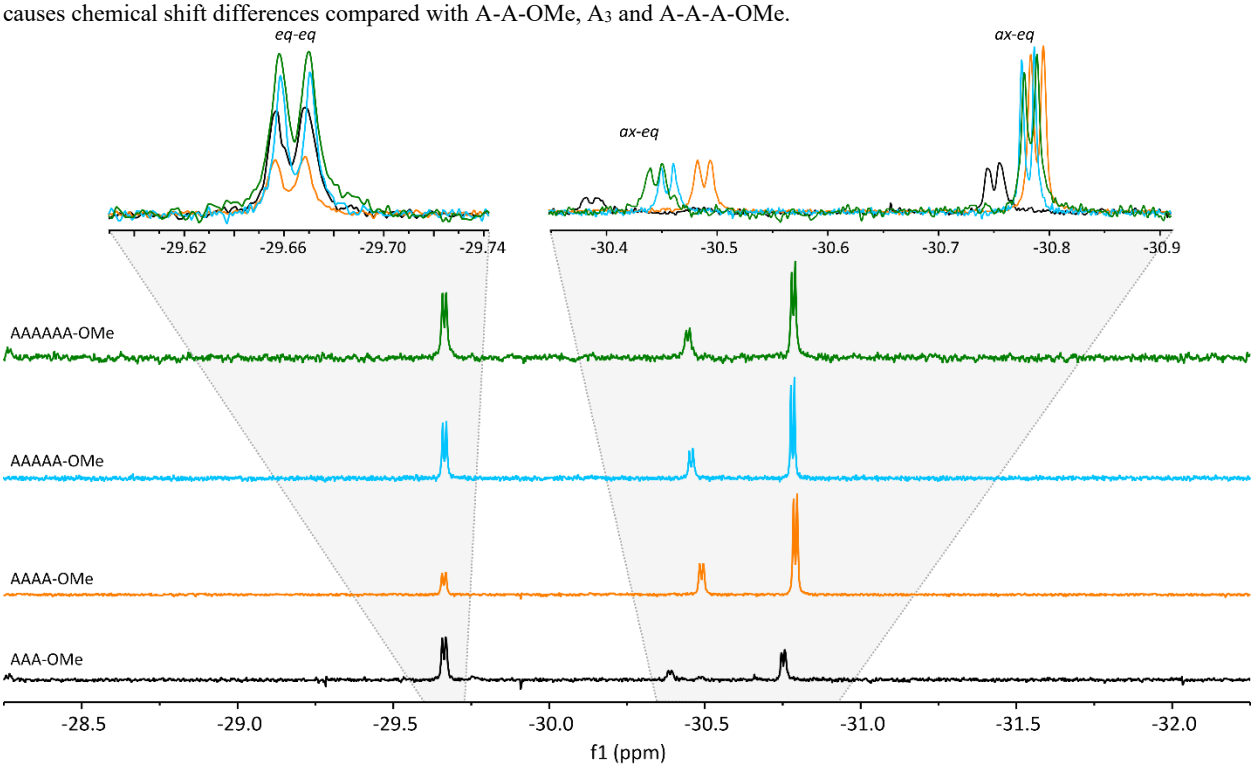


Figure S11. nhPHIP H_A spectra of alanine tri- to hexamer esters, recorded at 283 K. *eq-eq* signals resonate at the same frequency, while *ax-eq* signals vary slightly more in their chemical shifts.

2.5.Formation of equilibrium *ax-eq* complex

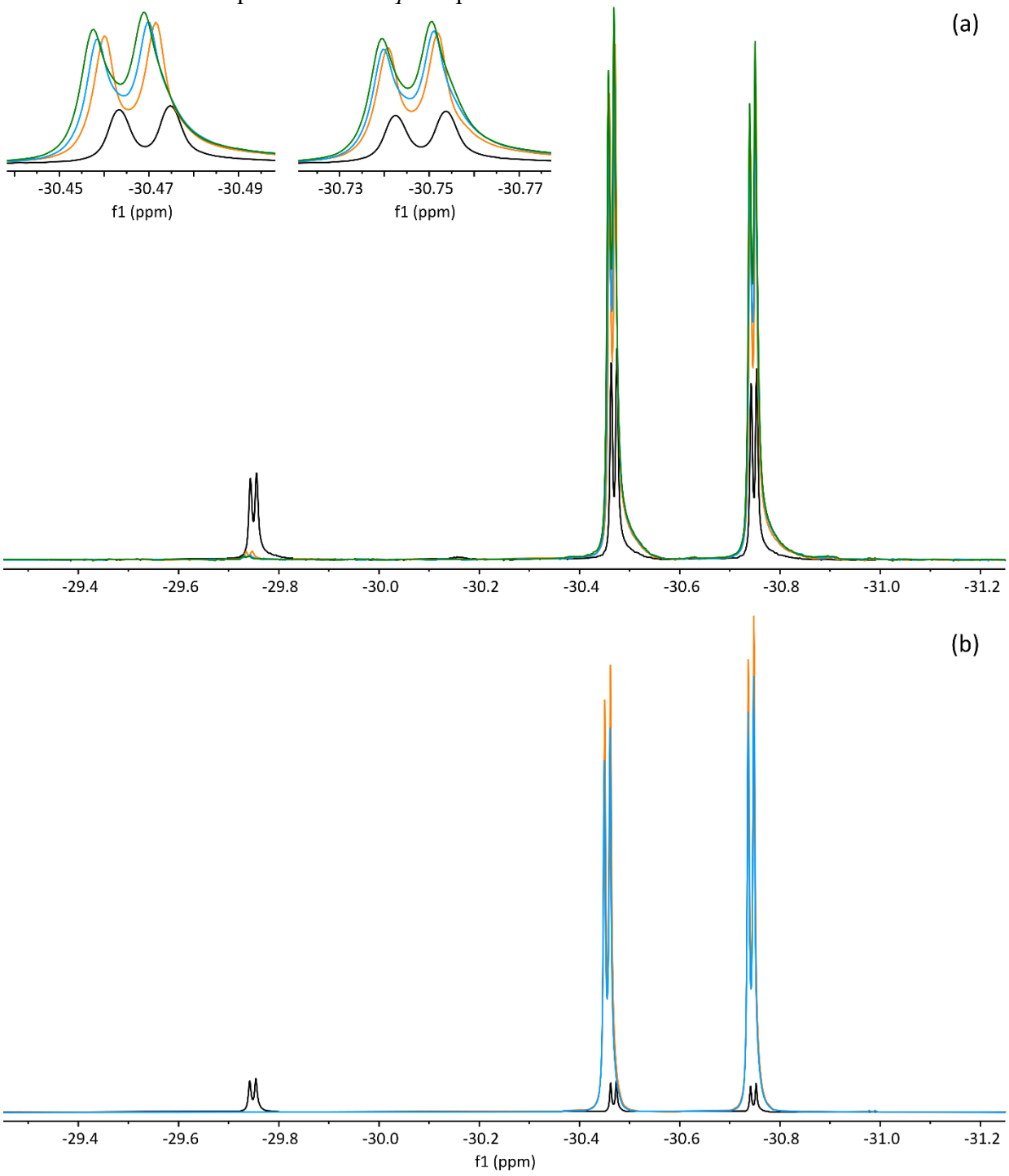


Figure S12. Superimposed nhPHIP H_A spectra (a) of A_3 non-heated (black) and after heating at 37.5 ˚C for 5 min (orange), additional 5 min (blue), and additional 5 min (green); (b) A_3 non-heated (black) and after heating at 50 ˚C for 5 min (orange) and additional 5 min (blue). Repeated warming of the sample does not increase *ax-eq* signals. Spectra recorded at 283 K.

2.5.1. nhPHIP enhancement

nhPHIP signal enhancement was estimated by comparing hydrides' signals of a thermally polarized spectrum (1 scan) and a nhPHIP spectrum (1 scan) of the same sample, using earlier published pulse sequences.[11] The enhancement of *ax-eq* signals at thermal equilibrium was similar for (a) low peptide concentration (0.1 mM) and (b) high peptide concentration (1 mM) samples.

$$enhancement = \frac{Integral_{nhPHIP}}{Integral_{thermal}}$$

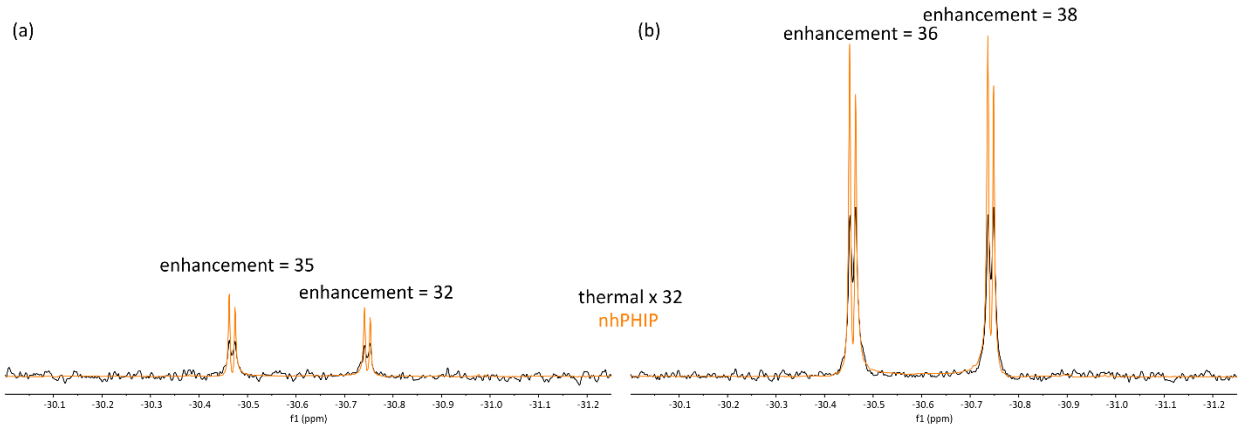


Figure S13. Superimposed spectra of thermally polarized (black) and nhPHIP hyperpolarized (orange) H_A of A_3, containing (a) 1:18:0.08 and (b) 1:18:0.8 ratio of catalyst : 3F4MePy : A_3, acquired in one scan at 283 K.

2.6.nhPHIP applications

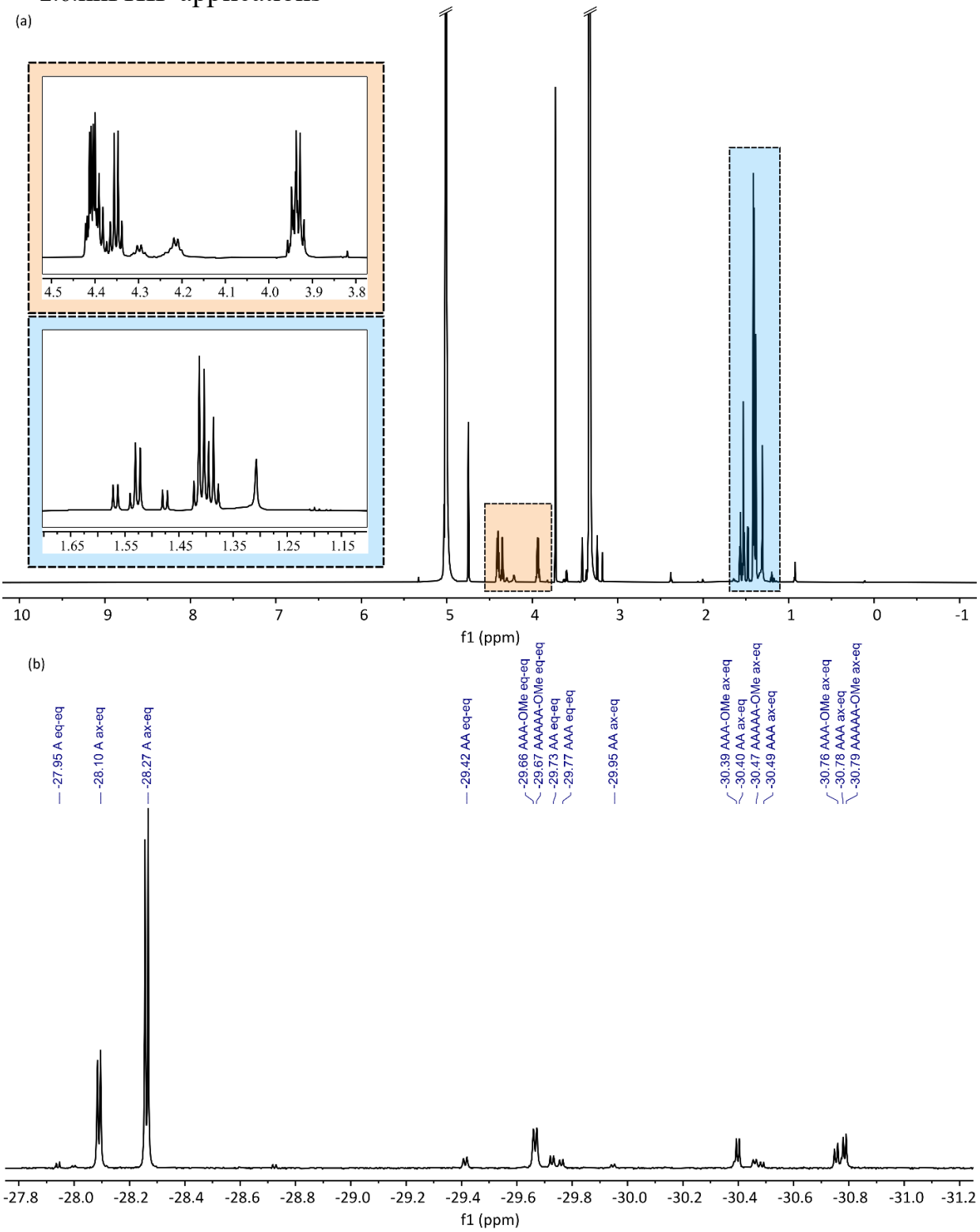


Figure S14. (a) Regular ^{1}H spectrum of a mixture of alanine monomer (A), dimer (A_2), trimer (A_3), trimer ester (A-A-A-OMe), and pentamer ester (A_5-OMe) acquired with 64 scans in 66 min (quantitative conditions). Analye signals overlap, hindering mixture analysis by regular 1D spectroscopy. (b) nhPHIP H_A spectrum of the same mixture acquired with 64 scans in 6 min. nhPHIP improves resolution and allows to annotate each signal by superimposing specific analyte nhPHIP spectrum on mixture spectrum (see Figure S15 below). Spectra recorded at 283 K.

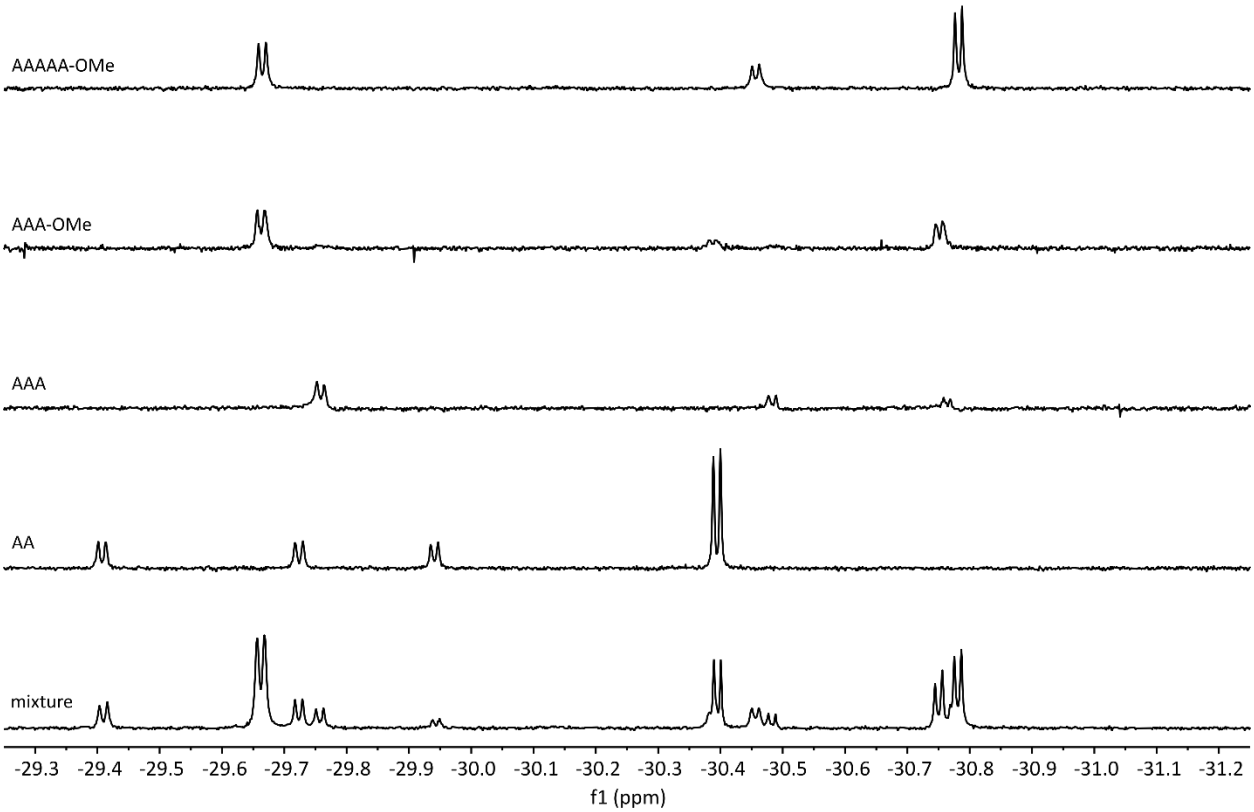


Figure S15. nhPHIP H_A spectrum of a mixture (bottom), which can be annotated by superimposing specific analyte nhPHIP spectrum on it. Spectra recorded at 283 K.

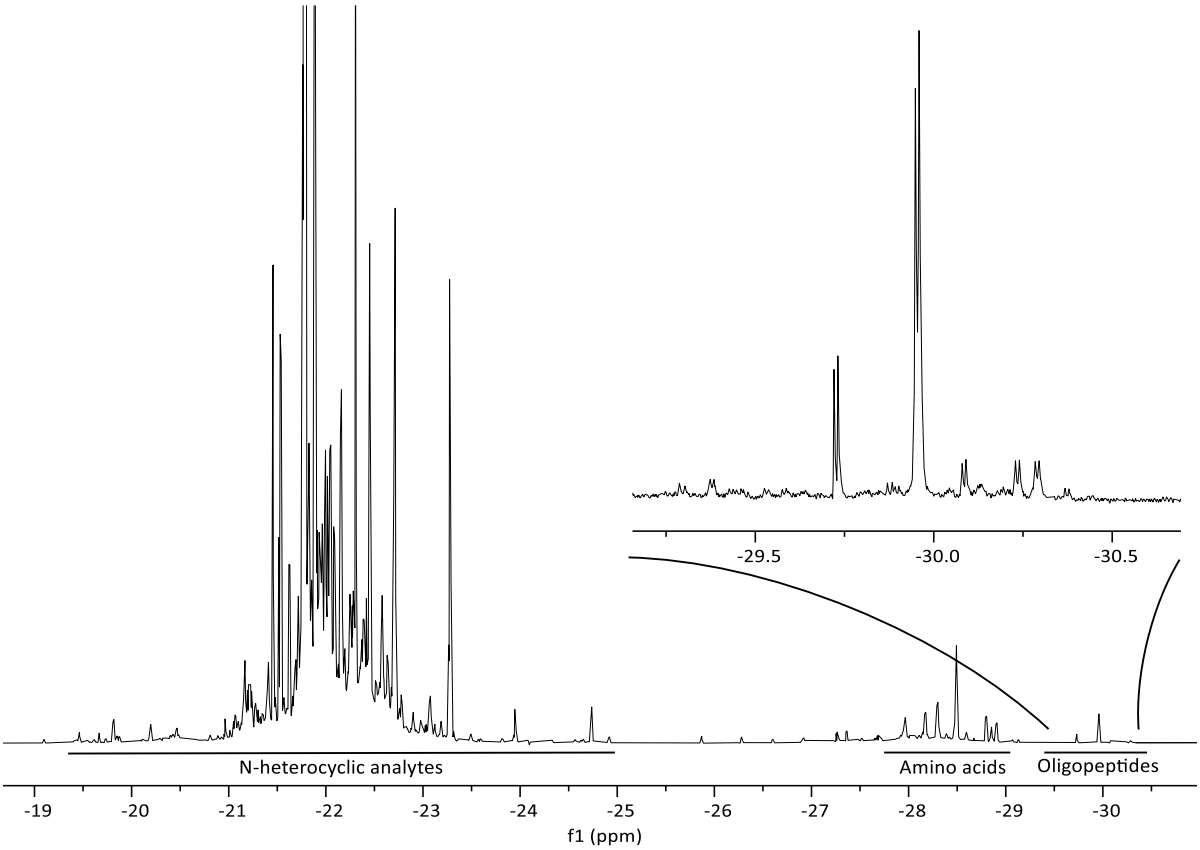


Figure S16. nhPHIP spectrum of a urine sample, annotated for different biomolecule classes that have been identified to date. Oligopeptides display their characteristic H_A signals in a distinct spectral region, whereas H_X signals appear within N-heterocycles' region, as expected for a hydride *trans* to an N-heterocyclic cosubstrate. Displayed spectrum was acquired from a whole urine sample, prepared as described by Ausmees et al.[12] The spectrum was acquired with 1-N-methytriazol (mtz) as cosubstrate instead of 3F4MePy. Therefore, H_A chemical shifts here are not directly equal to oligopeptide related signals in main text, but they can be safely assumed to be similar, as demonstrated by cosubstrate screening in Figure S1. While mtz is not optimal for oligopeptides, it is a more universal cosubstrate, giving better access to several classes of analytes. Recorded at 298 K.

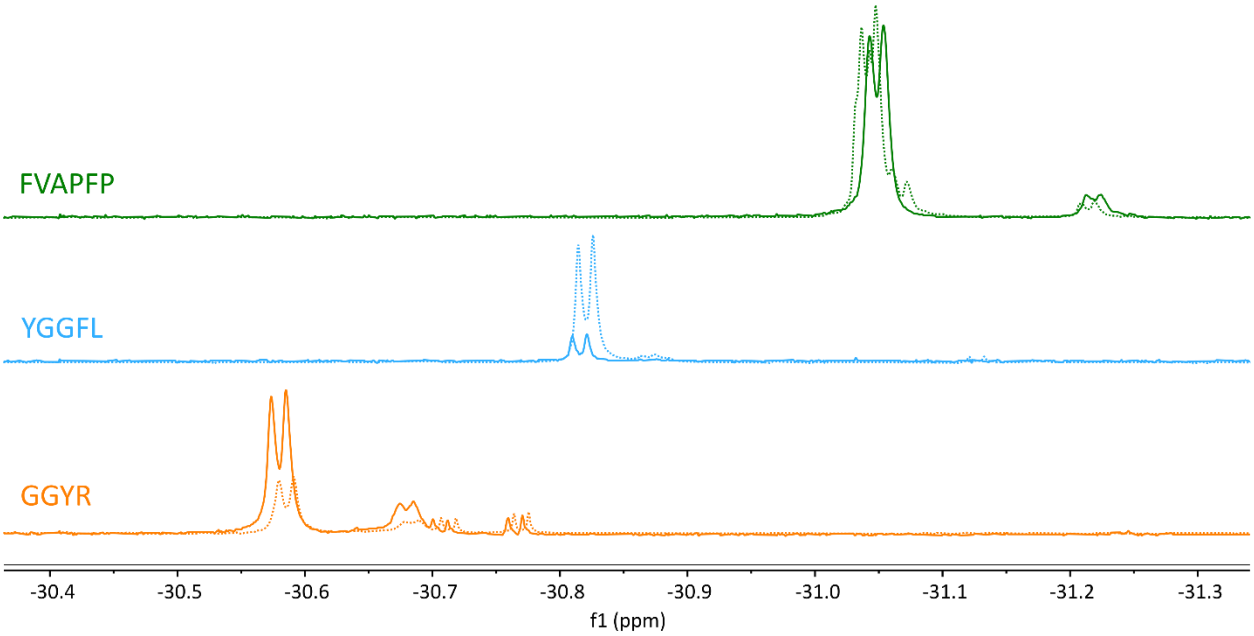


Figure S17. nhPHIP H_A signals of commercial oligopeptides papain inhibitor (GGYR), neuropeptide Leu-enkephalin (YGGFL), and cosmeceutical peptide hexapeptide-11 (FVAPFP), measured at 298 K. Two sample preparation protocols were followed: a) peptide was dissolved in water, pH corrected to 7.4-7.9, lyophilized and dissolved in methanol-d_4 prior nhPHIP measurement – solid lines; b) peptide was dissolved in methanol-d_4 as supplied – dashed lines. Sample consisted of (a) 1:18:0.08 and (b) 1:8:0.08 ratio of catalyst : 3F4MePy : peptide. The chemical shifts suggest bidentate binding involving N- and O- sites, demonstrated in the main text for alanine oligomers. It should be noted that these three peptides showed more intensive signals at 25 °C instead of 10 °C like alanine oligomers and maximum nhPHIP enhancement may not have been achieved under the used conditions. Therefore, nhPHIP parameters optimization should be done for each peptide individually.

3. Peptide synthesis

3.1. HR-MS analysis conditions

An Acquity UPLC I Class plus (Waters) chromatograph connected in series to an autosampler, a column heater and interfaced to a Vion ion mobility detector coupled with quadrupole time-of-flight mass spectrometer (Vion IMS qTOF, Waters) was employed. The column heater and autosampler were set at 45 ˚C and 8 ˚C, respectively. Samples were analyzed using a C18 Waters Premier HSS T3 2.1x150 mm column. An injection volume of 3 µL was used. Flow rate was set to 0.3 mL/min using gradient elution with mobile phase A (*MilliQ* water with 0.1% formic acid) and B (acetonitrile 100%).

3.2. Oligopeptide synthesis

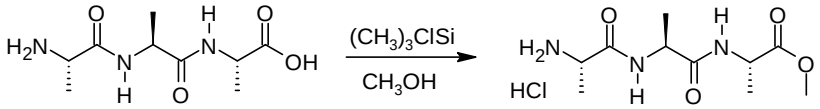
Oligopeptides were synthesized according to literature procedures.[4–6]

1 eq of the appropriate alanine oligomer methyl ester salt (HCl or TFA) and 1.1 eq of appropriate Boc-protected oligomer were dissolved in $CHCl_3$ or $CHCl_3$/DMF or DMF and cooled to 0 ˚C. 1.3 eq of 1-hydroxybenzotriazole (HOBt•H2O) and 1.3-1.5 eq N-methylmorpholine (NMM) were added and allowed to stir for 15 minutes. 1.3 eq of dicyclohexylcarbodiimide (DCC) in $CHCl_3$ was added and stirred at 0 ˚C for 2 h and then 24 h at room temperature. The reaction mixture was filtered and the obtained solid was washed with $CHCl_3$ and purified by column chromatography ($CHCl_3$/MeOH/Petroleum ether) for tetra- and pentamer. Hepta-, octa- and decamer were washed several times with ethyl acetate and/or methanol, solids were centrifuged and the liquid was discarded.

The Boc-protective group was removed by stirring in neat trifluoroacetic acid (TFA) for 1 h. The final product TFA salt was co-evaporated several times with CH_2Cl_2 to remove excess TFA and dried in a freeze drier.

3.3. Analytical data for oligopeptides and intermediates

3.3.1. Preparation of methyl (2S)-2-{(2S)-2-[(2S)-2-aminopropanamido]propanamido}-propanoate hydrochloride (A-A-A-OMe)



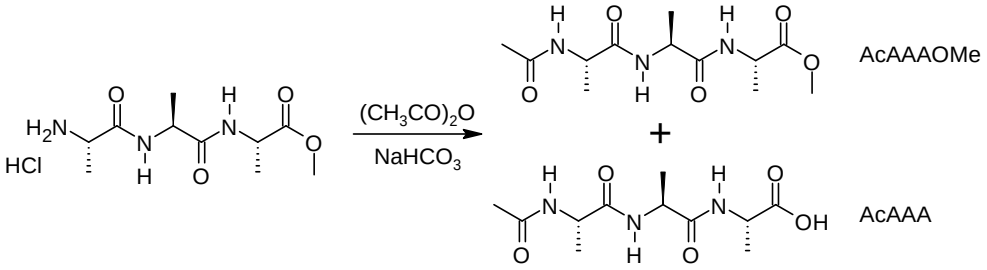
Prepared according to literature procedure.[7] Yield 91%.

^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (d, *J* = 6.7 Hz, 1H), 8.40 (d, *J* = 7.1 Hz, 1H), 8.23 (s, 3H), 4.47 – 4.34 (m, 2H), 3.96 (q, *J* = 7.1 Hz, 1H), 3.71 (s, 3H), 1.52 (d, *J* = 7.1 Hz, 3H), 1.40 (d, *J* = 4.7 Hz, 3H), 1.39 (d, *J* = 4.9 Hz, 3H).

^{13}C NMR (125 MHz, CD_3OD) δ 174.5, 174.4, 170.7, 52.8, 50.3, 50.1, 49.4, 18.1, 17.6, 17.3.

HRMS (ESI/Q-TOF) m/z: $[M+H]^+$ Calcd for $C_{10}H_{20}N_3O_4$ 281.7365, found 246.1454.

3.3.2. Preparation of methyl (2S)-2-{(2S)-2-[(2S)-2-acetamidopropanamido]propanamido}-propanoate (Ac-A-A-A-OMe) and (2S)-2-{(2S)-2-[(2S)-2-acetamidopropanamido]-propanamido}propanoic acid (Ac-A-A-A)



Prepared according to a literature procedure.[8]

Ac-A-A-A-OMe:

Yield 55%.

^{1}H NMR (500 MHz, CD_3OD) δ 4.41-4.34 (m, 2H), 4.31 (q, *J* = 7.2 Hz, 1H), 3.71 (s, 3H), 1.97 (s, 3H), 1.39 (d, *J* = 7.3 Hz, 3H), 1.36 (d, *J* = 7.1 Hz, 3H), 1.34 (d, *J* = 7.2 Hz, 3H).

^{13}C NMR (125 MHz, CD_3OD) δ 174.9, 174.7, 174.5, 173.3, 52.7, 50.5, 50.0, 49.4, 22.4, 18.0, 17.9, 17.3.

Ac-A-A-A:

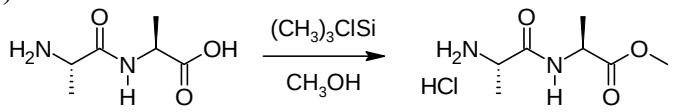
Yield 39%.

^{1}H NMR (500 MHz, CD_3OD) δ 4.33 (q, *J* = 7.3 Hz, 1H), 4.26 (q, *J* = 7.2 Hz, 1H), 4.16 (q, *J* = 7.1 Hz, 1H), 2.07 (s, 3H), 1.40 (d, *J* = 7.2 Hz, 3H), 1.40 (d, *J* = 7.3 Hz, 3H), 1.36 (d, *J* = 7.1 Hz, 3H).

^{13}C NMR (125 MHz, CD_3OD) δ 180.5, 175.8, 174.4, 174.1, 51.7, 51.6, 50.8, 22.7, 18.6, 17.6, 17.5.

HRMS (ESI/Q-TOF) m/z: $[M+Na]^+$ Calcd for $C_{11}H_{19}N_3NaO_5$ 296.1217, found 296.1220.

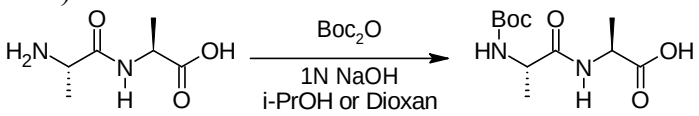
3.3.3. Preparation of methyl (2S)-2-[(2S)-2-aminopropanamido]propanoate hydrochloride (A-A-OMe)



Prepared according to literature procedure.[7] ^{1}H NMR in agreement with literature. Yield 93%.

^{1}H NMR (800 MHz, CD_3OD) δ 4.46 (q, *J* = 7.3 Hz, 1H), 3.96 (q, *J* = 7.1 Hz, 1H), 3.72 (s, 3H), 1.53 (d, *J* = 7.1 Hz, 3H), 1.42 (d, *J* = 7.3 Hz, 3H).

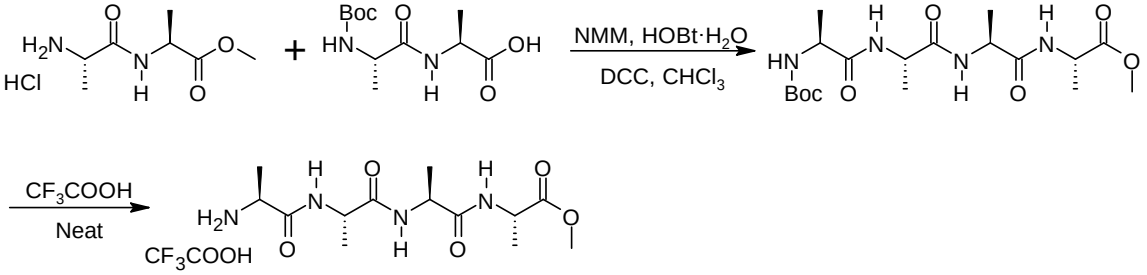
3.3.4. Preparation of (2S)-2-{(2S)-2-[(tert-butoxycarbonyl)amino]propanamido}propanoic acid (Boc-A-A)



Prepared according to a literature procedure.[4,9] ^{1}H NMR in agreement with literature. Crude reaction yield 88%.

^{1}H NMR (800 MHz, CD_3OD) δ 8.12 (d, *J* = 7.0 Hz, 1H), 4.39 (p, *J* = 7.2 Hz, 1H), 4.13 – 4.06 (m, 1H), 1.44 (s, 9H), 1.40 (d, *J* = 7.3 Hz, 3H), 1.31 (d, *J* = 7.2 Hz, 3H).

3.3.5. Preparation of methyl (2S)-2-[(2S)-2-[(2S)-2-[(2S)-2-aminopropanamido]-propanamido]propanamido]propanoate trifluoroacetic acid salt (A_4-OMe)



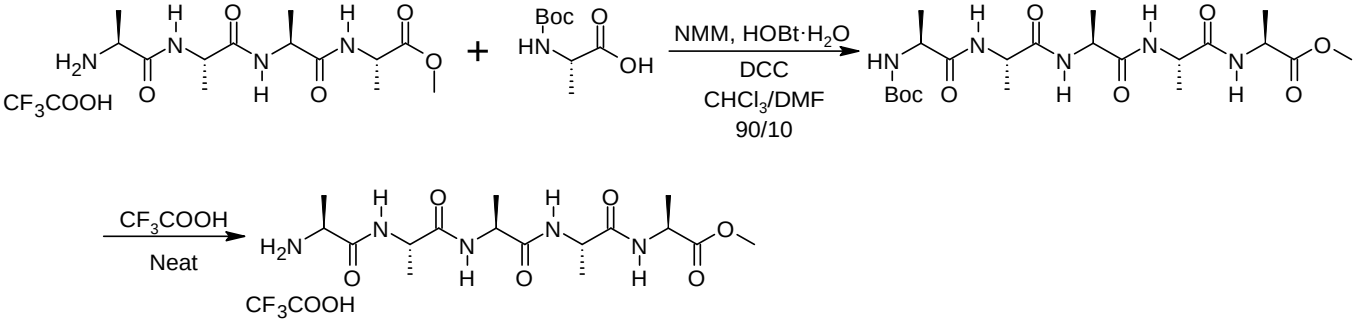
Yield over two steps 65%.

^{1}H NMR (800 MHz, DMSO-d_6) δ 8.52 (d, *J* = 7.3 Hz, 1H), 8.30 (d, *J* = 7.1 Hz, 1H), 8.05 (s, 3H) 8.04 (d, *J* = 7.1 Hz, 1H), 4.36 (p, *J* = 7.1 Hz, 1H), 4.29 (p, *J* = 7.1 Hz, 1H), 4.26 (p, *J* = 7.3 Hz, 1H) 3,85 (hept, *J* = 6.9 Hz, 1H), 3.61 (s, 3H), 1.33 (d, *J* = 6.9 Hz, 3H), 1.27 (d, *J* = 7.3 Hz, 3H), 1.23 (d, *J* = 7.1 Hz, 3H), 1.21 (d, *J* = 7.1 Hz, 3H).

^{13}C NMR (200 MHz, DMSO-d_6) δ 172.9, 172.0, 171.2, 169.0, 158.0 (q, *J* = 33.8 Hz), 116.4 (q, *J* =295.5 Hz), 51.9, 48.1, 48.0, 47.7, 47.5, 18.2 (2C), 17.2, 16.9.

HRMS (ESI/Q-TOF) m/z: $[M+H]^+$ Calcd for $C_{15}H_{25}N_4O_5$ 317.18195, found 317.1824.

3.3.6. Preparation of penta-L-Alanine methyl ester (A_5-OMe)



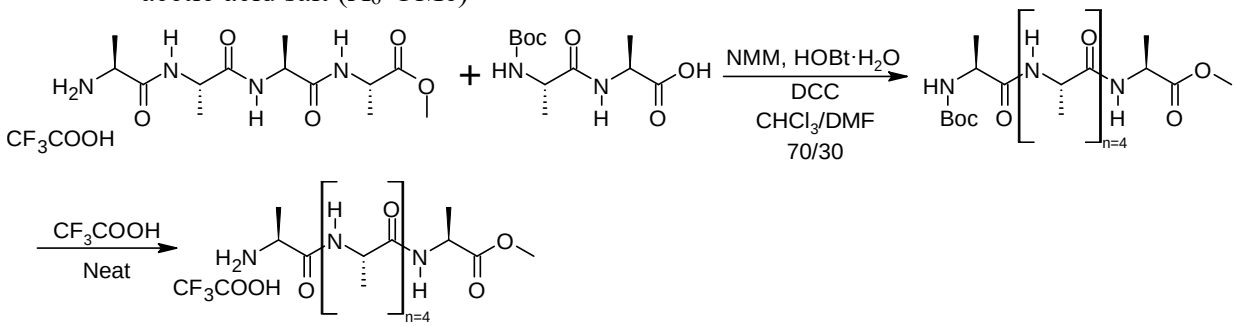
Yield over two steps 56%.

^{1}H NMR (500 MHz, DMSO-d_6) δ 8.54 (d, *J* = 7.5 Hz, 1H), 8.28 (d, *J* = 7.0 Hz, 1H), 8.11-8.03 (m, 4H), 7.90 (d, *J* = 7.6 Hz, 1H), 4.35 (p, *J* = 7.1 Hz, 1H), 4.31-4.21 (m, 3H), 3.89-3.80 (m, 1H), 3.61 (s, 3H), 1.33 (d, *J* = 6.9 Hz, 3H), 1.27 (d, *J* = 7.3 Hz, 3H), 1.24 (d, *J* = 7.0 Hz, 3H), 1.20 (d, *J* = 7.1 Hz, 3H), 1.20 (d, *J* = 7.2 Hz, 3H).

^{13}C NMR (125 MHz, DMSO-d_6) δ 172.9, 172.0, 171.5, 171.3, 169.1, 51.9, 48.1, 48.0, 48.0, 47.6, 47.5, 18.2, 18.2, 18.1, 17.2, 16.8.

HRMS (ESI/Q-TOF) m/z: $[M+H]^+$ Calcd for $C_{18}H_{30}N_5O_6$ 388.2191, found 388.2209.

3.3.7. Experimental procedure for the preparation of hexa-L-Alanine methyl ester trifluoro acetic acid salt (A_6-OMe)



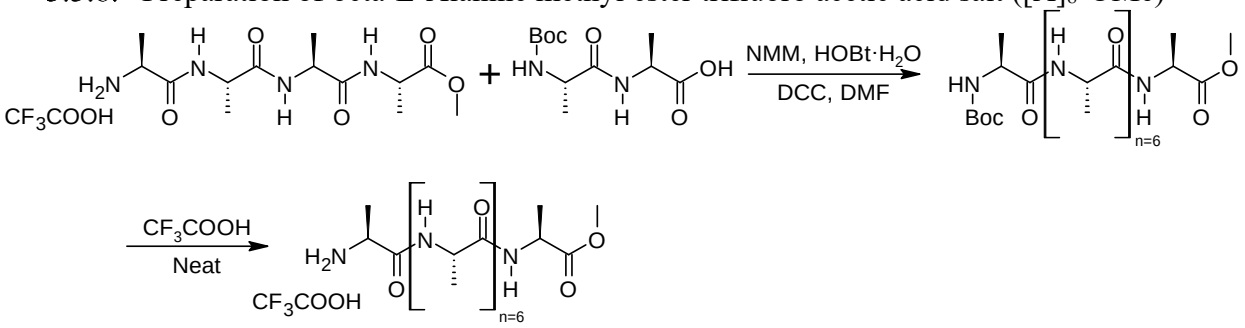
Yield over two steps 53%.

^{1}H NMR (800 MHz, DMSO-d_6) δ 8.53 (d, *J* = 7.5 Hz, 1H), 8.26 (d, *J* = 7.0 Hz, 1H), 8.09 (d, *J* = 7.2 Hz, 1H), 8.04 (d, *J* = 5.4 Hz, 3H), 7.94 (d, *J* = 7.3 Hz, 1H), 7.89 (d, *J* = 7.6 Hz, 1H), 4.35 (p, *J* = 7.2 Hz, 1H), 4.30 – 4.22 (m, 4H), 3.85 (hept, *J* = 6.9 Hz, 1H), 3.61 (s, 3H), 1.33 (d, *J* = 6.9 Hz, 3H), 1.27 (d, *J* = 7.2 Hz, 3H), 1.24 (d, *J* = 7.1 Hz, 3H), 1.20 (d, *J* = 7.1 Hz, 6H), 1.19 (d, *J* = 7.1 Hz, 3H).

^{13}C NMR (200 MHz, DMSO-d_6) δ 172.9, 172.0, 171.7, 171.5, 171.3, 169.0, 51.9, 48.1, 48.0, 48.0, 47.9, 47.6, 47.5, 18.2 (2C), 18.1, 18.1, 17.2, 16.8.

HRMS (ESI/Q-TOF) m/z: $[M+H]^+$ Calcd for $C_{21}H_{35}N_6O_7$ 459.2562, found 459.2579.

3.3.8. Preparation of octa-L-Alanine methyl ester trifluoro acetic acid salt ([A]$_8$-OMe)



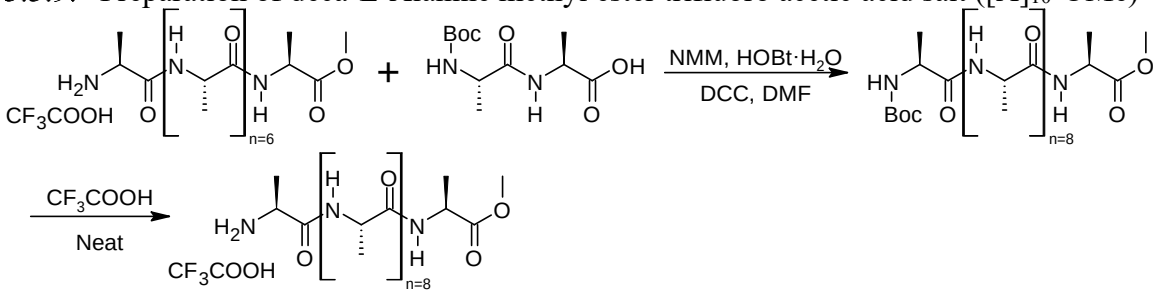
Yield over two steps 46%.

^{1}H NMR (800 MHz, DMSO-d$_6$) δ 8.52 (d, *J* = 7.5 Hz, 1H), 8.24 (d, *J* = 7.0 Hz, 1H), 8.09 (d, *J* = 7.3 Hz, 1H), 8.02 (d, *J* = 4.9 Hz, 3H), 7.96 (d, *J* = 7.3 Hz, 1H), 7.95 (d, *J* = 7.3 Hz, 1H), 7.92 (d, *J* = 7.4 Hz, 1H), 7.87 (d, *J* = 7.6 Hz, 1H), 4.36 (p, *J* = 7.1 Hz, 1H), 4.30 – 4.21 (m, 6H), 3.84 (hept, *J* = 7.0 Hz, 1H), 3.61 (s, 3H), 1.33 (d, *J* = 7.0 Hz, 3H), 1.27 (d, *J* = 7.4 Hz, 3H), 1.24 (d, *J* = 7.1 Hz, 3H), 1.22 – 1.18 (m, 15H).

^{13}C NMR (200 MHz, DMSO-d$_6$) δ 172.9, 172.0, 171.76, 171.74, 171.70, 171.6, 171.3, 169.0, 51.9, 48.1, 48.02, 47.99 (2C), 47.95, 47.6, 47.5, 18.24, 18.19, 18.13, 18.10 (2C), 18.08 (2C), 17.2, 16.8.

HRMS (ESI/Q-TOF) m/z: $[M+H]^+$ Calcd for $C_{27}H_{45}N_8O_9$ 601.3304, found 601.3330.

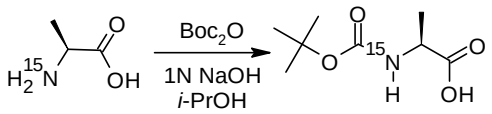
3.3.9. Preparation of deca-L-Alanine methyl ester trifluoro acetic acid salt ([A]$_{10}$-OMe)



Yield over two steps 63%.

HRMS (ESI/Q-TOF) m/z: $[M+H]^+$ Calcd for $C_{33}H_{55}N_{10}O_{11}$ 743.4046, found 743.4058.

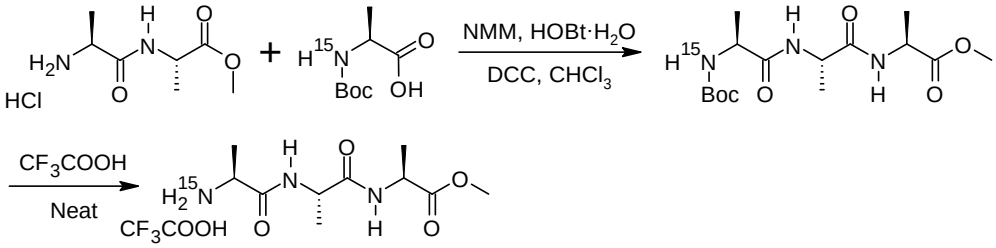
3.3.10. Experimental procedure for the preparation of (2S)-2-{[(tert-butoxy)carbonyl]-amino}propanoic acid-^{15}N



Crude reaction yield 94%.

^{1}H NMR (500 MHz, CD_3OD) δ 4.11 (m, 1H), 1.44 (s, 9H), 1.35 (dd, *J* = 7.3, 3.0 Hz, 3H).

3.3.11. Experimental procedure for the preparation of methyl (2S)-2-{(2S)-2-[(2S)-2-amino-propanamido]propanamido}propanoate-^{15}N trifluoro acetic acid salt (A[15N]-A-A-OMe)



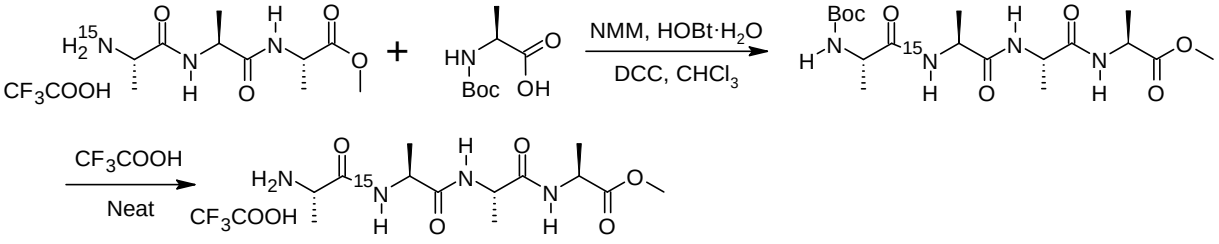
Yield over two steps 74%.

^{1}H NMR (500 MHz, DMSO-d_6) δ 8.54 (d, *J* = 7.6 Hz, 1H), 8.41 (d, *J* = 7.0 Hz, 1H), 8.04 (bs, 3H), 4.36 (p, *J* = 7.6, 7.1 Hz, 1H), 4.26 (p, *J* = 7.0, 7.3 Hz, 1H), 3.84 (q, *J* = 6.9 Hz, 1H), 3.62 (s, 3H), 1.32 (dd, *J* = 6.9, 2.9 Hz, 3H), 1.28 (d, *J* = 7.3 Hz, 3H), 1.25 (d, *J* = 7.1 Hz, 3H).

^{13}C NMR (125 MHz, DMSO-d_6) δ 172.9, 171.7, 169.1, 51.9, 48.0 (d, *J* = 48.0 Hz), 48.0, 47.9, 47.5, 18.2, 17.2, 16.8.

HRMS (ESI/Q-TOF) m/z: $[M+H]^+$ Calcd for $C_{10}H_{20}N_2[^{15}N]O_4$ 247.1419; Found 247.1410.

3.3.12. Experimental procedure for the preparation of methyl (2S)-2-[(2S)-2-[(2S)-2-[(2S)-2-amino-propanamido]propanamido]propanamido]propanoate-^{15}N trifluoro acetic acid salt (A-A[15N]-A-A-OMe)



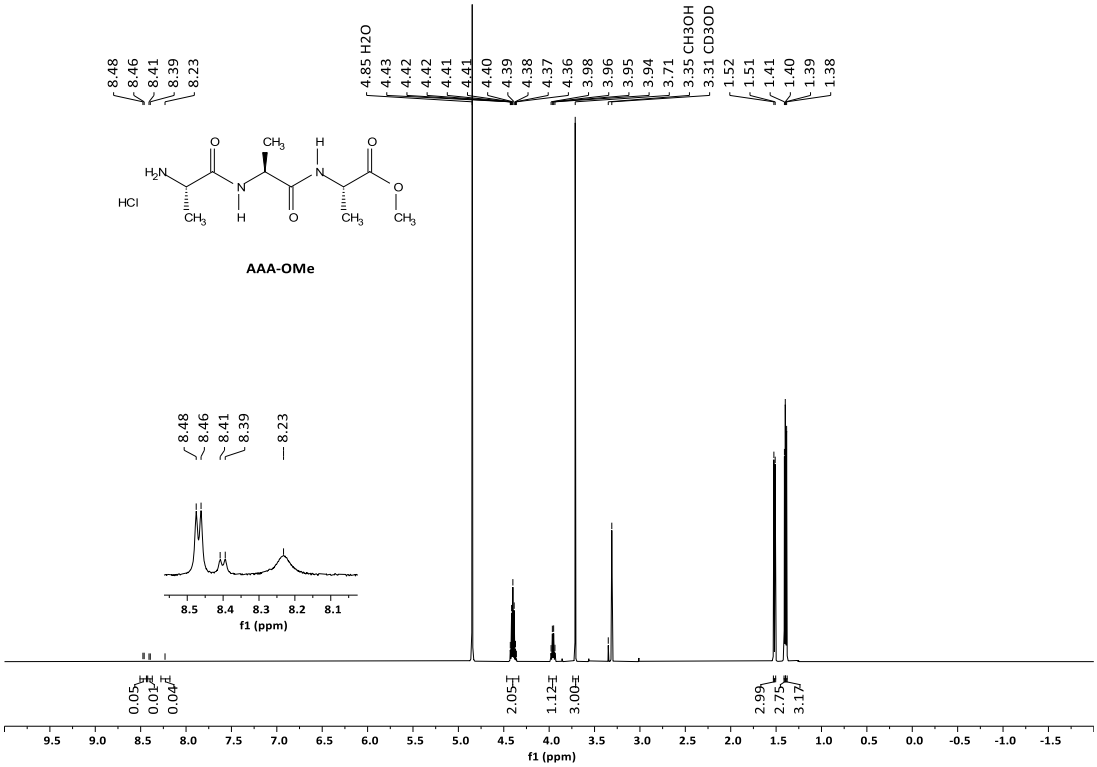
Yield over two steps 69.8%.

^{1}H NMR (500 MHz, DMSO-d_6) δ 8.52 (dd, *J* = 92.1, 7.5 Hz, 1H), 8.30 (d, *J* = 7.0 Hz, 1H), 8.06 (bs, 3H), 8.04 (d, *J* = 7.5 Hz, 1H), 4.35 (pd, *J* = 7.5, 7.16 1.2 Hz, 1H), 4.27 (m, 2H), 3.89 – 3.80 (m, 1H), 3.61 (s, 3H), 1.33 (d, *J* = 7.0 Hz, 3H), 1.27 (d, *J* = 7.3 Hz, 3H), 1.23 (dd, *J*=7.16, 2.29 Hz, 3H), 1.21 (d, *J*=7.1 Hz, 3H).

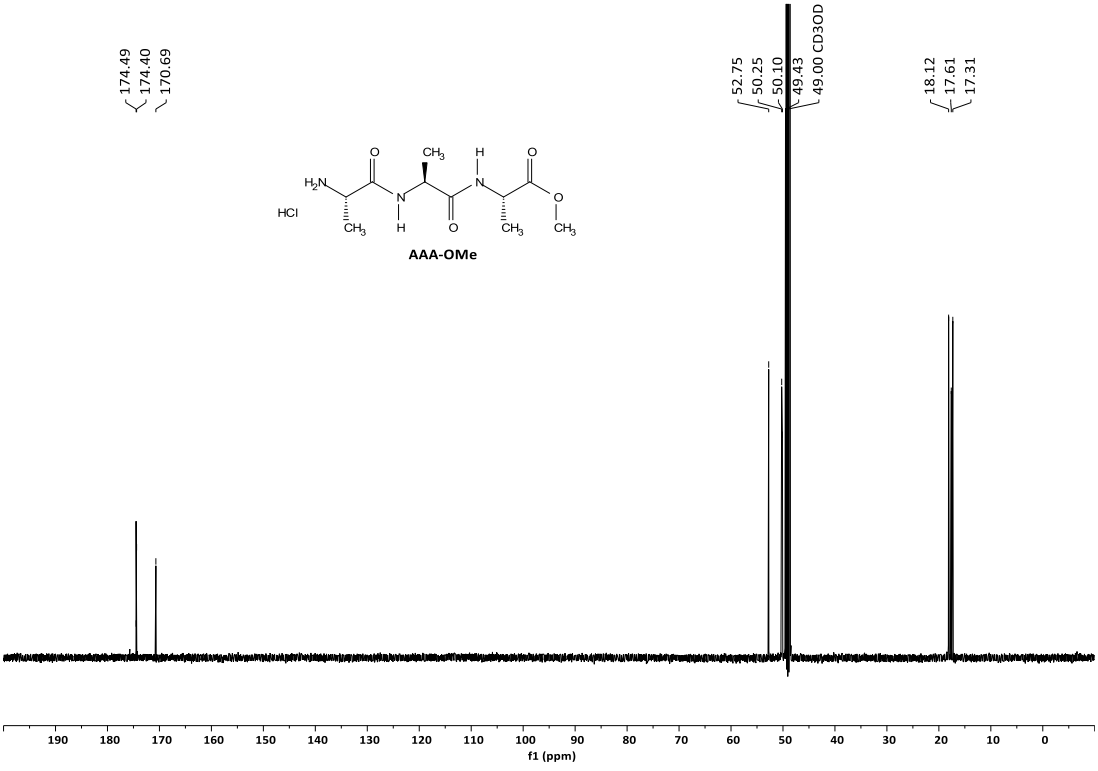
^{13}C NMR (125 MHz, DMSO-d_6) δ 173.0, 172.0, 171.2, 169.1 (d, *J* = 15.7 Hz), 158.0, 157.8, 51.9, 48.1 (d, *J* = 10.5 Hz), 48.0 (d, *J* = 9.15 Hz), 47.7, 47.5, 18.2 (d, *J* = 1.57 Hz), 17.2, 16.9.

3.4. NMR spectra of synthesized oligopeptides and intermediates

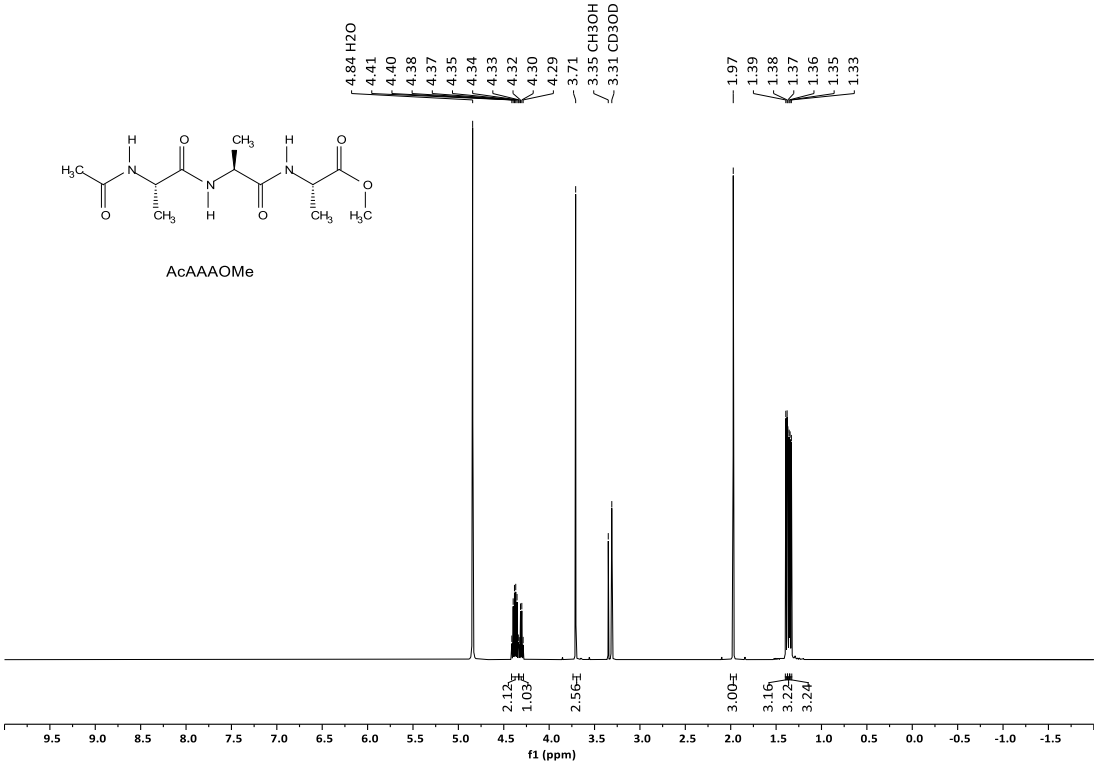
^{1}H NMR (500 MHz, CD_3OD) for **A-A-A-OMe**



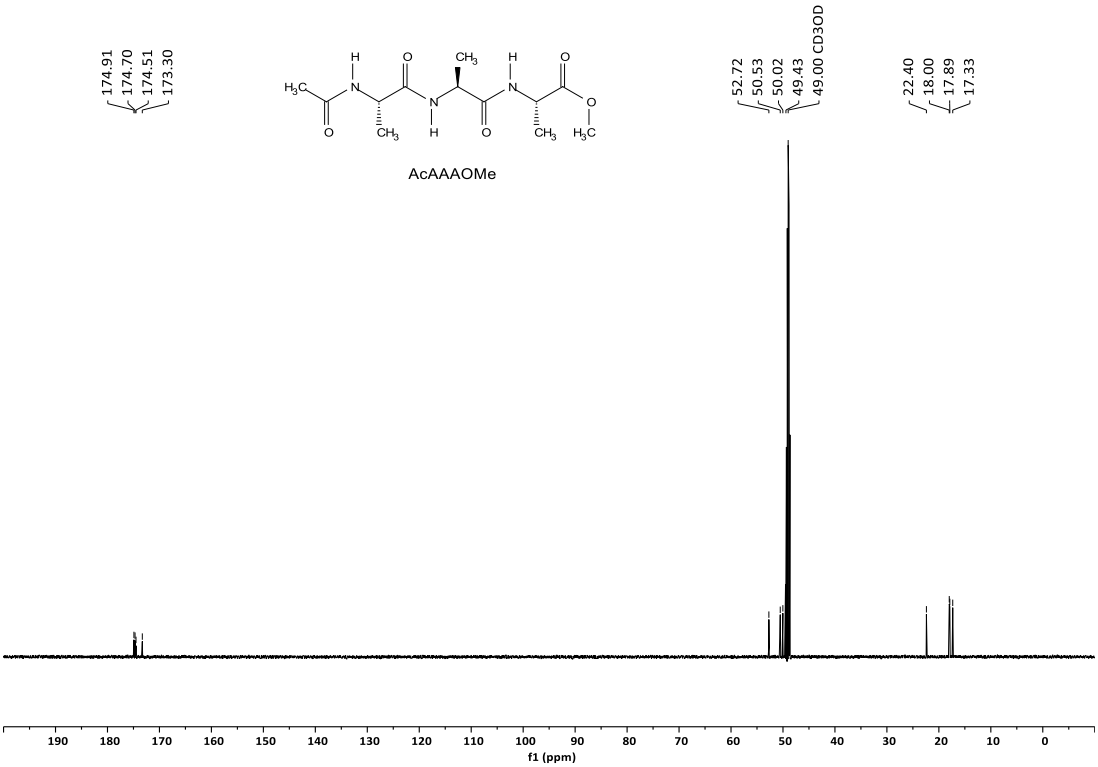
^{13}C NMR (125 MHz, CD_3OD) for **A-A-A-OMe**



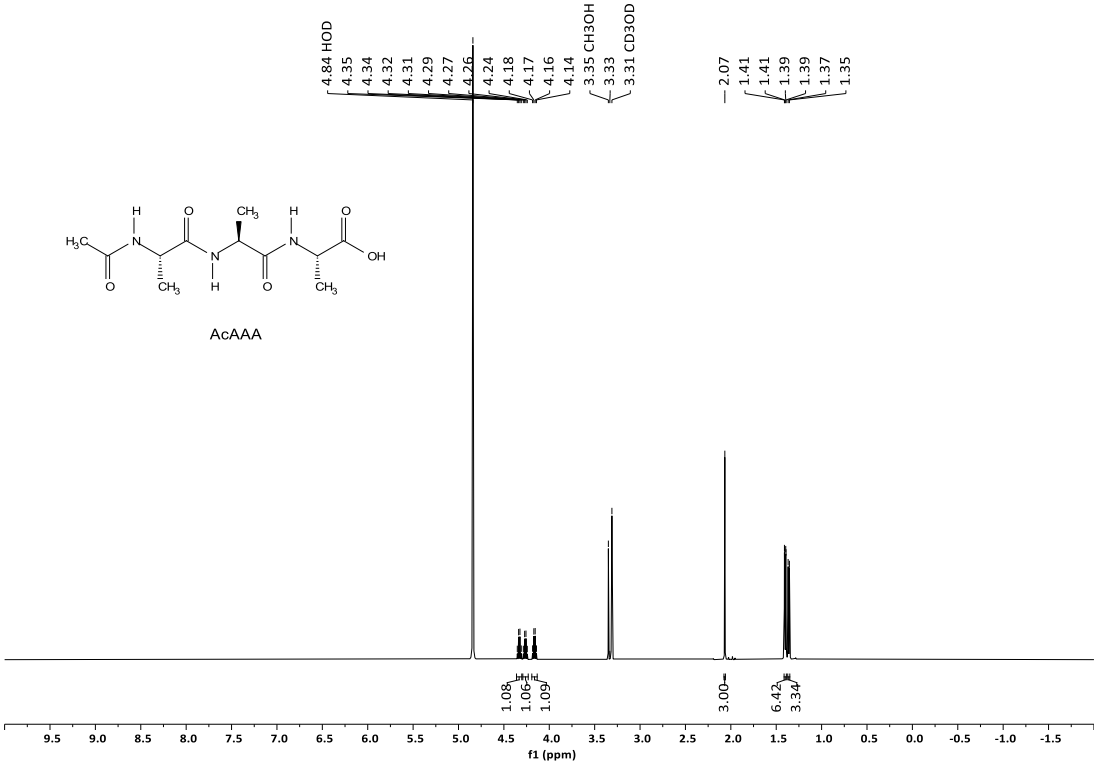
^{1}H NMR (500 MHz, CD_3OD) for **Ac-A-A-A-OMe**



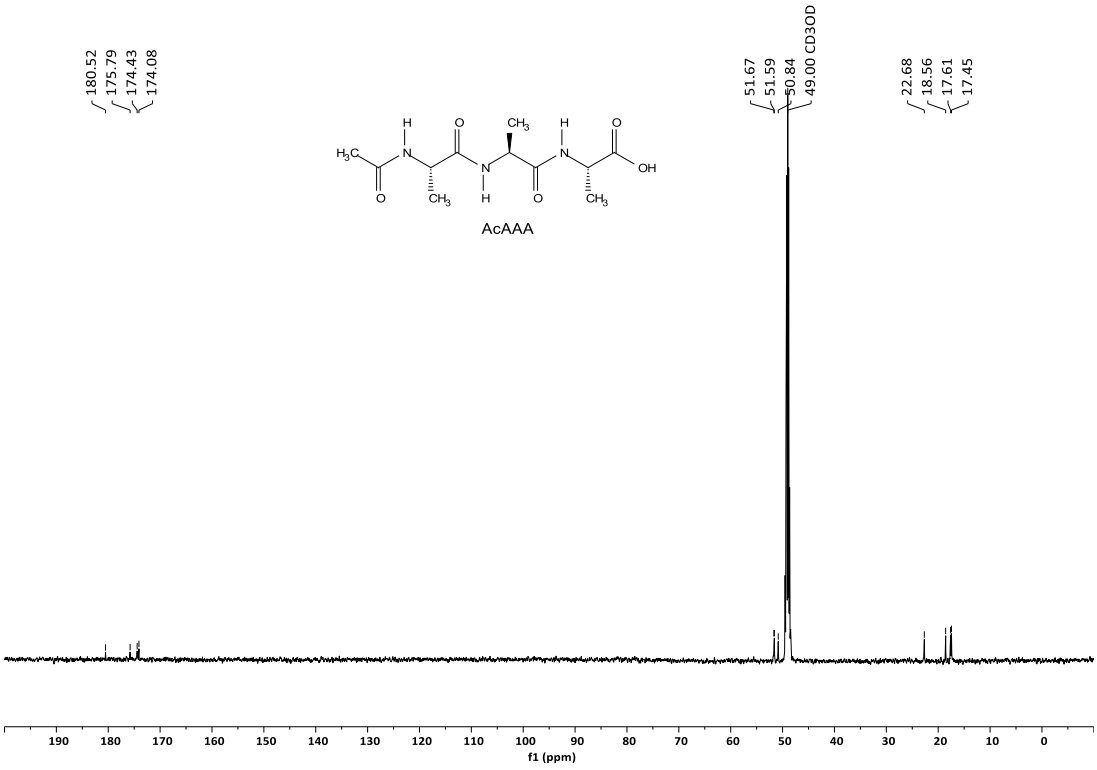
^{13}C NMR (125 MHz, CD_3OD) for **Ac-A-A-A-OMe**



^{1}H NMR (500 MHz, CD_3OD) for **Ac-A-A-A**

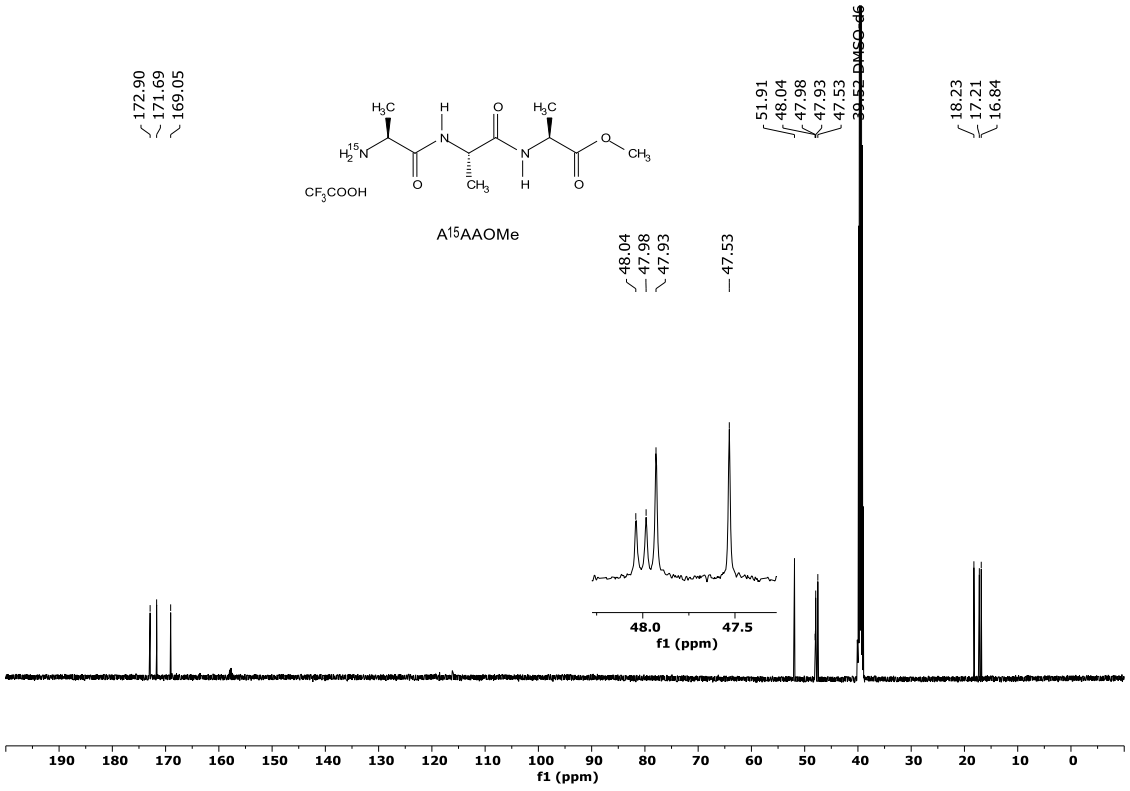


^{13}C NMR (125 MHz, CD_3OD) for **Ac-A-A-A**

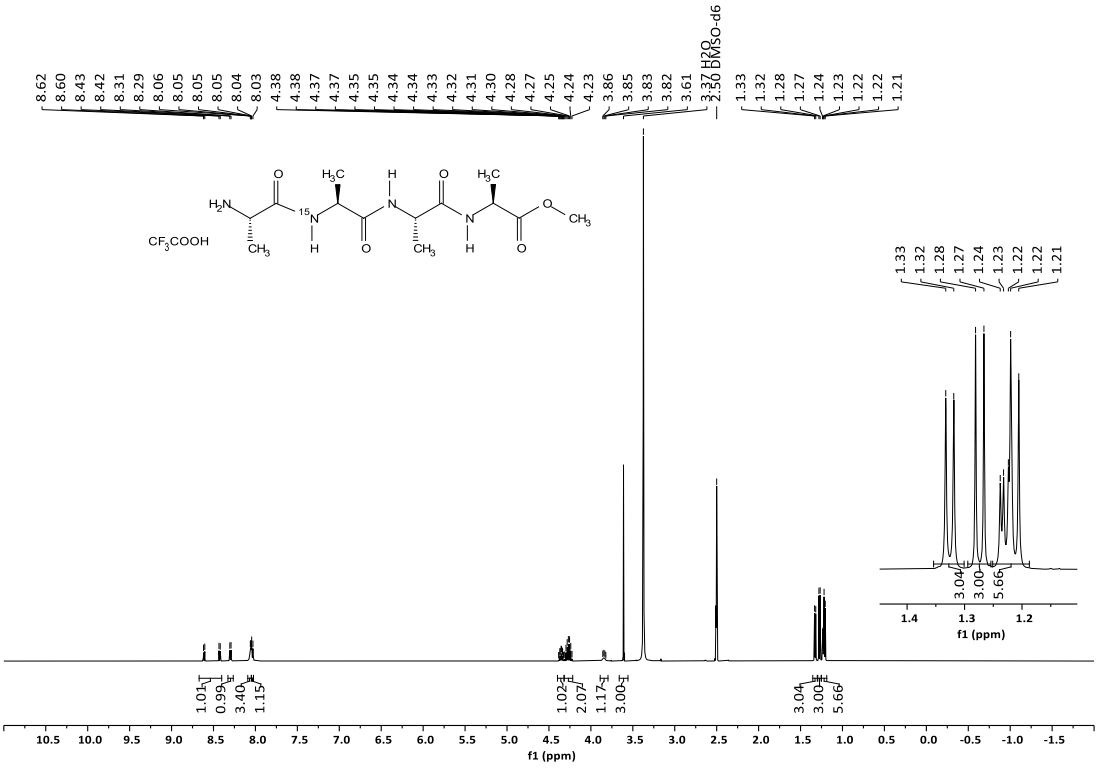


^{1}H NMR (500 MHz, DMSO-d$_6$) for **A[15N]-A-A-OMe**

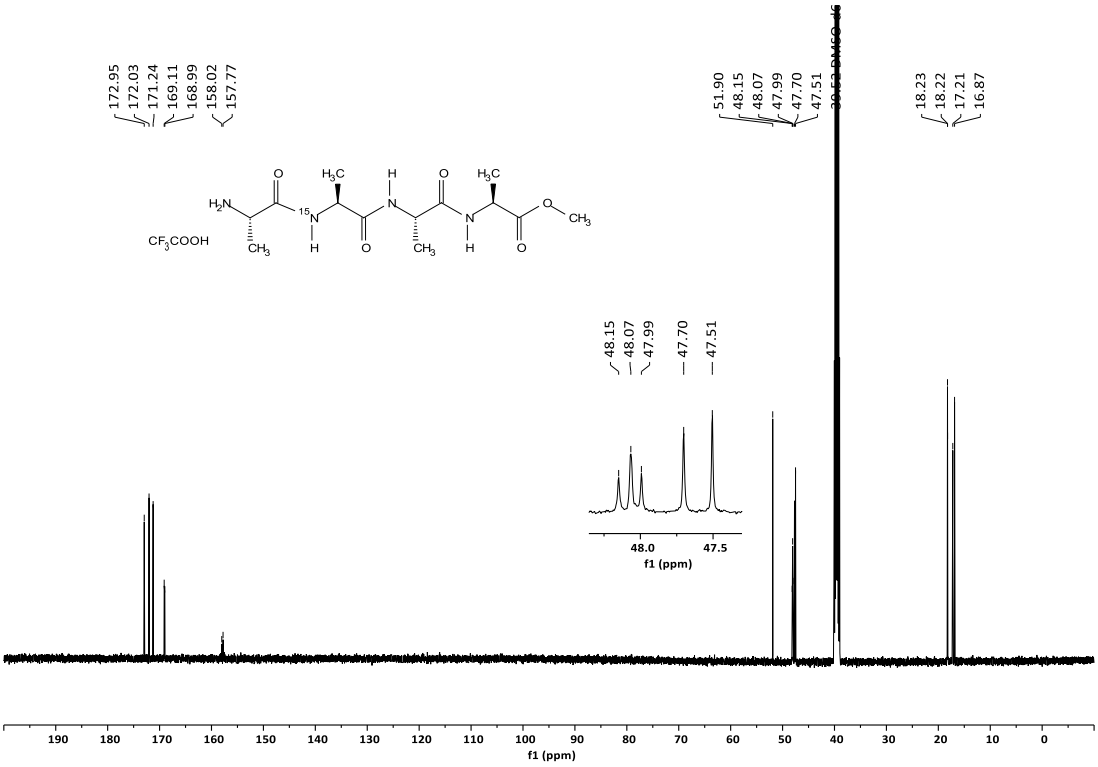
^{13}C NMR (125 MHz, DMSO-d$_6$) for **A[15N]-A-A-OMe**



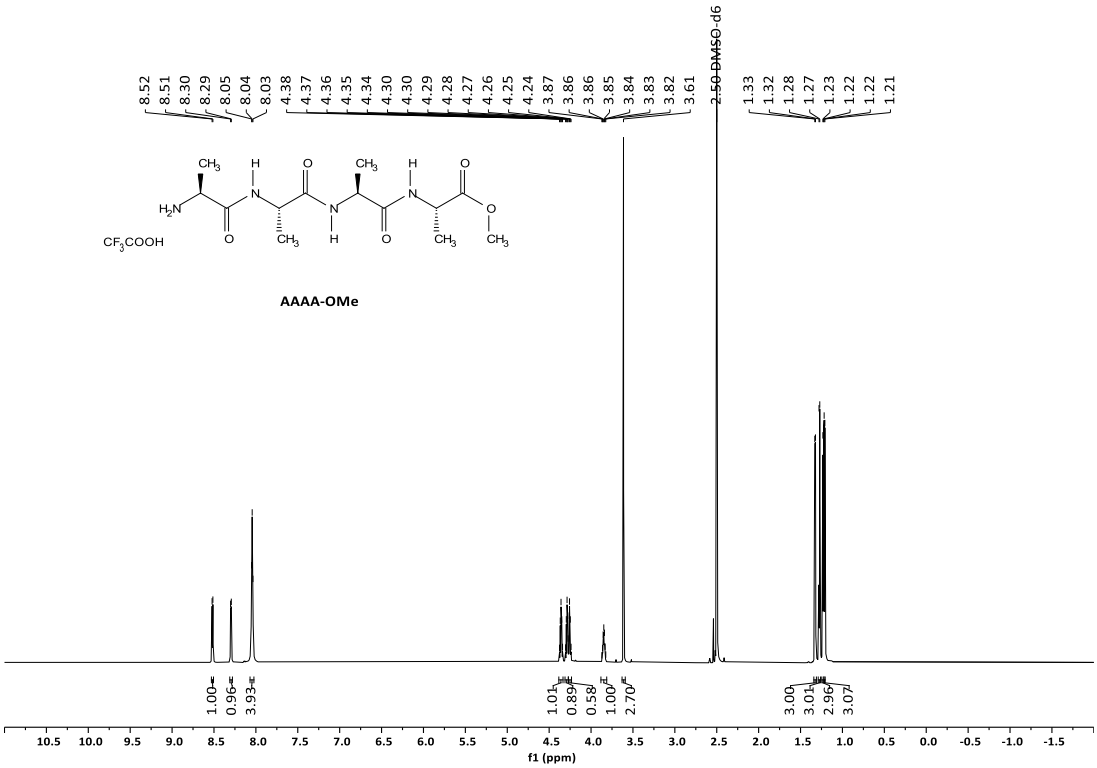
^{1}H NMR (500 MHz, DMSO-d$_6$) for **A-A[15N]-A-A-OMe**



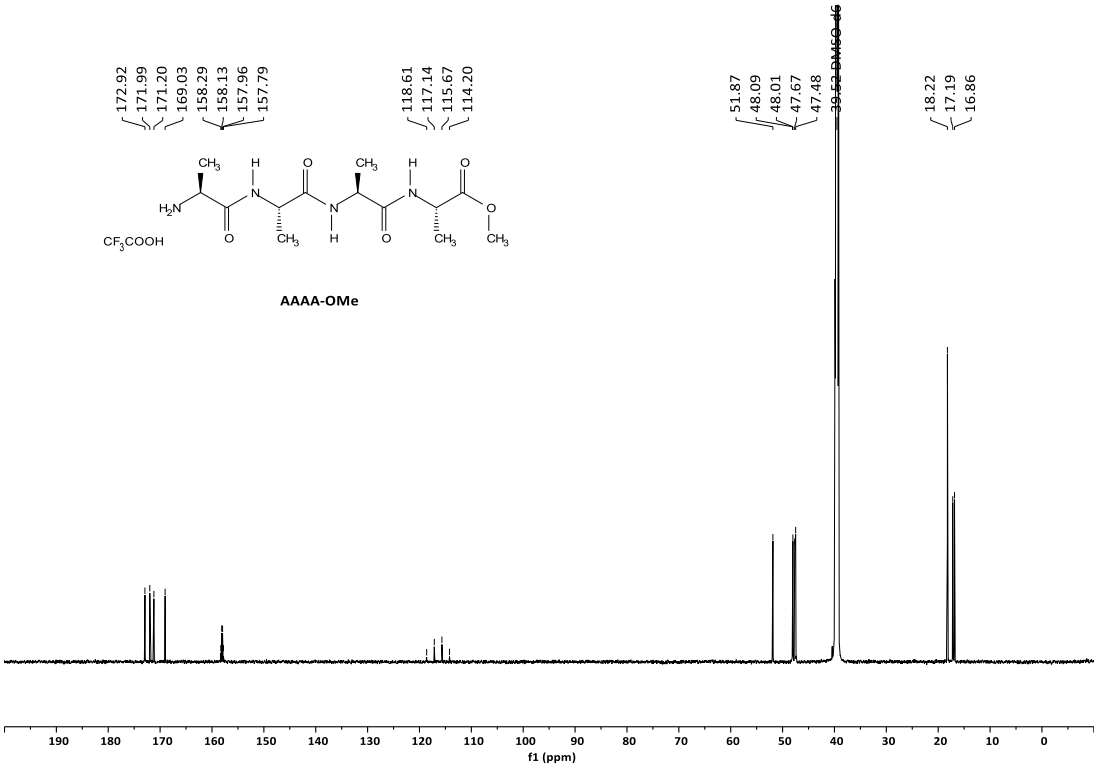
^{13}C NMR (125 MHz, DMSO-d$_6$) for **A-A[15N]-A-A-OMe**



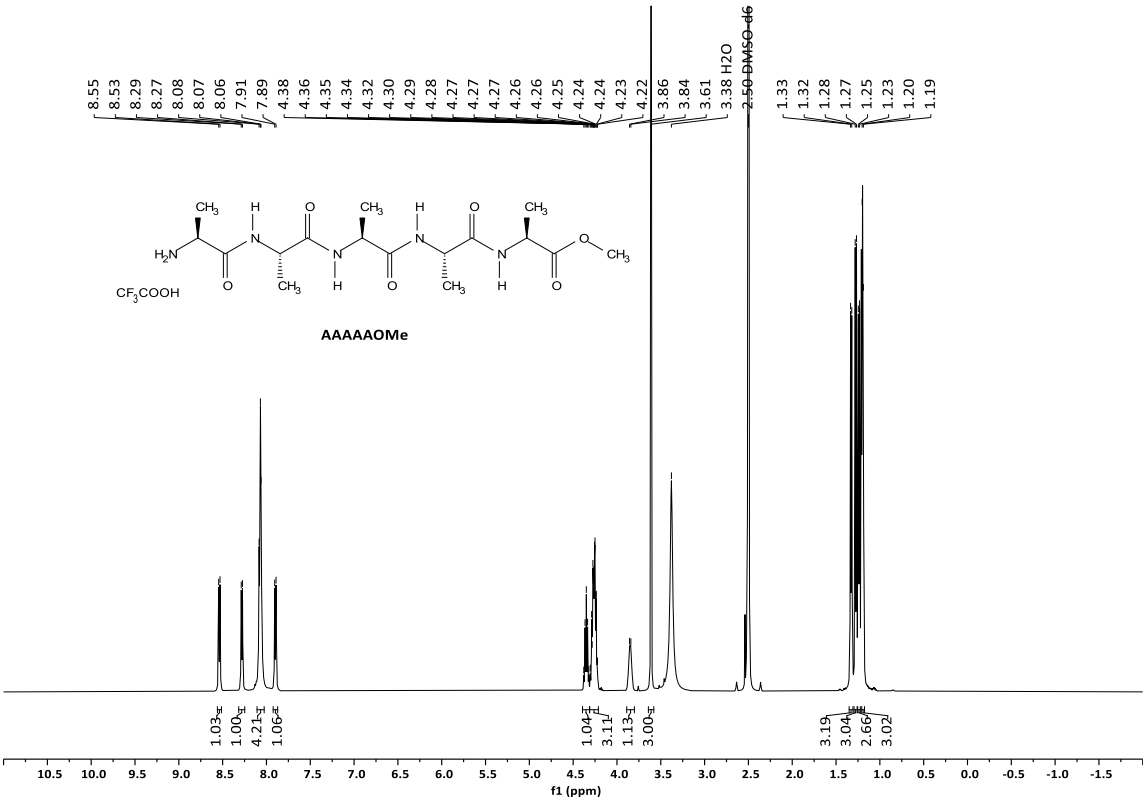
^{1}H NMR (800 MHz, DMSO-d$_6$) for **A$_4$-OMe**



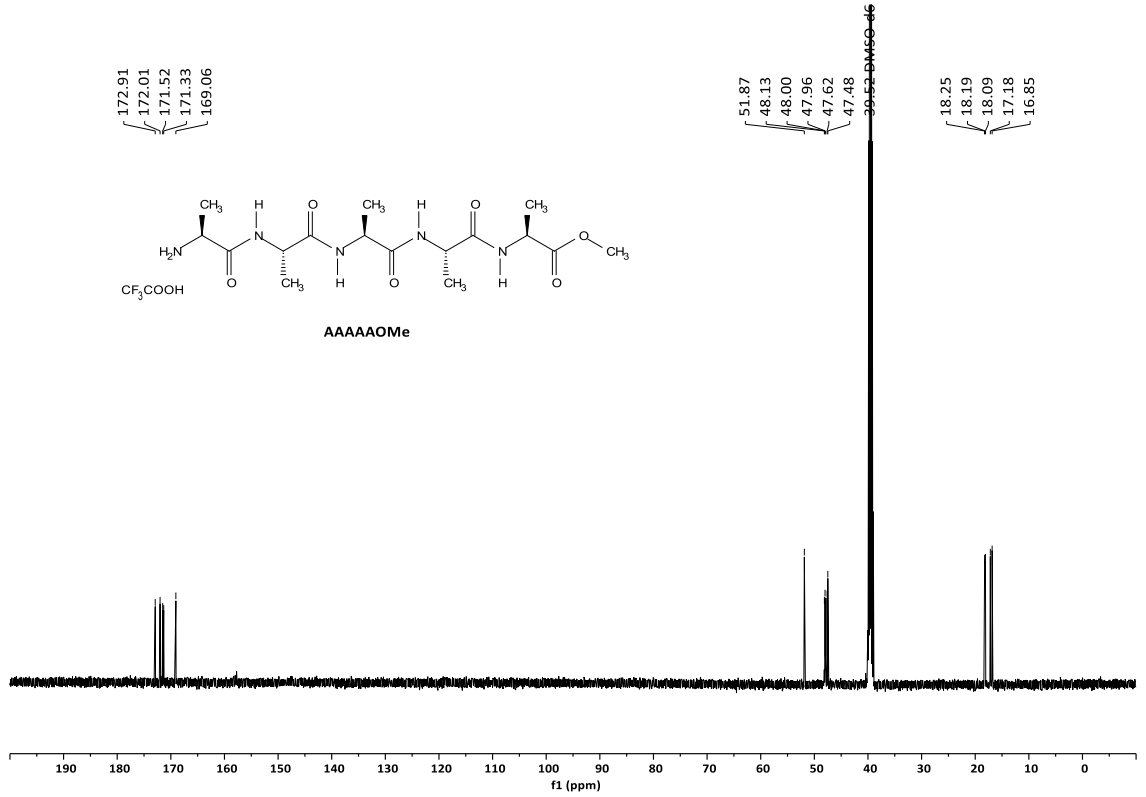
^{13}C NMR (200 MHz, DMSO-d$_6$) for **A$_4$-OMe**



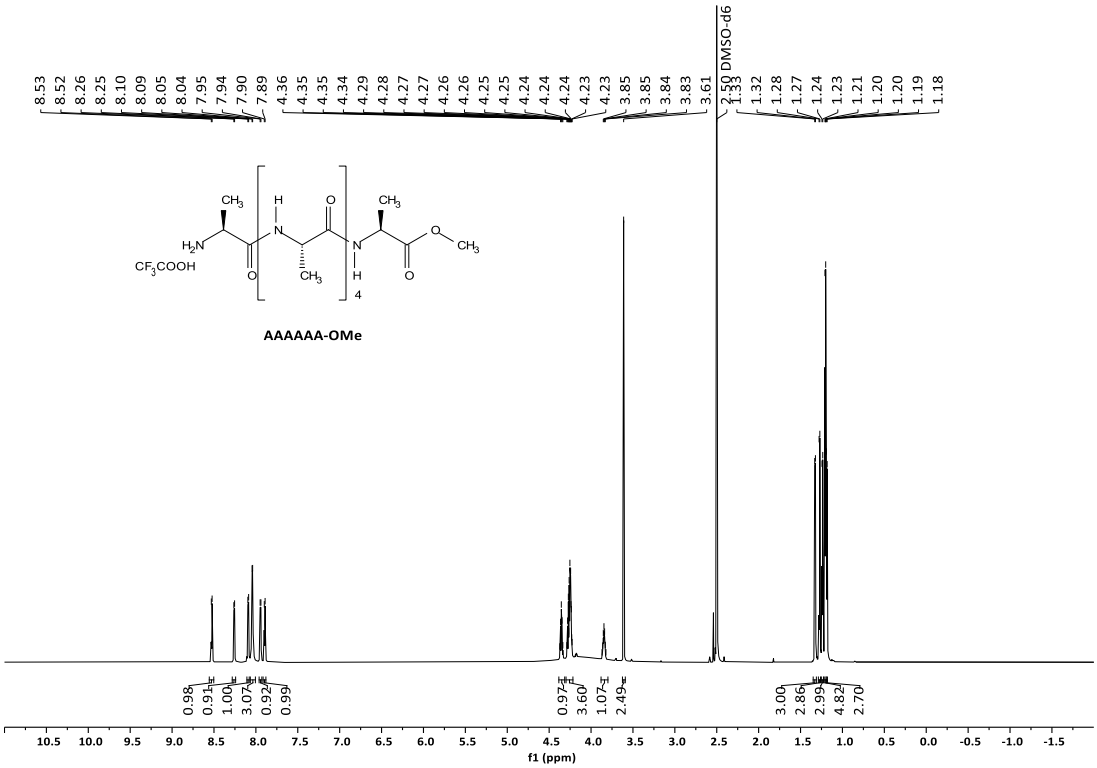
^{1}H NMR (500 MHz, DMSO-d$_6$) for **A$_5$-OMe**



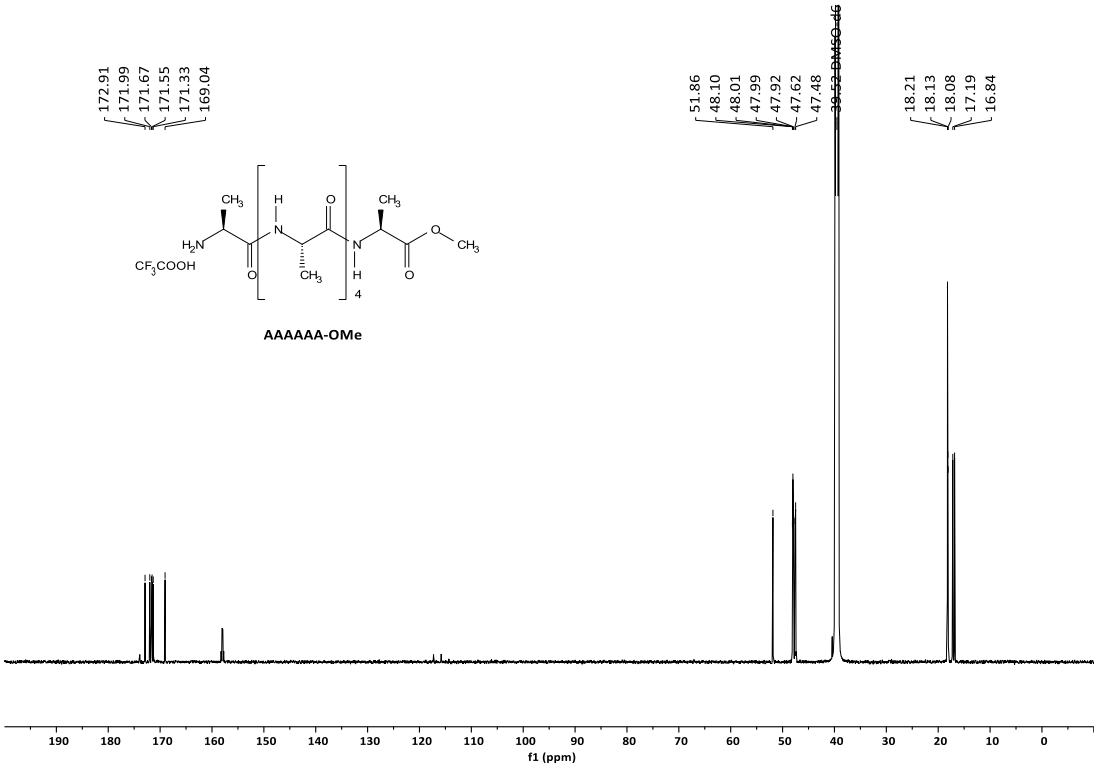
^{13}C NMR (125 MHz, DMSO-d$_6$) for **A$_5$-OMe**



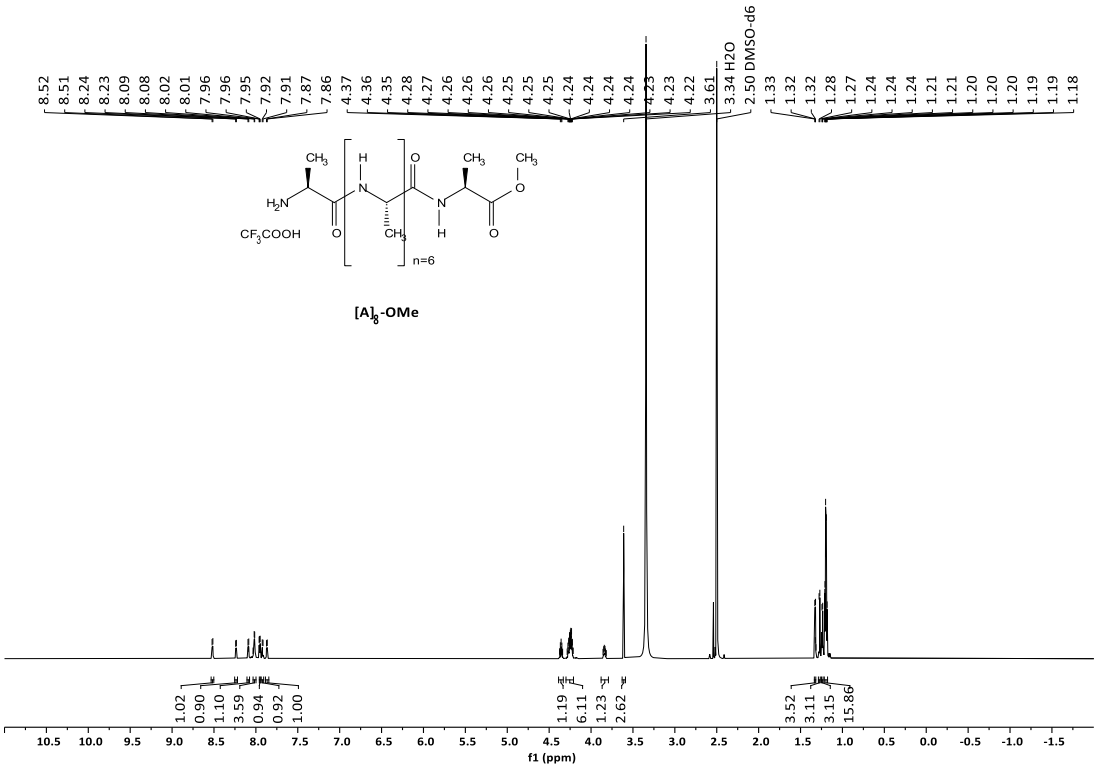
^{1}H NMR (800 MHz, DMSO-d_6) for **A_6-OMe**



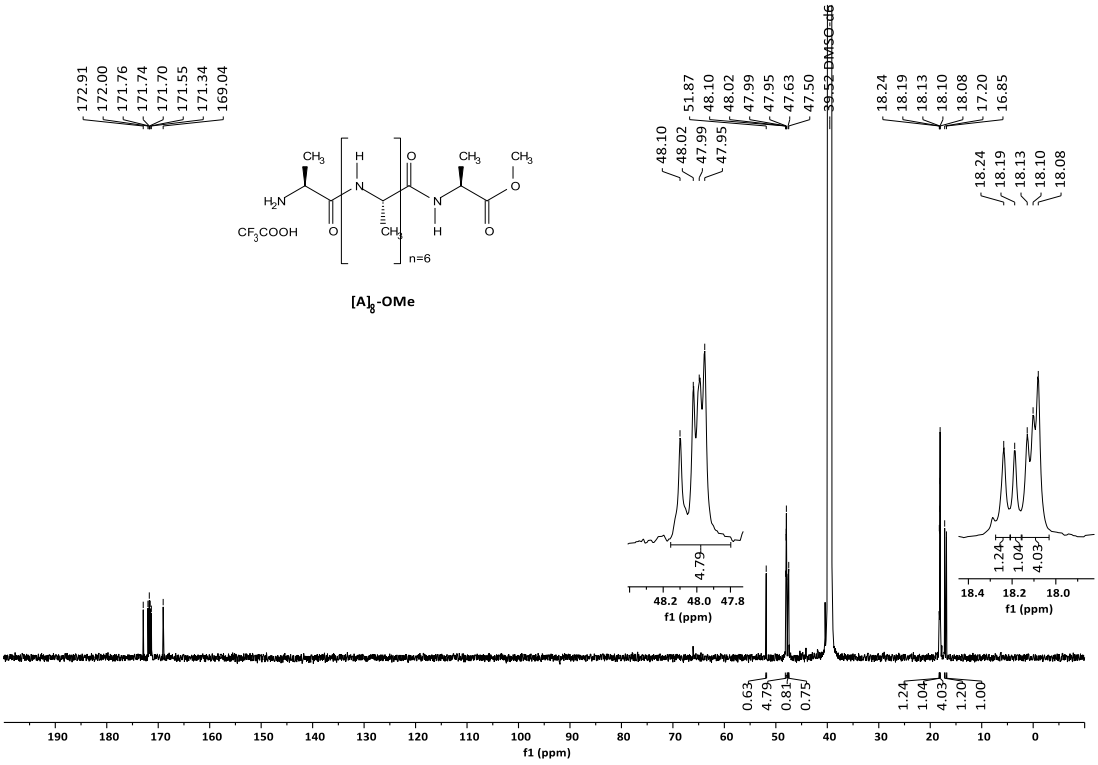
^{13}C NMR (200 MHz, DMSO-d_6) for **A_6-OMe**



^{1}H NMR (800 MHz, DMSO-d$_6$) for **[A]$_8$-OMe**



^{13}C NMR (200 MHz, DMSO-d$_6$) for **[A]$_8$-OMe**



4. References

(1) Savka, R.; Plenio, H. Facile Synthesis of [(NHC)MX(Cod)] and [(NHC)MCl(CO)2] (M = Rh, Ir; X = Cl, I) Complexes. *Dalt. Trans.* **2014**, *44* (3), 891–893. https://doi.org/10.1039/c4dt03449j.

(2) Cowley, M. J.; Adams, R. W.; Atkinson, K. D.; Cockett, M. C. R.; Duckett, S. B.; Green, G. G. R.; Lohman, J. A. B.; Kerssebaum, R.; Kilgour, D.; Mewis, R. E. Iridium N-Heterocyclic Carbene Complexes as Efficient Catalysts for Magnetization Transfer from Para-Hydrogen. *J. Am. Chem. Soc.* **2011**, *133* (16), 6134–6137. https://doi.org/10.1021/ja200299u.

(3) Seefeld, M. A.; Rouse, M. B.; Heerding, D. A.; Peace, S.; Yamashita, D. S. McNulty, K. C. Inhibitors of AKT Activity, WO2008/098104 A1, 2008.

(4) Bodanszky, M.; Bodanszky, A. *The Practice of Peptide Synthesis*; Reactivity and Structure Concepts in Organic Chemistry; Springer Berlin Heidelberg: Berlin, Heidelberg, 1984; Vol. 21. https://doi.org/10.1007/978-3-642-96835-8.

(5) Bonora, G. M.; Toniolo, C. Linear Oligopeptides, 60. Synthesis by the Liquid-Phase Method and Characterization by Amino Acid Analyses of Two Complete Monodispersed Homologous Series Derived from L-Alanine (to the Octapeptide) and L-Valine (to the Hexapeptide). *Die Makromol. Chemie* **1979**, *180* (9), 2095–2099. https://doi.org/10.1002/MACP.1979.021800904.

(6) Eriksson, U.; Peterson, L. W.; Kashemirov, B. A.; Hilfinger, J. M.; Drach, J. C.; Borysko, K. Z.; Breitenbach, J. M.; Kim, J. S.; Mitchell, S.; Kijek, P.; McKenna, C. E. Serine Peptide Phosphoester Prodrugs of Cyclic Cidofovir: Synthesis, Transport, and Antiviral Activity. *Mol. Pharm.* **2008**, *5* (4), 598–609. https://doi.org/10.1021/MP8000099/SUPPL_FILE/MP8000099-FILE002.PDF.

(7) Li, J.; Sha, Y. A Convenient Synthesis of Amino Acid Methyl Esters. *Molecules* **2008**, *13* (5), 1111–1119. https://doi.org/10.3390/MOLECULES13051111.

(8) Gamon, L. F.; White, J. M.; Wille, U. Oxidative Damage of Aromatic Dipeptides by the Environmental Oxidants $NO_2^{\cdot}$ and O3. *Org. Biomol. Chem.* **2014**, *12* (41), 8280–8287. https://doi.org/10.1039/C4OB01577K.

(9) Dahiya, R. SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF A GLYCINE-RICH PEPTIDE - CHERIMOLACYCLOPEPTIDE E. *J. Chil. Chem. Soc.* **2007**, *52* (3), 1224–1229. https://doi.org/10.4067/S0717-97072007000300006.

(10) Reimets, N.; Ausmees, K.; Vija, S.; Reile, I. Developing Analytical Applications for Parahydrogen Hyperpolarization: Urinary Elimination Pharmacokinetics of Nicotine. *Anal. Chem.* **2021**, *93* (27), 9480–9485. https://doi.org/10.1021/acs.analchem.1c01281.

(11) Sellies, L.; Reile, I.; Aspers, R. L. E. G.; Feiters, M. C.; Rutjes, F. P. J. T.; Tessari, M. Parahydrogen Induced Hyperpolarization Provides a Tool for NMR Metabolomics at Nanomolar Concentrations. *Chem. Commun.* **2019**, *55* (50), 7235–7238. https://doi.org/10.1039/C9CC02186H.

(12) Ausmees, K.; Reimets, N.; Reile, I. Parahydrogen Hyperpolarization of Minimally Altered Urine Samples for Sensitivity Enhanced NMR Metabolomics. *Chem. Commun.* **2022**, *58* (3), 463–466. https://doi.org/10.1039/D1CC05665D.