Conformational propensity and biological studies of proline mutated LR peptides inhibiting human thymidylate synthase and ovarian cancer cell growth.

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SUPPORTING INFORMATION

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Table S1. Peptide percentage of inhibition (%I) of recombinant hTS enzymatic activity for tested peptides at 100 µM.

Peptide	sequence	% inhibition at 100 µM
LR	LSCQLYQR	50 ± 5.12
[Pro^{1}]LR	PSCQLYQR	39 ± 1.89
[Pro^{2}]LR	LPCQLYQR	56 ± 2.49
[Pro^{3}]LR	LSPQLYQR	25± 2.55
[Pro^{4}]LR	LSCPLYQR	40± 1.58
[Pro^{5}]LR	LSCQPYQR	41± 1.73
[Pro^{6}]LR	LSCQLPQR	24± 5.28
[Pro^{7}]LR	LSCQLYPR	38± 7.18
[Pro^{8}]LR	LSCQLYQP	45± 7.17

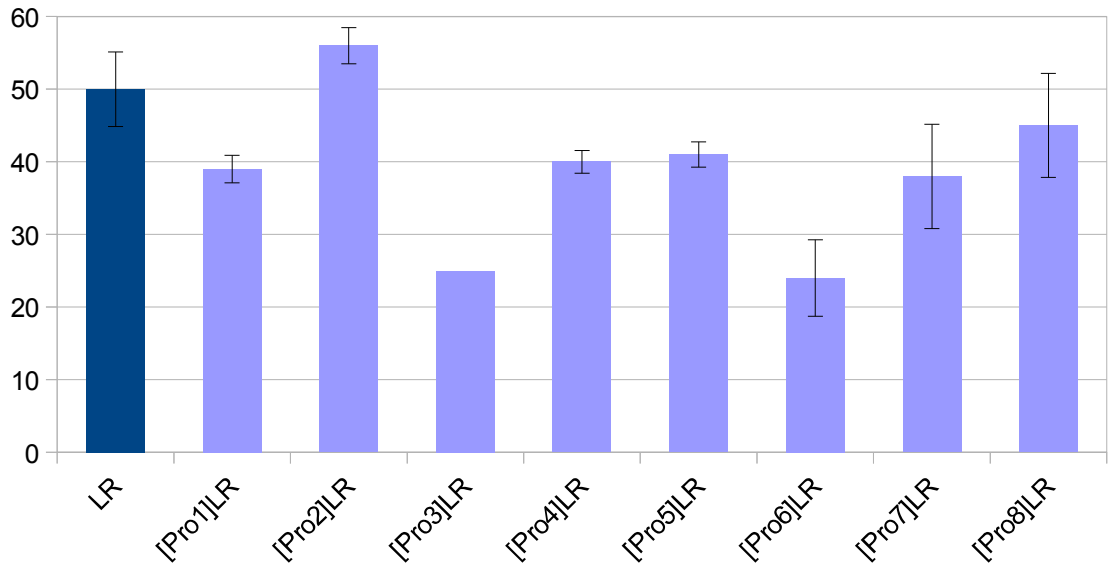

Figure S1. Peptide percentage of inhibition (%I) of recombinant hTS enzymatic activity for tested peptides at 100 µM.

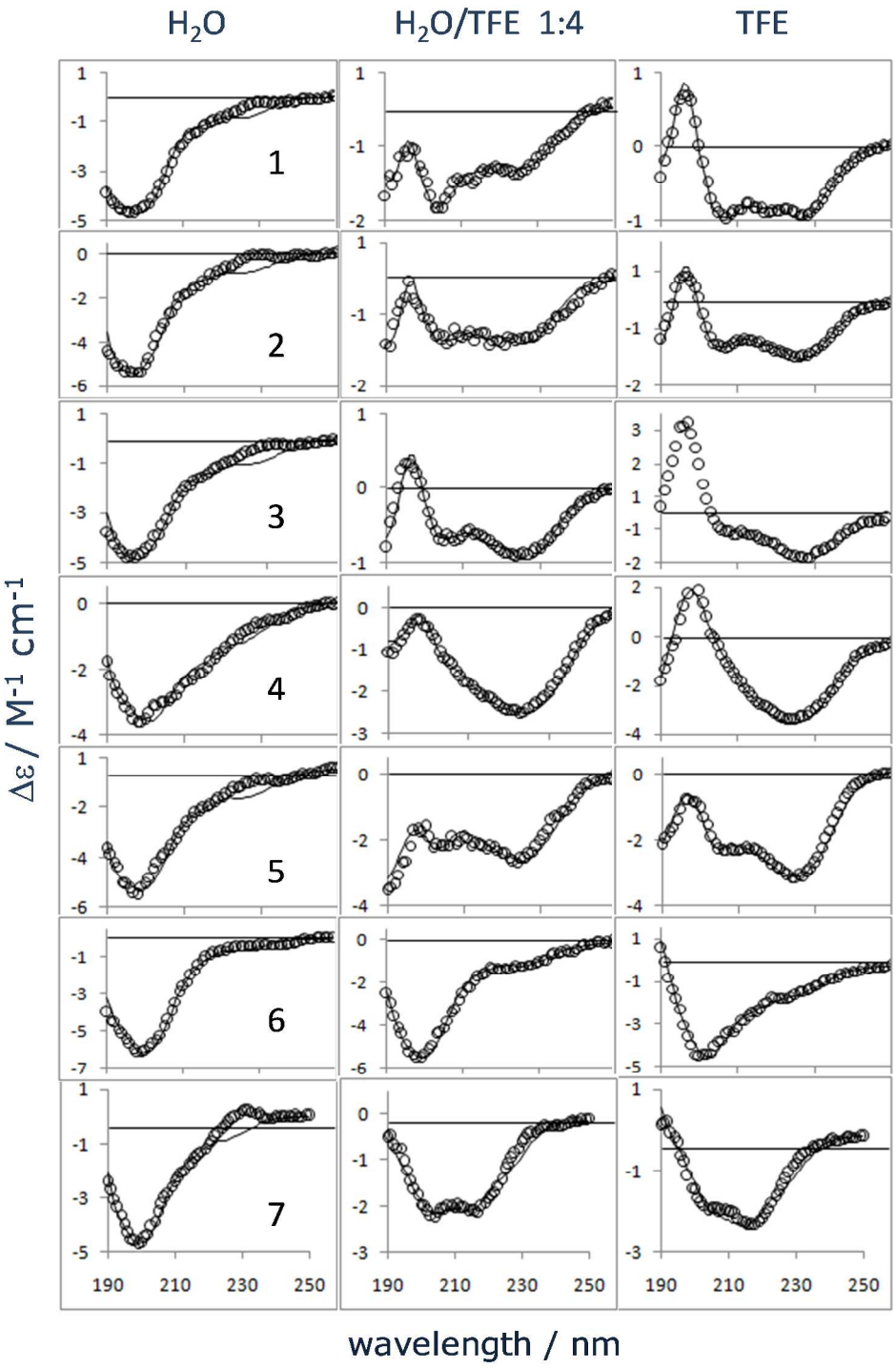

Figure S2. CD spectra (circles) of, from top to bottom, **[Pro^1]LR** to **[Pro^7]LR** in water, a 1:4 v/v DDW/TFE mixture and in TFE. Simulations in terms of the spectra of several secondary structure motifs are shown as solid lines (see text for details).

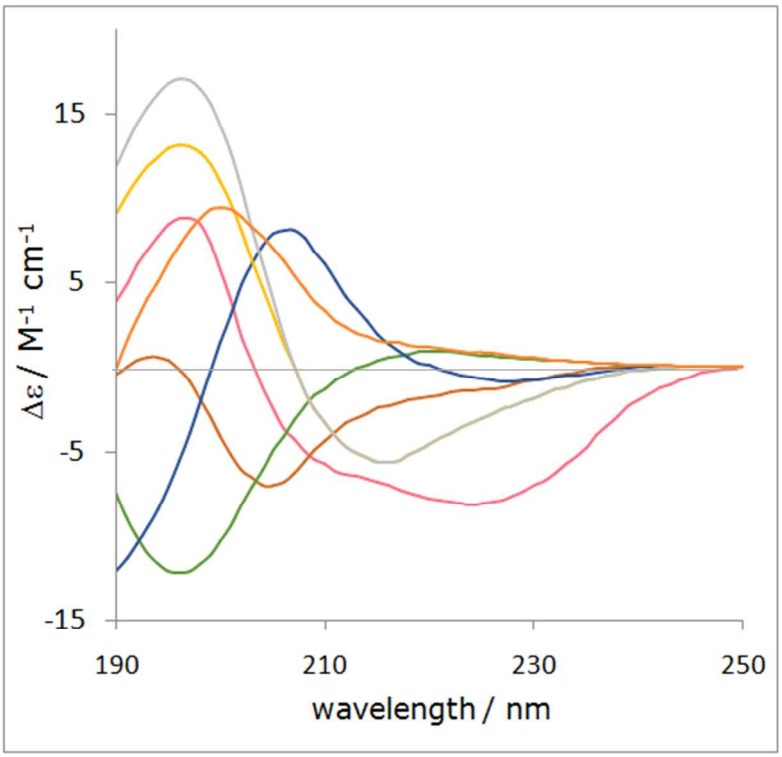

Figure S3. CD spectra of secondary structure motifs used for the simulation of the spectra of peptides **[Pro^1]LR** to **[Pro^7]LR**. Yellow: β-sheet; grey: β-sheet [twisted]; pink: type IA β-turn; brown: type IB β-turn; blue: type II β-turn; orange: type II β-turn; green: irregular.[1,2]

Table S2. Contributions of CD signatures of secondary structural motifs in the simulation of the CD spectra of peptides **[Pro^1]LR** to **[Pro^7]LR** in water (W), 1:4 v/v water/TFE mixture and in TFE.
* spectrum assumed to correspond to the spectrum of **[Pro^3]LR** in TFE

peptide	solvent	irregular	type IA β-turn	type IB β-turn	type II β-turn	β-strand	3(10) helix*
LR	TFE	6		9		2	84
[Pro^1]LR	W	60		12	4	18	6
	W/TFE	30				12	58
	TFE	20				9	71
[Pro^2]LR	W	65		8	4	13	10
	W/TFE	26			3	11	60
	TFE	18			2	7	73
[Pro^3]LR	W	63		8	3	15	10
	W/TFE	20			4	8	68
	TFE						100
[Pro^4]LR	W	55	7	15	2	21	
	W/TFE	34	26	9	20	10	
	TFE	17	41	8	31	5	
[Pro^5]LR	W	60	12	12	1	15	
	W/TFE	27	28	25	20		
	TFE	27	36	20	17		
[Pro^6]LR	W	58	4	30		8	
	W/TFE	55	9	22	5	8	
	TFE	45	10	29	2	14	
[Pro^7]LR	W	58	4	19		18	
	W/TFE	39		23	9	29	
	TFE	37	2	21	8	32	

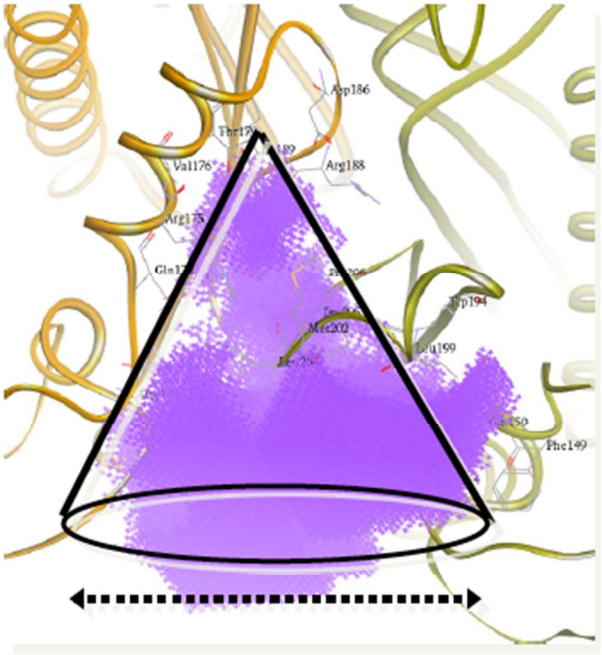

Figure S4. The peptide binding active cavity has the shape of an inverted cone (highlighted in violet color), narrower at the top and broader at the bottom.

Table S3. Proline mutated LR peptidesIC_{50} values against 2008, C13* and IGROV-1 human ovarian cancer cell lines. The values for the reference drug 5FU, were already reported.[6]The error values were calculated with statistical significance t value 95% ($p< 0.05$).

	2008 cells	**C13* cells**	**IGROV-1 cells**
5FU	6.1±2.0	12.1±1.9	8.2±0.6
LR	4.6±0.8	9.5±3.0	10.6±1.0
[D-Gln^4]LR	2.6±0.8	5.1±0.8	2.6±1.1
[Pro^1]LR	*(31.7±4.1)*[a]	*(38.6±1.7)*[a]	*(15.9±2.2)*[a]
[Pro^2]LR	*(41.3±3.4)*[a]	*(40.9±3.1)*[a]	*(24.4±4.7)*[a]
[Pro^3]LR	1.57±0.6	8.1±2.0	6.2±0.5
[Pro^4]LR	0.96±0.3	3.55±1.6	12.1±2.0
[Pro^5]LR	*(33.8±2.7)*[a]	*(35.6±2.8)*[a]	*(24.1±4.2)*[a]
[Pro^6]LR	*(27.6±5.2)*[a]	*(23.8±4.1)*[a]	*(17.2±2.7)*[a]
[Pro^7]LR	1.2±0.6	6.6±1.7	4.3±1.0
[Pro^8]LR	nottested	not tested	not tested

[a]For peptides not reaching the 50% of cancer cell growth inhibition, it is reported, in parenthesis, the cell growth inhibition percentages at 5 μM.

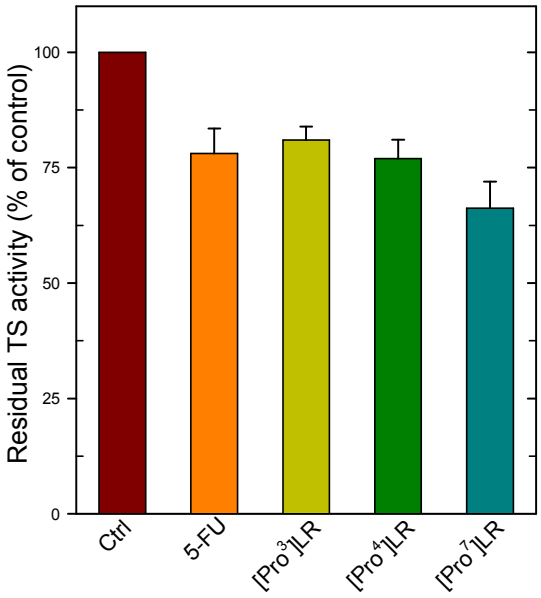

Figure S5. Inhibition of cellular TS activity in IGROV-1 cells treated for 72 h with 5µM 5FU or 5µM of peptides (transfected by SAINT-PhD system). After 72hr, cells were harvested and 150 µg of cellular proteins have been used for the TS activity assay. Bars are the mean ± S.E.M of two separate experiments performed in duplicate.

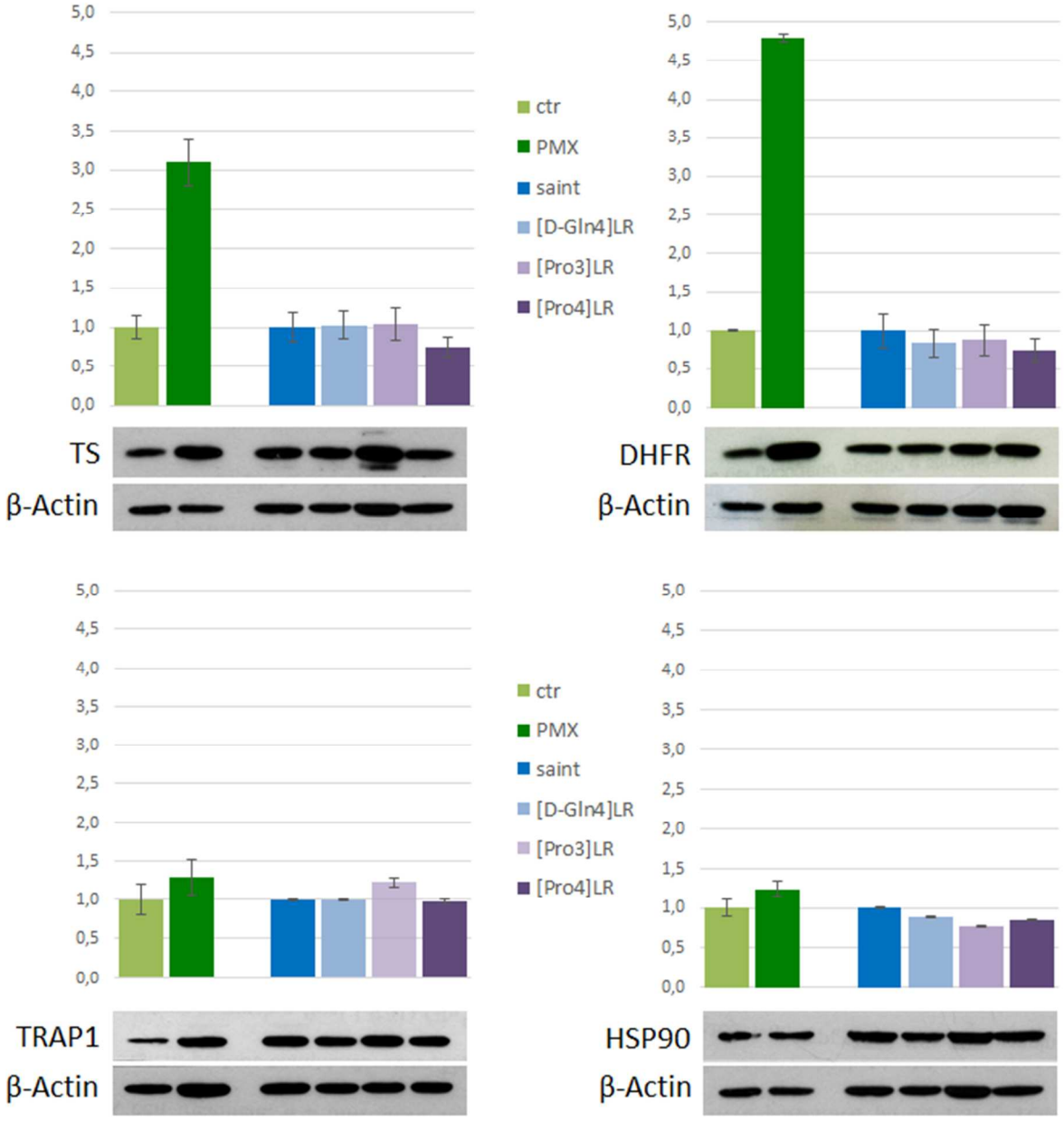

Figure S6. Protein expression modulation profile of the tested compounds in IGROV-1 human cisplatin-sensitive ovarian cancer cell line.

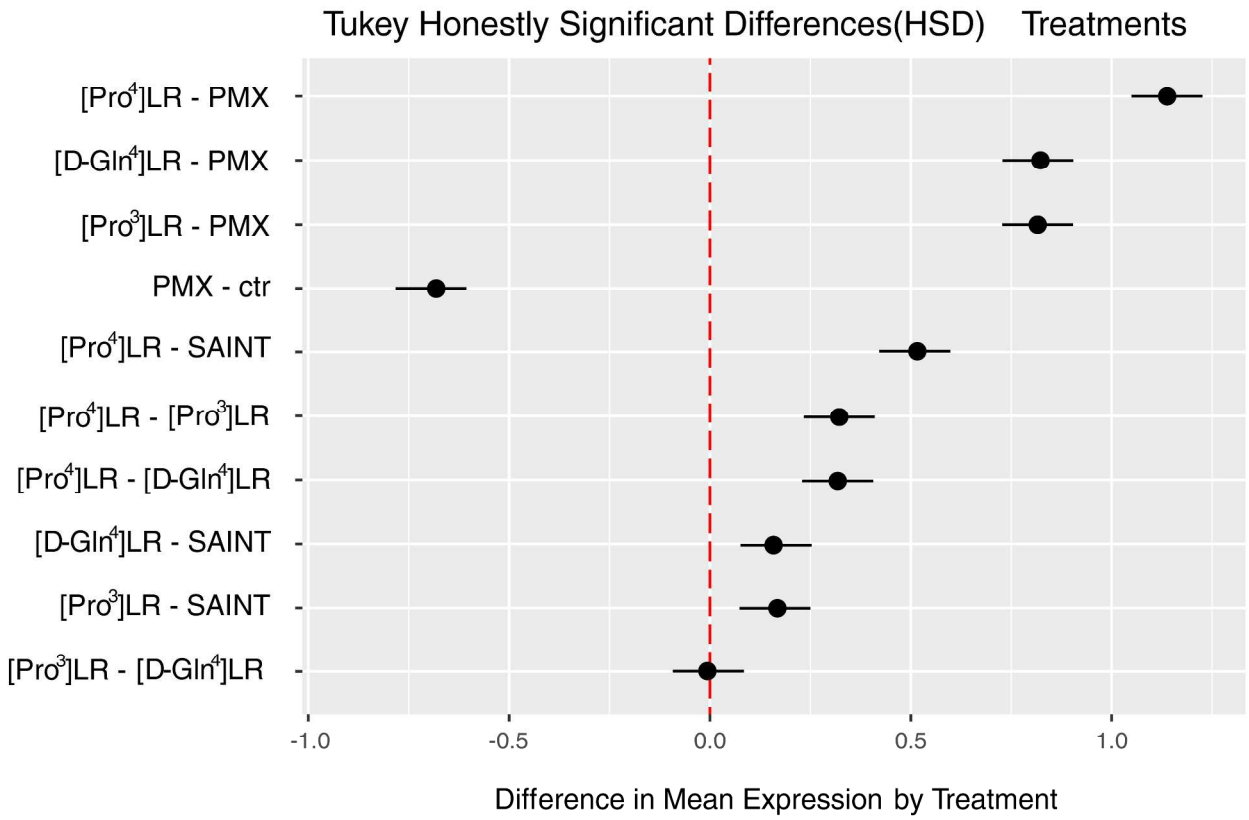

Figure S7. Comparison of expression levels modulation by pair of treatments. Significant differences are reported, plotted by the decreasing absolute value of their difference. The vertical dashed red line corresponds to a null difference and the black horizontal segments represents the 95% confidence interval (CI). Only the interaction pairs with CI not crossing the "null difference line" may be considered significantly different, with a p-value < 0.05.

HPLC profiles of peptide characterization

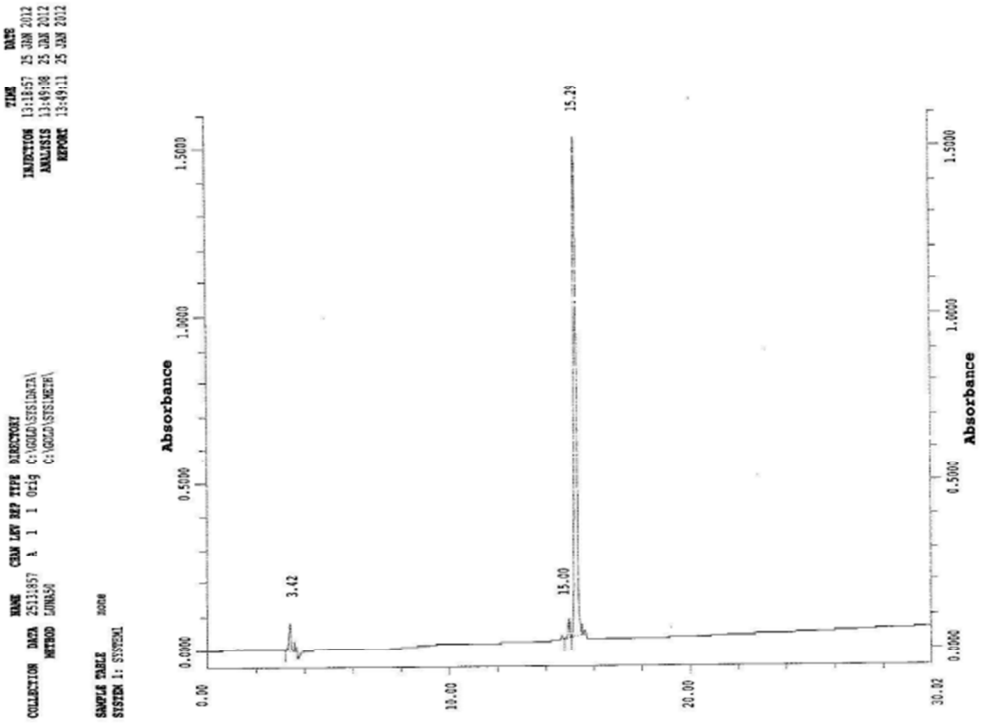

Figure S8. [Pro[1]]LR: analytical HPLC chromatogram

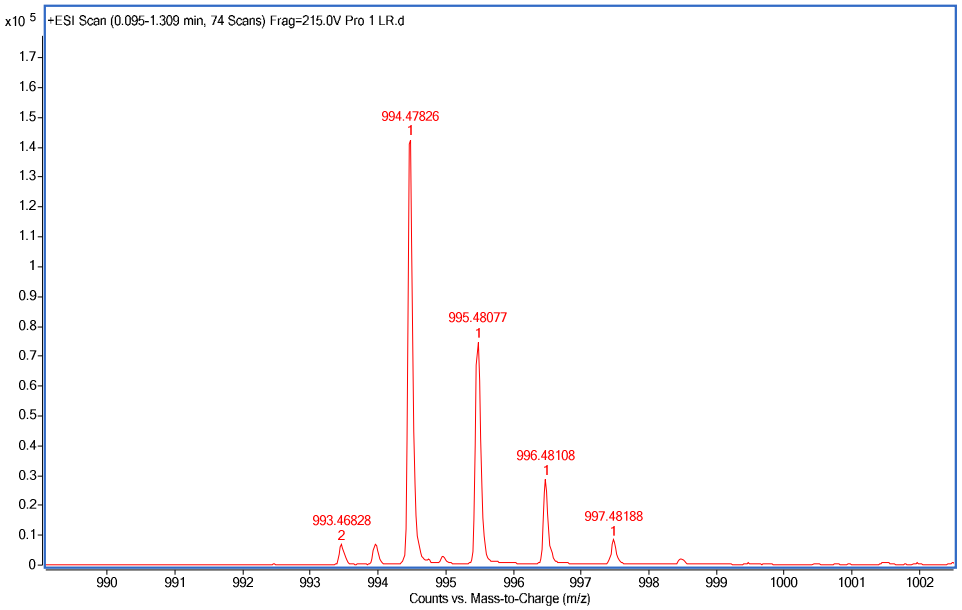

Figure S9. [Pro[1]]LR: HRMS $[MH]^+$ calculated= 994.4775

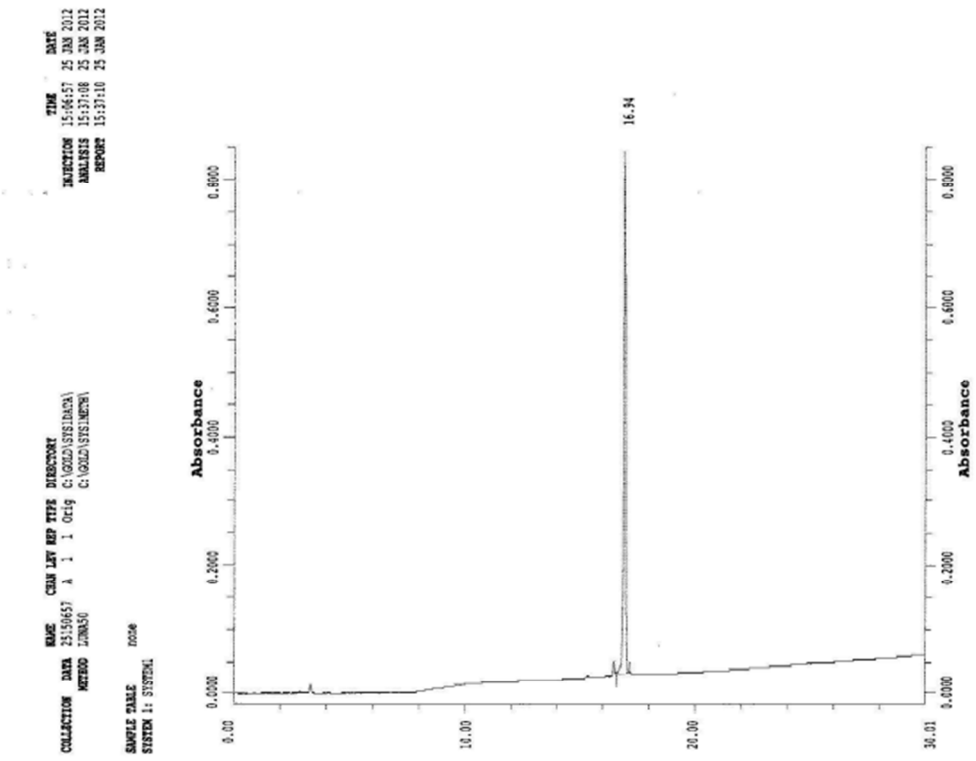

Figure S10. [Pro^2]LR: analytical HPLC chromatogram

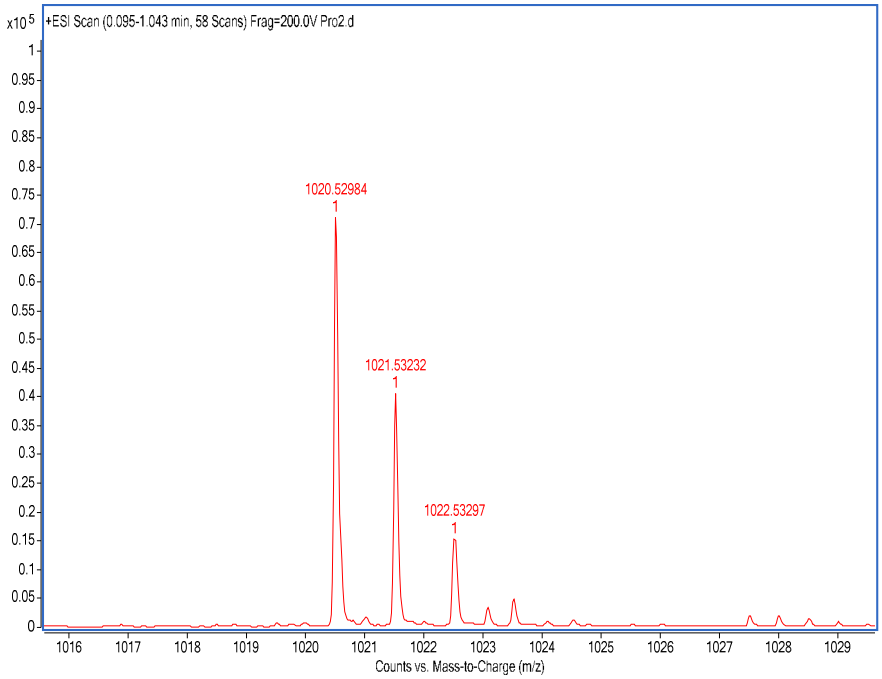

Figure S11. [Pro^2]LR: HRMS $[MH]^+$ calculated= 1020.5295

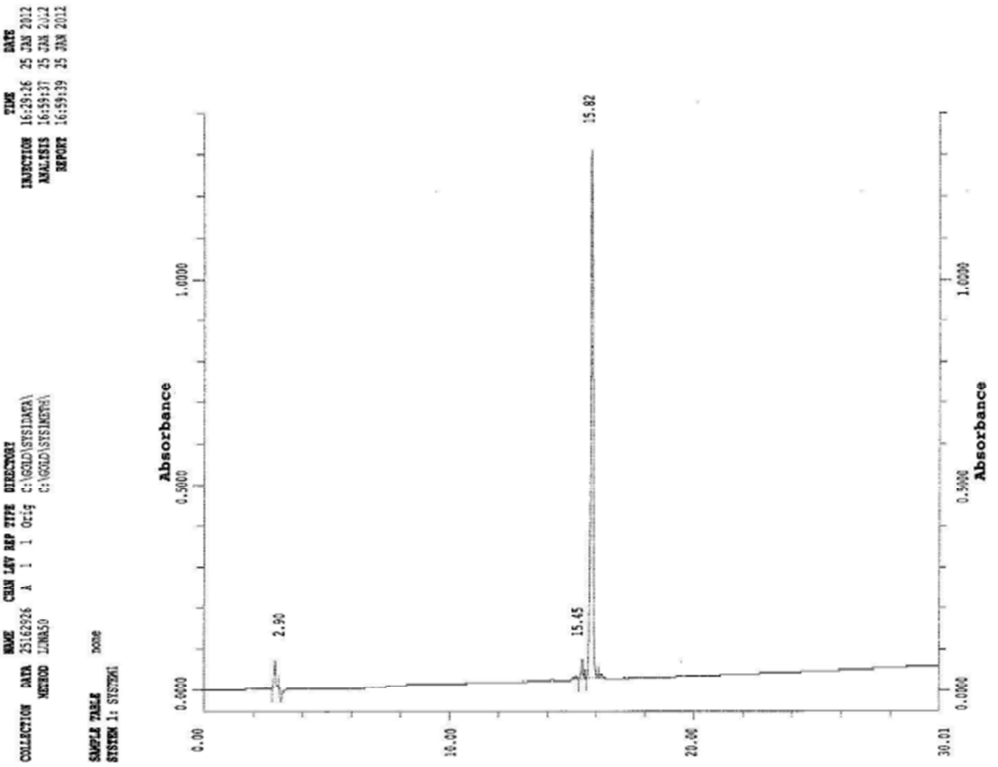

Figure S12[Pro^3]LR: analytical HPLC chromatogram

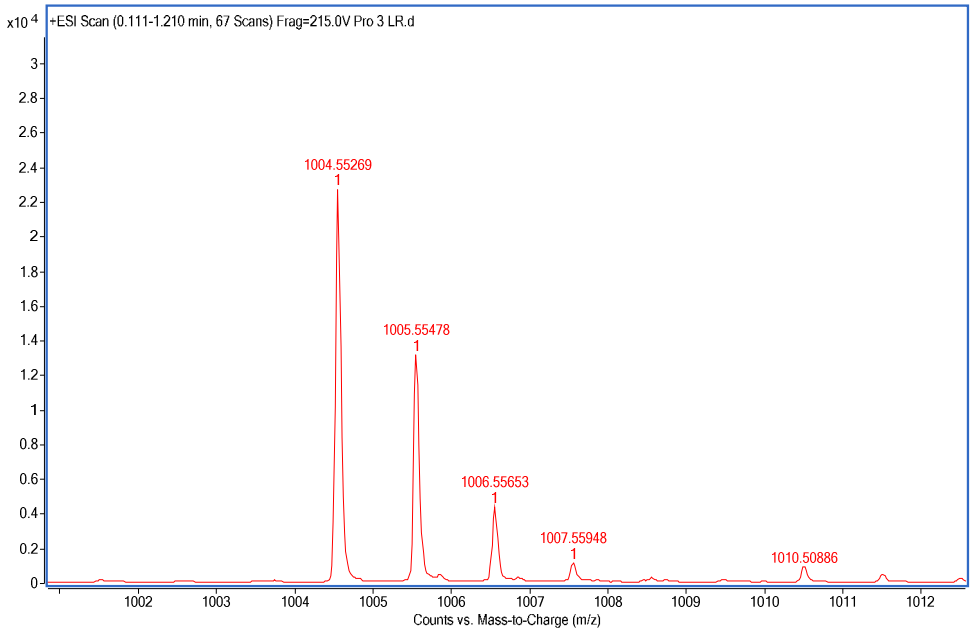

Figure S13[Pro^3]LR: HRMS $[MH]^+$ calculated=1004.5525

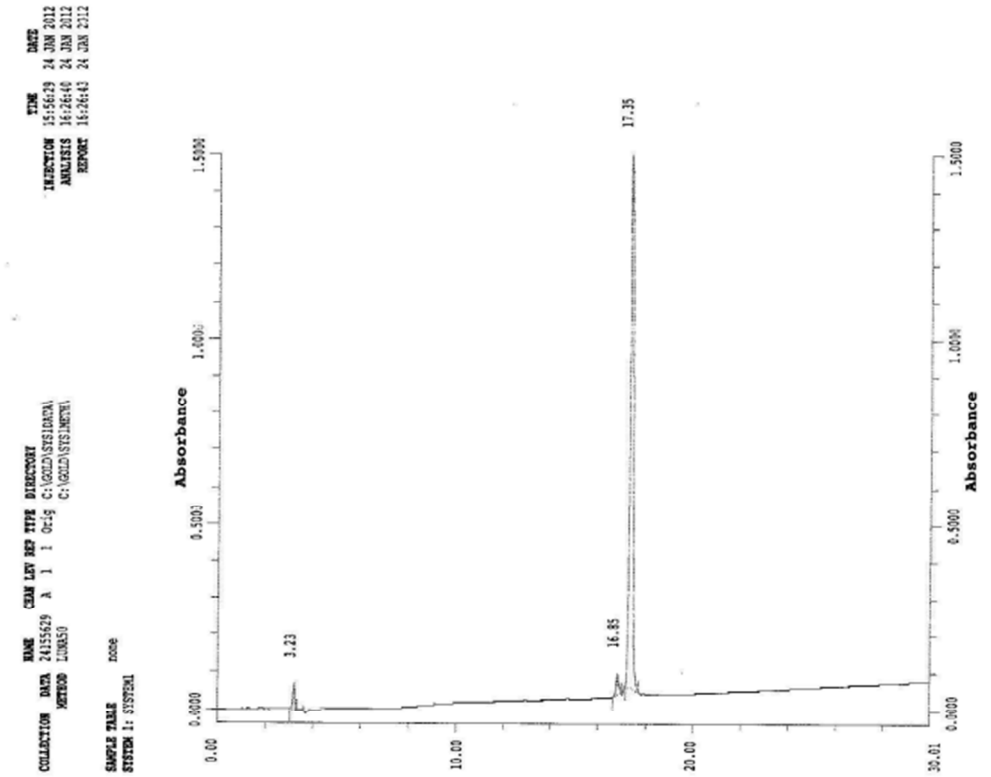

Figure S14[Pro^4]LR: analytical HPLC chromatogram

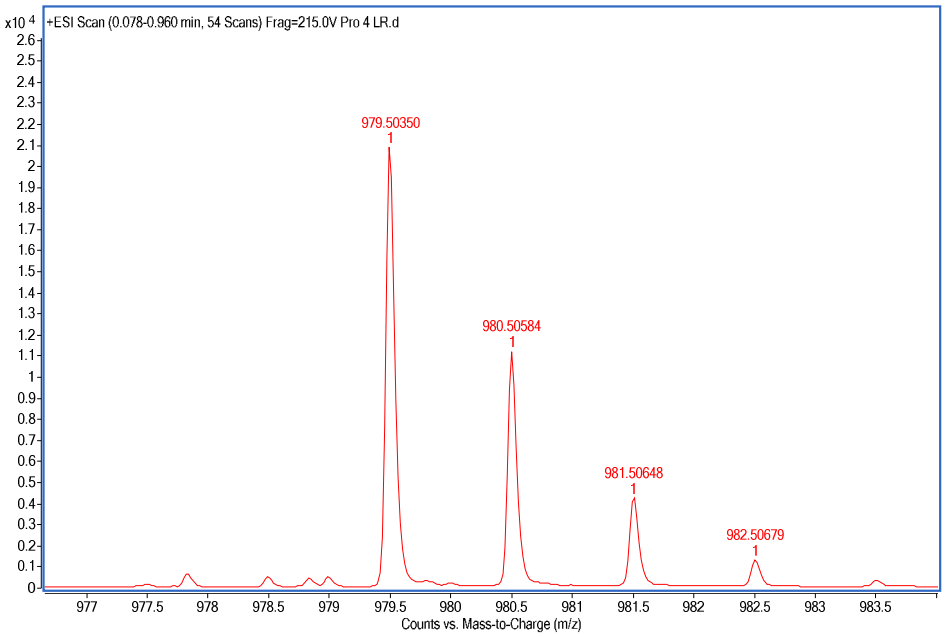

Figure S15[Pro^4]LR : HRMS $[MH]^+$ calculated= 979.5030

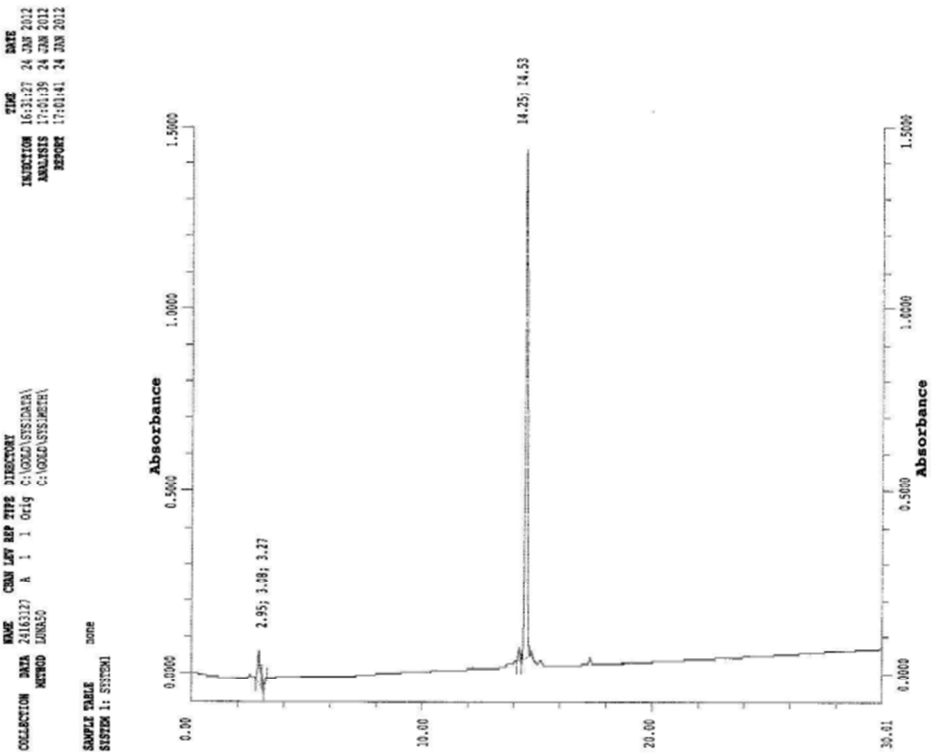

Figure S16 [Pro^5]LR: analytical HPLC chromatogram

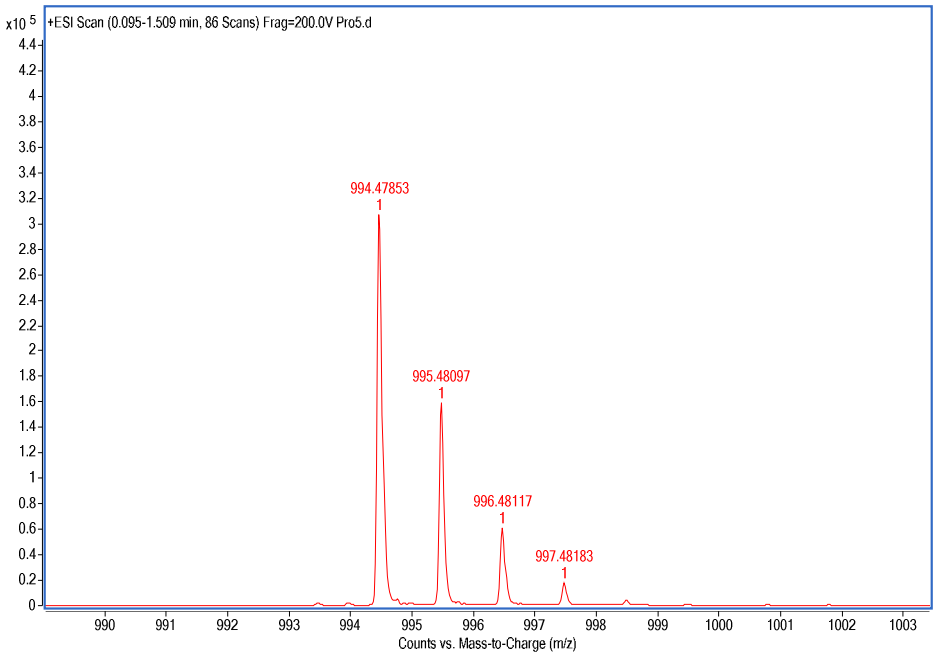

Figure S17 [Pro^5]LR : HRMS $[MH]^+$ calculated=994.4775

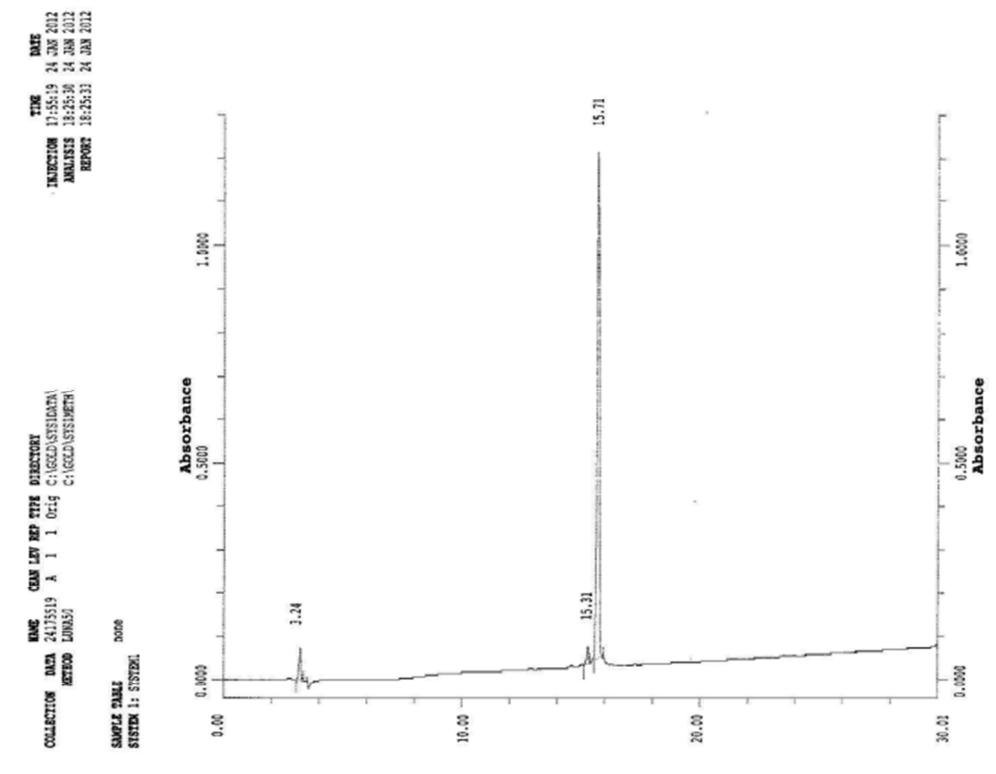

Figure S18 [Pro^6]LR: analytical HPLC chromatogram

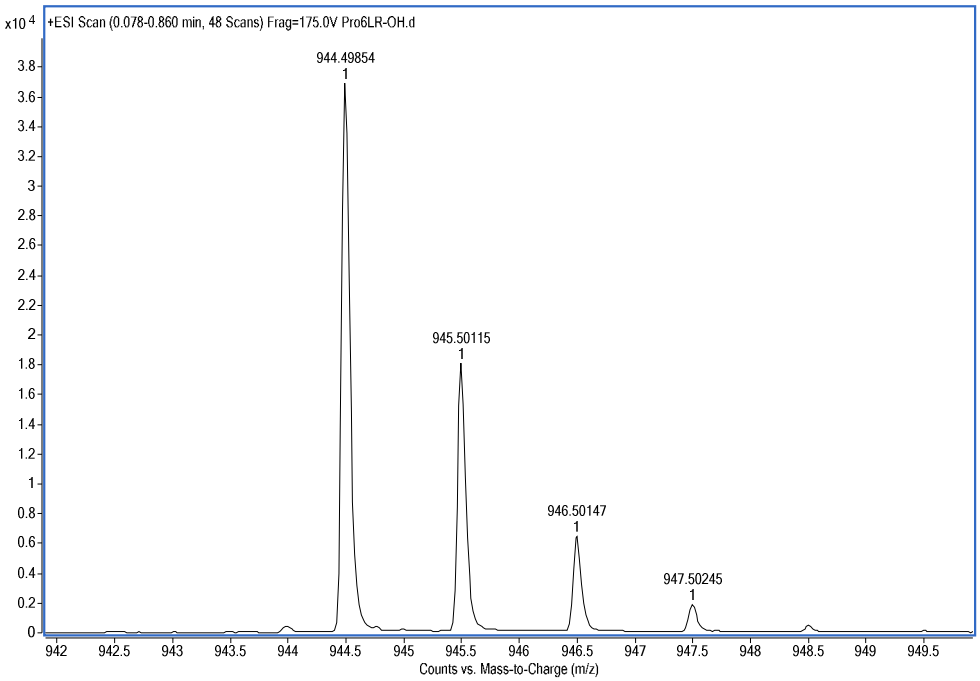

Figure S19 [Pro^6]LR: HRMS $[MH]^+$ calculated = 994.4982

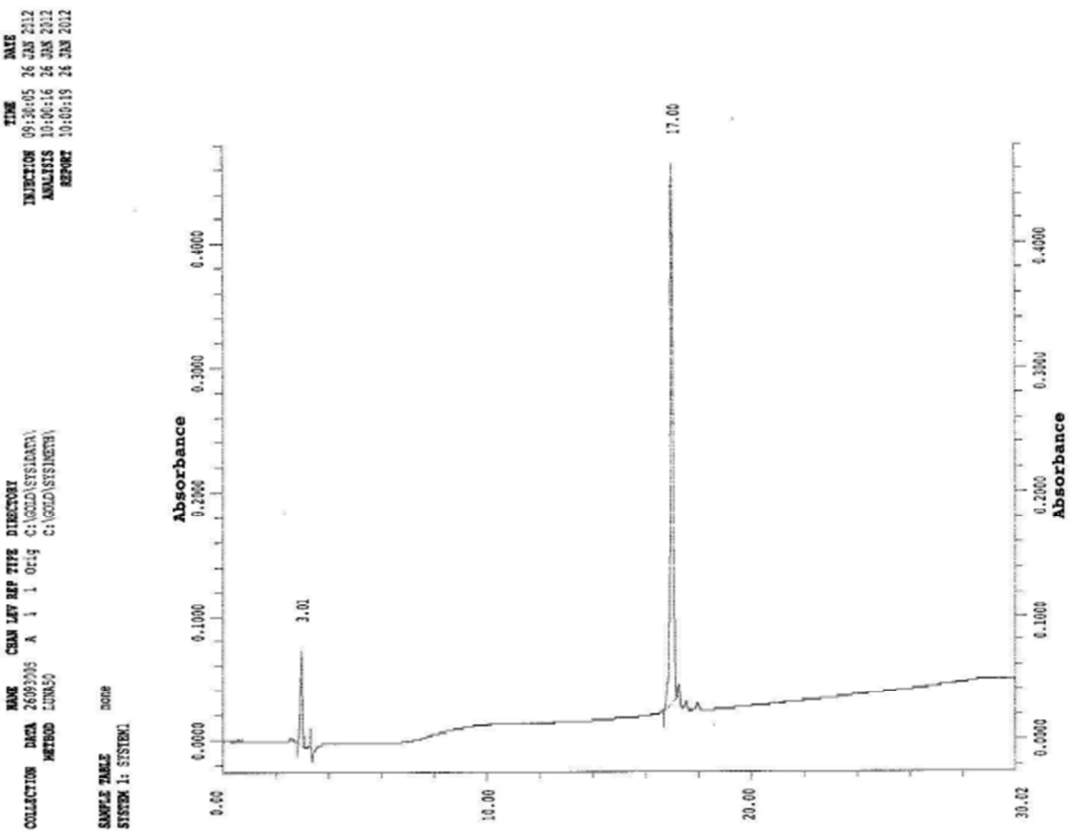

Figure S20 [Pro^7]LR: analytical HPLC chromatogram

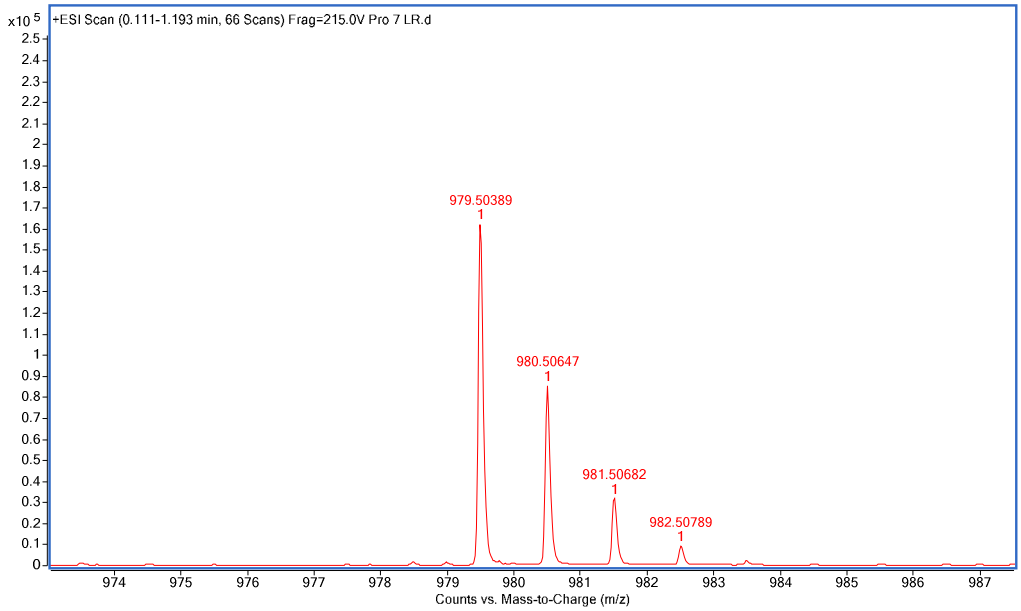

Figure S21 [Pro^7]LR: HRMS $[MH]^+$ calculated= 979.5030

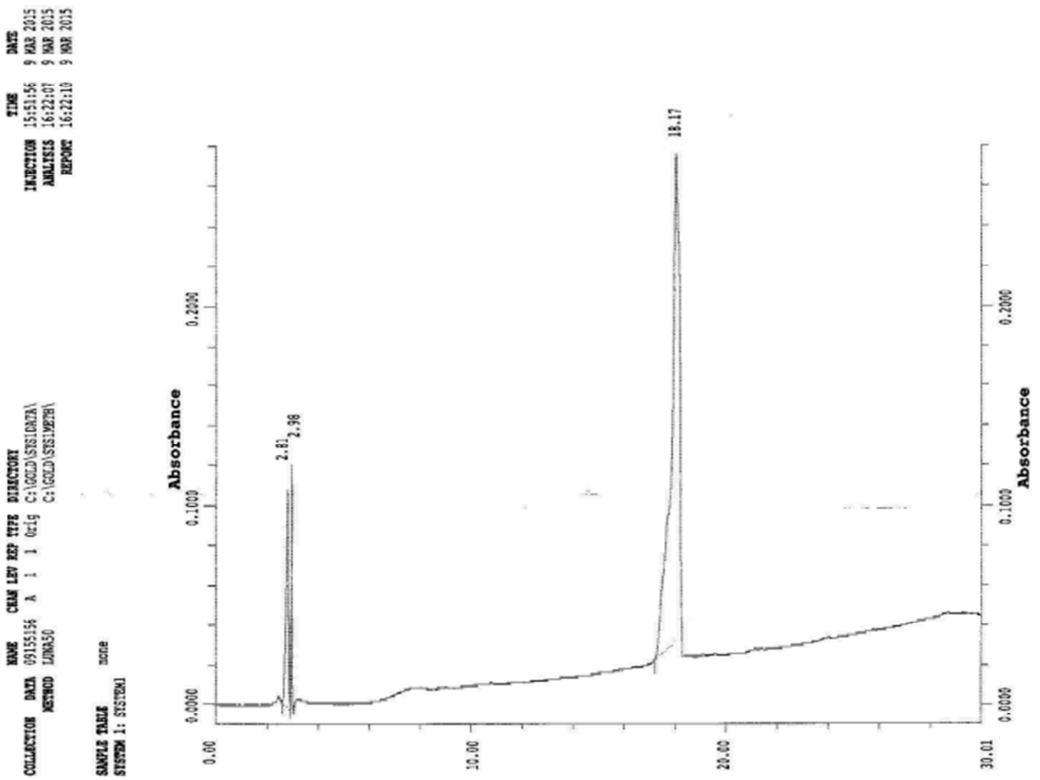

Figure S22 [Pro^8]LR: analytical HPLC chromatogram

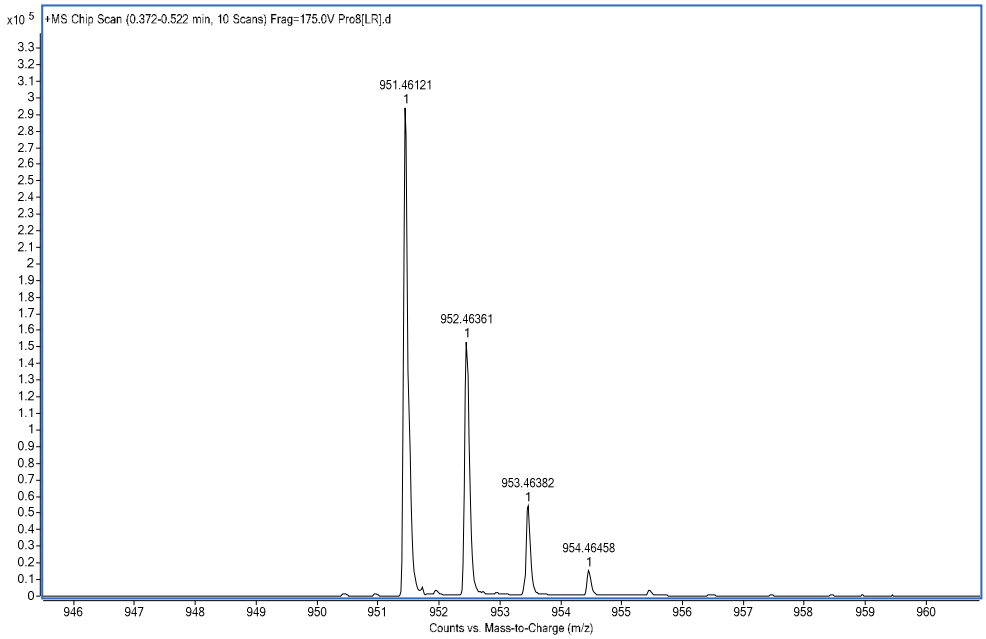

Figure S23 [Pro^8]LR: HRMS $[MH]^+$ calculated= 951.46043

Experimental section details

Peptide purification

Crude peptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 3000 system with a Jupiter column C18 (250 x 30 mm, 300 angstrom, 15 mm spherical particle size). The column was perfused at a flow rate of 20 mL/min with a mobile phase containing solvent A (0.1% TFA in H_2O), and a linear gradient from 5 to 50% of solvent B (60%, v/v, acetonitrile/0.1% TFA in H_2O) over 25 min for the elution of peptides. Analytical HPLC analyses were performed on a Beckman 116 liquid chromatograph equipped with a Beckman 166 diode array detector. Analytical purity of the peptides were determined using a Luna C18 column (4.6 x 100 mm, 3 μm particle size) with the above solvent system (solvents A and B) programmed at a flow rate of 0.5 mL/min using a linear gradient from 0% to 80% B over 25 min. All analogues showed ≥ 95% purity when monitored at 220 nm. Accurate mass of final compounds were determined using an Agilent 6520 ESI/Q-TOF mass spectrometer and the data obtained are reported as supporting information (Figures S8-S23).

Enzymatic assay details. hTS was purified as previously reported and stored at -80°C.[3]Enzyme solution was thawed the day of the experiment and enzyme concentration was determined by UV-Vis using ε_{280} = 89000 and MW=74229. Thawed protein solution was kept constantly at 4°C. In this condition, enzyme was able to reproduce normal kinetic activity values ($K_{M\ dUMP}$ = 10-12 μM, Km_{mTHF} = 4-6 μM, k_{cat} = 0,8 - 0,9 s^{-1}). Protein activity inhibition studies were performed as previously reported.[4]

CD experiments details. The cell plus solvent contributions were measured and subtracted from the averaged spectra. Concentrated peptide solutions were prepared in TFE and deionized water and were diluted to the concentrations required for the measurements (ca. 70 μM) by adding suitable volumes of water and/or TFE obtaining the needed solvent mixtures. All samples were checked by UV−visible (UV−vis) absorption spectroscopy using a Varian Cary 100 spectrophotometer. Peptide concentrations were determined spectrophotometrically using the absorbance at 280 nm (ε_{tyr} = 1450 M^{-1} cm^{-1}) for all peptides except **[Pro^6]LR** that was determined at 195 nm (ε = 105000 M^{-1} cm^{-1}). Fused-silica cuvettes (1 mm) were employed, and measurements were performed at 20−22 °C about 20 min after sample preparation.

MD simulation details.MD simulation details. The two complexes, hTSin complex with**[Pro^2]LR**and**[Pro^6]LR**, were built starting from the crystal structure of hTS complexed with **LR** (PDBid: 3N5E) replacing the second and sixth acids of **LR** peptide with a proline residue, respectively. The disulfide bond between Cys^{192} of hTS chain A and Cys^3 of the peptides was removed, and the two cysteine residues were reinstated. Both the complexes totally comprise the 785 residues of the hTS dimer and the 8 residues of the **[Pro^2]LR**and **[Pro^6]LR** peptides. The MD simulations were carried out using GROMACS (version 5.1.4)[5] [S]software package and the GROMOS'96 atom force field was applied; parameters and protocols were set as in previous similar published studies.[6] Both complexes were solvated with approximatively 43860 SPC water molecules in a cubic box of dimension 20 Å edge length. To make the systems electrostatically neutral 27 Na^+ ions were added using the *genion* utility, included in GROMACS suite of utilities. In the initial step, both systems were subjected to prior minimization protocol until the maximum force is less than 100 kJ/(mol-nm). During this step, the atoms of the peptide as well as the protein were kept fixed with a force constant of 1000 kJ/(mol-nm^2), whereas the solvent water molecules were allowed to relax. The resulting system was then put up for an

equilibrium phase of 1 ns, with a time-step (δt) of 1 fs, with no restraints applied, followed by a production run of 20 ns, without any restraints, and δt being 1 fs.
The overall simulations were conducted at a constant temperature of 300 K and a constant pressure of 1 atm, being controlled by Berendsen algorithm. Periodic Boundary Conditions were implemented in both systems. The long-range interactions were treated with particle-mesh Ewald (PME) method within a radius of 15 Å, while the short-range interactions were calculated with the Leannard-Jones potential with a cutoff of 15 Å.
Analysis of Root mean square deviation (rmsd) of C-alpha carbon atoms and the radius of gyration were made using *g_rms* and *g_gyrate* utilities, respectively. The resulting trajectories were hence visualized by VMD software (version 1.9.1).[7] The hydrogen bonds were studied by using the default parameters of *HBonds* option, provided in the software.

Cell lines growth and treatment details. 2008, C13*, and IGROV-1 human ovarian carcinoma cells were grown in RPMI 1640 medium (Lonza) containing 10% heat-inactivated fetal bovine serum. The cells were incubated at 37°C under 5% CO_2 for at least 24 h before treatment with the peptides. Treatments with peptides were performed according to the standard transfection protocol of the SAINT PhD delivery system (Synvolux Therapeutics, NL). 0.8 mM stock solution of the peptide was prepared in HBS and stored at −80 °C before use. The methods have been already reported.[6] Delivery system−peptide complexes were prepared, adding 20 μL/mL of SAINT PhD to a diluted peptide solution to reach a final concentration of 1, 2, and 5 μM of peptide. Each SAINT-PhD/peptide complex was added to the cells, and after 4h the cells were added with complete RPMI. Then cells were growth for 48 or 72h for cytotoxicity assay and protein quantification respectively.[8] Cells treated with a delivery system solution at the same concentration used in other treatments were used as control.
For **PMX** cell treatment (purchased from Ely Lilly), a 2 mM stock solution was obtained by solubilizing the drug in DMSO and then stored at −20 °C before use. The drug was diluted to a 5 μM final concentration in complete RPMI medium, and cells were incubated for 48h. Cells treated with 1% DMSO were used as control.

Cellular TS activity evaluation details. Cells used for the enzyme assay were harvested by trypsinisation when they were in an exponential growth phase, washed with PBS and used or stored at -20°C. Cell pellets were thawed by the addition of ice-cold lysis buffer (200 mM Tris-HCl, pH 7.4, 20 mM 2-mercaptoethanol, 100 mM NaF and 1% Triton X-100), sonicated (three x 5 s), and subsequently centrifuged at 14,000 x g for 15 min at 4°C. The supernatant was used for enzyme assays. The TS catalytic assay was conducted as previously reported;[6] the assay determined the catalytic activity of TS by measuring the amounts of ^3H released during the TS catalyzed conversion of [5-^{3}H]dUMP to dTMP. Briefly, the assay consisted of the enzymes in assay buffer (lysis buffer without Triton X-100) and 650 μM mTHF in a final volume of 50 μL. The reaction was started by adding [5-^{3}H]dUMP (1 μM final concentration, specific activity 5 mCi/mol), followed by incubation at 37°C for 60 min and stopped by adding 50 μL of ice-cold 35% trichloroacetic acid. Residual [5-^{3}H]dUMP was removed by adding 250 μL of 10% neutral activated charcoal. The charcoal was removed by centrifugation at 14,000 X g for 15 min at 4°C, and a 150-μL sample of the supernatant was assayed for tritium radioactivity by liquid scintillation counting in the liquid scintillation analyzer Tri-Carb 2100 (Packard). The linearity of [5-^{3}H]dUMP conversion with respect to amount of protein and time was established.

Western Blot analysis details. Western blot assays was used for the identification and the semi-quantification of TS, DHFR, TRAP1 and HSP90. After treatment, cells were washed twice in ice-cold PBS, lysed in RIPA buffer (20 mM TRIS-HCl, pH 7.5; 150 mM NaCl; 1 mM Na_2EDTA; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; 1 mM Na_3VO_4; 1 mM PMSF; Complete Mini Protease

inhibitor cocktail (Roche); and Phosphatase Inhibitor Cocktail l and 2 (Sigma)) and then centrifuged at 14000 x g at 4 °C for 30 min to remove debris.[9]The protein concentration in each lysate was determined using the Bradford protein assay reagent (Sigma-Aldrich). Forty/sixty micrograms of the cell extracts was subsequently loaded on a polyacrylamide gel after denaturation according to the method of Laemmli.[10]After SDS-PAGE, blotting was performed on PVDF membranes (Hybond-P, Amersham). The membranes were blocked in nonfat dry milk (2%) in TBS buffer containing 0.1% Tween-20 at room temperature for 1h. Anti-DHFR (clone A-4, Santa Cruz Biotechnology, 1:250 dilution), anti-TS (clone TS106, Abnova, 1:500 dilution), anti-HSP90 (clone 4F10, Abnova, 1:5000 dilution), and anti-TRAP1 (clone TR-1A, Santa Cruz Biotech, 1:2000 dilution) antibodies were incubated overnight in non-fat dry milk (2-5%) in PBS buffer containing 0.1% Tween-20. After washing with 0.1% TBST, membranes were incubated with a horseradish peroxidase-conjugated with a sheep anti-mouse secondary antibody (Amersham Biosciences, 1:5000 dilution) or with a horseradish peroxidase-conjugated sheep anti-goat antibody (Sigma Aldrich, 1:8000 dilution) for one hour at room temperature. Antibody staining was performed with a chemiluminescent detection system (ECL Plus, Amersham), and the signal was detected with X-ray film (Amersham). Densitometric quantification of the obtained western blot bands was performed using an Epson Photo Perfection 4180 scanner (Epson) and ImageJ densitometry software, v. 1.47 (National Institutes of Health, Bethesda, MD). The protein levels were normalized against the housekeeping protein actin and Red Ponceau.

Statistical analysis details. Data analysis has been performed by means of R program v. 3.3.1.[11]The added packages were “readxl”, “reshape2”, “data.table”, “car” “scales” ,”RColorBrewer” and “ggplot2”, providing support to the application of the methods of analysis. The data didn’t show normality of residuals so the expression variable has been transformed with a power function (newExpr = oldExpr-$^{1.896}$) in order to satisfy the normality assumption, the ANOVA test has been carried out with the new expression variable. Two-way ANOVA test and Tukey HSD multiple comparisons of means have been applied to analyze the difference of mean protein expression modulation caused by each treatment.

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