Supporting Information

Macrocyclization of an all-D linear α-helical peptide imparts cellular permeability

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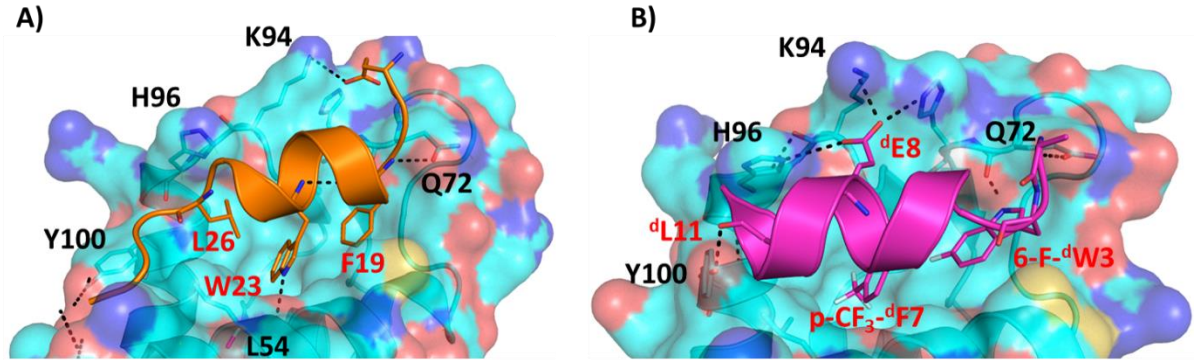

Figure S1: Structural representation of crystal structures of (A) p53-Mdm2 (PDB: 1YCR) and (B) ^{d}PMI-δ – Mdm2 (PDB: 3TPX) complex. Mdm2 is shown as surface and bound peptide is shown as cartoon with interacting residues highlighted in sticks. Hydrogen bond interactions are shown as dotted lines (black).

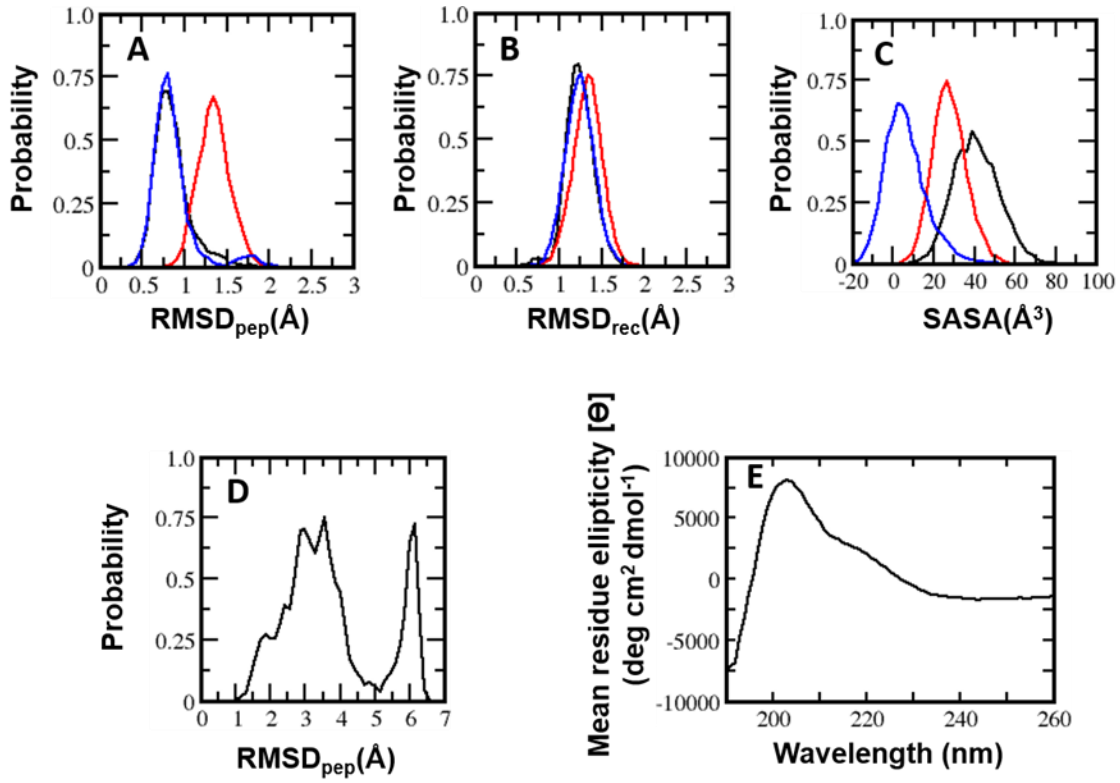

Figure S2: (A) Probability distributions of RMSD (root mean square deviation) (A, B, three colours correspond to triplicate simulations), (B) SASA (solvent accessible surface area) of 6-F-^{d}W3 (black), p-CF3-^{d}F7 (red), ^{d}L11 (blue) (C) of conformations sampled during MD simulations of the ^{d}PMI-δ – Mdm2 complex . (D) Probability distribution of RMSD of peptide conformations sampled during BP-REMD simulations in the absence of Mdm2. (E) CD spectra of the ^{d}PMI-δ peptide Note that this spectra is inverted, as expected for a peptide consisting of D-amino acids only

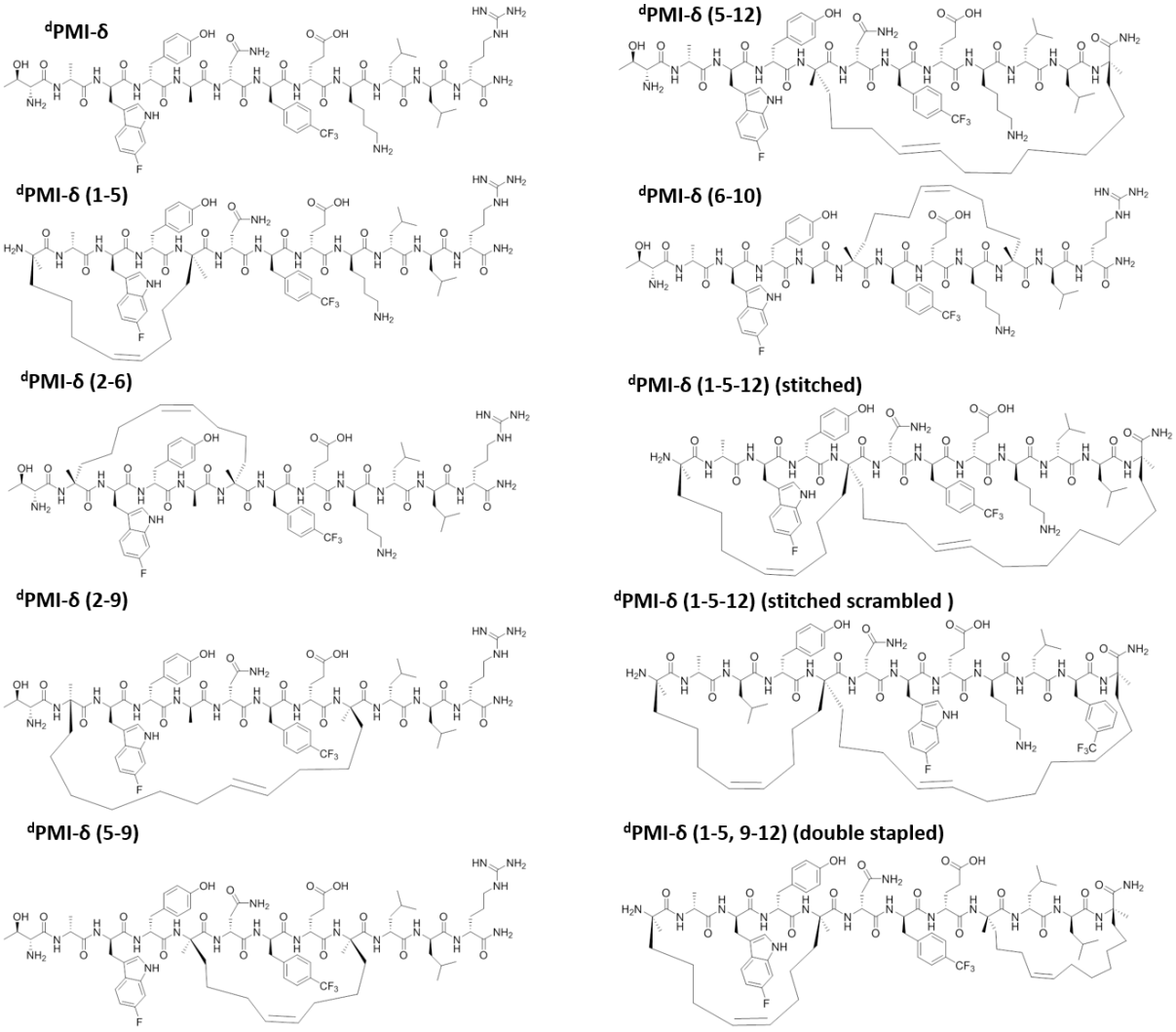

Figure S3: Chemical structures of linear, stapled and stitched [d]PMI-δ peptides

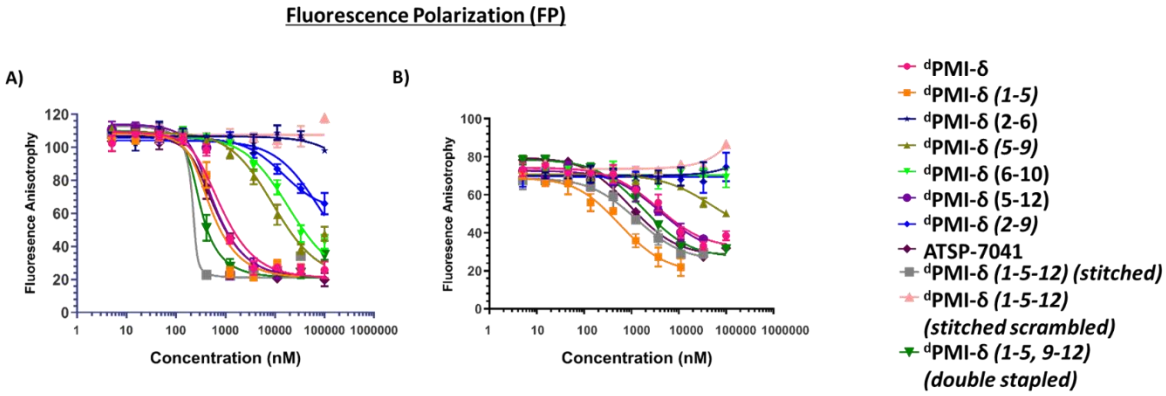

Figure S4: *Fluorescence polarization (FP) (0.1% Tween-20) binding analysis of linear, stapled, stitched* ^{d}*PMI-δ peptides and (A) Mdm2 (B) Mdm4 protein.*

Table S1: *Binding of linear, stapled, stitched ^{d}PMI-δ and ATSP-7041 peptides determined through Fluorescence polarization (FP) (0.1% Tween-20) assay. Error values are given in parenthesis.*

Peptide	FP Mdm2 Kd (nM)	FP Mdm4 Kd (nM)
^{d}PMI-δ	51.2 (1.1)	43.8 (10.8)
^{d}PMI-δ*(1-5)*	18.3 (1.3)	4.5 (6.4)
^{d}PMI-δ*(2-6)*	> 10000	> 10000
^{d}PMI-δ*(2-9)*	2430 (1.1)	> 10000
^{d}PMI-δ*(5-9)*	961.2 (1.3)	> 10000
^{d}PMI-δ*(6-10)*	2378 (1.1)	> 10000
^{d}PMI-δ*(5-12)*	23.1 (1.4)	29.5 (14.6)
^{d}PMI-δ*(1-5-12) Stitched*	20.5 (1.2)	12.5 (5.1)
^{d}PMI-δ*(1-5-12) stitched scrambled*	> 10000	> 10000
^{d}PMI-δ*(1-5, 9-12) double stapled*	6.9 (1.3)	2.1 (4.1)
ATSP-7041	79.4 (1.3)	4.5 (3.7)

Table S2: *Binding of linear, stapled, ^{d}PMI-δ peptides determined through Isothermal Calorimetry (ITC).*

Peptide	ITC (kcal/mol)		
	ΔH	-TΔS	ΔG
^{d}PMI-δ	-13.80	3.04	-10.8
^{d}PMI-δ*(1-5)*	-15.8	4.2	-11.6
^{d}PMI-δ*(2-6)*	n.d	n.d	n.d
^{d}PMI-δ*(2-9)*	n.d	n.d	n.d
^{d}PMI-δ*(5-9)*	n.d	n.d	n.d
^{d}PMI-δ*E(5-12)*	-8.25	-1.83	-10.1
^{d}PMI-δ*(6-10)*	-9.35	-1.86	-11.2

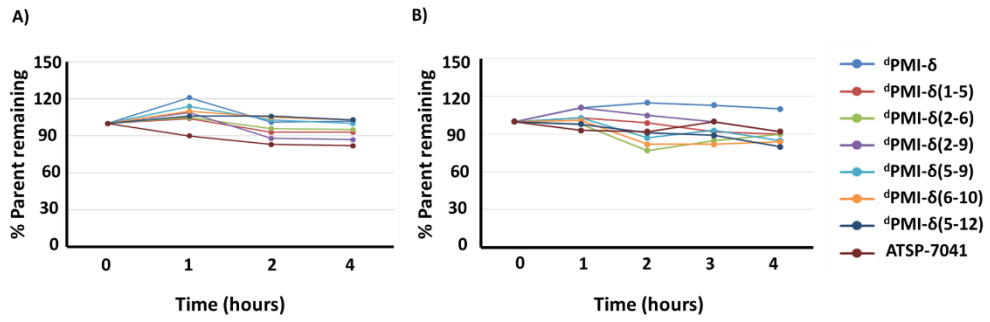

Figure S5: (A) Stability of stapled and linear ^{d}PMI-δ peptides in whole cell homogenate. Parent sequences. (B) Plasma stability of the stapled ^{d}PMI-δ peptides quantified over 4 hrs.

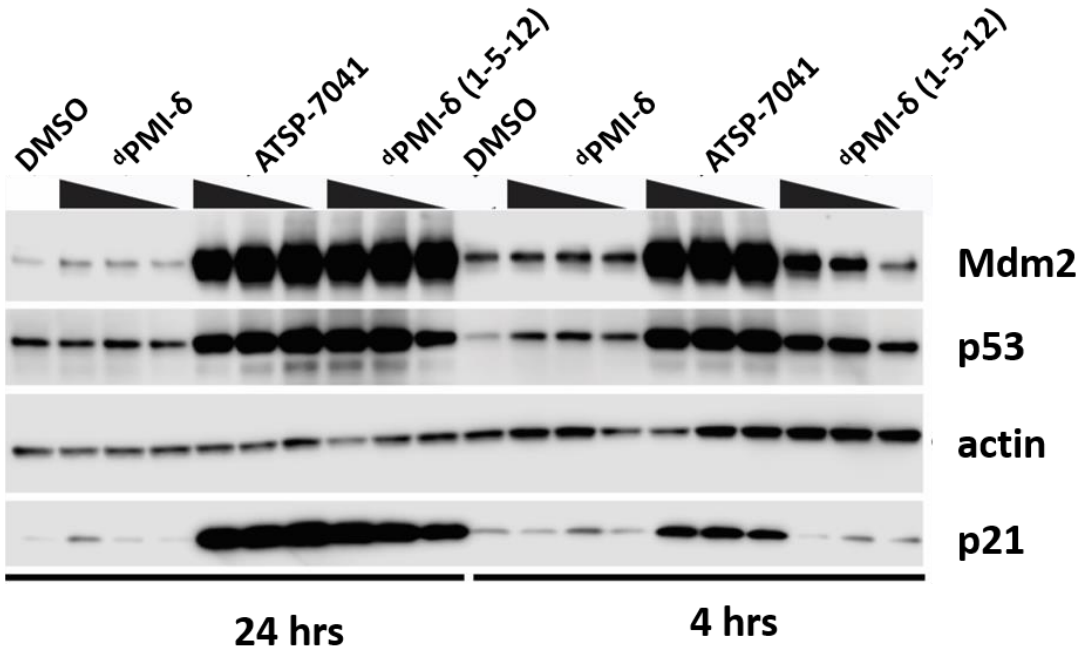

Figure S6: Western blot analysis of HCT-116 cells treated with either vehicle control (1% DMSO) or with 6.123 µM, 12.5 µM and 25 µM of the stated compound for either 4 or 24 hours. Compounds treatments contained a residual DMSO concertation of 1% v/v DMSO**.**

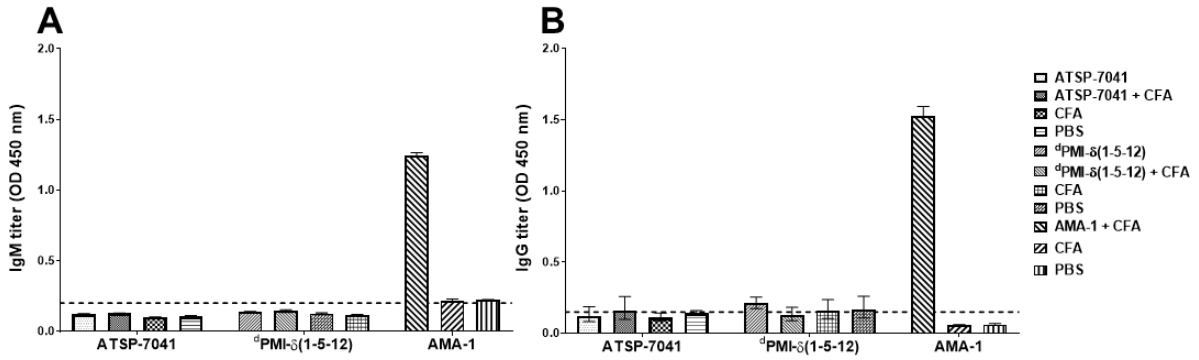

Figure S7: Antibody profiles of peptide-specific antibodies in vaccinated mice. Total peptide-specific IgM (A) and IgG (B) antibody titres in mouse sera at dilution (1:20) were determined by ELISA using ^{d}PMI-δ(1-5-12) or ATSP-7041 peptides or the AMA-1 protein . Non-immunized mouse sera were used as control. Data are presented as geometric mean ± SD.

Materials and Methods

Computational

The available crystal structure of the linear ^{d}PMI (^{d}PMI-δ) peptide co-crystalized with Mdm2 [pdb 3TPX] [1] was used to model the stapled (single and double) and stitched peptides. All models were subjected to molecular dynamics (MD) simulations for further refinement. MD simulations were carried out on free peptide and also on the peptide – Mdm2 complexes. The Xleap module of the Amber18 program [2] was used to prepare the system for the MD simulations. Hydrogen atoms were added and the C- terminus of the peptide was capped with the NHE moiety. The parameters for the staple linkers were taken from our previous study [3]. All simulation systems were neutralized with an appropriate number of counter ions. The neutralized system was solvated in an octahedral box with TIP3P [4] water molecules, leaving at least 10 Å between the solute atoms and the borders of the box. MD simulations were carried out with the pmemd module of the Amber18 package in combination with the ff14SB force field [5]. All MD simulations were carried out in explicit solvent at 300 K. During all the simulations the long-range electrostatic interactions were treated with the particle mesh Ewald [6] method using a real space cut off distance of 9 Å. The settle [7] algorithm was used to constrain bond vibrations involving hydrogen atoms, which allowed a time step of 2 fs during the simulations. Solvent molecules and counter ions were initially relaxed using energy minimization with restraints on the protein and peptide atoms. This was followed by unrestrained energy minimization to remove any steric clashes. Subsequently the system was

gradually heated from 0 to 300 K using MD simulations with positional restraints (force constant: 50 kcal mol^{-1} $Å^{-2}$) on protein and peptides over a period of 0.25 ns allowing water molecules and ions to move freely followed by gradual removal of the positional restraints and a 2 ns unrestrained equilibration at 300 K. The resulting systems were used as starting structures for the respective production phase of the MD simulations. For each case, three independent (using different initial random velocities) MD simulations were carried out starting from the well equilibrated structures. Each MD simulation was carried out for 250 ns and conformations were recorded every 4 ps. To enhance the conformational sampling, each of these peptides were subjected to Biasing Potential Replica Exchange MD (BP-REMD) simulations. The BP-REMD technique is a type of Hamiltonian -REMD methods which includes a biasing potential that promotes dihedral transitions along the replicas [8, 9]. For each system, BP-REMD was carried out with eight replicas including a reference replica without any bias. BP-REMD was carried out for 50 ns with exchange between the neighbouring replicas attempted for every 2 ps and accepted or rejected according to the metropolis criteria. Conformations sampled at the reference replica (no bias) was used for further analysis. Simulation trajectories were visualized using VMD [10] and figures were generated using Pymol [11].

Binding Energy calculations and energy decomposition analysis

Molecular Mechanics Poisson Boltzmann Surface Area (MMPBSA) methods were used for the calculation of binding free energies between the peptides and their partner proteins [12, 13] 250 conformations extracted from the last 50 ns of the simulations were used for the binding energy calculations. Entropy calculations are computationally intensive and do not converge easily and hence are ignored. The effective binding energies were decomposed into contributions of individual residues using the MMGBSA energy decomposition scheme. The MMGBSA calculations were carried out in the same way as in the MMPBSA calculations. The polar contribution to the solvation free energy was determined by applying the generalized born (GB) method (igb =2) [2], using mbondi2 radii. The non-polar contributions were estimated using the ICOSA method [2] by a solvent accessible surface area (SASA) dependent term using a surface tension proportionality constant of 0.0072 kcal/mol $Å^2$. The contribution of peptide residues was additionally explored by carrying out in-silico alanine scanning in which each peptide residue is mutated to D-alanine in each conformation of the MD simulation and the change with respect to the binding energy of the wild type peptide is calculated using MMPBSA and averaged over all the conformations.

Materials

All peptides were sourced from CPC Scientific. The purity and identity of the peptides was confirmed by analytic HPLC and mass spectrometry. All the final peptides have ≥90% purity. All peptides are dissolved in neat DMSO as 10 mM stock solution and diluted thereof for subsequent experiments.

Peptide Synthesis

Peptides were synthesized using Rink Amide MBHA resin and Fmoc-protected amino acids, coupled sequentially with DIC/HOBt activating agents. Double coupling reactions were performed on the first amino acid and also at the stapling positions. At these latter positions, the activating reagents were switched to DIEA/HATU for better coupling efficiencies. Ring closing metathesis reactions were performed by first washing the resin three times with DCM, followed by the addition of the first-generation Grubbs Catalyst (20 mol % in DCM and allowed to react for 2 h; all steps with Grubbs Catalyst were performed in the dark). The RCM (ring closing metathesis) reaction was repeated to ensure a complete reaction. After the RCM was complete, a test cleavage was performed to ensure adequate yield. Peptides were cleaved and then purified as a mixture of cis-trans isomers by RP-HPLC.

Mdm2 Protein Production

For use in the peptide binding assay, a human Mdm2 1–125 sequence was cloned into a pNIC-GST vector. The TEV (tobacco etch virus) cleavage site was changed from ENLYFQS to ENLYFQG to give a fusion protein with the following sequence:

MSDKIIHSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLE
FPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRI
AYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLY
MDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKLEV
LFQGHMHHHHHHSSGVDLGTENLYFQGMCNTNMSVPTDGAVTTSQIPASEQETLVR
PKPLLLKLLKSVGAQKDTYTMKEVLFYLGQYIMTKRLYDEKQQHIVYCSNDLLGDL
FGVPSFSVKEHRKIYTMIYRNLVVVNQQESSDSGTSVSEN-.

The corresponding plasmid was transformed into BL21 (DE3) Rosetta T1R Escherichia coli cells and grown under kanamycin selection. Bottles of 750 mL Terrific Broth, supplemented

with appropriate antibiotics and 100 µL of antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA, were inoculated with 20 mL seed cultures grown overnight. The cultures were incubated at 37 °C in the LEX system (Harbinger Biotech, Toronto, Canada) with aeration and agitation through the bubbling of filtered air through the cultures. The LEX system temperature was reduced to 18 °C when culture OD600 reached 2, and the cultures were induced after 60 min with 0.5 mM IPTG. Protein expression was allowed to continue overnight. Cells were harvested by centrifugation at 4000× g, at 15 °C for 10 min. The supernatants were discarded and the cell pellets were resuspended in a lysis buffer (1.5 mL per gram of cell pellet). The cell suspensions were stored at −80 °C before purification work.

The re-suspended cell pellet suspensions were thawed and sonicated (Sonics Vibra-Cell, Newtown, CO, USA) at 70% amplitude, 3 s on/off for 3 min, on ice. The lysate was clarified by centrifugation at 47,000× g, 4 °C for 25 min. The supernatants were filtered through 1.2 µm syringe filters and loaded onto the AKTA Xpress system (GE Healthcare, Fairfield, CO, USA). The purification regime is briefly described as follows. The lysates were loaded onto a 1 mL Ni-NTA Superflow column (Qiagen, Valencia, CA, USA) that had been equilibrated with 10 column volumes of wash 1 buffer. Overall buffer conditions were as follows: Immobilized metal affinity chromatography (IMAC) wash 1 buffer—20 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 500 mM NaCl, 10 mM Imidazole, 10% (v/v) glycerol, 0.5 mM TCEP (Tris(2-carboxyethyl)phosphine), pH 7.5; IMAC wash 2 buffer—20 mM HEPES, 500 mM NaCl, 25 mM Imidazole, 10% (v/v) glycerol, 0.5 mM TCEP, pH 7.5; IMAC Elution buffer—20 mM HEPES, 500 mM NaCl, 500 mM Imidazole, 10% (v/v) glycerol, 0.5 mM TCEP, pH 7.5. The sample was loaded until air was detected by the air sensor, 0.8 mL/min. The column was then washed with wash 1 buffer for 20 column volumes, followed by 20 column volumes of wash 2 buffer. The protein was eluted with five column volumes of elution buffer. The eluted proteins were collected and stored in sample loops on the system and then injected into gel filtration (GF) columns. Elution peaks were collected in 2 mL fractions and analyzed on SDS-PAGE gels. The entire purification was performed at 4 °C. Relevant peaks were pooled, TCEP was added to a total concentration of 2 mM. The protein sample was concentrated in Vivaspin 20 filter concentrators (VivaScience, Littleton, MA, USA) at 15 °C to approximately 15 mg/mL. (<18 kDa—5 K MWCO, 19–49 kDa—10 K MWCO, >50 kDa—30 K MWCO). The final protein concentration was assessed by measuring absorbance at 280 nm on Nanodrop ND-1000 (Thermo Fisher, Waltham, MA, USA). The final protein purity was

assessed on SDS-PAGE gel. The final protein batch was then aliquoted into smaller fractions, frozen in liquid nitrogen and stored at −80 °C.

For x-ray crystallography, Mdm2 (6–125) was cloned as a GST-fusion protein using the pGEX-6P-1 GST expression vector (GE Healthcare). The GST-fused Mdm2 (6–125) construct was then transformed into Escherichia coli BL21(DE3) pLysS (Thermo Fisher, Waltham, MA, USA) competent cells. Cells were grown in Luria-Bertani (LB) medium at 37 °C and induced at OD600 nm of 0.6 with 0.5 mM Isopropyl β- D -1-thiogalactopyranoside (IPTG) at 16 °C. After overnight induction, the cells were harvested by centrifugation, resuspended in binding buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl), and lysed by sonication. After centrifugation for 60 min at 19,000× g at 4 °C, the cell lysate was then applied to a 5 mL GSTrap FF column (GE Healthcare) pre-equilibrated in wash buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT). The GST-fused Mdm2 (6–125) was then cleaved on-column by PreScission protease (GE Healthcare) overnight at 4 °C and eluted off the column with wash buffer. The protein sample was then dialyzed into a buffer A solution (20 mM Bis-Tris, pH 6.5, 1 mM DTT) using HiPrep 26/10 Desalting column, and loaded onto a cation-exchange Resource S 1 mL column (GE Healthcare), pre-equilibrated in buffer A. The column was then washed in six column volumes of buffer A and the bound protein was eluted with a linear gradient in buffer comprising 1 M NaCl, 20 mM Bis-Tris pH 6.5, and 1 mM DTT over 30 column volumes. Protein purity as assessed by SDS-PAGE was ~95%, and the proteins were concentrated using Amicon-Ultra (3 kDa MWCO) concentrator (Millipore, Burlington, MA, USA). Protein concentration was determined using 280 nm absorbance measurements.

Mdm4 protein production

MDM4 protein was cloned into pNIC-GST vector and expressed in LEX system (Harbinger Biotech) at Protein Production Platform (PPP) at NTU School of Biological Sciences. Using glycerol stocks, inoculation cultures were started in 20mL Terrific Broth with 8g/L glycerol supplemented with Kanamycin. The cultures were incubated at 37 °C, 200rpm overnight. The following morning, bottles of 750mL Terrific Broth with 8g/L glycerol supplemented with Kanamycin and 100uL of antifoam 204 (Sigma-Aldrich) were inoculated with the cultures. The cultures were incubated at 37 °C in the LEX system with aeration and agitation through the bubbling of filtered air through the cultures. When the OD600 reached ~2, the temperature was reduced to 18 °C and the cultures were induced after 30 to 60 min with 0.5mM IPTG. Protein expression was allowed to continue overnight. The following morning, cells were harvested by

centrifugation at 4200 rpm at 15 °C for 10min. The supernatants were discarded and the cells were re-suspended in lysis buffer (100 mM HEPES, 500 mM NaCl, 10 mM Imidazole, 10 % glycerol, 0.5 mM TCEP, pH 8.0 with Benzonase (4uL per 750mL cultivation) and 250U/uL Merck Protease Inhibitor Cocktail Set III, EDTA free (1000x dilution in lysis buffer) from Calbiochem) at 200 rpm, 4 °C for approximately 30min and stored at -80 °C. The re-suspended cell pellet suspensions were thawed and sonicated (Sonics Vibra-cell) at 70 % amplitude, 3s on/off for 3min, on ice. The lysate was clarified by centrifugation at 47000g, 4 °C for 25min. The supernatants were filtered through 1.2 µm syringe filters and loaded onto AKTA Xpress system (GE Healthcare) with a 1mL Ni-NTA Superflow (Qiagen) IMAC column. The column was washed with 20 column volume (CV) of wash buffer 1 (20 mM HEPES, 500 mM NaCl, 10 mM Imidazole, 10 % (v/v) glycerol, 0.5 mM TCEP, pH 7.5) and 20 CV of wash buffer 2 (20 mM HEPES, 500 mM NaCl, 25 mM Imidazole, 10 % (v/v) glycerol, 0.5 mM TCEP, pH 7.5) or until a stable baseline for 3 min and delta base 5mAU (0.8mL/min) was obtained respectively. Mdm4 protein was eluted with elution buffer (20 mM HEPES, 500 mM NaCl, 500 mM Imidazole, 10 % (v/v) glycerol, 0.5 mM TCEP, pH 7.5) and eluted peaks (start collection: >50mAU, slope >200mAU/min, stop collection: <50mAU, stable plateau of 0.5min, delta plateau 5mAU) were collected and stored in sample loops on the system and then injected into equilibrated Gel Filtration (GF) column (HiLoad 16/60 Superdex 200 prep grade (GE Healthcare)) and eluted with 20 mM HEPES, 300 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP, pH 7.5 at a flowrate of 1.2mL/min. Elution peaks (start collection: >20mAU, slope >10mAU/min, stop collection: < 20mAU, slope >10mAU/min, minimum peak width 0.5min) were collected in 2 mL fractions. The entire purification was performed at 4 °C. Relevant peaks were pooled and TCEP was added to a final concentration of 2 mM. The protein sample was concentrated in Vivaspin 20 filter concentrators (VivaScience) at 15 °C to approximately 15mg/mL. The final protein concentration was assessed by measuring absorbance at 280nm on Nanodrop ND-1000 (Nano-Drop Technologies). The final protein purity was assessed by SDS-PAGE and purified MDM4 protein was frozen in liquid nitrogen and stored at -80 °C.

Competitive Fluorescence Anisotropy Assays (Mdm2 and Mdm4)

Purified Mdm2 (1-125) protein was titrated against 50 nM carboxyfluorescein (FAM)-labeled 12/1 peptide13 (FAM-RFMDYWEGL-NH2). Dissociation constants for titrations of Mdm2

and Mdm4 against FAM-labeled 12/1 peptide were determined by fitting the experimental data to a 1:1 binding model equation shown below:

Equation 1:

$$r = r_o + (r_b - r_o) \times \frac{(K_d + [L]_t + [P]_t) - \sqrt{K_d + [L]_t + [P]_t)^2 - 4[L]_t[P]_t}}{2[L]_t}$$

[P] is the protein concentration (Mdm2), [L] is the labeled peptide concentration, r is the anisotropy measured, r_0 is the anisotropy of the free peptide, r_b is the anisotropy of the Mdm2–FAM-labeled peptide complex, K_d is the dissociation constant, $[L]_t$ is the total FAM labeled peptide concentration, and $[P]_t$ is the total Mdm2 concentration. The apparent K_d values for FAM-labeled 12/1 peptide against Mdm2 and Mdm4 were determined to be 13.0 nM and 4.0 nM, respectively. These values were then used to determine apparent K_d values of the respective competing ligands in subsequent competition assays in fluorescence anisotropy experiments.

Mdm2 and Mdm4 competition experiments were performed with their respective concentrations held constant at 250 nM and 75 nM, in the presence of 50 nM of FAM-labeled 12/1. The competing molecules were then titrated against the complex of the FAM-labeled peptide and protein. Apparent Kd values were determined by fitting the experimental data to the equations shown below:

$$r = r_o + (r_b + r_o) \times \frac{2\sqrt{(d^2 - 3e)}\cos(\theta/3) - 9}{3K_{d1} + 2\sqrt{(d^2 - 3e)}\cos(\theta/3) - d}$$

$$d = K_{d1} + K_{d2} + [L]_{st} + [L]_t - [P]_t$$

$$e = ([L]_t - [P]_t)K_{d1} + ([L]_{st} - [P]_t)K_{d2} + K_{d1}K_{d2}$$

$$f = -K_{d1}K_{d2}[P]_t$$

$$\theta = ar\cos\left[\frac{-2d^3 + 9de - 27f}{2\sqrt{(d^2 - 3e)^3}}\right]$$

$[L]_{st}$ and $[L]_t$ denote labeled ligand and total unlabeled ligand input concentrations, respectively. K_{d2} is the dissociation constant of the interaction between the unlabeled ligand and the protein. In all competition experiments, it is assumed that $[P]t > [L]_{st}$, otherwise considerable amounts of free labeled ligand would always be present and would interfere with measurements. K_{d1} is the apparent K_d for the labeled peptide used and has been experimentally determined as described in the previous paragraph. The FAM-labeled peptide was dissolved in dimethyl sulfoxide (DMSO) at 1 mM and diluted into experimental buffer. Readings were carried out with an Envision Multilabel Reader (PerkinElmer). Experiments were carried out in PBS (2.7 mM KCl, 137mM NaCl, 10 mM Na2HPO4 and 2 mM KH2PO4 (pH 7.4)) and 0.1% Tween-20 buffer. All titrations were carried out in triplicate. Curve-fitting was carried out using Prism 4.0 (GraphPad). To validate the fitting of a 1:1 binding model we carefully ensured that the anisotropy value at the beginning of the direct titrations between Mdm2 and the FAM-labeled peptide did not differ significantly from the anisotropy value observed for the free fluorescently labeled peptide. Negative control titrations of the ligands under investigation were also carried out with the fluorescently labeled peptide (in the absence of Mdm2) to ensure no interactions were occurring between the ligands and the FAM-labeled peptide. In addition, we ensured that the final baseline in the competitive titrations did not fall below the anisotropy value for the free FAM-labeled peptide, which would otherwise indicate an unintended interaction between the ligand and the FAM-labeled peptide to be displaced from the Mdm2 binding site. Measurements were taken a minimum of three times (biological replicates) and values are reported as geometric means of the replicates.

Whole Cell Homogenate Stability

Peptides at a concentration of 1 μM were incubated at 37 °C with HCT116 whole cell homogenates prepared from 1 million lyzed cells/mL. The reaction was stopped at 0, 1, 2, and 4 hours and 22 hours with an organic solvent followed by centrifugation. The resulting supernatant was injected to LC/MS for the detection of tested peptide. The remaining percentage of each compound was normalized to the 0 hour amount and reported.

Plasma Stability

Peptide was incubated with human plasma at the concentration of 1 mM at 37 C for 1, 2, 3, and 4 hours. The incubation was stopped at indicated time points by addition of organic solvent

followed by centrifugation. Parent compound in supernatant was analyzed by LC/MS. Percent remaining of peptide was calculated against the amount of compound at time 0.

p53 Beta-Lactamase Reporter Gene Cellular Functional Assay

HCT116 cells were stably transfected with a p53 responsive β-lactamase reporter and expanded in McCoy's 5A Medium with 10% fetal bovine serum (FBS), Blasticidin, and Penicillin/Streptomycin and then transferred to 1.5 ml freezing vials and stored under liquid nitrogen in growth media containing 5% DMSO. One day prior the assay, a vial of banked cells was recovered in a cell culture flask and incubated for 24 hours, followed by removal of cell growth media and replacement with Opti-MEM containing 2% FBS. The cells were then seeded into a 384-well plate at a density of 8000 cells per well. Peptides were then dispensed to each well using a liquid handler, ECHO 555, and incubated 16 h. The final working concentration of DMSO was 0.5%. β-lactamase activity was detected using the ToxBLAzer Dual Screen (Invitrogen), as per the manufacturer's instructions. Measurements were made using the Envision multiplate reader (Perkin–Elmer). Maximum p53 activity was defined as the amount of β-lactamase activity induced by 50 μM azide-ATSP-7041. This was determined as the highest amount of p53 activity induced by azide-ATSP-7041 from titrations on HCT116 cells. Measurements were taken a minimum of three times (biological replicates) and values are reported as geometric means of the replicates.

Lactate Dehydrogenase (LDH) Release Assay

HCT116 cells were stably transfected with a p53 responsive β-lactamase reporter and expanded in McCoy's 5A Medium with 10% fetal bovine serum (FBS), Blasticidin, and Penicillin/Streptomycin and then transferred to 1.5 ml freezing vials and stored under liquid nitrogen in growth media containing 5% DMSO. One day prior the assay, a vial of banked cells was recovered in a cell culture flask and incubated for 24 hours, followed by removal of cell growth media and replacement with Opti-MEM containing 2% FBS. The cells were then seeded into a 384-well plate at a density of 8000 cells per well. Peptides were then dispensed to each well using a liquid handler, ECHO 555, and incubated 16 h. The final working concentration of DMSO was 0.5%. Lactate dehydrogenase release was detected using the CytoTox-ONE Homogenous Membrane Integrity Assay Kit (Promega), as per the manufacturer's instructions. Measurements were carried out using the Tecan plate reader. Maximum LDH release was defined as the amount of LDH released as induced by the lytic

peptide (iDNA79) and used to normalize the results. Measurements were taken a minimum of three times (biological replicates) and values are reported as geometric means of the replicates.

Tetracycline Beta-Lactamase Reporter Gene Cellular Assay (Counterscreen)

This assay was based on Jump-In™ T-REx™ CHO-K1 BLA cells containing a stably integrated β-lactamase under the control of an inducible cytomegalovirus (CMV) promoter. Cells were maintained in Dulbecco's Minimal Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), Blasticidin, and Penicillin/Streptomycin and then transferred to 1.5 ml freezing vials and stored under liquid nitrogen in growth media containing 5% DMSO. One day prior the assay, a vial of banked cells was recovered in a cell culture flask and incubated for 24 hours, followed by removal of cell growth media and replacement with Opti-MEM containing 2% FBS. Cells were seeded into a 384-well plate at a density of 4000 cells per well. Peptides were then dispensed to each well using a liquid handler, ECHO 555 and incubated for 16 h. The final working concentration of DMSO was 0.5%. β-lactamase activity was detected using the ToxBLAzer Dual Screen (Invitrogen), as per the manufacturer's instructions. Measurements were carried out using the Envision multiplate reader (Perkin–Elmer). Counterscreen activity was defined as the amount of β-lactamase activity induced by tetracycline. Measurements were taken a minimum of three times (biological replicates) and values are reported as geometric means of the replicates.

Yeast 2-hybrid (Y2H) assay

The Y2H assay was performed as previously described [14]. Briefly, yeast cultures that were grown overnight to saturation in SD-Leu-Trp medium were washed and diluted at OD_{600} =0.2 in 200 µL SD-Leu-Trp-His-Ade medium containing the desired concentration of the peptide in duplicatein a 96- well microplate. The cultures were then placed in a 30 °C incubator with shaking for 3 days. Growth of the cultures was measured by recording the OD_{600} at different time points using a Gen 5^{TM}(BIO-TEK Instrument, Vermont, USA) microplate reader. Average of the duplicate OD_{600} readings was calculated and used in the analysis.

Isothermal Titration Calorimetry (ITC)

Overnight dialysis of protein and peptides were carried out in buffer containing 1× phosphate-buffered saline (PBS) pH 7.2, 3% DMSO, and 0.001% Tween-20. Approximately 100–200 µM of peptide was titrated into 20 µM of purified recombinant human Mdm2 protein (amino acids 1–125), over 40 injections of 1 µL each. Reverse ITC (200 µM of Mdm2 protein titrated into

20 µM of peptide) was carried out for peptides that are insoluble at high concentrations. All experiments were performed in duplicates using the MicroCal PEAQ-ITC Automated system. Data analysis was carried out using the MicroCal PEAQ-ITC Analysis Software.

Circular Dichroism (CD)

A total of 5 µL of the 10 mM stock peptide was mixed with 45 µL of 100% methanol, and dried for 2 h in the SpeedVac concentrator (Thermo Scientific). The dried peptide was reconstituted in a buffer (1 mM Hepes pH 7.4 and 5% methanol) to a concentration of 1 mM. The peptide sample was placed in a quartz cuvette with a path length of 0.2 cm. The peptide concentration was determined by the absorbance of the peptide at 280 nM. The CD spectrum was recorded from 300 to 190 nm using the Chirascan-plus qCD machine (Applied Photophysics, Surrey, UK), at 25 °C. All experiments were done in duplicates. The CD spectrum was converted to mean residue ellipticity, before deconvolution and estimation of the secondary structure components of the peptide using the CDNN software (distributed by Applied Photophysics). Measurements were taken at least twice and were reported as arithmetic means.

Surface Plasmon Resonance (SPR)

SPR experiments were performed with Biacore T100 (GE Healthcare) at 25 °C. The site-specific mono-biotinylated Mdm2 was prepared by sortase-mediated ligation. The SPR buffer consisted of 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM DTT, 0.05% Tween-20, and 3% DMSO. The CM5 chip was first conditioned with 100 mM HCl, followed by 0.1% SDS, 50 mM NaOH, and then water, all performed twice with 6 sec injection at a flow rate of 100 µL/min. With the flow rate set to 10 µL/min, streptavidin (S4762, Sigma-Aldrich) was immobilized on the conditioned chip through amine coupling, as described in the Biacore manual. Excess protein was removed by 30 s injection of the wash solution (50 mM NaOH + 1 M NaCl) at least eight times. The immobilized level was ~3000 RU. The biotinylated Mdm2 was captured by streptavidin, up to a level of ~400 RU. A flow cell consisting of only streptavidin was used as the reference surface. Using a flow rate of 30 µL/min, peptides dissolved in the SPR buffer were injected for 180 s. The dissociation was monitored for 300 s. For each peptide concentration, the peptide injection was followed by a similar injection of SPR buffer to allow the surface to be fully regenerated (though not completely for peptides with an extremely slow off-rate). After the run, responses from the target protein surface were transformed by: (i) correcting with the DMSO calibration curve, (ii) subtracting the responses obtained from the

reference surface, and (iii) subtracting the responses of the buffer injections from those of peptide injections. The last step is known as double referencing, which corrects the systematic artefacts. The resulting responses were subjected to kinetic analysis by global fitting with a 1:1 binding model to obtain the *KD*, k_a (M-1 s-1), and k_d (s-1). Binding responses that did not have enough curvature during the association and dissociation were subjected to steady-state analysis, and the *KD* was obtained by fitting a plot of response at equilibrium against the concentration.

HCT-116 Western blot analysis

Preparation of compound Stock and working Solutions: 10 mM or 1 mM stock solutions of compounds were prepared in 100% DMSO. Each compound was then serially diluted in 100% DMSO and further diluted 10-fold into HPLC grade sterile water to prepare 10X working solutions in 10% DMSO/water of each compound. Depending on the required volume used in the relevant assay, compounds were added to yield final concentrations as indicated in the relevant figure with a residual DMSO concentration of 1% v/v.

HCT116 cells (Thermo Fisher Scientific) were cultured in DMEM cell media, which was supplemented with 10% foetal calf serum (FBS) and penicillin/streptomycin. All cell lines were maintained in a 37 °C humidified incubator with 5% CO_2 atmosphere. HCT116 cells were seeded into 96 well plates at a cell density of 60,000 cells per well and incubated overnight. Cells were also maintained in DMEM cell media with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cell media was then removed and replaced with cell media containing the various compounds/vehicle controls at the concentrations indicated in DMEM cell media with 2% FCS. After the stated incubation time (4 or 24 hours) cells were rinsed with PBS and then harvested in 100 µl of 1x NuPAGE LDS sample buffer supplied by Invitrogen (NP0008). Samples were then sonicated, heated to 90 °C for 5 mins, sonicated twice for 10 s and centrifuged at 13, 000 rpm for 5 minutes. Protein concentrations were measured by BCA assay (Pierce). Samples were resolved on Tris-Glycine 4-20% gradient gels (BIORAD) according to the manufacturer's protocol. Western transfer was performed with an Immuno-blot PVDF membrane (Bio-Rad) using a Trans-Blot Turbo system (BIORAD). Western blot staining was then performed using antibodies against actin (AC-15, Sigma) as a loading control, p21 (118 mouse monoclonal), Mdm2 (2A9 mouse monoclonal) and p53 (DO-1 mouse monoclonal).

Crystallization and data Collection

Mdm2(6-125) was concentrated to 4.6 mg/mL and then incubated with thedouble stapled peptide at a 1:3 molar ratio of protein to peptide at 4°C overnight. The lyophilized double stapled peptide Mdm2-^{d}PMI-δ(1-5, 9-12) was first dissolved in DMSO to make a 100 mM stock solution before direct addition to the protein solution. The sample was clarified by centrifugation before crystallization trials at 16°C using the sitting drop vapour diffusion method. Crystals of Mdm2(6-125) in complex with ^{d}PMI-δ(1-5, 9-12) were obtained by mixing the protein-peptide complex with the reservoir solution in a ratio of 1:1, with the reservoir solution containing 800 mM Sodium di-hydrogen phosphate, 800 mM di-Potassium hydrogen phosphate, 100 mM HEPES pH 7.5. Mdm2-^{d}PMI-δ(1-5, 9-12) complex crystals were frozen in an equivalent mother liquor solution containing 15% (v/v) glycerol and then flash frozen in liquid nitrogen. X-ray diffraction was collected at the Australian synchrotron (Aus.). See Table 3 for data collection statistics.

Table 3: Data collection statistics and refinement Parameters

	6KZU
Resolution (Å)	43.90 – 1.79 (1.82 – 1.79)
Space Group	C 12_12
Unit Cell Dimensions (Å)	a= 80.95, b= 43.38 Å, c= 34.54 Å (α= 90° β=94.82°, γ=90°)
Temp (K)	100
Redundancy	20.7
Unique Collected Reflections	10913(599)
Completeness (%)	100 (99.4)
R Pim (%)	1.4 (39.0)
I/sigma	25.6 (1.6)
R factor (%)	19.90
R free (%)	23.61
RMS Bonds (Å)	0.00091
RMS Angles (°)	1.562
Wilson B-factor (Å^2)	40.39
Average Refined B Factors	
Chain A (Mdm2)	33.99
Chain B (Peptide)	29.11
Waters	43.30
Number of Water Molecules	61
Ramachandran Data (Rampage). Number of Residues in(%) :	

Favoured Region	98.8
Allowed Region	1.2
Outlier Region	0.0

Structure Solution and Refinement

X-ray datasets were processed and scaled with the XDS[15] and CCP4 [16] packages. The structures were solved by molecular replacement with the program PHASER [17] using the human Mdm2 (6-125) structure from the PDB:4UMN (chain A) as a search model. The starting model was built and refined by iterative cycles of manual and automatic building with Coot [18] and restrained refinement with Refmac [19]. The d-amino acid peptide was manually buit into the electron density using COOT [18]. The geometric restraints for the non-natural amino acids constituting the hydrocarbon staples and the covalent bond linking their respective side chains together, to form the macrocyclic linkages constraining the stapled peptide, were defined and generated using JLigand [20]. The final model was validated using RAMPAGE [21] and the MOLPROBITY [22] webserver. Structural overlays and analysis was performed using PYMOL[11]. See table 3 for data collection and refinement statistics. The Mdm2-^{d}PMI-δ(1-5, 9-12) complex structure has been deposited in the PDB with following accession code 6KZU.

Immunogenicity assay

Mice

6 weeks old female ICR mice purchased from Invivos (Singapore) were used in this study under specific pathogen-free environment in the A*STAR Biological Resource Centre (BRC), Singapore. The experiments and procedures were performed under the approval of Institutional Animal Care and Use Committee (IACUC), IACUC #181314, in accordance to the Animal & Veterinary Service (AVS) and National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore.

Immunization

ICR mice were immunized with the peptides (^{d}PMI-δ(1-5-12) or ATSP-7041) or with an *E.coli* recombinant form of the AMA-1 protein of the malaria parasite *Plasmodium yoelii* (used as a positive control and kindly provided Dr Julien Lescar, NTU, Singapore) (n=3 per group) as follows: one dose of 50 µg peptide or the protein in 100 µL of phosphate-buffered saline (PBS) with or without 50% Freund's Adjuvant, Complete (CFA) (Sigma-Aldrich) followed by two

subsequent booster doses of 50 µg peptide or protein in 100 µL PBS with or without Freund's Adjuvant, Incomplete (IFA) (Sigma-Aldrich) at day 14 and 21. Control mice were immunized with PBS with or without 50% CFA and PBS with or without IFA for the first dose and subsequent booster doses respectively. Mouse sera were collected at day 28 for peptide-based enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay (ELISA)

Briefly, polystyrene 96-well flat-bottom plates (MaxiSorp, Nunc) were coated with peptide (15µM in Carbonate-Bicarbonate Buffer; 100 µL per well) or protein (3µg in Carbonate-Bicarbonate Buffer; 100 µL per well) by overnight incubation at 4°C. Wells were blocked with PBS containing 0.05% Tween-20 and 3% non-fat milk (PBST-milk) and incubated for 1 hours at 37°C. Mouse sera were then diluted in PBST-milk and incubated for 2 hour at 37°C. Horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (Invitrogen) were used to detect mouse antibodies bound to peptide-coated wells. Reactions were developed using 3,3',5,5'-tetramethylbenzidine substrate (BioFX™) and terminated by 1M HCl. Absorbance was measured at 450 nm.

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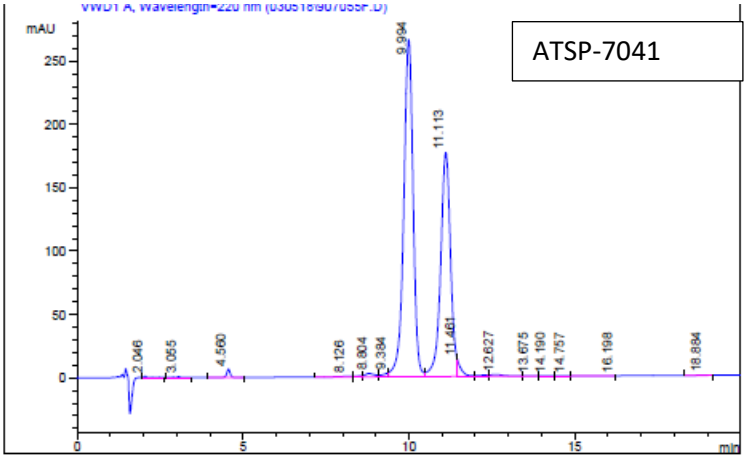

ATSP-7041

Signal 1:VWD1 A, Wavelength=220 nm

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1	2.046	MM	1.095	0.128	8.422	0.091
2	3.055	MM	0.836	0.161	8.072	0.087
3	4.560	VV	6.475	0.122	48.343	0.522
4	8.126	VV	0.587	0.353	14.796	0.160
5	8.588	VV	0.916	0.185	10.154	0.110
6	8.804	VV	2.681	0.304	56.365	0.609
7	9.384	VV	3.392	0.176	35.871	0.387
8	9.994	VV	265.678	0.299	5225.314	56.439
9	11.113	VF	176.585	0.318	3665.268	39.589
10	11.461	VV	13.495	0.131	106.104	1.146
11	12.393	VV	0.965	0.350	20.240	0.219
12	12.627	VV	1.325	0.460	42.362	0.458
13	13.675	VV	0.192	0.388	5.724	0.062
14	14.190	VV	0.139	0.376	3.709	0.040
15	14.757	VV	0.060	0.386	1.797	0.019
16	16.198	VV	0.017	2.655	2.654	0.029
17	18.884	VV	0.142	0.316	3.068	0.033

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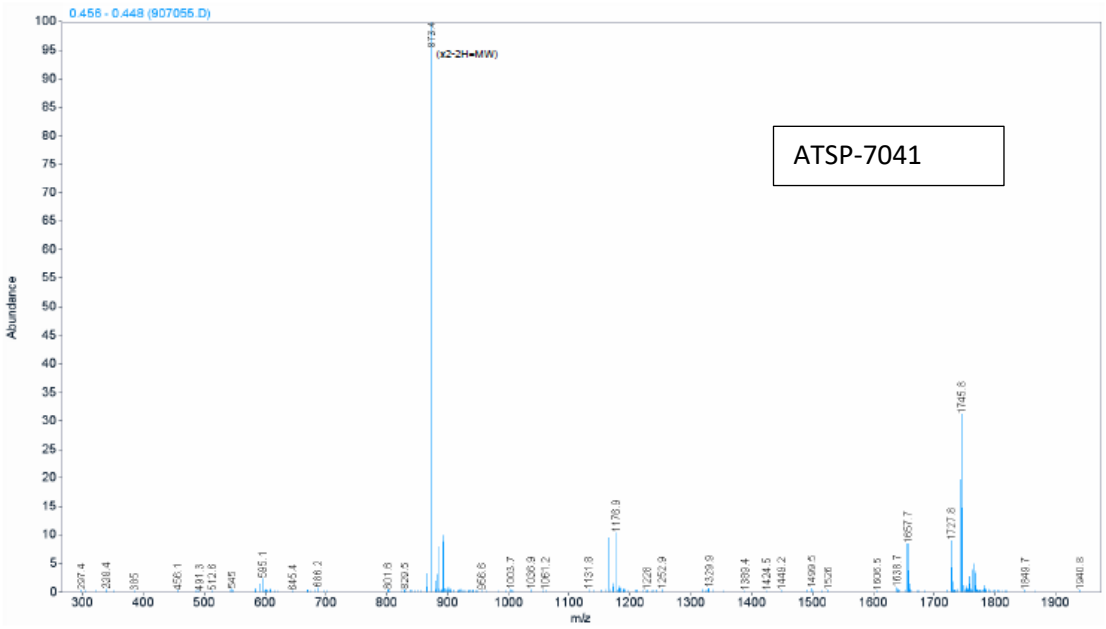
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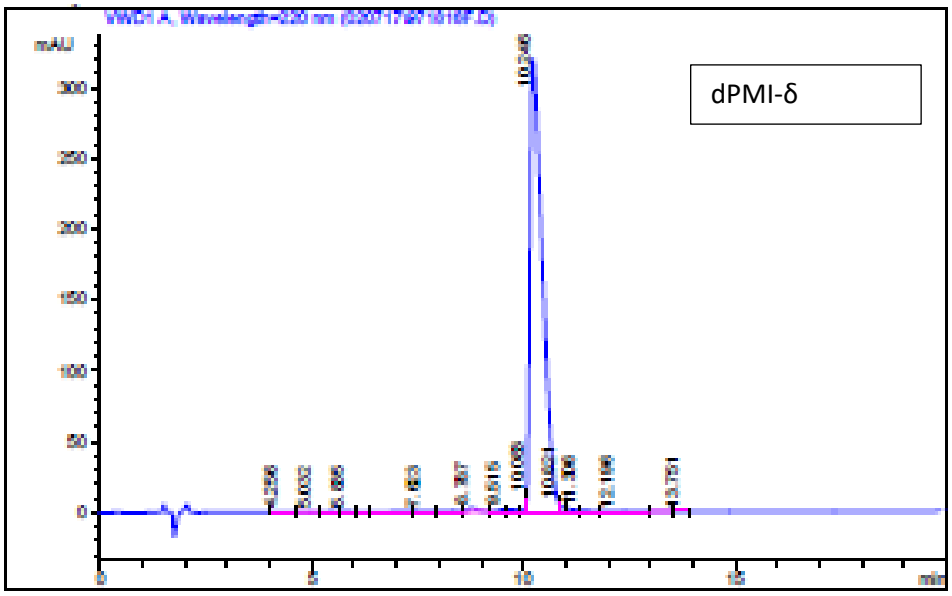
ATSP-7041
Abundance
m/z
(x2-2H+MW)



Signal 1:VWD1 A, Wavelength=220 nm

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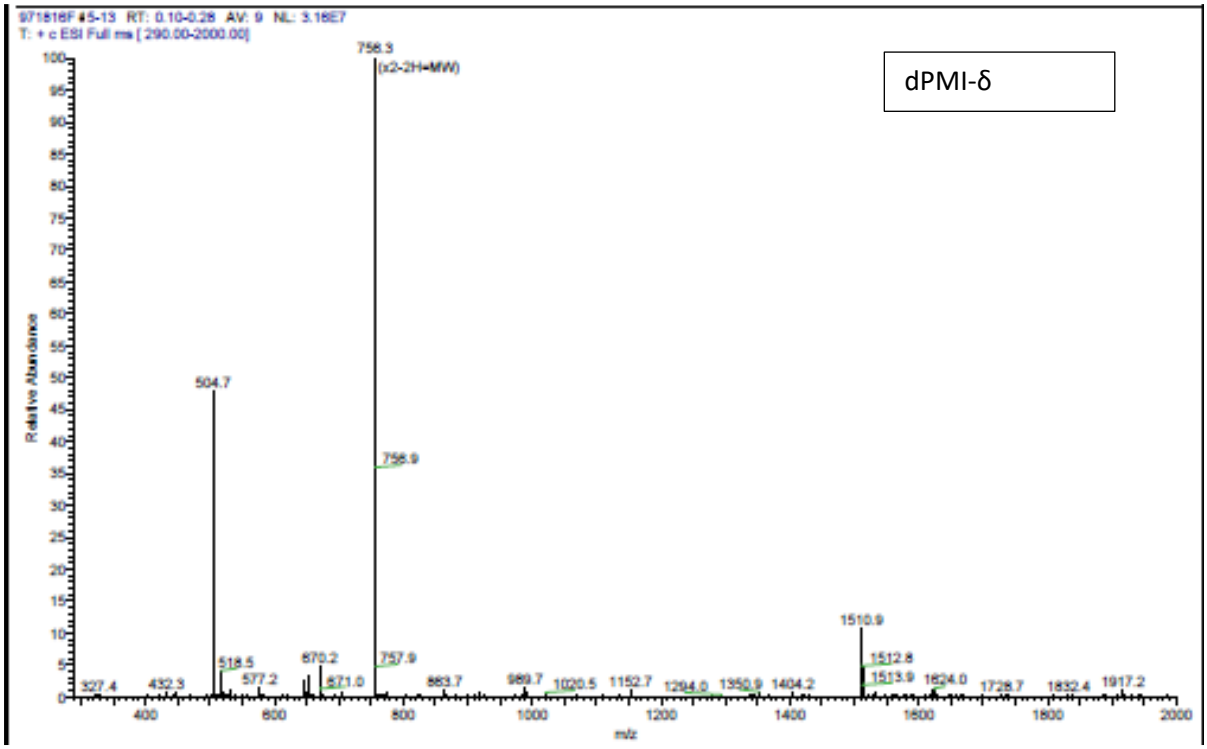
2	5.032	VV	0.098	0.377	2.714	0.040
3	5.462	VV	0.264	0.190	3.510	0.052
4	5.835	VV	0.452	0.169	5.211	0.077
5	6.132	VV	0.105	0.236	1.648	0.024
6	7.160	VV	0.232	0.370	6.554	0.096
7	7.623	VV	0.271	0.317	6.284	0.092
8	8.340	VV	0.593	0.346	14.051	0.206
9	8.787	VV	2.855	0.244	46.859	0.688
10	9.515	VV	1.396	0.202	19.292	0.283
11	9.745	VV	1.633	0.200	22.235	0.327
12	10.083	VV	10.614	0.034	21.913	0.322
13	10.246	VF	319.626	0.309	6549.090	96.192
14	10.821	VF	7.461	0.096	43.037	0.632
15	11.015	VF	1.987	0.213	25.399	0.373
16	11.306	VV	0.894	0.314	16.864	0.248
17	12.196	VV	0.398	0.590	18.344	0.269
18	13.286	VV	0.063	0.378	1.753	0.026
19	13.751	VV	0.076	0.315	1.062	0.016

*** End of Report ***

Page 1 of 1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(x2-2H+MW)
504.7
756.9
757.9
1510.9
1512.8
1513.9
518.5
577.2
670.2
671.0
883.7
969.7
1020.5
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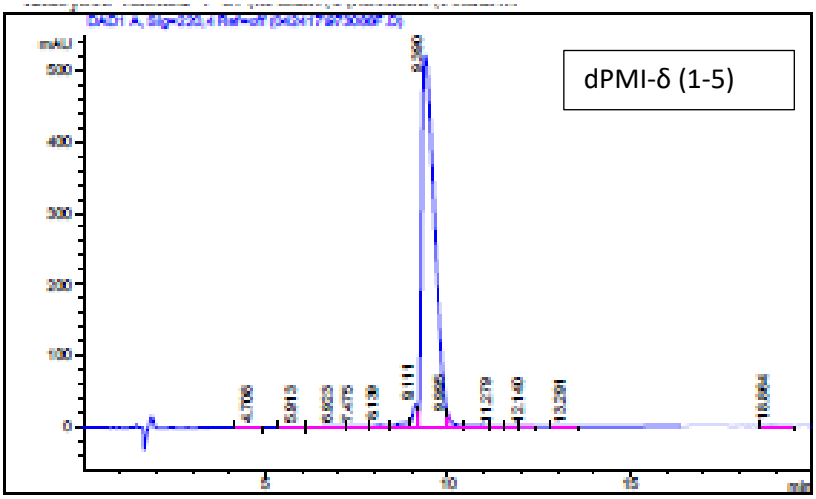

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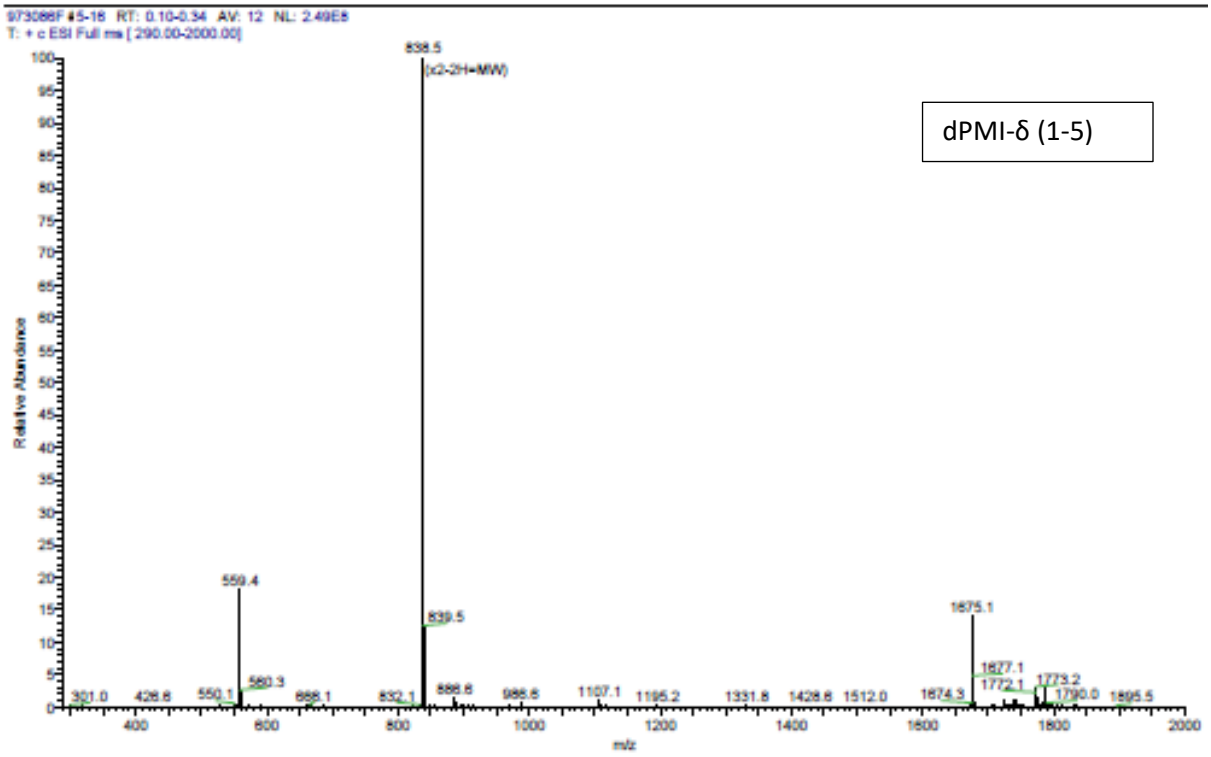
2	5.913	VV	0.342	0.315	8.503	0.065
3	6.923	VV	1.273	0.434	42.459	0.324
4	7.475	VV	1.027	0.396	33.122	0.253
5	8.139	VV	2.506	0.305	51.809	0.395
6	8.873	VV	6.053	0.265	124.024	0.946
7	9.111	VV	31.210	0.158	337.394	2.574
8	9.390	VP	516.885	0.366	12224.842	93.246
9	9.955	VP	17.008	0.124	126.397	0.964
10	10.422	VV	1.433	0.611	52.587	0.401
11	11.279	VV	0.898	0.230	14.274	0.109
12	11.718	VV	0.463	0.252	8.240	0.063
13	12.140	VV	0.462	0.245	7.404	0.056
14	13.291	VV	0.631	0.401	20.312	0.155
15	16.864	VP	2.564	0.278	49.812	0.380

*** End of Report ***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dPMI-δ (1-5)
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839.5
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1827.1
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1773.2
1790.0
1895.5
321.0
426.6
550.1
580.3
668.1
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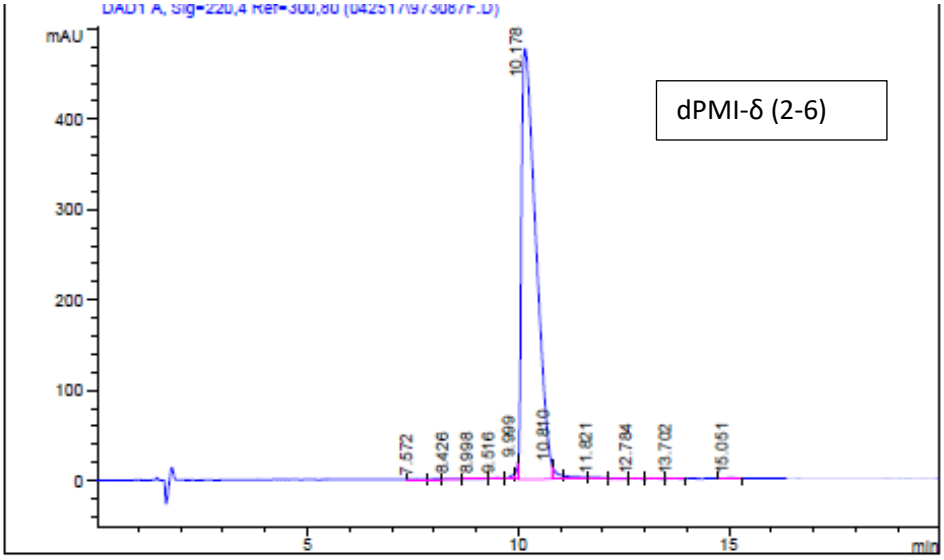
Relative Abundance
m/z



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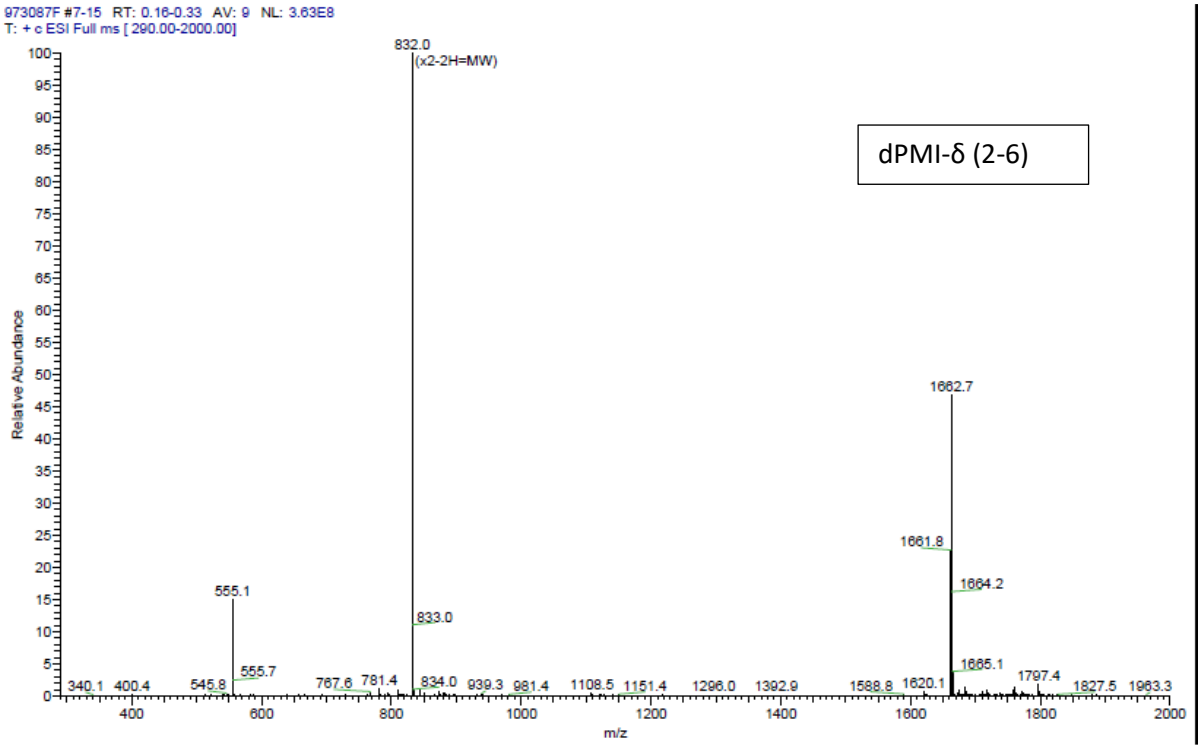
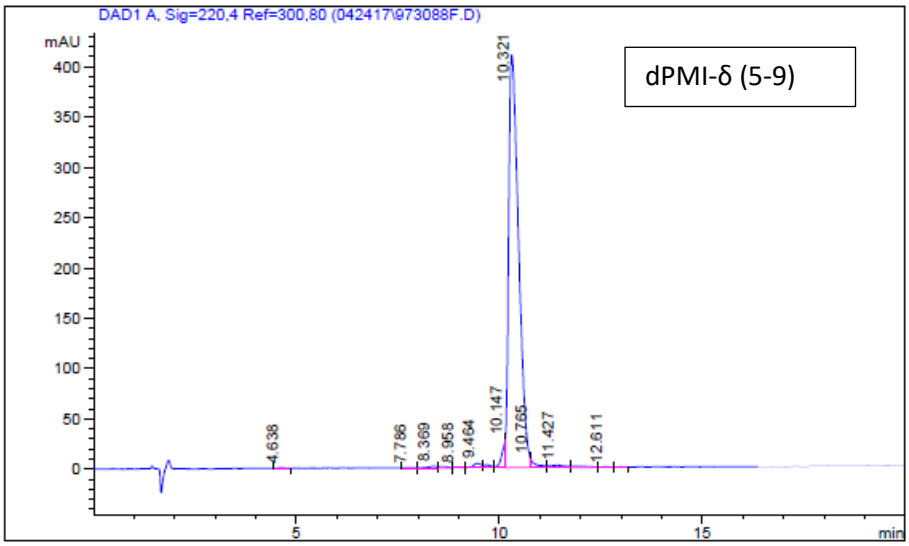
1	7.572	VV	0.410	0.278	8.178	0.070
2	8.023	VV	0.421	0.241	7.064	0.060
3	8.426	VV	0.621	0.261	12.177	0.104
4	8.998	VV	0.698	0.396	22.165	0.189
5	9.516	VV	1.453	0.243	24.631	0.210
6	9.914	VV	5.701	0.120	40.994	0.350
7	9.999	VV	19.012	0.045	51.529	0.440
8	10.178	VF	476.458	0.359	11270.159	96.137
9	10.810	VF	15.215	0.116	105.643	0.901
10	11.066	VV	3.715	0.371	82.613	0.705
11	11.821	VF	1.792	0.364	46.059	0.393
12	12.154	VV	0.786	0.357	16.830	0.144
13	12.784	VV	0.516	0.260	8.967	0.076
14	13.224	VV	0.445	0.265	8.152	0.070
15	13.702	VV	0.376	0.228	6.325	0.054
16	15.051	VV	0.721	0.256	11.493	0.098

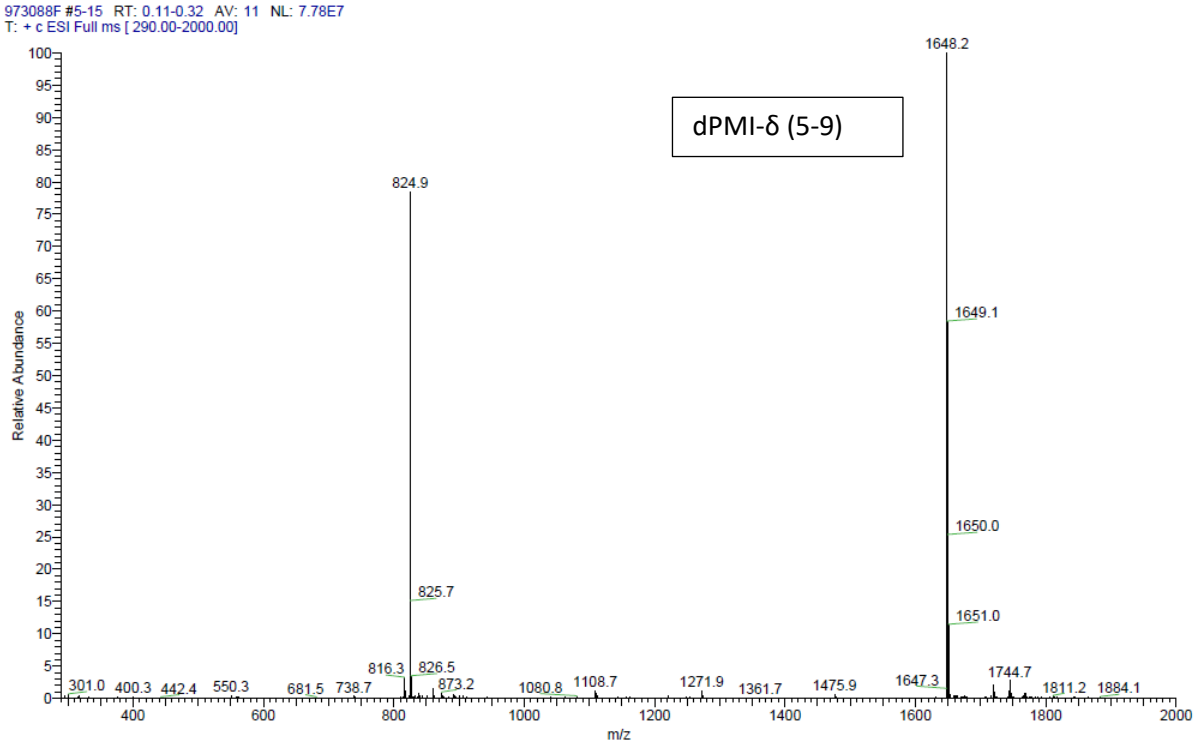
*** End of Report ***

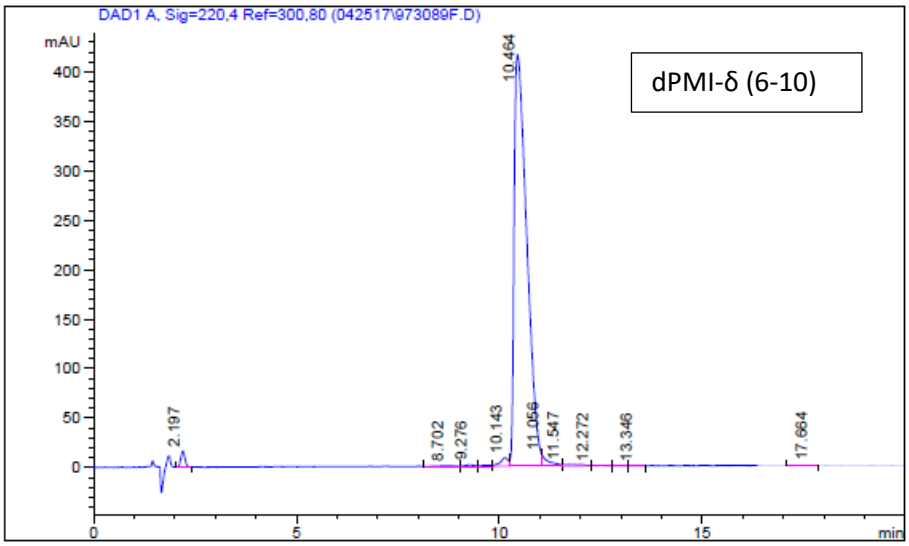

973087F #7-15 RT: 0.16-0.33 AV: 9 NL: 3.63E8
T: + c ESI Full ms [290.00-2000.00]
832.0
(x2-2H=MW)
dPMI-δ (2-6)
1662.7
1661.8
1664.2
555.1
833.0
1665.1
1797.4
555.7
340.1 400.4 545.8 767.6 781.4 834.0 939.3 981.4 1108.5 1151.4 1296.0 1392.9 1588.8 1620.1 1827.5 1963.3
Relative Abundance
m/z



Signal 1:DAD1 A, Sig=220,4 Ref=300,80

Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	4.638	VV	0.487	0.222	6.860	0.092
2	7.786	VV	0.317	0.219	4.909	0.066
3	8.369	VV	1.649	0.226	25.605	0.344
4	8.615	VV	1.286	0.199	17.650	0.237
5	8.958	VV	0.506	0.220	8.154	0.110
6	9.464	VF	4.358	0.207	58.375	0.784
7	9.592	VV	2.487	0.246	36.776	0.494
8	10.147	VV	28.521	0.085	145.652	1.957
9	10.321	VF	409.174	0.269	6959.339	93.500
10	10.765	VV	9.627	0.133	76.930	1.034
11	11.427	VF	2.391	0.330	57.232	0.769
12	11.768	VV	1.070	0.501	32.152	0.432
13	12.611	VV	0.463	0.238	8.160	0.110
14	13.010	VV	0.359	0.207	5.389	0.072

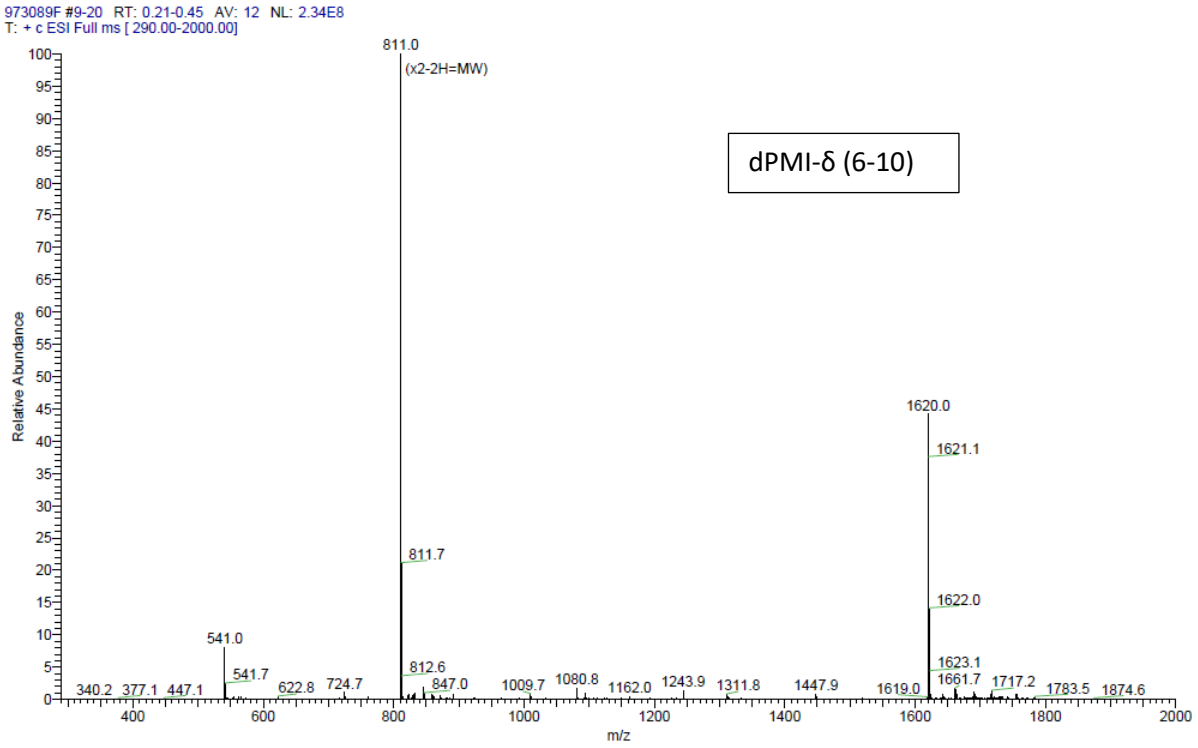

973088F #5-15 RT: 0.11-0.32 AV: 11 NL: 7.78E7
T: + c ESI Full ms [290.00-2000.00]
dPMI-δ (5-9)
Relative Abundance
m/z
1648.2
824.9
1649.1
1650.0
825.7
1651.0
816.3
826.5
873.2
1744.7
301.0
400.3
442.4
550.3
681.5
738.7
1080.8
1108.7
1271.9
1361.7
1475.9
1647.3
1811.2
1884.1

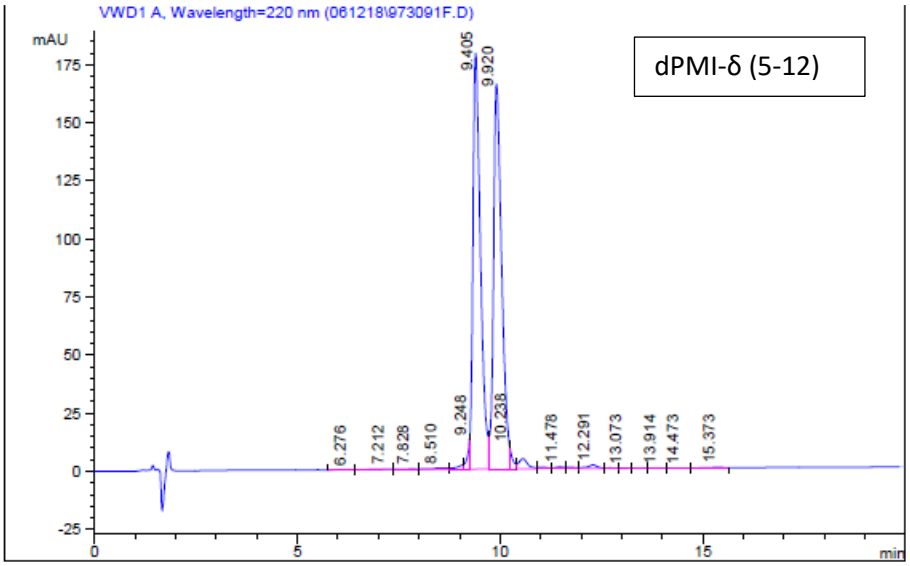


Signal 1:DAD1 A, Sig=220,4 Ref=300,80

Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	2.197	MM	16.381	0.115	112.946	1.188
2	8.702	VV	0.846	0.354	22.440	0.236
3	9.276	VV	1.525	0.243	24.168	0.254
4	9.696	VV	0.900	0.274	18.190	0.191
5	10.143	VV	8.686	0.194	115.938	1.219
6	10.464	VF	415.540	0.336	8995.650	94.613
7	11.056	VF	11.398	0.182	124.756	1.312
8	11.547	VF	1.674	0.556	55.888	0.588
9	12.272	VV	0.630	0.352	13.287	0.140
10	12.943	VV	0.374	0.242	6.534	0.069
11	13.346	VV	0.443	0.167	4.896	0.051
12	17.664	MM	0.543	0.403	13.129	0.138

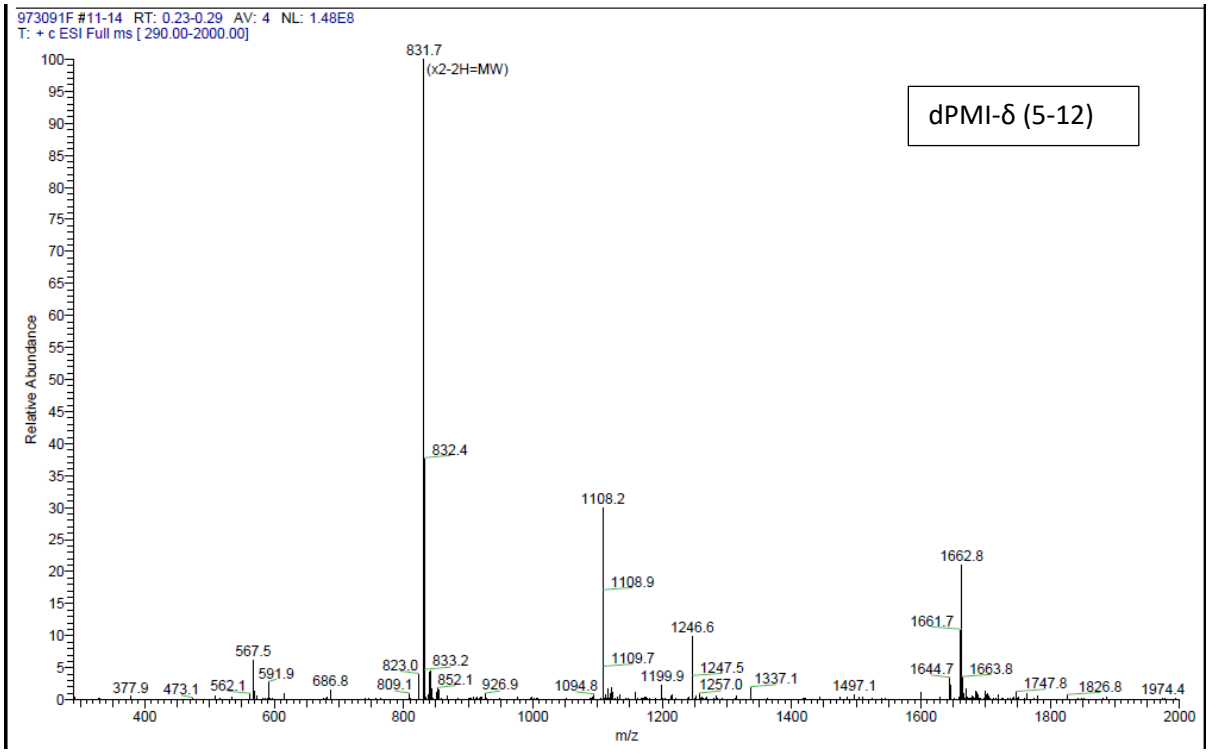
*** End of Report ***

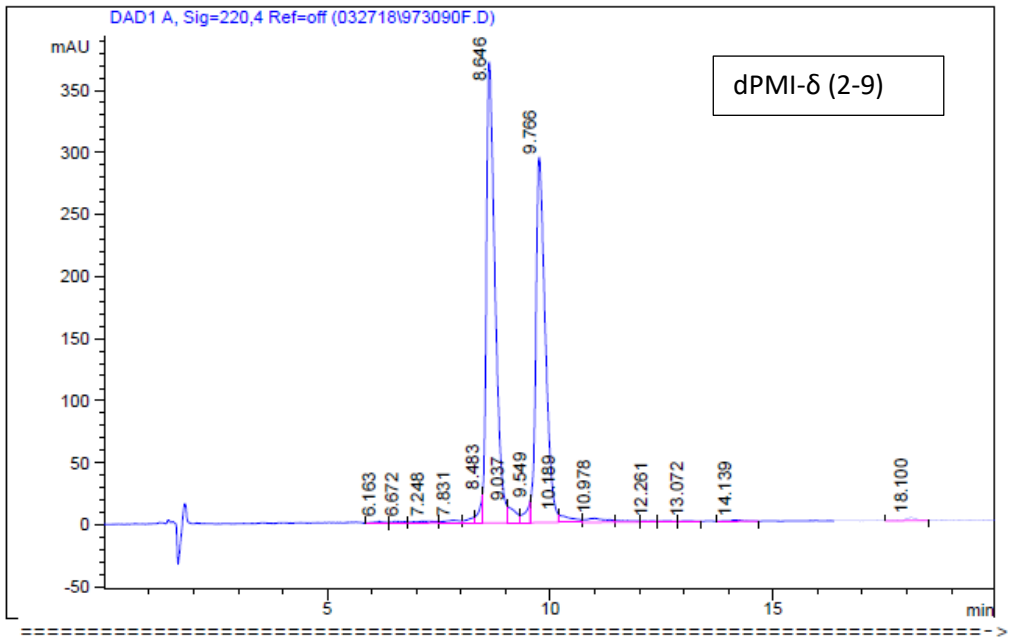

973089F #9-20 RT: 0.21-0.45 AV: 12 NL: 2.34E8
T: + c ESI Full ms [290.00-2000.00]
811.0
(x2-2H=MW)
dPMI-δ (6-10)
1620.0
1621.1
811.7
1622.0
541.0
812.6
1623.1
541.7
847.0
1661.7
340.2
377.1
447.1
622.8
724.7
1009.7
1080.8
1162.0
1243.9
1311.8
1447.9
1619.0
1717.2
1783.5
1874.6
Relative Abundance
m/z



Signal 1:VWD1 A, Wavelength=220 nm

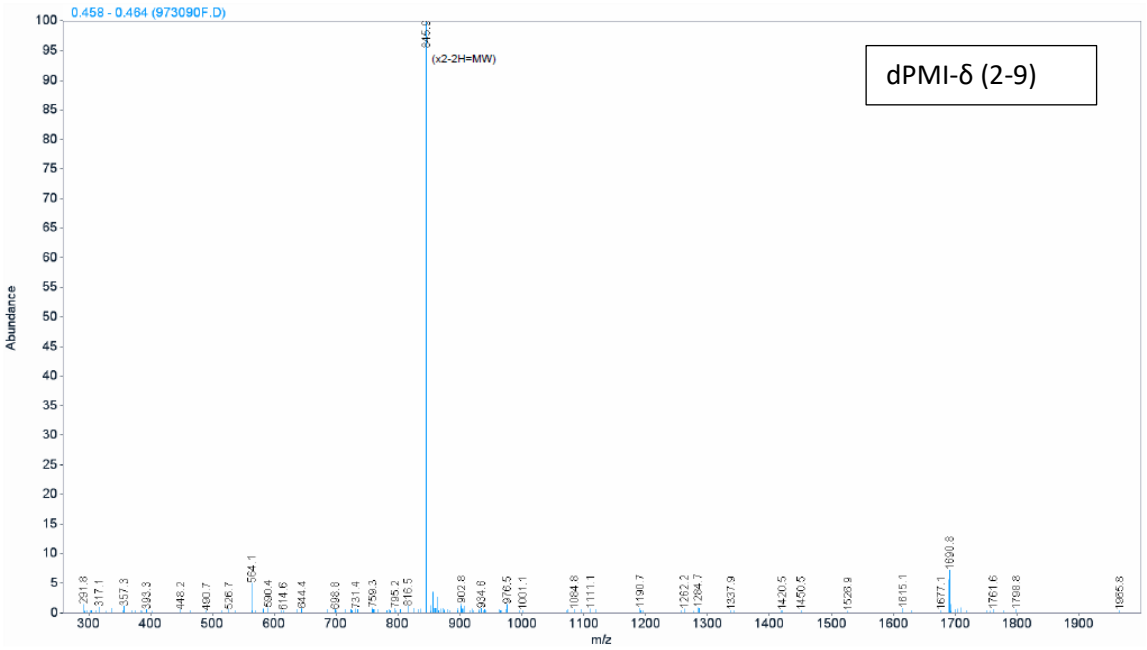
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	6.276	VV	0.061	0.266	1.175	0.024
2	7.212	VV	0.126	0.342	3.251	0.066
3	7.828	VV	0.170	0.367	4.671	0.095
4	8.510	VV	0.592	0.353	15.550	0.318
5	9.093	VV	1.967	0.160	18.826	0.385
6	9.248	VV	12.774	0.061	46.594	0.952
7	9.405	VV	177.865	0.198	2302.192	47.021
8	9.920	VF	162.823	0.218	2313.046	47.243
9	10.238	VV	9.570	0.076	43.353	0.885
10	10.562	VV	4.383	0.230	66.578	1.360
11	11.050	VV	0.615	0.264	11.163	0.228
12	11.478	VV	0.751	0.232	11.929	0.244
13	11.723	VV	0.578	0.214	8.863	0.181
14	12.291	VV	1.524	0.270	28.405	0.580
15	12.710	VV	0.261	0.253	4.616	0.094
16	13.073	VV	0.175	0.269	3.252	0.066
17	13.446	VV	0.124	0.302	2.791	0.057
18	13.914	VV	0.098	0.332	2.349	0.048
19	14.473	VV	0.079	0.392	2.137	0.044
20	15.373	VV	0.186	0.428	5.347	0.109

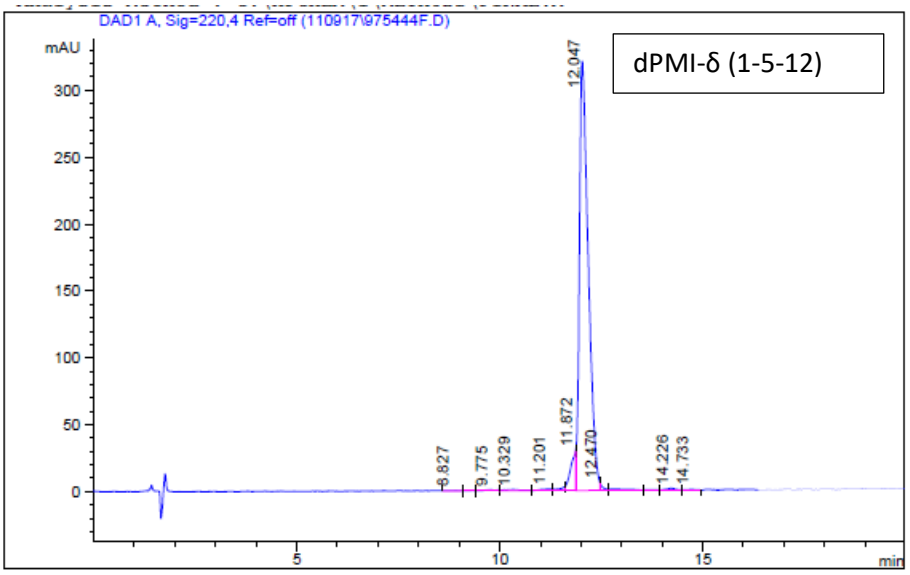

973091F #11-14 RT: 0.23-0.29 AV: 4 NL: 1.48E8
T: + c ESI Full ms [290.00-2000.00]
dPMI-δ (5-12)
831.7
(x2-2H=MW)
832.4
1108.2
1108.9
1662.8
1661.7
1246.6
567.5
591.9
823.0
833.2
852.1
1109.7
1199.9
1247.5
1257.0
1337.1
1497.1
1644.7
1663.8
1747.8
1826.8
1974.4
377.9
473.1
562.1
686.8
809.1
926.9
1094.8
Relative Abundance
m/z



Signal 1: DAD1 A, Sig=220,4 Ref=off

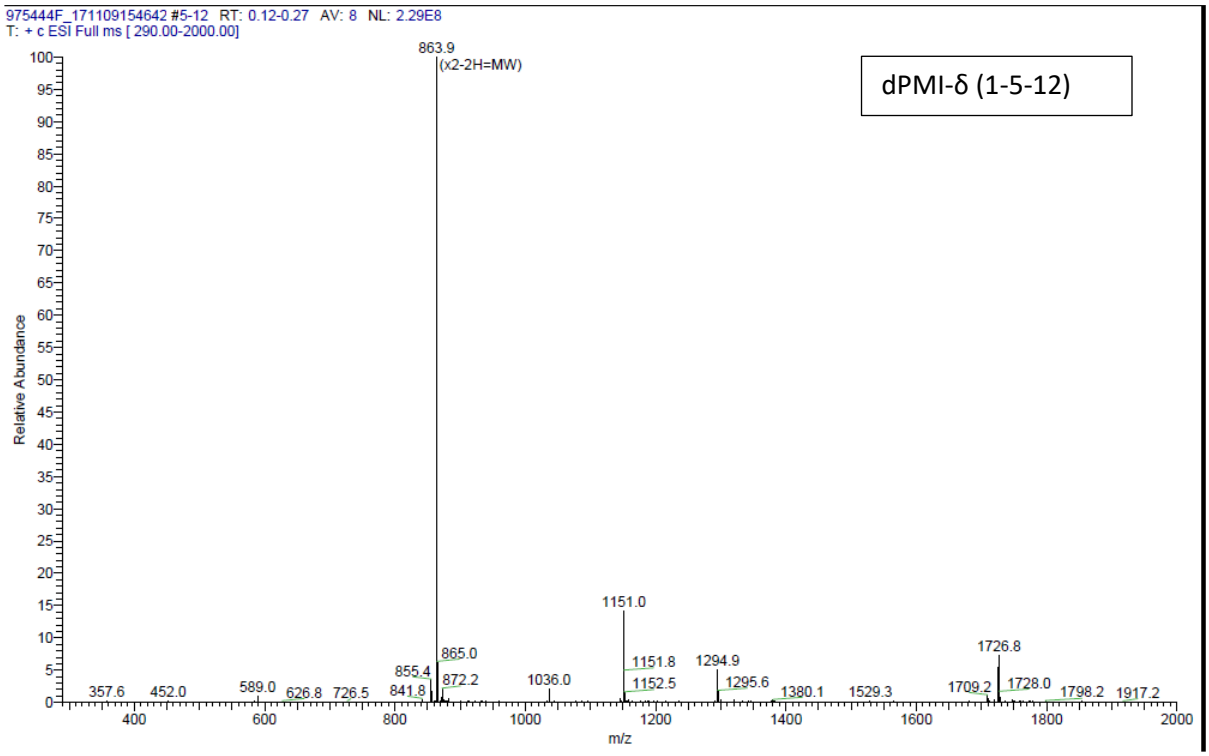
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	6.163	VP	1.497	0.223	24.543	0.238
2	6.672	VV	1.370	0.253	26.737	0.259
3	7.248	VV	1.585	0.412	49.856	0.483
4	7.831	VV	2.139	0.344	50.193	0.486
5	8.312	VV	4.925	0.179	52.924	0.513
6	8.483	VV	23.428	0.077	108.672	1.052
7	8.646	VF	361.702	0.213	5028.284	48.697
8	9.037	VV	13.832	0.205	169.772	1.644
9	9.549	VV	16.836	0.117	118.605	1.149
10	9.766	VF	292.953	0.223	4321.612	41.853
11	10.189	VV	6.444	0.300	116.015	1.124
12	10.978	VF	3.172	0.439	100.202	0.970
13	11.448	VV	1.244	0.433	32.350	0.313
14	12.261	VV	0.840	0.245	15.333	0.148
15	12.593	VV	0.761	0.263	15.049	0.146
16	13.072	VV	0.640	0.301	14.046	0.136
17	14.139	VP	0.903	0.414	29.553	0.286
18	18.100	VV	2.637	0.287	51.845	0.502

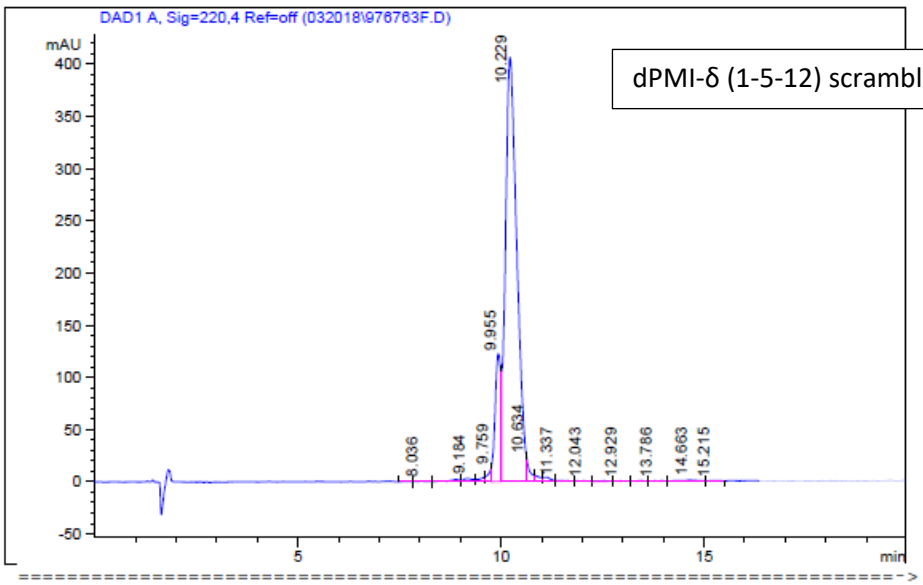

0.458 - 0.464 (973090F.D)
dPMI-δ (2-9)
(x2-2H=MW)
Abundance
m/z



Signal 1:DAD1 A, Sig=220,4 Ref=off

Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	8.827	VV	0.331	0.245	6.215	0.116
2	9.321	VV	0.289	0.248	5.504	0.103
3	9.775	VV	0.742	0.299	16.567	0.310
4	10.329	VV	0.752	0.438	24.918	0.467
5	11.201	VV	1.178	0.261	22.441	0.420
6	11.595	VV	2.656	0.154	24.465	0.458
7	11.872	VV	29.650	0.139	247.374	4.634
8	12.047	VF	318.981	0.230	4891.597	91.628
9	12.470	VV	6.004	0.075	27.015	0.506
10	12.869	VV	1.132	0.426	35.717	0.669
11	13.788	VV	0.442	0.240	7.130	0.134
12	14.226	VV	1.367	0.252	21.990	0.412
13	14.733	VV	0.495	0.212	7.634	0.143


975444F_171109154642 #5-12 RT: 0.12-0.27 AV: 8 NL: 2.29E8
T: + c ESI Full ms [290.00-2000.00]
863.9
(x2-2H=MW)
dPMI-δ (1-5-12)
Relative Abundance
m/z
1151.0
865.0
855.4
872.2
1151.8
1294.9
1152.5
1295.6
1036.0
1726.8
357.6
452.0
589.0
626.8
726.5
841.8
1380.1
1529.3
1709.2
1728.0
1798.2
1917.2



dPMI-δ (1-5-12) scrambled

Signal 1: DAD1 A, Sig=220,4 Ref=off

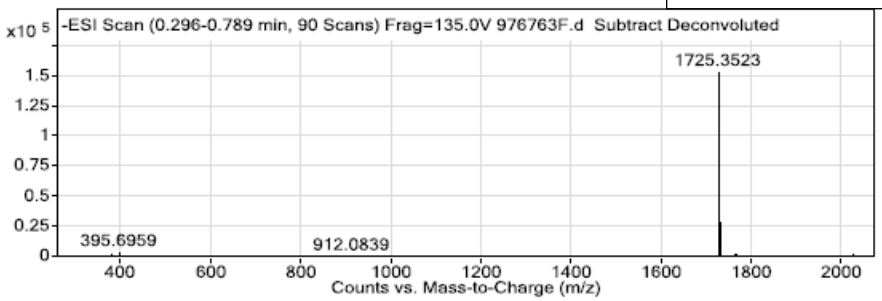
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	7.625	VV	0.523	0.209	7.351	0.076
2	8.036	VV	0.579	0.238	9.905	0.103
3	8.898	VV	2.084	0.265	39.302	0.407
4	9.184	VV	2.899	0.236	47.501	0.492
5	9.588	VV	4.042	0.149	36.088	0.374
6	9.759	VV	12.978	0.092	71.921	0.745
7	9.955	VV	121.092	0.156	1222.180	12.653
8	10.229	VF	405.060	0.298	7908.353	81.873
9	10.634	VF	22.531	0.087	117.477	1.216
10	10.825	VF	5.822	0.158	55.072	0.570
11	11.039	VF	3.900	0.223	52.099	0.539
12	11.337	VV	0.824	0.317	15.680	0.162
13	12.043	VV	0.473	0.274	9.568	0.099
14	12.514	VV	0.459	0.260	8.471	0.088
15	12.929	VV	0.443	0.244	7.312	0.076
16	13.461	VV	0.465	0.246	8.533	0.088
17	13.786	VV	0.556	0.201	8.046	0.083
18	14.663	VV	0.984	0.357	27.400	0.284
19	15.215	VV	0.428	0.224	7.032	0.073

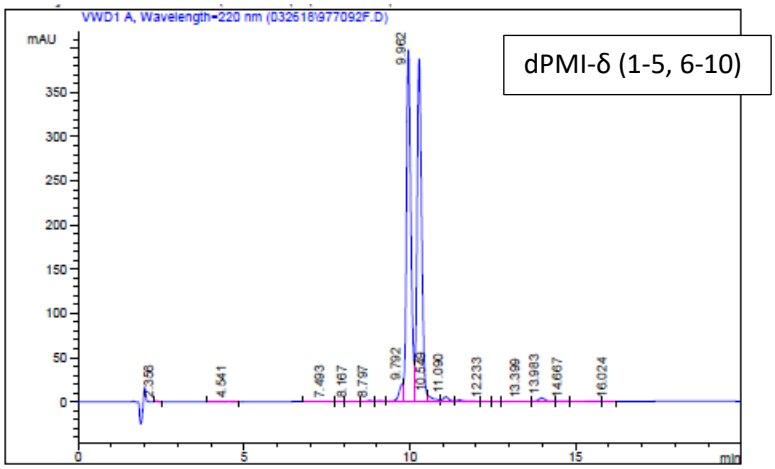
*** End of Report ***

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
135	0	ESI

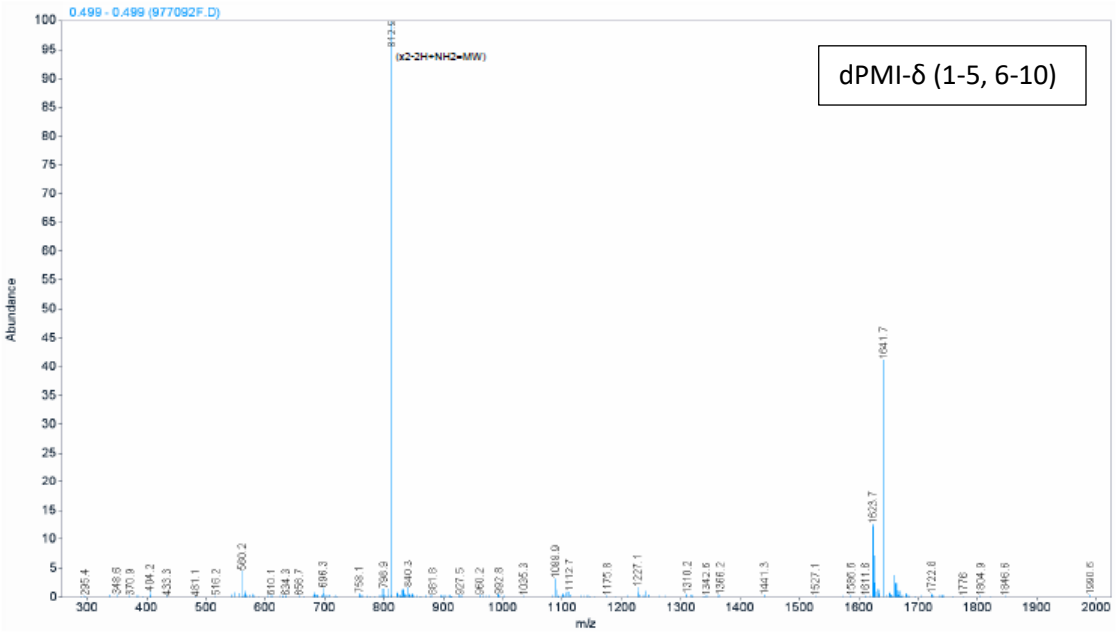
dPMI-δ (1-5-12) scrambled





Signal 1:VWD1 A, Wavelength=220 nm

Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	2.356	MM	1.369	0.063	5.172	0.066
2	4.541	MM	0.204	0.179	2.193	0.028
3	7.493	VV	0.308	0.185	3.959	0.050
4	7.921	VV	0.112	0.194	1.475	0.019
5	8.167	VV	0.082	0.329	1.987	0.025
6	8.797	VV	1.246	0.154	12.740	0.162
7	9.094	VV	1.181	0.184	14.465	0.184
8	9.792	VV	20.236	0.132	160.014	2.038
9	9.962	VV	389.946	0.151	3675.221	46.798
10	10.286	VF	388.759	0.153	3743.800	47.671
11	10.549	VV	6.847	0.183	75.211	0.958
12	11.090	VV	5.295	0.186	66.092	0.842
13	11.498	VV	1.585	0.246	27.835	0.354
14	12.233	VV	0.144	0.245	2.442	0.031
15	12.588	VV	0.094	0.244	1.598	0.020
16	13.399	VV	0.059	0.664	3.203	0.041
17	13.983	VV	3.844	0.206	50.678	0.645
18	14.667	VV	0.078	0.337	2.006	0.026
19	15.613	VV	0.046	0.715	2.683	0.034
20	16.024	VV	0.027	0.274	0.588	0.007


0.499 - 0.499 (977092F.D)
dPMI-δ (1-5, 6-10)
(x2-2H+NH2=MW)
Abundance
m/z