Solid Phase Synthesis of an Insect Pyrokinin Analog Incorporating an Imidazoline Ring as Isosteric Replacement of a *trans* Peptide Bond

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SUPPORTING INFORMATION

Materials and Methods

All solvents purchased were HPLC or anhydrous p.a. grade (SIGMA-Aldrich, Milipore, ABI) and were used without purification. Fmoc-Ala-OSu, Fmoc-OSu, H-Ala-NH_2, HBTU, HOBt, DIEA and Fmoc-Dap(Fmoc)-OH were purchased from ChemImpex, Inc. (Chicago, IL) or IRIS Biotech (Germany). All other organic reagents were supplied by Aldrich.

General automated SPPS (ABI Synthesizer): Rink resin (0.25 mmol) was initially swollen in NMP with vortexing for 10 min at rt. (i) Fmoc removal: 20% piperidine in NMP 3x 2,5 min while vortexing, rt. Completeness of deprotection is checked comparing difference in value of conductivity between the last deprotection with the preceding one with conductivity meter, and if this value is higher than was set up in programming, machine is repeating deprotections (up to three more). (ii) Washing 6x NMP. (iii) Coupling: Fmoc-AA-OH 1 mmol, 4 equivalents) in the cartridge, to which HBTUHOBt (3.6 eq., 0.45 M in DMF) and DIEA (7.2 equivalents, 2.0 M in NMP) were added; solution in the cartridge was agitated with gentle stream on nitrogen for 10 min, after which was transferred to the washed resin. The resulting suspension was vortexed 45 min at rt. The reaction vessel was then drained, and resin was thoroughly washed with NMP three times while vortexing. (iv). Capping: solution of 5ml NMP solution containing acetic anhydride/DIEA/HOBt (19 mL/9.5 mL/800mg diluted with NPM to 400 mL) was added to the resin and reaction vessel was vortexed for 15 min, drained, and resin was thoroughly washed with DCM six times.

General Peptide Cleavage Method: The resin was soaked with DCM and cleaved using 50% TFA/DCM cleavage cocktail at rt for 2 h. A cocktail, which mostly used for this purpose, TFA/TIS/H2O, 95:2.5:2.5 was found as being not compatible with imidazoline moiety. The resin was then filtered from the cleavage cocktail solution and the solution was concentrated by evaporation under vacuum. The residual TFA solution was poured into diethyl ether (at least 10 times the volume of the residual solution) to precipitate the crude peptide. The crude product was sedimented by gentle centrifugation and then ether phase was decanted. Suspending of crude peptide in ether and decantation was repeated twice. The crude peptide PPK-Jo was dissolved in 20% acetonitrile: water and purified by reverse-phase preparative HPLC using method A (below). Fractions containing peptide of expected molecular weight were dried, dissolved in 80% acetonitrile-water and further purified by normal-phase preparative HPLC using method B.

All NMR spectra of the obtained compound were acquired using a Bruker Avance II Plus 16.4 T spectrometer (Bruker BioSpin, Germany). All experiments were performed at 300 K. The operating frequencies were 700 and 175 MHz for ^{1}H and ^{13}C experiments. The instrument was equipped with 5 mm Z-gradient broadband decoupling inverse probe. The precise ^{1}H and ^{13}C chemical shifts assignments were performed by 2D NMR experiments. All chemical shifts were referenced to the DMSO signal at 2.50 ppm. 2D NMR spectra were processed with TopSpin 2.1 (Bruker).

HRMS measurements were performed using Synapt G2-Si mass spectrometer (Waters) equipped with an ESI source and quadrupole-Time-of-flight mass analyzer. The mass spectrometer was operated in the positive ion detection mode. To ensure accurate mass measurements, data were collected in centroid mode and mass was corrected during acquisition using leucine enkephalin solution as an external reference (Lock-SprayTM) which generated reference ion at m/ z 556.2771 Da ($[M+H]^{+}$) in positive ESI mode. The results of the measurements were processed using the MassLynx 4.1 software (Waters) incorporated with the instrument.

The identity of the analog during purification steps was confirmed via MALDI-MS on a Kratos Kompact Probe MALDI-MS machine (Kratos Analytical, Ltd., Manchester, UK).

Experimental

Fmoc-Ala-NH_2 1

Method A. To the solution of Fmoc-Ala-OSu (4.084g, 10 mmol, IRIS Biotech, Germany) in Methanol (50mL) at rt was added dropwise solution of 7N Ammonia in Methanol (1.5 mL, 10.5 mmol, 1.05 eqiv, Aldrich). Reaction solution was then stirred overnight, and it was poured into 250 mL of cold water while stirring. After sitting overnight in refrigerator, a white solid was filtered, washed thoroughly with water and dried in desiccator overnight over P_2O_5. Yield 3.073 g (99.0%). MW calc. for $C_{18}H_{18}N_2O_3$ 310.353, found MH^+ 311.5 (MALDI-MS, KRATOS). RP-HPLC purity on C_{18} column in gradient 40-80% B (B 0.1% TFA/80% acetonitrile/20% water) was over 99%. This product was used in the next step without further purification. CAS 136497-80-8.

This reaction was repeated a few times and scaled up to 50 mmol without changing quality of received product.

Method B. To the solution of Fmoc-OSu (6.746 g, 20 mmol, ChemImpex) in 100 mL of dioxane was added H-Ala-NH_2 (1.762 g, 20 mmol, IRIS Biotech, Germany)) and stirred overnight. RP-HPLC showed disappearance of Fmoc-OSu. Reaction solution was poured slowly to 500 mL 0.01 M $KHSO_4$ in water to remove traces of residual substrate. Precipitated white solid was filtered and washed thoroughly with water and dried in desiccator overnight over P_2O_5. Yield 6.120 g (98.6%). MW calc. for $C_{18}H_{18}N_2O_3$ 310.353, found MH^+ 311.4 (MALDI-MS, KRATOS). RP-HPLC purity on C_{18} column in gradient 40-80% B (B 0.1% TFA/80% acetonitrile/20% water) was higher than 99%. This product was used in the next step without further purification.

Iminoether 2

Fmoc-Ala-NH_2 **1** (1.55g, 5 mmol) was added to cooled with ice-bath solution of triethyloxonium tetrafluoroborate (1.045g, 5.5 mmol, 1.1 equiv, Aldrich) in dry DCM. Resulting suspension was stirred at this temperature for 2 h, after which suspension disappeared. Reaction mixture was washed once with 1M $KHCO_3$ to destroy excess of reagent and DCM layer was dried over anhydrous $MgSO_4$ overnight. After filtration resulting solution was concentrated on rotary evaporator under vacuum giving quantitatively almost colorless oil, which solidified after sitting in refrigerator overnight. This product was used in the next step without further purification. During prolonged storage even as solid in refrigerator compound slowly decomposes turning its color to yellow then orange, so should be kept in the freezer.

Cyclization reaction to imidazoline ring on solid support

To 0.1 mmol H-Dap-Arg(Pmc)-Leu-Rink resin obtained from ABI Peptide synthesizer placed in 8 mL propylene syringe equipped with 20 μm frit, and swollen in dry DCM, was added a solution of iminoether **2** (0.3 mmol, 3 equiv) in DCM. After addition of DIEA (0.05 mmol, 0.5 equiv), resulting suspension was shaken overnight at rt, then filtered, washed 3 times with DCM, 3 times with MeOH and 3 times with DCM. Protection of imidazoline nitrogen was performed also in the syringe adding solution of di-*tert*-butyl pyrocarbonate in DCM (0.2 mmol, 2 equiv, ChemImpex, Illinois) to the resin. After being shaken for 3 h at rt, resin was filtered through frit and washed consecutively with DCM (3x), MeOH (3x) and dried. Then was placed again in reaction vessel of ABI Peptide Synthesizer and automatic synthesis was continued. After finishing automated synthesis, the resin was transferred back

to the polypropylene syringe, washed with MeOH (3x) and dried. Yield of the crude peptide attached to the polymer support, calculated from weight gain was in the range of 90-95% in several trials on the scale from 0.05 mmol to 0.125 mmol.

Purification and Amino Acid Analysis

Method A. A Waters C_{18} Sep Pak cartridge, and a Delta-Pak C_{18} reverse-phase column (8 x 100 mm, 15 μm particle size, 100 A pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. Delta-Pak C-18 retention time: t_R= 4.5 min.

Method B. A Waters Protein Pak I 125 column (7.8 x 300 mm). Conditions: isocratic using 80% aqueous acetonitrile containing no TFA; flow rate 2 ml/min. Waters Protein Pak retention time: 6.0 min.

The first purification step was performed on C_{18} Waters cartridge (Method A), where fractions containing a peak of correct MS value were collected. The identity of the analog during purification steps was confirmed via MALDI-MS on a Kratos Kompact Probe MALDI-MS machine (Kratos Analytical, Ltd., Manchester, UK) with the presence of the molecular ion 778.11 [MH^+]

The peptide analog for biological activity studies was subjected to final purification step (Method B).

Amino acid analysis was used to quantify the peptides and to confirm identity, leading to the following analysis: F[1.0], L[1.1], R[1.2], Y[1.2];.

HRMS measurements from repurified sample were performed using Synapt G2-Si mass spectrometer (Waters) equipped with an ESI source and quadrupole-Time-of-flight mass analyzer. The mass spectrometer was operated in the positive ion detection mode. To ensure accurate mass measurements, data were collected in centroid mode and mass was corrected during acquisition using leucine enkephalin solution as an external reference (Lock-SprayTM) which generated reference ion at m/ z 556.2771 Da ([$M+H]^+$) in positive ESI mode. The results of the measurements were processed using the MassLynx 4.1 software (Waters) incorporated with the instrument. Calc. for $C_{38}H_{56}N_{11}O_7$ = 778.4264 [MH^+], found 778.4254 [MH^+] and 379.7226 [MH_2^{2+}] for single and doubly charged ions, respectively. (Fig. 1 and Fig. 2)

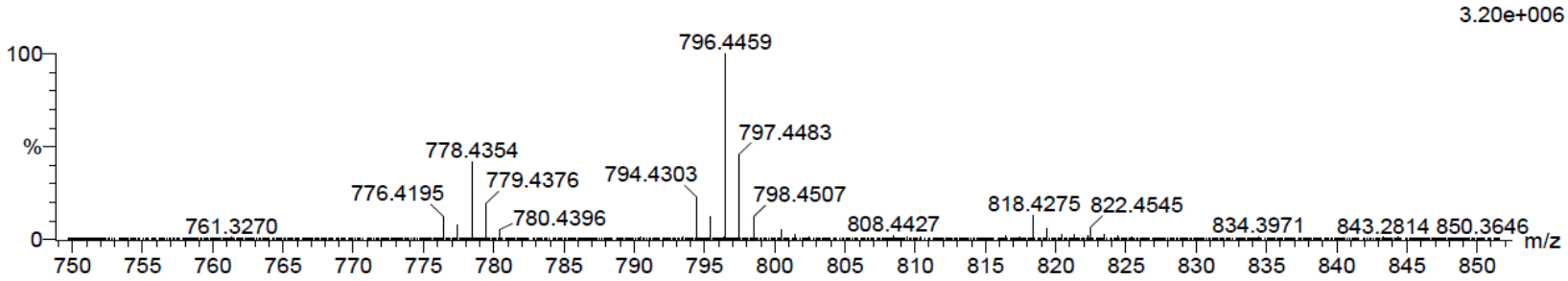
Elemental Composition Report **Page 1**

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -50.0, max = 80.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 5

Monoisotopic Mass, Even Electron Ions
200 formula(e) evaluated with 2 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-40 H: 0-60 N: 0-14 O: 0-10

181128_peptydA 14 (0.160) Cm (13:17-(3:8+32:72))
TOF MS ES+



Minimum:				-50.0				
Maximum:		15.0	5.0	80.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
778.4354	778.4364	-1.0	-1.3	16.5	913.9	1.490	22.54	C38 H56 N11 O7
	778.4324	3.0	3.9	12.5	912.7	0.255	77.46	C33 H56 N13 O9

Figure 1. HRMS of PPK-Jo displayed with computed possible empirical formulas.

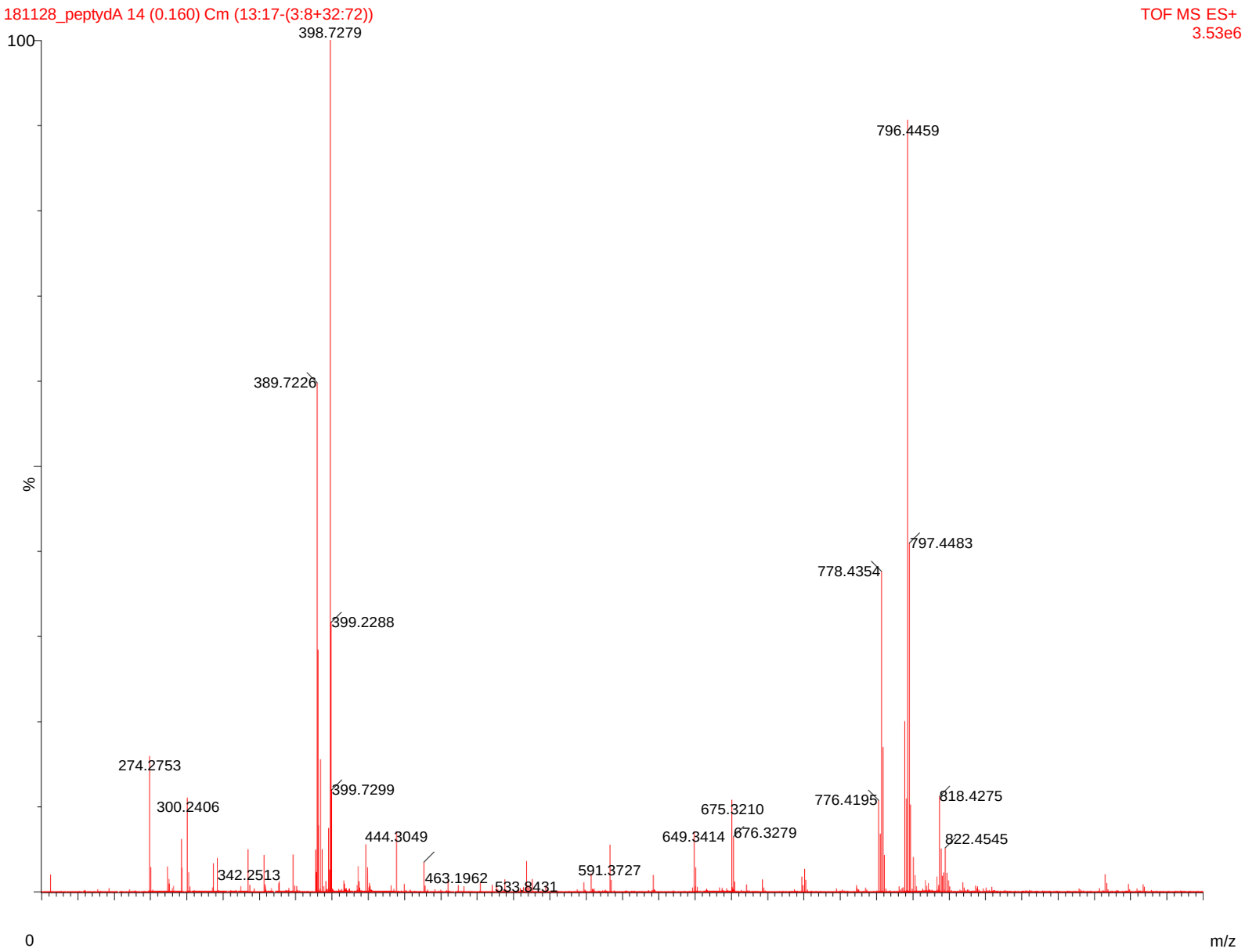


Fig. 2. HRMS measurements. Calc. for $C_{38}H_{56}N_{11}O_7$ = 778.4264 [MH^+], found 778.4254 [MH^+] and 379.7226 [MH_2^{2+}] for single and doubly charged ions, respectively.

NMR experiments

All NMR spectra of the obtained compound were acquired using a Bruker Avance II Plus 16.4 T spectrometer (Bruker BioSpin, Germany). All experiments were performed at 300 K. The operating frequencies were 700 and 175 MHz for ^{1}H and ^{13}C experiments. The instrument was equipped with 5 mm Z-gradient broadband decoupling inverse probe. The precise ^{1}H and ^{13}C chemical shifts assignments were performed by 2D NMR experiments. All chemical shifts were referenced to the DMSO signal at 2.50 ppm. 2D NMR spectra were processed with TopSpin 2.1 (Bruker). Due to the low concentration of the compound it was not possible to assign all of the chemical shifts of the quaternary carbons. The presence of the imidazoline ring was confirmed on the basis of 2D NMR spectroscopy. Homonuclear correlated spectra (COSY) were acquired using a standard pulse sequence (cosygpqf). Spectra were recorded with acquisition of 32 transients for each of 512 increments with 2K data points. The spectral widths for ^{1}H were 8417 Hz. Total correlated spectra (TOCSY) were acquired using a standard pulse sequence (mlevph). Spectra were recorded with acquisition of 48 transients for each of 512 increments with 2K data points. The spectral widths for ^{1}H was 8417 Hz. Two-dimensional ^{1}H-^{13}C HSQC (Fig. 6) spectra were recorded using Bruker pule sequence - hsqcetgpsi2. The spectral widths for ^{1}H and ^{13}C were 8417 Hz and 31706 Hz sampled with 4096 and 256 complex points, respectively. Number of scans was 64. Two-dimensional ^{1}H-^{13}C HMBC spectra (Fig. 4) were recorded using Bruker pule sequence - hmbcgplpndqf. The spectral widths for ^{1}H and ^{13}C were 9090 Hz and 39062 Hz sampled with 4096 and 256 complex points, respectively. Number of scans was 640. All spectra were recorded in two solvents D_2O and DMSO.

On the HMBC spectrum (Fig. 4) we observed correlation peak between the methine proton (CH, 4.82 ppm) and the quaternary carbon atom of the imidazoline ring (C=N, 146.50 ppm). Moreover, this carbon has also correlations with CH_2 (3.72, 4.10 ppm), CH_3 (1.44 ppm) and CH (4.08 ppm) groups.

^{1}H (DMSO-d_6, 300K) δ, ppm: 0.85 (3H, H^{δ}, Leu), 0.89 (3H, H^{δ}, Leu), 1.27 (1H, H^{γ}, Leu), 1.44 (3H, Imid.), 1.45 (2H, H^{γ}, Arg), 1.47 (1H, H^{β}, Leu), 1.54 (1H, H^{β}, Arg), 1.60 (1H, H^{β}, Leu), 1.76 (1H, H^{β}, Arg), 1.93 (3H, Ac), 2.83 (1H, H^{β}, Phe), 2.92 (1H, H^{β}, Phe), 3.03 (1H, H^{β}, Tyr), 3.10 (2H, H^{δ}, Arg), 3.16 (1H, H^{β}, Tyr), 3.42 (2H, NH_2, Arg), 3.44 (2H, NH_2, Leu), 3.72 (1H, CH_{2ring}, Imidazoline), 4.08 (1H, CH, Imidazoline), 4.10 (1H, CH_{2ring}, Imidazoline), 4.23 (1H, H^{α}, Tyr), 4.25 (1H, H^{α}, Leu), 4.34 (1H, H^{α}, Arg), 4.51 (1H, H^{α}, Phe), 4.58 (1H, OH, Tyr), 4.82 (1H, CH_{ring}, Imidazoline), 6.61 (2H, H^{γ}, Tyr), 6.95 (2H, H^{δ}, Tyr), 7.16 (1H, H^{γ}, Phe), 7.23 (1H, H^{ε}, Phe), 7.25 (1H, NH, Arg), 7.28 (1H, H^{δ}, Phe), 7.48 (1H, NH, Imidazoline), 8.00 (1H, NH, Leu), 8.03 (1H, NH, Arg), 8.08 (1H, NH, Tyr), 8.10 (1H, NH, Arg), 8.13 (1H, NH, Phe).

^{13}C (DMSO-d_6, 300K) δ, ppm: 21.63 (CH_3, Ac), 22.09 (C^{δ}, Leu), 23.35 (C^{δ}, Leu), 24.75 (C^{β}, Arg), 25.19 (C^{γ}, Leu), 25.20 (CH_3, Imidazoline), 25.65 (C^{γ}, Arg), 34.7 (C^{β}, Phe), 40.14 (C^{δ}, Arg), 41.39 (C^{β}, Leu), 41.94 (C^{β}, Tyr), 47.30 (CH, Imidazoline), 50.84 (C^{α}, Phe), 51.28 (C^{α}, Leu), 51.43 (C^{α}, Tyr), 51.92 (CH_{2ring}, Imidazoline), 55.01 (C^{α}, Arg), 56.40 (CH_{ring}, Imidazoline), 115.31 (C^{γ}, Tyr), 126.20 (C^{ε}, Phe), 127.40 (C^{δ}, Phe), 129.51 (C^{γ}, Phe), 130.13 (C^{δ}, Tyr), 146.50 (C=N, Imidazoline), 169.60 (C=O, Ac), 170.82 (C=O, Tyr), 172.91 (C=O, Leu), 173.01 (C=O, Phe), 173.26 (C=O(NH2)), 173.50 (C=O, Arg)

	H/C	δ, ppm $[H \rightarrow N]$	δ, ppm $[H \rightarrow {}^{\alpha}C]$	δ, ppm $[H \rightarrow {}^{\beta}C]$	δ, ppm $[H \rightarrow {}^{\gamma}C]$	δ, ppm $[H \rightarrow {}^{\delta}C]$	δ, ppm $[H \rightarrow {}^{\varepsilon}C]$	δ, ppm $[C = O]$	δ, ppm $[NH_2]$	δ, ppm $[CH_3]$	δ, ppm $[OH]$
Arg	H	8.03	4.34	1.76, 1.54	1.45	3.10	-	-	3.42	-	-
	C	-	55.01	24.75	25.65	40.14	-	173.50	-	-	-
Leu	H	-	4.25	1.60, 1.47	1.27	0.85, 0.89	-	-	3.44	-	-
	C	-	51.28	41.39	25.19	22.09, 23.35	-	172.91	-	-	-
Phe	H	8.13	4.51	2.92, 2.83	7.16	7.28	7.23	-	-	-	-
	C	-	50.84	34.37	129.51	127.40	126.20	173.01	-	-	-
Tyr	H	8.08	4.23	3.03, 3.16	6.61	6.95	-		-	-	4.58
	C	-	51.43	41.94	115.31	130.13	-	170.82	-	-	-
Ac	H	-	-	-	-	-	-	-	-	1.93	-
	C	-	-	-	-	-	-	169.60	-	21.63	-

Imidazoline	δ, ppm $[NH]$	δ, ppm $[CH_{ring}]$	δ, ppm $[CH_{2ring}]$	δ, ppm $[C =_{ring}]$	δ, ppm $[CH]$	δ, ppm $[CH_3]$
H	7.48	4.82	3.72, 4.10	-	4.08	1.44
C		56.40	51.92	146.50	47.30	25.20

Table 1. ^{1}H and ^{13}C chemical shifts for all atoms in PPK-Jo.

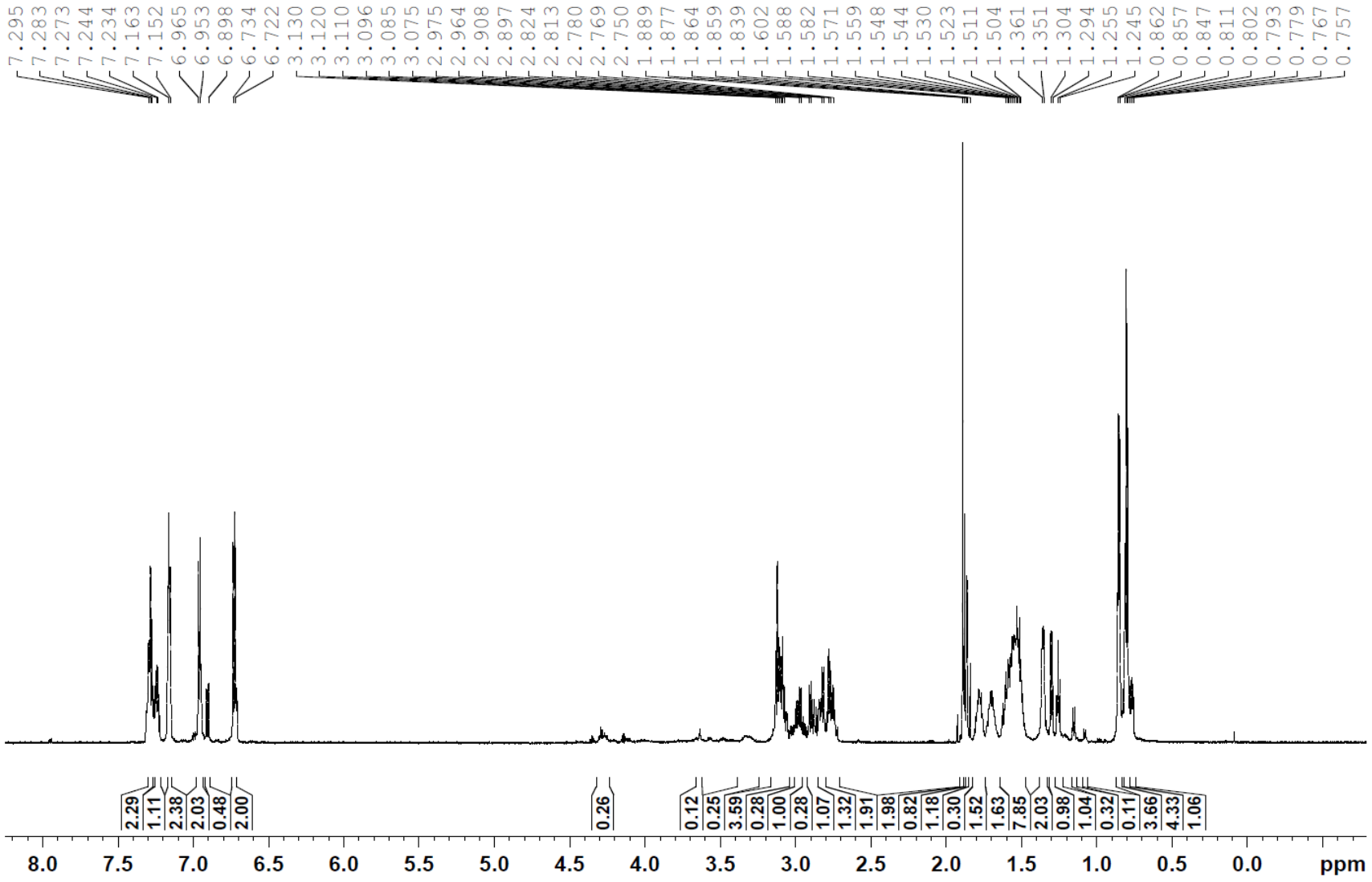


Figure 3. ^{1}H NMR spectrum of PPK-Jo in D_2O.

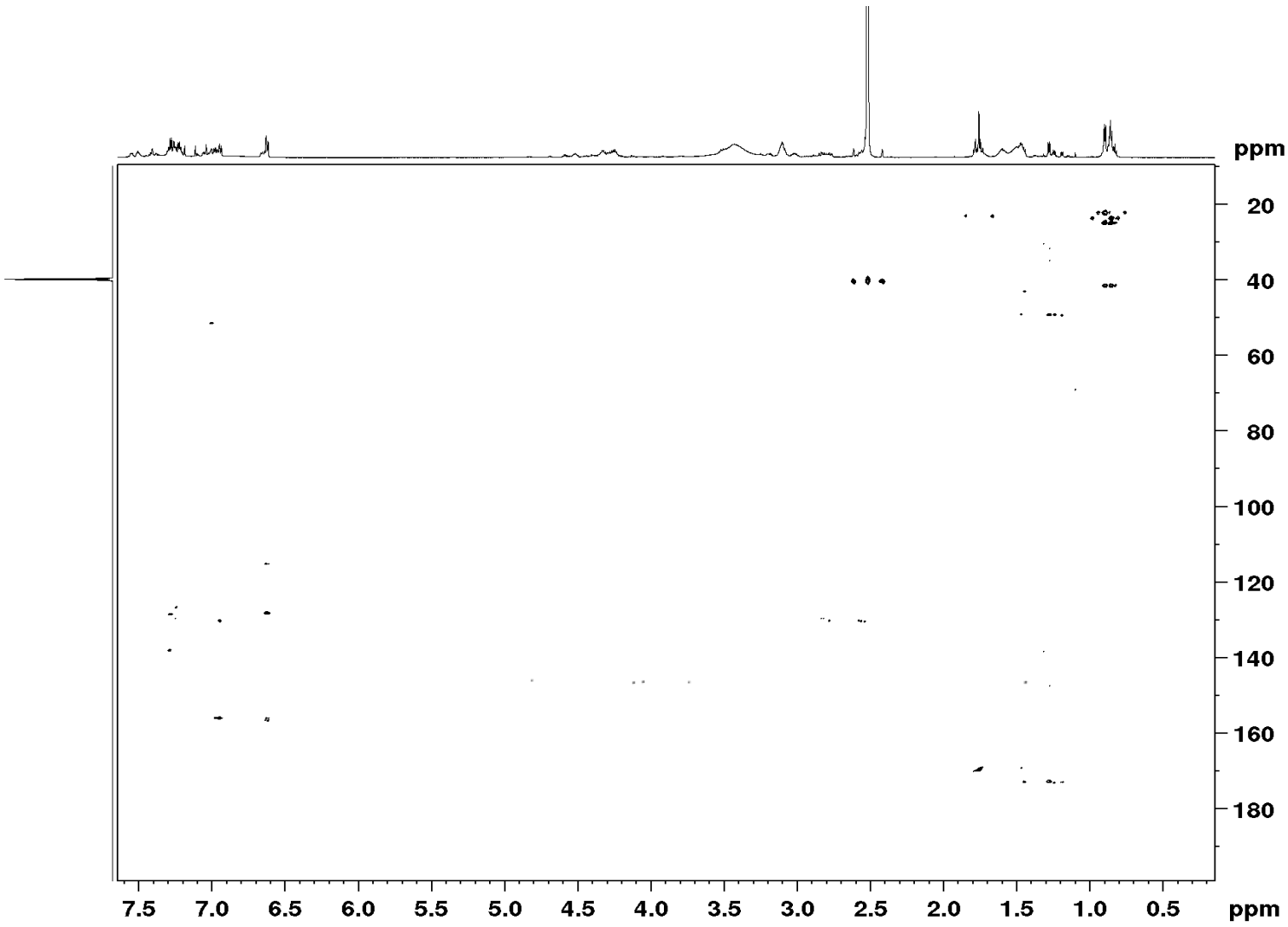


Figure 4. ^{1}H-^{13}C HMBC spectrum of PPK-Jo in DMSO.

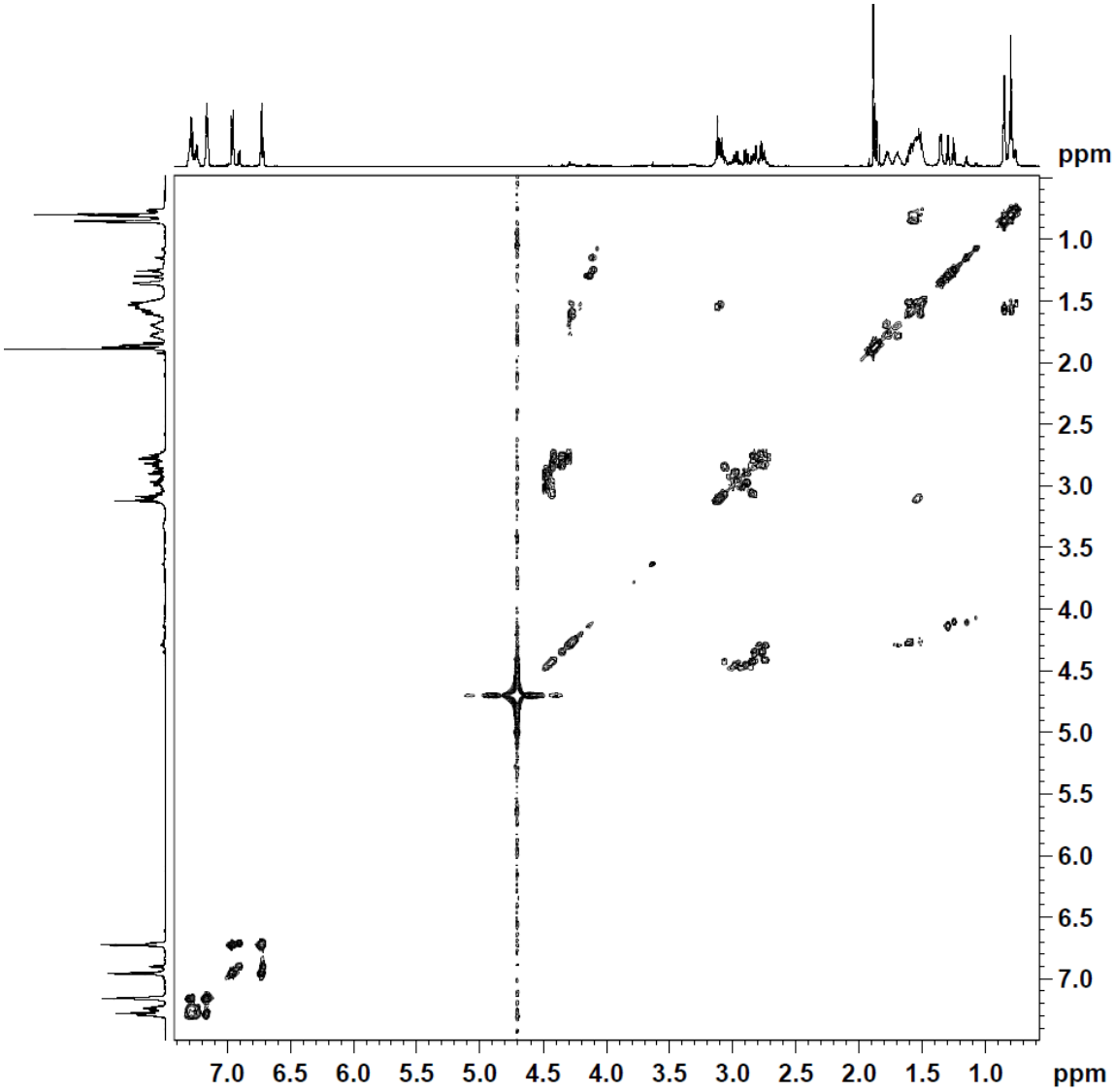


Figure 5. ^{1}H-^{1}H COSY spectrum of PPK-Jo in D_2O.

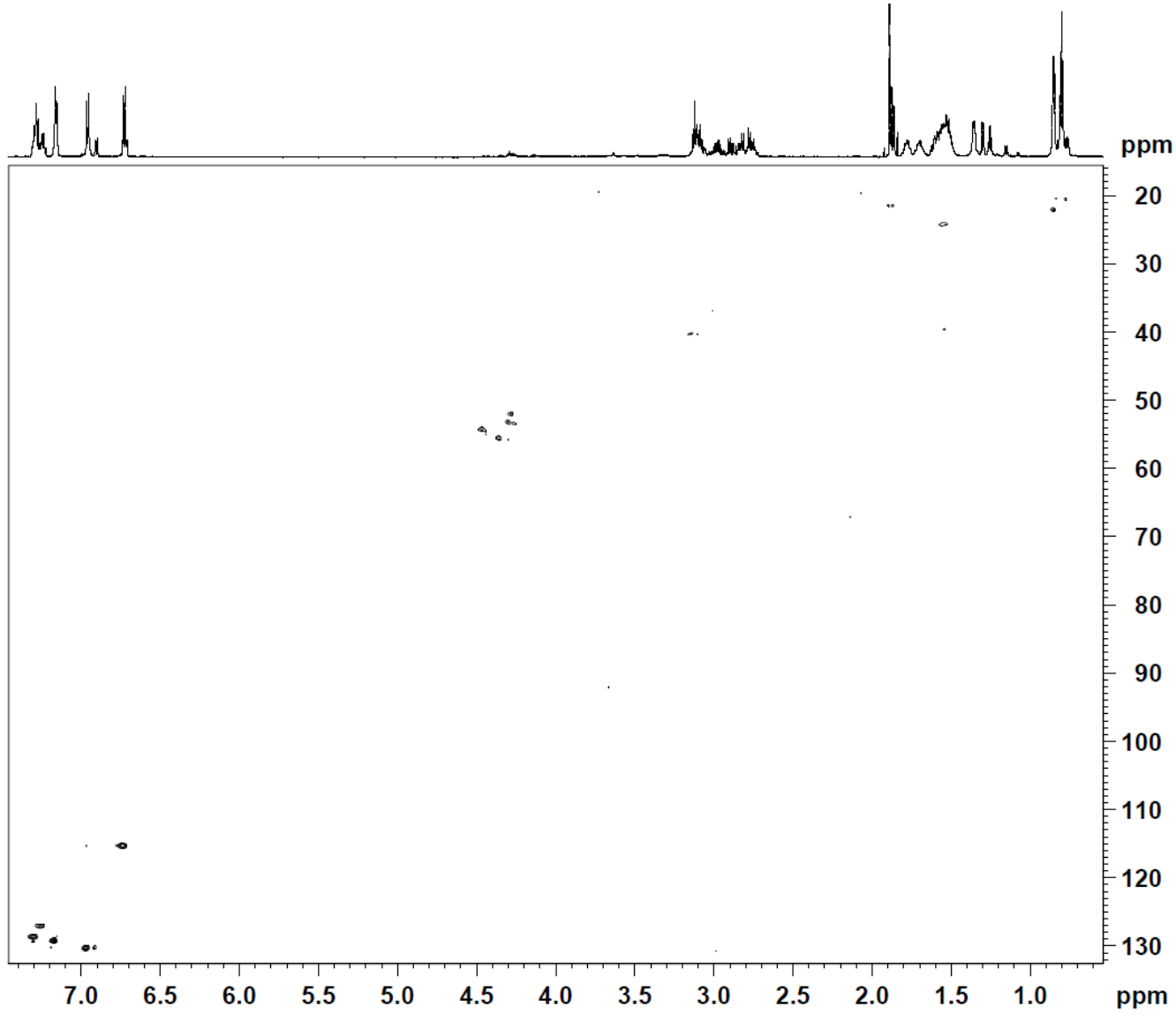


Figure 6. ^{1}H-^{13}C HSQC spectrum of PPK-Jo in D_2O.

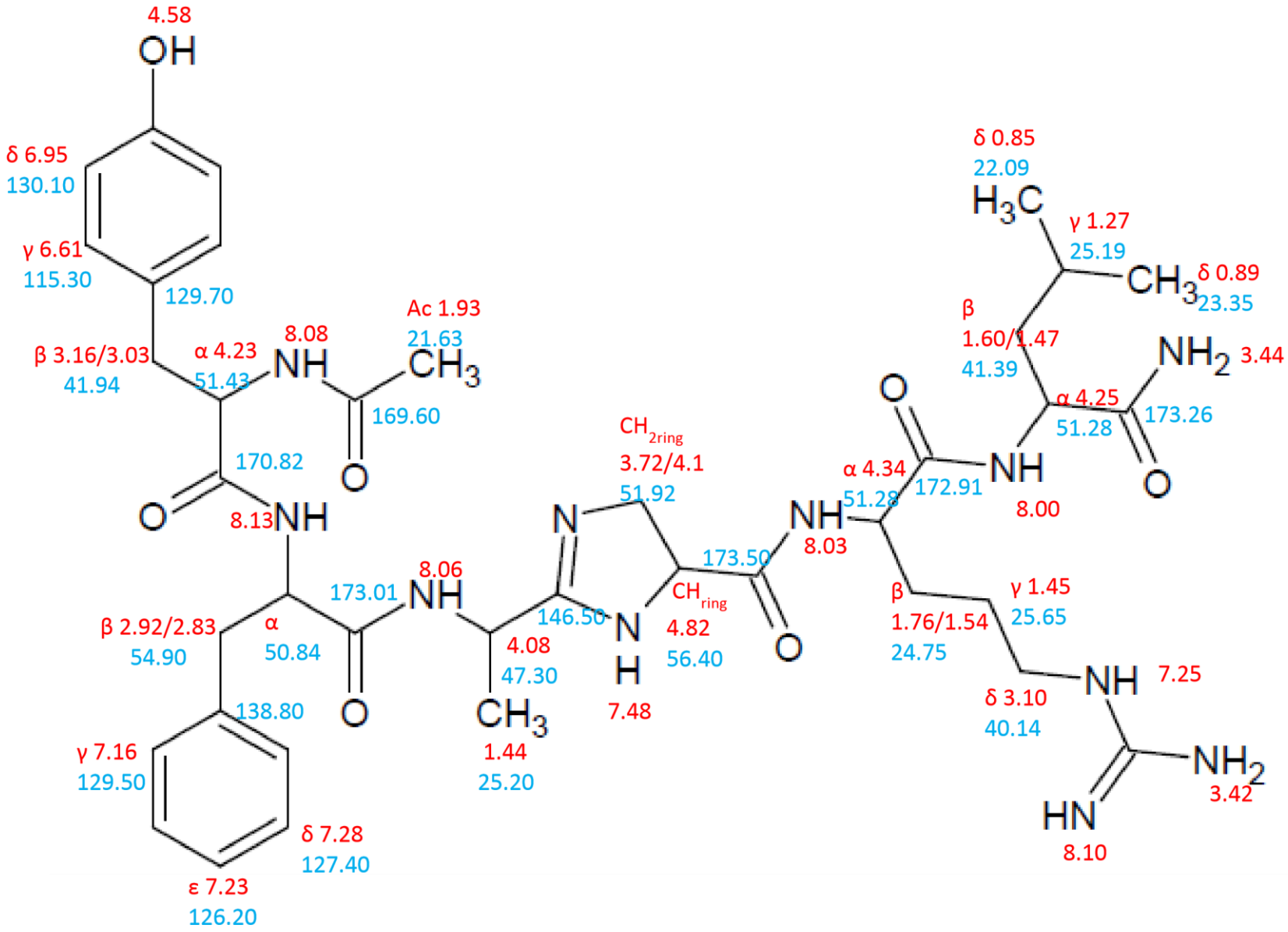


Figure 7. Structure of PPK-Jo with shifts of H and C atoms displayed.