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Cell penetrating peptides containing 2,5-diketopiperazine (DKP) scaffolds as shuttles for anti-cancer drugs: conformational studies and biological activity

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EXPERIMENTAL PROCEDURES

Reagents

All chemicals, reagents and consumables used in this work were purchased from Acros Organics (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), Applichem (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), Fluka Analytical (Taufkirchen, Germany), IRIS Biotech GmbH (Marktredwitz, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sarstedt (Nürmbrecht, Germany), Sigma-Aldrich (Taufkirchen, Germany) and VWR BDH Prolabo (Radnor, USA), if not specified otherwise. The following side chain protecting groups were used: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg; Trityl (Trt) for Asn; tert-butyloxycarbonyl (Boc) for Lys. For the selective deprotection of side chains also Fmoc-Lys(Dde)-OH was used. Daunorubicin hydrochloride was a gift from Prof. Dr. Gábor Mező (Budapest, Hungary).

For NMR studies all compounds used for the preparation of the buffer (i.e. sodium phosphate mono- and dibasic) as well as deuterated water were purchased from Sigma-Aldrich and d_{25}-SDS (98% deuterated) from Cambridge Isotopes Laboratories.
Synthesis of DKP scaffolds. DKP1 and DKP3 scaffolds were synthesized according to previously reported procedures and their analytical data were in agreement with those already published.[1]

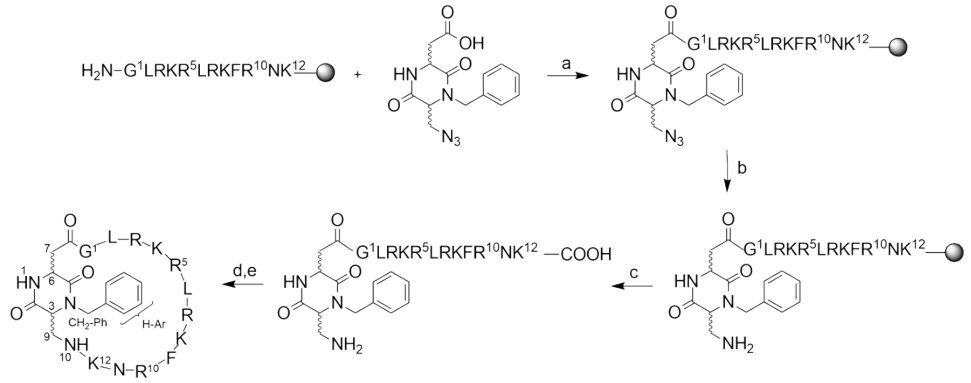
Peptides and conjugates syntheses. In general, the peptides used were synthesized on H-l-Lys(Boc) preloaded 2-Chlorotrityl resin (200-400 mesh, loading: 0.74 mmol/g) by automated solid-phase peptide synthesis (SPPS) on a multiple Syro II peptide synthesizer (MultiSynTech, Witten, Germany) following Fmoc/tBu-strategy using a double-coupling procedure and in situ activation with Oxyma/DIC.

Synthesis of CAP18(106-117) (sC18*). Achieved as described previously.[2]

Synthesis of DKP-sC18* linear peptides (1=DKP1-sC18*; 2=DKP3-sC18*). The linear peptide sC18* was synthesized by automated Fmoc/tBu-based solid-phase peptide synthesis (SPPS). Chlorotrityl resin was used as solid support because of the very mild conditions required to cleave the protected peptide fragment from the resin. Then, the DKP scaffold was manually coupled to the *N*-terminal sC18* peptide chain, using 3 eq. of the reagent (DKP), 3 eq. Oxyma and 3 eq. DIC overnight. The success of the coupling was then controlled by Kaiser test.[3] For the reduction of the azido group, the resin was treated with DTT (2 M) in 500 µL of DCM (Scheme 1).Then DIPEA (1 M) was added and the reaction was left shaking at room temperature for 2 h. Then the solvent was removed and the resin was washed and dried. The peptide was finally cleaved from the resin using TFA:water:TIS (95:2.5:2.5, v/v/v). After 3 h reaction at room temperature, the peptides were precipitated in ice cold diethyl ether and then washed and centrifuged 5 times at 4 C, 5000 rpm. The pellet was lyophilized from water:tert-butyl alcohol (3:1 v/v) and analyzed by RP-HPLC-ESI-MS on a Chromolith ® Performance RP-18e column, 100 x 4.6 mm from Merck (Darmstadt, Germany) using linear gradients of 10-60% B in A (A=0.1% FA in water; B= 0.1% FA in acetonitrile) over 15 minutes and a flow rate of 0.6 ml min-1. Further purification of the peptides was achieved by preparative HPLC, Hitachi Elite LaChrom (VWR, Darmstadt, Germany) on RP18 column Nucleodur C18ec, 100-5 from Macherey-Nagel (Düren, Germany) at 6 ml min-1 and 220 nm detection. Acetonitrile/ water with 0.1% TFA were used as eluents with a gradient of 10-60% ACN in 45 min. The collected fractions were evaporated, analyzed with LC-MS and lyophilized to obtain the purified peptides with purities >95%. **DKP1-sC18*= linear DKP1-sC18*:** ESI-MS (m/z) $C_{83}H_{141}N_{31}O_{17}$: calcd, 1845.3; found 1845.7. **DKP3-sC18*= linear DKP3-sC18*:** ESI-MS (m/z) $C_{83}H_{141}N_{31}O_{17}$: calcd, 1845.3; found 1845.7.

Synthesis of DKP-sC18* cyclic peptides (3, c[DKP1- sC18*]= Cyclic [DKP1-sC18*]; 4, c[DKP3- sC18*]= Cyclic [DKP3-sC18*]). (Scheme 1) For the cyclization the amino acid side chains of the linear analogue synthesized as described above, have to be protected and the cleavage from the resin occurs, consequently, under milder conditions. The resin was treated with a solution of DCM:TFE:Acetic acid 8:1:1 for 2 h at rt. The solution was then transferred to a flask and the resin was washed twice with the same cleavage solution. The solvent was evaporated under reduced pressure and hexane was added to remove acetic acid as azeotrope. The crude was washed and digested with 5% $NaHCO_3$, filtered and washed with distilled H_2O to remove all the salts. After freeze-drying, the product was subjected to the cyclization step. 1 eq of the full protected linear peptide was dissolved in DMF (0.2 mM). DIPEA was added till the solution reached pH 8. BOP (6 eq) and HOBt (6 eq) were added under these conditions. The reaction was left under stirring at rt. After 6 hours, BOP was added again and the reaction was left stirring overnight. For the treatment of the cyclization reaction, the mixture was diluted with EtOAc and extracted with brine and saturated $NaHCO_3$. The organic phase was then dried at reduced pressure, freeze-dried and subjected to full cleavage. The crude was treated with TFA/phenol/water/Thioanisole/EDT 82.5:5:5:5:2.5 (1 ml in total). The reaction was kept under stirring at rt for 3 h. Then the mixture was put in a vial containing 10 ml of cold Et_2O in order to precipitate the peptide. The mixture was centrifuged and washed five times with cold Et_2O. The crude was freeze-dried and then dissolved in H_2O:ACN 90:10 + 0.1% TFA and purified on semipreparative RP-HPLC as previously described. The peptide was freeze-dried from water obtaining a white solid (22% to 36%

yield). **c[DKP1- sC18*]= Cyclic [DKP1-sC18*]:** ESI-MS (m/z) $C_{83}H_{139}N_{31}O_{16}$: calcd, 1827.3; found 1827.7. **c[DKP3- sC18*]= Cyclic [DKP3-sC18*]:** ESI-MS (m/z) $C_{83}H_{139}N_{31}O_{16}$: calcd, 1827.3; found 1827.7.

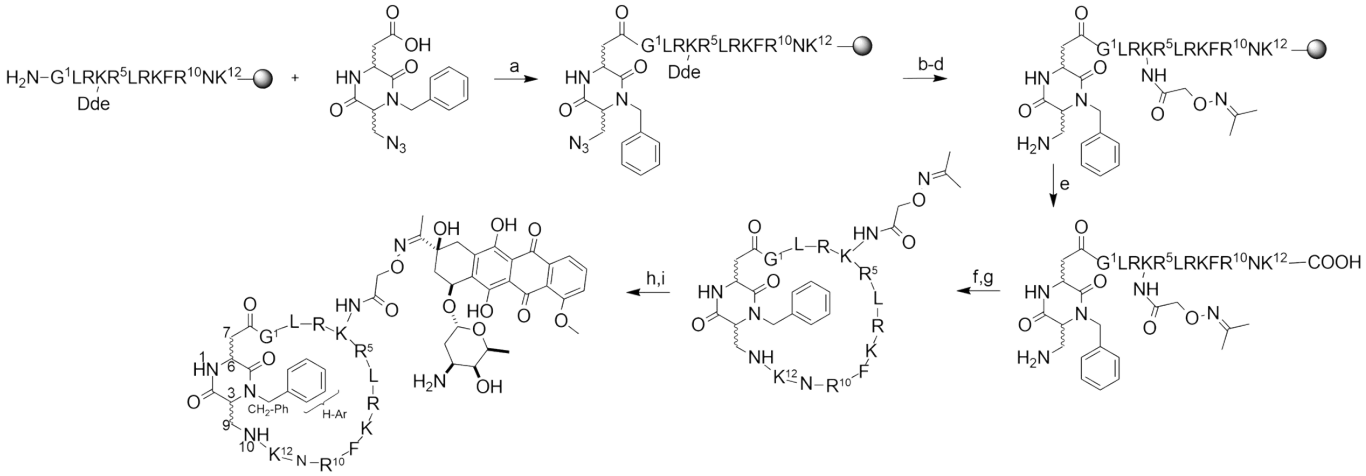


Scheme S1: Synthesis of the **DKP** scaffold-containing linear and cyclic peptides **1-4**. Reaction conditions: a) Oxyma, DIC, overnight; b) DTT (2 M), DIPEA (1 M), DCM; c) acetic acid/TFE/DCM 1/1/8, 2 h; d) BOP (6 eq.), HOBt (6 eq.), DIPEA (12 eq.), DMF (0.2 mM), rt; e) full cleavage with TFA/phenol/H_2O/thioanisol/EDT 82.5/5/5/5/2.5.

Synthesis of Daunorubicin-peptides conjugates

Synthesis of Daunorubicin-conjugated sC18* peptide (5=sC18*(Lys^4-Aoa=Dau)). A molecule of Bis-boc aminooxyacetic acid was coupled to the side chain of lysine 4 (3 eq with oxyma and DIC overnight). The success of the coupling was checked by Kaiser test.[3] The cleavage from the resin occurred with the standard scavengers but, as already described by Mezö et al,[4] 10 eq. of Boc-aminooxyacetic acid were added in the cleavage cocktail in order to avoid the formation of the acetone adduct with mass +40. After precipitation, washing and purification, the peptide was dissolved in ammonium acetate buffer 0.2 M at pH 5 reaching a concentration of 10 mg peptide/ml for the coupling to the drug. Daunorubicin was added in excess of about 30% and the reaction was stirred overnight. In order to remove excess of daunorubicin, the reaction solution was directly injected into the HPLC, Hitachi Elite LaChrom (VWR, Darmstadt, Germany) as before. **5=sC18*(Lys^4-Aoa=Dau):** ESI-MS (m/z) $C_{112}H_{169}N_{33}O_{27}$: calcd 2154.5; found 2155.2.

Synthesis of Daunorubicin-conjugated DKP3-sC18* cyclic peptide (6=c[DKP3-sC18*(Lys^5-Aoa=Dau)]). (Scheme 2) Instead of the bis-boc aminooxyacetic acid as for the linear version, an isopropylidene protected aminooxyacetic acid was coupled manually to the peptide using 3 eq. of the reagent, 3 eq. Oxyma and 3 eq. DIC overnight. This was prepared by stirring for 30 minutes carboxymethoxylamine hemihydrochloride in acetone. The conjugation to the drug occurred after the cyclization and the deprotection of the aminooxyacetic acid with a solution of 1M methoxylamine containing NH_4OAc-buffer (0.2 M, pH 5). **5=c[DKP3-sC18*(Lys^5-Aoa=Dau)]:** ESI-MS (m/z) $C_{98}H_{156}N_{30}O_{25}$: calcd 2409.8; found 2410.5.



Scheme S2: Synthesis of the DKP scaffold-containing daunorubicin conjugate **c[DKP3-sC18*-Dau].** Reaction conditions: a) 3 eq. Oxyma, 3 eq. DIC, overnight; b) 2% hydrazine in DMF (10x); c) 5 eq. >=Aoa, 5 eq. oxyma, 5 eq. DIC, overnight; d) DTT (2 M), DIPEA (1 M), DCM, 3 h; e) acetic acid/TFE/DCM 1:1:8, 2 h; f) BOP (6 eq.), HOBt (6 eq.), DIPEA (12 eq.), DMF (0,2 mM), rt, overnight; g) full cleavage with TFA/phenol/H_2O/thioanisol/EDT 82.5:5:5:5:2.5, 3h; h) Methoxylamine 1 M, NH_4OAc 0.2 M, pH 5; i) 30% excess daunorubicin, 0.2 M NH_4OAc, pH 5, 10 mg/ml.

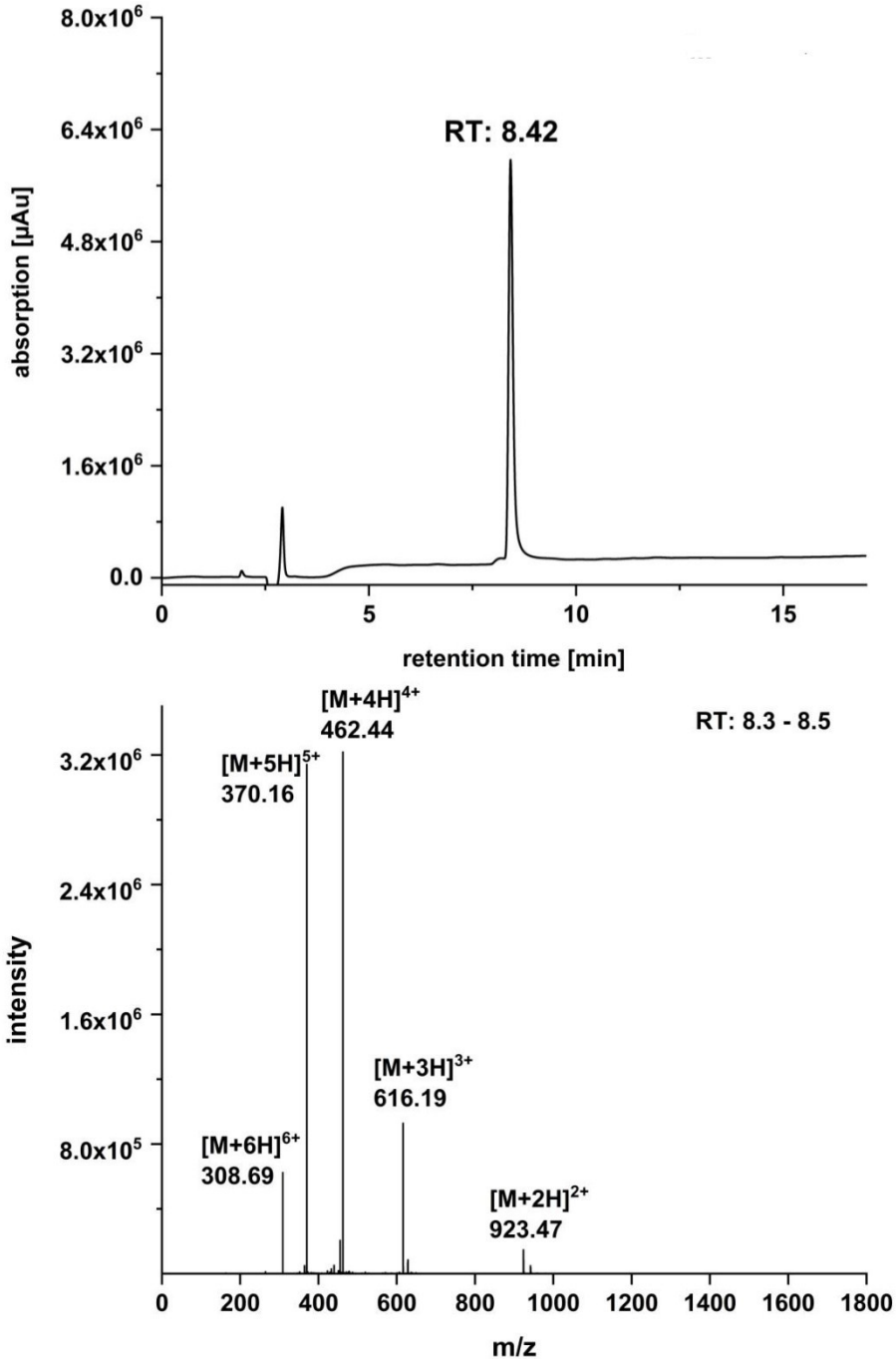


Figure S1. LC-MS analysis of peptide 1 (DKP1-sC18*). MW calculated: 1845.3 g/mol. Purity: 97%. UV chromatogram (220 nm) and ESI-MS mass spectrum. Gradient: 10-60% B in A in 15 min, 0.6 ml/min. A: 0.1% TFA in H_2O; B: 0.1% TFA in ACN.

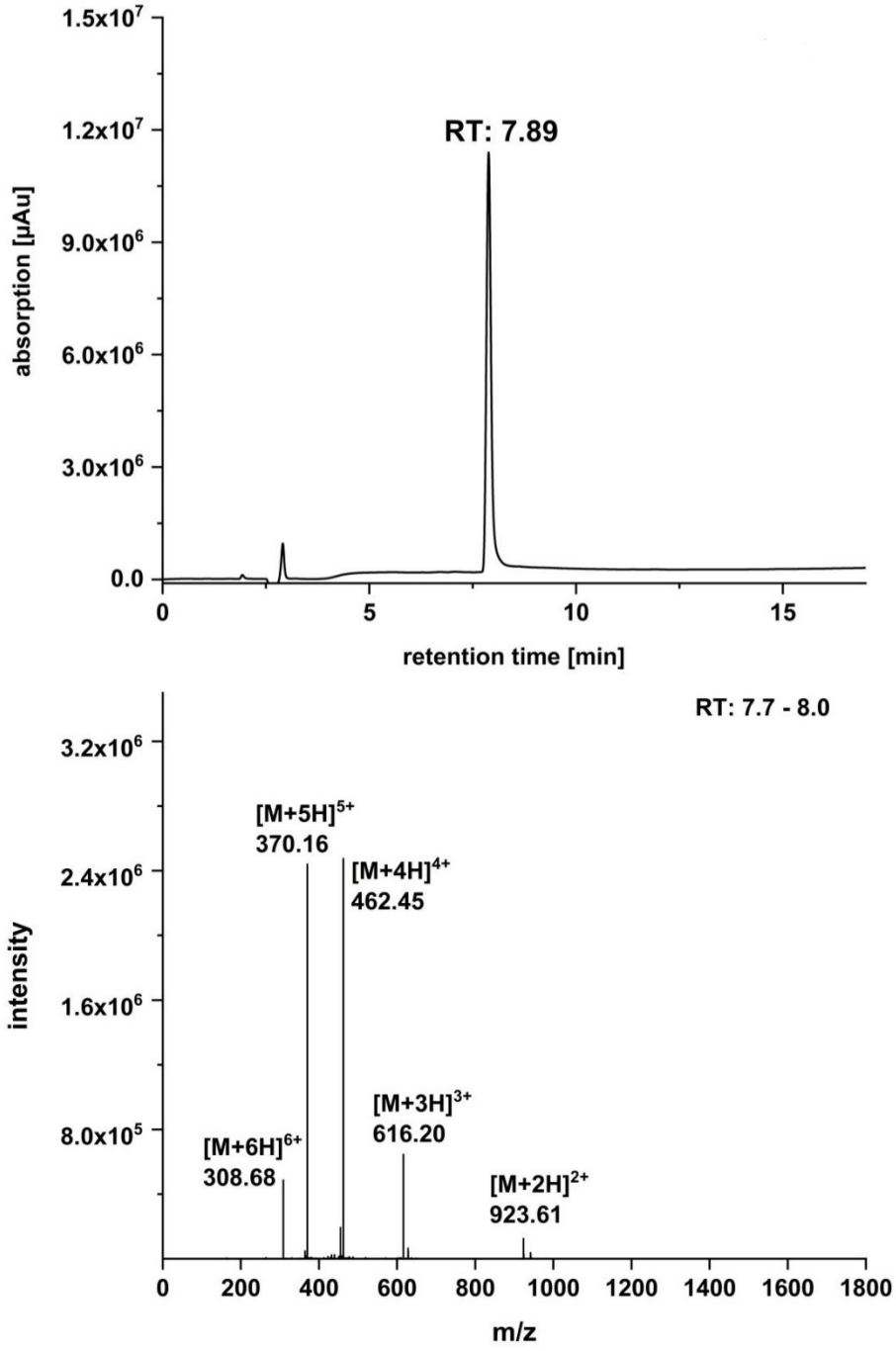


Figure S2. LC-MS analysis of peptide 2 (DKP3-sC18*). MW calculated: 1845.3 g/mol. Purity: 99%. UV chromatogram (220 nm) and ESI-MS mass spectrum. Gradient: 10-60% B in A in 15 min, 0.6 ml/min. A: 0.1% TFA in H_2O; B: 0.1% TFA in ACN.

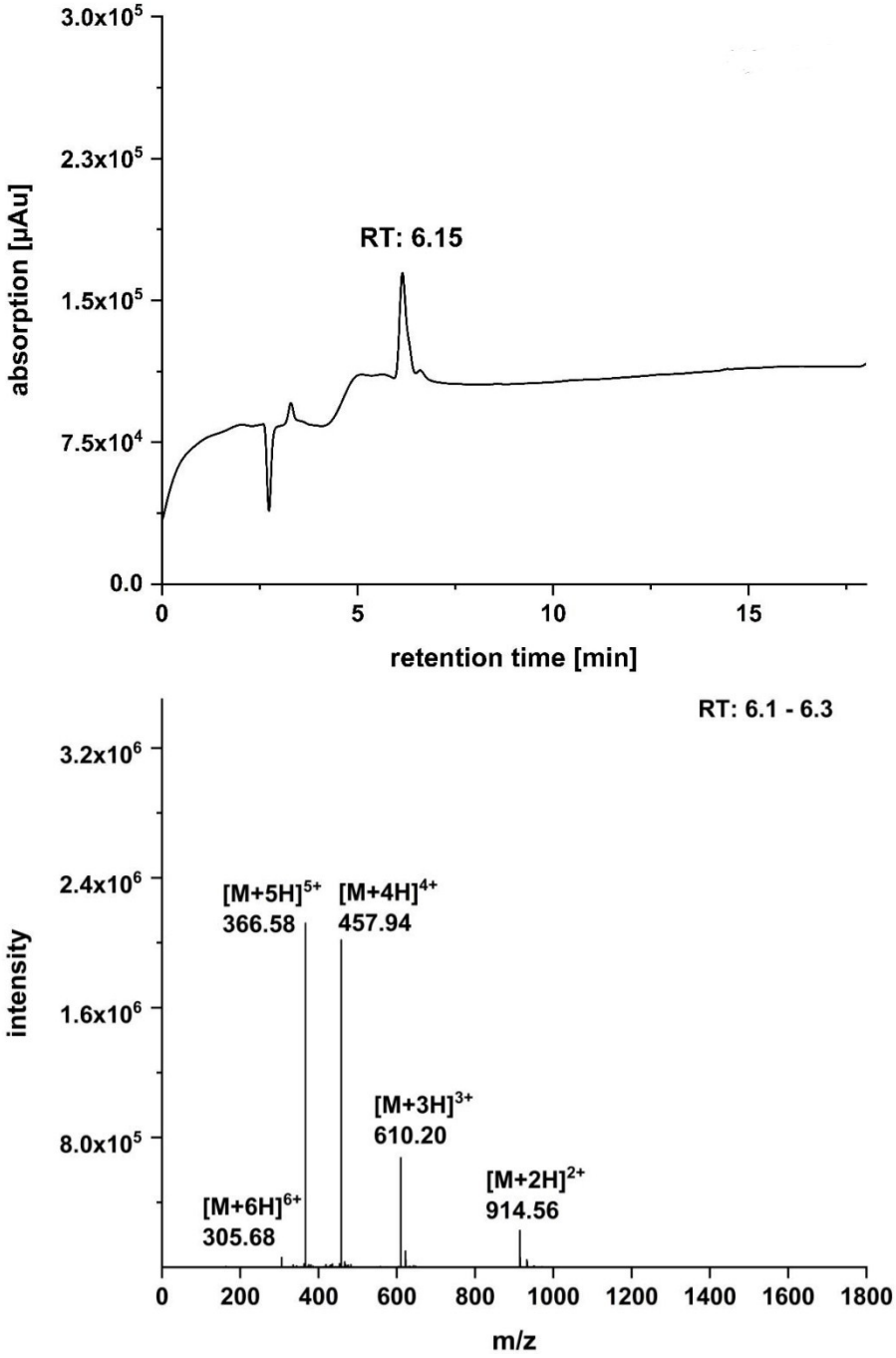


Figure S3. LC-MS analysis of peptide 3 (c[DKP1-sC18*]). MW calculated: 1827.3 g/mol. Purity: 99%. UV chromatogram (195 nm) and ESI-MS mass spectrum. Gradient: 5-55% B in A in 15 min, 0.6 ml/min. A: 0.1% FA in H_2O; B: 0.1% FA in ACN.

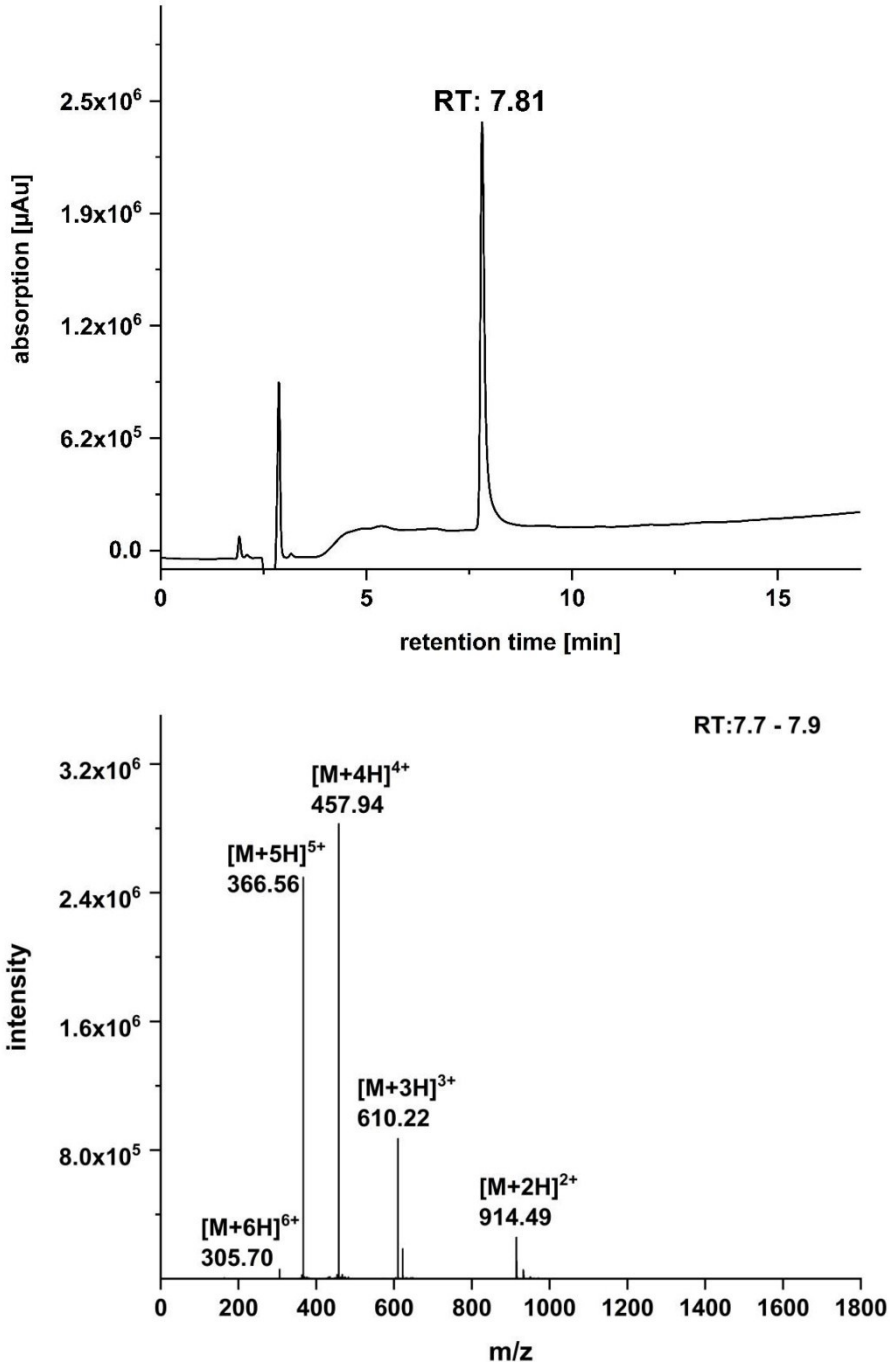


Figure S4. LC-MS analysis of peptide 4 (c[DKP3-sC18*]). MW calculated: 1827.3 g/mol. Purity: >99%. UV chromatogram (220 nm) and ESI-MS mass spectrum. Gradient: 10-60% B in A in 15 min, 0.6 ml/min. A: 0.1% TFA in H_2O; B: 0.1% TFA in ACN.

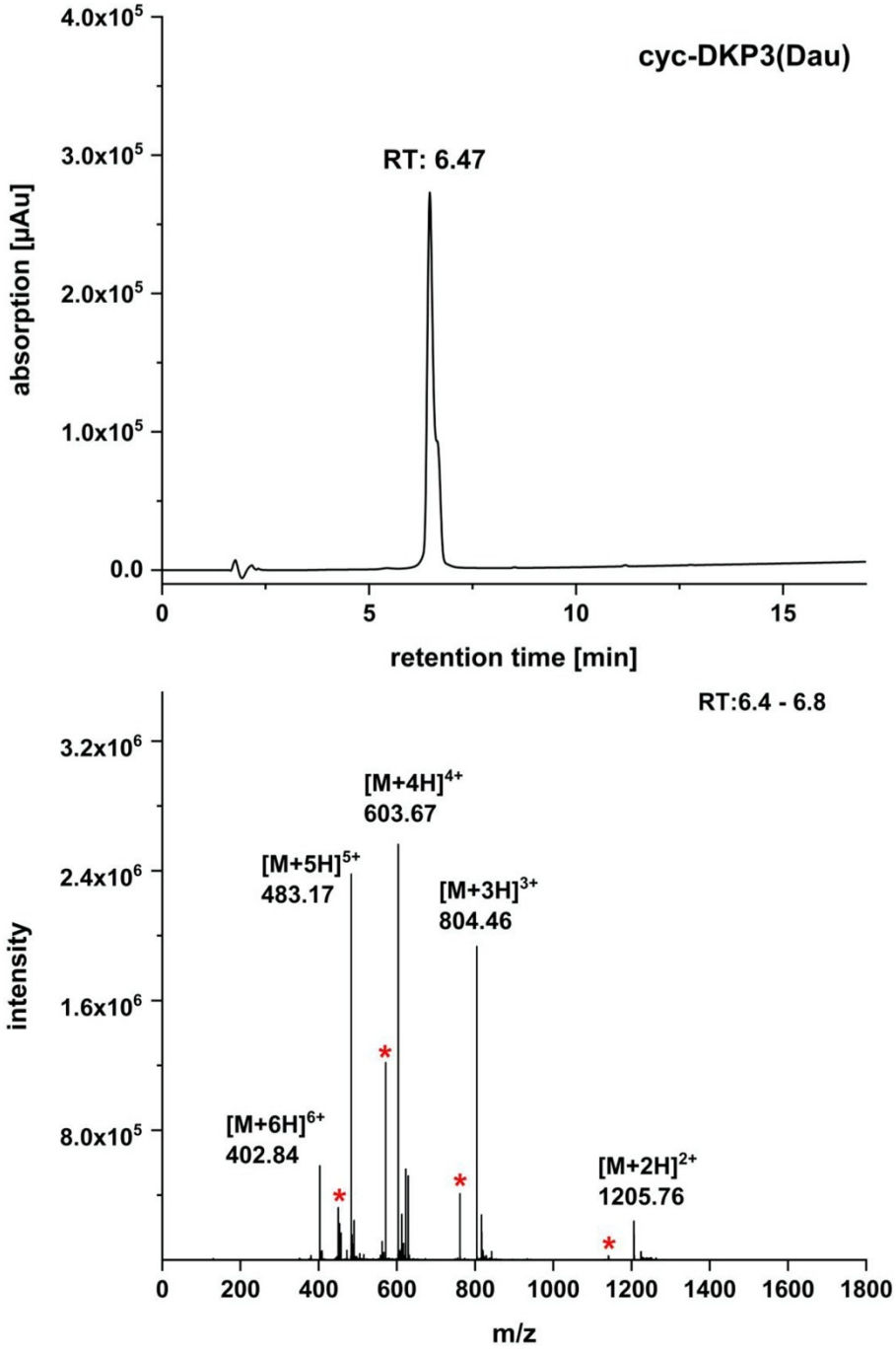


Figure S5. LC-MS analysis of peptide 5 (cyc[DKP3-sC18*(K[4]-Aoa-Dau)]). UV chromatogram (220 nm) and ESI-MS mass spectrum. Gradient: 10-60% B in A in 15 min, 0.6 ml/min. A: 0.1% FA in H_2O; B: 0.1% FA in ACN. Red asterisks: peaks corresponding to the product without daunosamine after mass fragmentation.

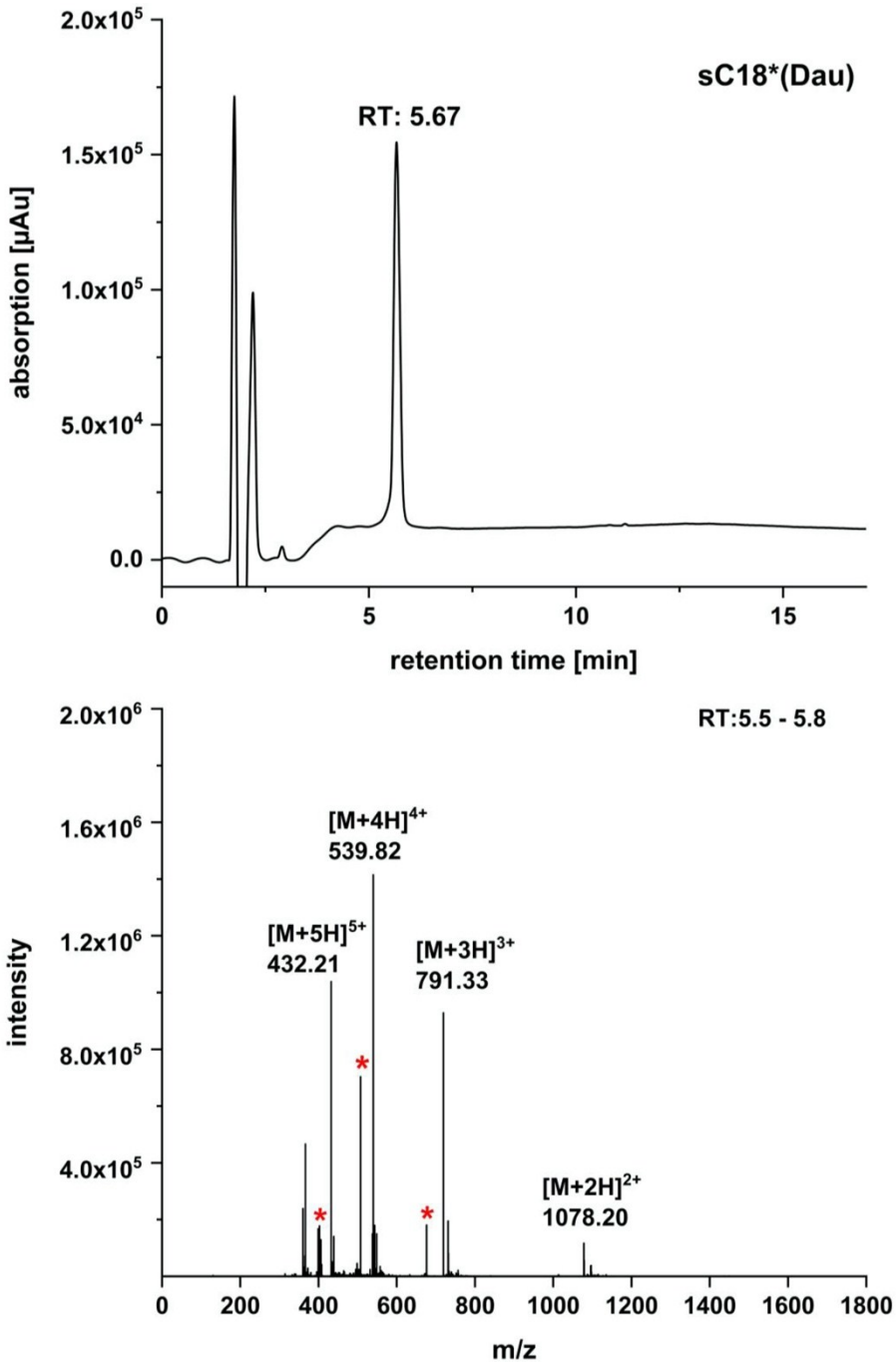


Figure S6. LC-MS analysis of peptide 6 (sC18*(Dau)). UV chromatogram (220 nm) and ESI-MS mass spectrum. Gradient: 10-60% B in A in 15 min, 0.6 ml/min. A: 0.1% FA in H_2O; B: 0.1% FA in ACN. Red asterisks: peaks corresponding to the product without daunosamine after mass fragmentation.

ANALYTICAL METHODS

Circular dichroism (CD) spectroscopy. CD spectra were recorded from 260 nm to 190 nm at 20 C using a Jasco J-715 spectropolarimeter purged with N_2 gas. Peptide samples were dissolved in 10 mM sodium phosphate buffer (pH 7) containing 0 or 50% (v/v) TFE. For measuring CD spectra the peptides were dissolved to a final concentration of 20 μM. Each measurement was repeated 4 times using a sample cell with a path length of 0.1 cm. Instrument parameters were: response time 2 s, scan speed 50 nm/min, sensitivity 100 mdeg, step resolution 0.5 nm and bandwidth 1.0 nm. All CD spectra were corrected by subtraction of the CD spectrum of the solvent.

NMR spectroscopy. DKP-sC18* peptides were dissolved in 200 ul PBS buffer (pH* 6.08, H_2O/D_2O, 9:1, final concentration ca. 1.3 mm, 3 mm NMR tubes). The concentrations of perdeuterated SDS (d_{25}-SDS) samples resulted in a peptide:SDS molecule ratio of 1:60-100.

NMR spectra were recorded on a Bruker Avance II+ spectrometer (^{1}H frequency of 600 MHz) equipped with a triple resonance high-resolution probe (TBI). All NMR data were acquired and processed using Topspin software (Bruker). The transmitter frequency was set on the HDO/H_2O signal, and the d_4-TSP (3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt) was used as chemical shift reference (^{1}H δ_{TSP} = 0 ppm).

For assignment 2D homonuclear spectra (i.e. 2D [^{1}H, ^{1}H]-TOCSY, [^{1}H, ^{1}H]-NOESY) were recorded using standard Bruker pulse sequences including excitation sculpting with gradients for solvent suppression.

The temperature dependence of the amide protons of peptides was calculated from the shift of the amide protons within the range of 283 K-298 K.

The DOSY experiments were performed using standard pulse sequence from Bruker library including water suppression (stebgp1s19). The diffusion delay Δ was set at 100 ms, the gradient strength, g (1 ms) was linearly incremented in 32 steps from 2% to 98% of its maximum value, and a longitudinal eddy currents delay of 5 ms and eight/sixteen scans were recorded for each experiment. Processing was achieved using 8K points in the F2 dimension and 32 points in F1. An exponential window function with 3 Hz line broadening was applied in the F2 dimension prior to Fourier transformation. After baseline correction, the diffusion dimension was processed with the DOSY processing program (Bruker TopSpin software 3.5).[5] A logarithmic scaling was applied in the diffusion axis, and a noise sensitivity factor of 4 and line width factor of 1 were used. The fitting of the diffusion dimension in the 2D-DOSY spectra was obtained using a single exponential fit.

The processed data were analyzed by using CARA software.[6] and the complete assignment of the backbone and side-chain 1H resonances was performed using standard sequential assignment procedures, according to the methodology developed by Wüthrich. [7] Three-dimensional structures were determined by the standard protocol of the CYANA program (version 2.1),[8] using seven cycles of combined automated NOESY assignment and structure calculations followed by a final structure calculation. Since the cyclic peptides contain non-standard residues, the corresponding libraries for CYANA were built using the MOLMOL program.[9] For each CYANA cycle, 1000 randomized conformers and the standard simulated annealing schedule were used. The 20 conformers with the lowest final score were retained for analysis and passed on to the next cycle. Weak restraints on ϕ/ψ–torsion-angle pairs and on side-chain torsion angles between tetrahedral carbon atoms were applied temporarily during the high temperature and cooling phases of the simulated annealing schedule in order to favor the permitted regions of the Ramachandran plot and staggered rotamer positions, respectively.

The list of upper- distance bonds for the final structural calculation consists of unambiguously assigned upper-distance bonds and does not require the possible swapping of diastereotopic pairs. Root-mean-square deviation (rmsd) values were calculated using CYANA for superpositions of the backbone N, Cα, and CO atoms; the heavy atoms over the whole peptide or the cyclic fragment. To obtain the rmsd of a structure represented by a bundle of conformers, all conformers were superimposed upon the first one and the average of the rmsd values between the individual conformers and their average coordinates was calculated.

Molecular modelling. All calculations were run by using the Schrödinger suite of programs (http://www.schrodinger.com) through the Maestro graphical interface.[10]

Molecular mechanic studies on the DKP-containing cyclic peptidomimetics were carried out using MacroModel from Schroedinger software suite.[11] Initial conformations were energy minimized using conjugate gradient method [12] with the OPLS_2005 force field.[13] The convergence criterion was set to 0.05 kJ mol-1 Å-1 on the energy gradient.

An unconstrained study for conformational preferences was then performed starting from this structure using the mixed-mode Metropolis Monte Carlo/Stochastic Dynamics (MC/SD) simulation method [14] with the same force field and convergence criterion. MC/SD simulations were performed at 300 K with the framework of MacroModel release 2019-2. Side-chain dihedral angles were defined as internal coordinate degrees of freedom in the Monte Carlo part of the algorithm. A time step of 0.5 fs was used for the stochastic dynamics (SD) part of the algorithm for 5 ns of simulation time. 51 structures were generated and energy minimized for analysis.

To select starting geometries for conformational search, clustering of these results by using the average linkage method on the basis of atomic rmsd was performed.

CELL CULTURE

All the cell experiments were carried out under a laminar flow hood Herasafe. The temperature (37 C) of the chemicals used was adjusted by Julabo SW22 heating bath. Cell culture was carried out at 5% CO2 at 37 C, using 100x20 mm Petri dishes. The medium used for Hela cells was RPMI, supplemented with 10% fetal bovine serum (FBS) and 2 mm l-Glutamine. For cell culturing Dulbecco´s phosphate buffered saline (PBS 1X) and trypsin-EDTA solution were used. All these reagents were obtained from Sigma Aldrich (St. Louis, USA).

CELL VIABILITY ASSAY

First, a cell suspension with a defined concentration (Hela 4,500 cells per well) was placed in the wells and filled with medium (with FBS) reaching a final volume of 200 µl. The next day, the culture medium was replaced by 100 µl of culture medium (without FBS) with a defined peptide concentration. Cells were incubated for 24 h with the peptide solution. After removing the solvent, 200 µl of fresh medium (with FBS) were added and the cells were incubated for further 48 h. Subsequently, the medium was removed and the cells were incubated with 10 µl resazurin in 90 µl medium (without FBS) for 1 h. As negative and positive controls untreated cells and cells treated 10 min with 70% EtOH in H2O were used. The fluorometrical measurement was performed on a Tecan infinite M200 (Männedorf, Switzerland) at 596 nm with excitation at 550 nm.

PEPTIDE INTERNALIZATION STUDIES

Peptide internalization studies by flow cytometry. Cells were seeded in a 24-well plate (Hela: 100,000 cells per well) and grown to 70–80% confluency. After incubation at 37°C for 30 min with labeled peptides (daunorubicin) in serum-free medium, the cells were washed twice with PBS, detached with indicator-free trypsin and resuspended in serum containing-RPMI medium. The cells suspension was moved into a 96-well FACS plate and the fluorescence was then measured by a Guava ® easyCyte flow cytometer (Merck) where 10,000 viable cells were counted. Cellular autofluorescence was subtracted. The experiments were performed twice in triplicate.

Confocal laser scanning microscopy. Cells were seeded in an eight-well (Ibidi) plate (30,000 cells per well) and grown to 70–80% confluency. The next day, cells were incubated with daunorubicin-labeled peptides in serum-free medium for 30 min at 37 °C. Because of a modest but detectable toxicity, the concentration of both peptides had to be lowered from 10 µM to 5 µM for these studies. Nuclei were stained for the last 10 min of incubation with Hoechst33342 nuclear dye. Finally, the solution was removed and cells were treated with 200 µl trypan blue solution (150 mM in 0.1 M acetate buffer, pH 4.15) for 15 s to quench all external fluorescence. After washing once with serum-free medium and adding fresh medium, images were taken by using a Leica SP8 confocal laser scanning microscope equipped with a 63x oil immersion objective. Images were recorded with Leica Mycrosystems software and adjusted with Fiji software.

STABILITY ASSAYS

Stability test with trypsin. A peptide solution (100 µM) in 0.1 M aqueous ammonium bicarbonate solution was prewarmed to 37 °C and incubated together with a trypsin solution (peptide / trypsin 500: 1 (w / w)) also preheated to 37 °C. The reaction was carried out in 1.5 ml reaction vessels in a thermomixer at 37 °C, shaking (1200 rpm). At defined times (5/15/30 min) 20 µl aliquots were taken and 3 µl of 10% formic acid were added to inactivate the enzyme by acidification. Samples were stored at -80 °C until analysis by LC-ESI-MS. The relative abundance of starting peptide and the correspondent fragments in the ion current was measured by integrating the peak area of the sequence of interest and calculating the percentage on the total surface area.

Stability test with cell culture supernatant. Supernatant from a HeLa cell culture, grown for 24 h in RPMI medium with FBS, was removed and stored at 37 °C until prompt use for the stability test. Peptides (1 mM stock solution) were diluted with the cell culture supernatant to 100 µM and incubated at 37 °C, shaking (500 rpm) in a thermomixer. At defined times (1/2 h), 20 µl aliquots were taken and 3 µl of 10% TFA were added. The relative abundance of starting peptide and the correspondent fragments in the ion current was measured by integrating the peak area of the sequence of interest and calculating the percentage on the total surface area.

Table S1. ^{1}H chemical shifts for **1 DKP1-sC18*** in 50 mM phosphate buffer (600 MHz, pH 6.08, water:D_2O 9:1, d_4-TSP ^{1}H δ=0 ppm).

Residue	^{1H} δ (ppm) 283 K				^{1H} δ (ppm) 298K		Temperature coefficient (ppb/K)
	HN	H^{α}		Others	HN	H^{α}	
Gly[1]	8.727	4.019, 3.973			8.633	4.019, 3.955	-6.30
Leu[2]	8.331	4.298	β γ δ	1.621 1.609 0.895, 0.858	8.221	4.298	-7.30
Arg[3]	8.483	4.286	β γ δ ε	1.832, 1.743 1.642 3.185 7.361	8.321	4.286	-10.8
Lys[4]	8.410	4.253	β γ δ ε	1.781 1.447 1.701 2.980	8.257	4.253	-10.2
Arg[5]	8.505	4.309	β γ δ ε	1.831, 1.782 1.646 3.188 7.377	8.365	4.309	-10.2
Leu[6]	8.467	4.353	β γ δ	1.657 1.586 0.948, 0.889	8.312	4.353	-9.60
Arg[7]	8.488	4.299	β γ δ ε	1.745 1.648 3.177 7.341	8.342	4.299	-9.70
Lys[8]	8.454	4.253	β γ δ ε	1.750, 1.689 1.283, 1.336 1.570 2.967	8.309	4.253	-9.70
Phe[9]	8.439	4.626	β ***o*** 7.270, ***m*** 7.355, ***p*** 7.328	3.032, 3.132	8.299	4.626	-9.30
Arg[10]	8.371	4.313	β γ δ ε	1.725, 1.824 1.576 3.182 7.320	8.275	4.313	-6.60
Asn[11]	8.558	4.647	β δ	2.774, 2.872 7.766, 7.039	8.459	4.647	-6.60
Lys[12]	7.996	4.150	β γ δ ε	1.725, 1.818 1.369 1.678 2.975	7.877	4.150	-7.90
DKP1			**H3** **H6** **H7** **H9** **CH_2Ph** **CH_2Ph**	4.445 4.570 3.121, 2.949 3.484, 3.439 4.692, 4.855 7.378, 7.433, 7.404			

Table S2. ^{1}H chemical shifts for **2 DKP3-sC18*** in 50 mM phosphate buffer (600 MHz, pH 6.08, water:D_2O 9:1, d_4-TSP 1H δ=0 ppm).

Residue	^{1H} δ (ppm) 283 K				^{1H} δ (ppm) 298K		Temperature coefficient (ppb/K)
	HN	H^{α}		Others	HN	H^{α}	
Gly[1]	8.557	3.959			8.432	3.946	-8.33
Leu[2]	8.296	4.339	β δ	1.614 0.896	8.184	4.324	-7.47
Arg[3]	8.515	4.291	β γ δ ε	1.747 1.556 3.170 2.980	8.389	4.279	–8.40
Lys[4]	8.433	4.257	β γ δ ε	1.785 1.418 1.720 2.980	8.277	4.365	-10.4
Arg[5]	8.481	4.360	β γ δ ε	1.664 1.581 3.194 7.252	8.382	4.299	-6.60
Leu[6]	8.463	4.270	β γ δ	1.645 1.569 0.932, 0.851	8.313	4.350	-10.0
Arg[7]	8.519	4.294	β γ δ ε	1.801, 1.741 1.614 3.171 7.252	8.336	4.371	-12.2
Lys[8]	8.468	4.264	γ δ ε	1.349 1.660 3.188	8.307	4.247	-10.7
Phe[9]	8.444	4.628	β ***o*** 7.270, ***m*** 7.355, ***p*** 7.328	3.126, 3.030	8.301	4.635	-9.53
Arg[10]	8.378	4.318	β γ δ ε	1.836, 1.731 1.574 3.199 7.255	8.288	4.314	-6.00
Asn[11]	8.564	4.658	β δ	2.868, 2.778 7.768, 7.044	8.457	4.663	-7.13
Lys[12]	8.007	4.140	β γ δ ε	1.833, 1.727 1.400 1.673 2.861	7.881	4.144	-8.40
DKP3			**H3** **H6** **H7** **H9** **CH_2Ph**	4.276 4.683 3.136, 2.959 3.506 5.153, 4.391			

Table S3. ^{1}H chemical shifts for **3 c(DKP1-sC18*)** in 50 mM phosphate buffer (600 MHz, pH 6.08, water:D_2O 9:1, d_4-TSP ^{1}H δ=0 ppm).

Residue	^{1H} δ (ppm) 283 K				^{1H} δ (ppm) 298K		Temperature coefficient (ppb/K)
	HN	H^{α}		Others	HN	H^{α}	
Gly[1]	8.643	3.975			8.556	3.963	-5.80
Leu[2]	8.444	4.349	β	1.609	8.316	4.334	-8.50
			γ	1.696			
			δ	0.851			
Arg[3]	8.445	4.308	β	1.850, 1.748	8.357	4.309	-5.87
			γ	1.607			
			δ	3.168			
			ε	7.391			
Lys[4]	8.349	4.232	β	1.660, 1.630	8.217	4.369	-8.80
			γ	1.380			
			δ	1.781			
			ε	2.967			
Arg[5]	8.380	4.223	β	1.845, 1.763	8.324	4.242	-3.73
			γ	1.606			
			δ	3.187			
			ε	7.396			
Leu[6]	8.360	4.378	β	1.714	8.190	4.377	-11.3
			γ	1.626			
			δ	0.891, 0.947			
Arg[7]	8.427	4.231	β	1.834, 1.749	8.315	4.337	-7.50
			γ	1.560			
			δ	3.173			
			ε	7.343			
Lys[8]	8.293	4.169	γ	1.146	8.172	4.179	-8.10
			δ	1.610			
			ε	2.895			
Phe[9]	8.134	4.689	β	3.185, 3.014	8.039	4.690	-6.30
Arg[10]	8.372	4.231	β	1.625, 1.602	8.284	4.240	-6.00
			γ	1.807			
			δ	3.171			
			ε	7.401			
Asn[11]	8.504	4.660	β	2.809, 2.852	8.387	4.664	-7.80
			δ	7.742, 7.029			
Lys[12]	8.322	4.367	β	1.672	8.210	4.234	-7.50
			γ	1.381			
			δ	1.809			
			ε	2.955			
DKP1			**H3**	4.168			
			H6	4.587			
			H7	2.918			
			H9	3.775			
			H10	8.418			
			CH_2Ph	4.366, 5.092			

Table S4. ^{1}H chemical shifts for **4 c(DKP3-sC18*)** in 50 mM phosphate buffer (600 MHz, pH 6.08, water:D_2O 9:1, d_4-TSP ^{1}H δ=0 ppm).

Residue	^{1H} δ (ppm) 283 K				^{1H} δ (ppm) 298K		Temperature coefficient (ppb/K)
	HN	H^{α}		Others	HN	H^{α}	
Gly[1]	8.474	3.848, 4.074			8.370	3.849, 4.067	-6.90
Leu[2]	8.360	4.352	β	1.711	8.232	4.351	-8.50
			γ	1.608			
			δ	0.879, 0.927			
Arg[3]	8.466	4.250		1.734, 1.838	8.360	4.254	-7.10
			β	1.592			
			δ	3.104			
			ε	7.363			
Lys[4]	8.334	4.148	β	1.755, 1.812	8.208	4.172	-8.40
			γ	1.391			
			δ	1.653			
			ε	2.962			
Arg[5]	8.416	4.246	β	1.809	8.310	4.240	7.10
			γ	1.598			
			δ	3.164			
			ε	7.356			
Leu[6]	8.347	4.382	β	1.734	8.188	4.394	-11.3
			γ	1.630			
			δ	0.896, 0.965			
Arg[7]	8.374	4.219	β	1.792	8.288	4.239	-5.70
			γ	1.573			
			δ	3.173			
Lys[8]	8.347	4.171	γ	1.126	8.187	4.166	-10.7
			δ	1.571			
			ε	2.883			
Phe[9]	8.132	4.706	β	3.026, 3.228	8.030	4.706	-6.80
			o	7.269, ***m*** 7.372			
Arg[10]	8.361	4.226	β	1.805	8.281	4.235	-5.30
			γ	1.582			
			δ	3.162			
			ε	7.415			
Asn[11]	8.570	4.645	β	2.830, 2.881	8.449	4.647	-8.10
			δ	7.046, 7.789			
Lys[12]	8.246	4.334	β	1.758, 1.848	8.138	4.338	-7.20
			γ	1.378, 1.454			
			δ	1.663			
			ε	2.965			
DKP3			**H1**	8.445			
			H3	4.030			
			H6	4.607			
			H7	2.891, 3.026			
			H9	3.738, 3.877			
			H10	8.531			
			CH_2Ph	4.162, 5.280			
			**CH_2Ph**	7.330			

Table S5. 1H chemical shifts for **1** (**DKP1-sC18***), **2** (**DKP3-sC18***) and **3** (**c[DKP3-sC18*]**) in 50 mM phosphate buffer and d_{25}-SDS micelles (600 MHz, 298 K, pH 6.08, water:D_2O 9:1, d_4-TSP 1H δ=0 ppm).

Residue	^{1H} δ (ppm) DKP1-sC18*				^{1H} δ (ppm) DKP3-sC18*				^{1H} δ (ppm) c[DKP1-sC18*]			
	HN	**Hα**		**Others**	**HN**	**Hα**		**Others**	**HN**	**Hα**		**Others**
Gly[1]	8.755	3.937			8.326	3.963, 4.130			8.418	3.978		
Leu[2]	7.799	4.053	β	1.823, 1.697	8.155	4.071	β	1.830	8.392	4.303	β	1.833
			γ	1.611			γ	1.644			γ	1.660
			δ	0.804			δ	0.908, 0.748			δ	0.854
Arg[3]	8.207	3.837	β	1.918, 1.691	8.277	3.911	β	1.938, 1.823	7.670	4.404	β	1.906, 1.763
			γ	1.623			γ	1.685			γ	1.608
			δ	3.141			δ	3.188			δ	3.270
			ε	7.232			ε	7.238			ε	7.266
Lys[4]	7.694	4.001	β	1.919, 1.756	7.690	4.068	β	1.874, 1.676	8.306	4.304	β	1.824
			γ	1.394			γ	1.419			γ	1.459
			δ	1.669			δ	1.491			δ	1.710
			ε	3.183			ε	3.003			ε	3.026
Arg[5]	7.845	4.147	β	1.872	7.873	4.189	β	1.951, 1.777	7.862	4.410	β	1.901, 1.760
			γ	1.670			γ	1.601			γ	1.605
			δ	3.176			ε	7.162			δ	3.263
			ε	7.153							ε	7.086
Leu[6]	8.052	4.164	β	1.954, 1.827	7.962	4.202	δ	0.912	7.922	4.239	β	1.696
			γ	1.668							γ	1.621
			δ	0.911							δ	0.895
Arg[7]	7.952	4.064	β	1.953, 1.827	7.853	4.108	β	1.898	7.771	4.268	β	1.782
			γ	1.698			γ	1.676			γ	1.579
			δ	3.181			δ	3.186			δ	3.160
			ε	7.305			ε	7.160			ε	7.057
Lys[8]	7.644	4.104	β	1.969, 1.828	7.686	4.110	β	1.811	7.886	4.334	β	1.712
			γ	1.341			γ	1.356			γ	1.266
			δ	1.664			δ	1.640			δ	1.623
			ε	2.945			ε	2.977			ε	2.992
Phe[9]	7.892	4.345	β	3.165	7.904	4.421	β	3.236, 3.178	7.983	4.552	β	3.202, 3.019
			o 7.298, ***m*** 7.235								***o*** 7.325, ***m*** 7.210	
Arg[10]	7.301	4.162	β	1.965, 1.759	7.883	4.216	β	1.805	7.980	4.429	β	1.979, 1.692
			γ	1.630			γ	1.667			γ	1.479
			δ	2.939			δ	3.186			δ	3.214
			ε	7.302							ε	7.169
Asn[11]	7.955	4.688	β	2.877, 2.688	8.024	4.718	β	2.889, 2.698	8.338	4.518	β	3.265, 2.783
			δ	7.558, 6.850			δ	7.562, 6.867			δ	7.596, 6.931
Lys[12]	7.650	4.105	β	1.892, 1.810	7.680	4.140	β	1.802	7.695	4.655	β	1.827
			γ	1.351			γ	1.334			γ	1.437
			δ	1.679			δ	1.631			δ	1.716
			ε	3.173			ε	2.951			ε	3.017
	DKP1				**DKP3**		**H1**	8.330	**cDKP1**		**H1**	8.419
			H3	4.469			**H3**	4.327			**H3**	4.216
			H6	4.553			**H6**	4.817			**H6**	4.602
			H7	2.942			**H7**	2.952			**H7**	2.904, 2.530
			H9	3.526			**H9**	3.447, 3.374			**H9**	3.808, 3.477
			CH_2Ph	4.962, 4.327			**CH_2Ph**	4.96, 4.551			**H10**	8.135
			CH_2Ph	7.379, 7.325, 7.250			**CH_2Ph**	7.409, 7.343			**CH_2Ph**	5.563, 4.058
											CH_2Ph	7.278

Table S6. Summary of structural statistics for the 20 final energy-minimized CYANA structures of peptides **1-4.**

Parameter	DKP1-sC18*			DKP3-sC18*			c(DKP1-sC18*)			c(DKP3-sC18*)	
	1[a]	1[b]	1[c]	2[a]	2[b]	2[c]	3[a]	3[b]	3[c]	4[a]	4[b]
NOE upper distance limits											
Short-range |i-j|≤ 1	98	102	157	122	104	139	145	80	167	157	113
Medium-range 1<|i-j|≤ 5	0	0	36	0	1	27	8	10	6	16	17
Long-range |i-j|≥5	0	7	0	0	0	3	16	8	15	10	20
Violations > 0.2Å	0	0	0	0	0	0	0	0	0	0	0
CYANA target function (Å²)	0.98	0.98	0.98	0.58	0.58	0.58	0.69	0.69	0.73	0.60	0.59
Rmsd to mean co-ordinates (Å)											
Backbone N, Cα, CO	2.93	2.53	0.85	2.61	2.48	1.06	0.89	1.09	1.54	1.00	0.72
All heavy atoms of residues	4.44	4.17	1.82	4.19	4.14	1.90	2.06	2.44	3.06	2.29	1.95
Ramachandran plot statistics											
Residues in most favored (%)	70.5	60.5	58.9	57.0	67.5	72.4	51.0	33.5	43.0	48.0	46.0
Residues in additionally (%)	29.5	39.5	40.8	43.0	32.5	27.6	48.5	65.0	56.0	51.5	52.0
Residues in generously (%)	0.0	0.0	0.5	0.0	0.0	0.0	0.5	1.5	0.5	0.5	2.0
Residues in disallowed (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0

[a] 50 mM phosphate buffer ,pH 6.08, water:D_2O 9:1, 283 K; [b] 50 mM phosphate buffer ,pH 6.08, water:D_2O 9:1, 298 K; [c]50 mM phosphate buffer and d_{25}-SDS micelles, pH 6.08, water:D_2O 9:1, 298 K.

Table S7. Results summary obtained after analysis of CD data of **1-4** with Dichroweb[a]. Programmes used were Contin-LL and CDSSTR with different reference data sets. Shown is the average of all the results obtained.

Peptide	Conformation	Phosphate buffer	50% TFE
linDKP1 (1)	α-helix	7	40
	β-sheet	9	16
	turns	8	18
	unordered	76	26
linDKP3 (2)	α-helix	4	37
	β-sheet	19	15
	turns	14	19
	unordered	63	29
cycDKP1 (3)	α-helix	5	11
	β-sheet	29	20
	turns	21	16
	unordered	45	53
cycDKP3 (4)	α-helix	7	10
	β-sheet	34	32
	turns	23	22
	unordered	37	36

[a] according to http://dichroweb.cryst.bbk.ac.uk.

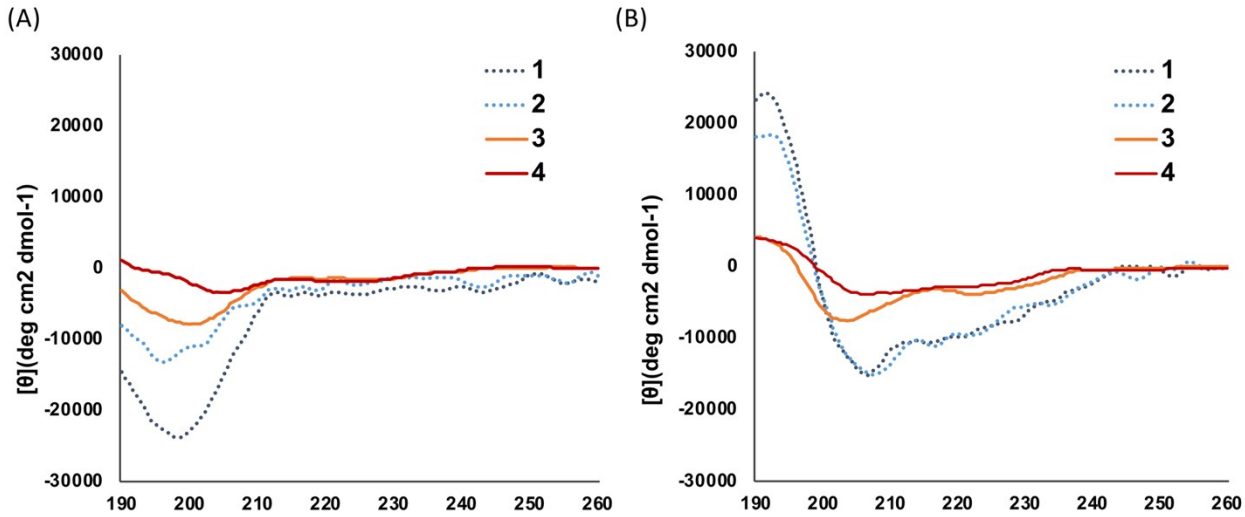


Figure S7. Circular dichroism spectra of peptides 1-4 (20 μM peptide concentration) in 10 mM phosphate buffer pH 7 (A) and in presence of 50% TFE (B) at 20 °C.

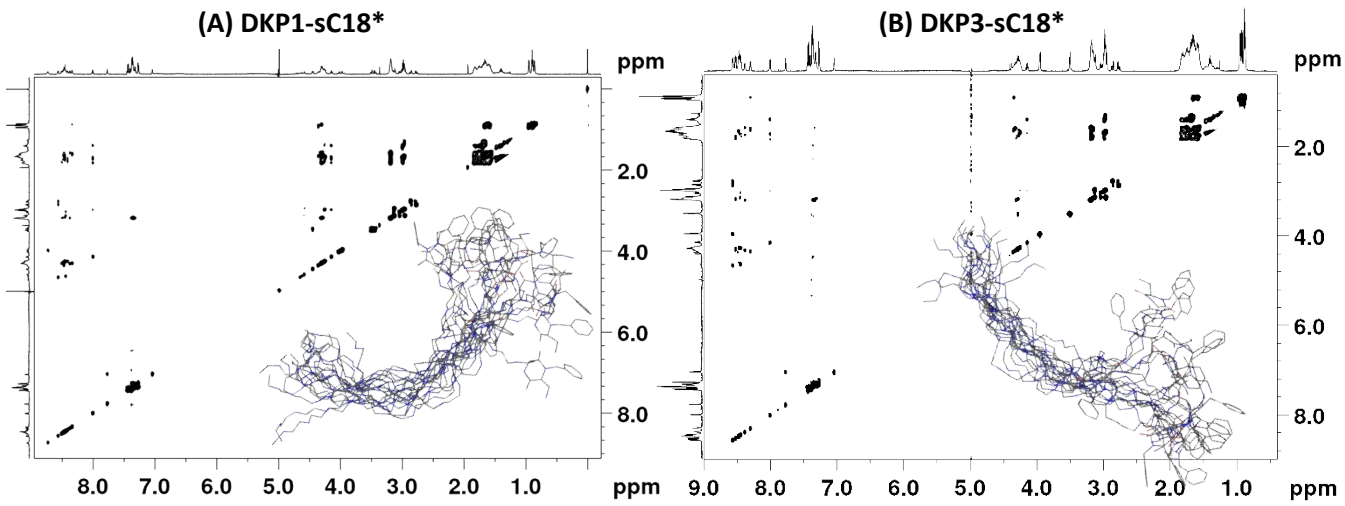


Figure S8. 2D ^{1}H,^{1}H-TOCSY spectra of linear peptides **1** (A) and **2** (B) in solution at r.t. (50mM phosphate buffer, pH 6.08, water:D_2O 9:1, mixing time 200 ms, 283K, 600 MHz) with integrated ensemble of 10 final energy-minimized superimposed CYANA structures.

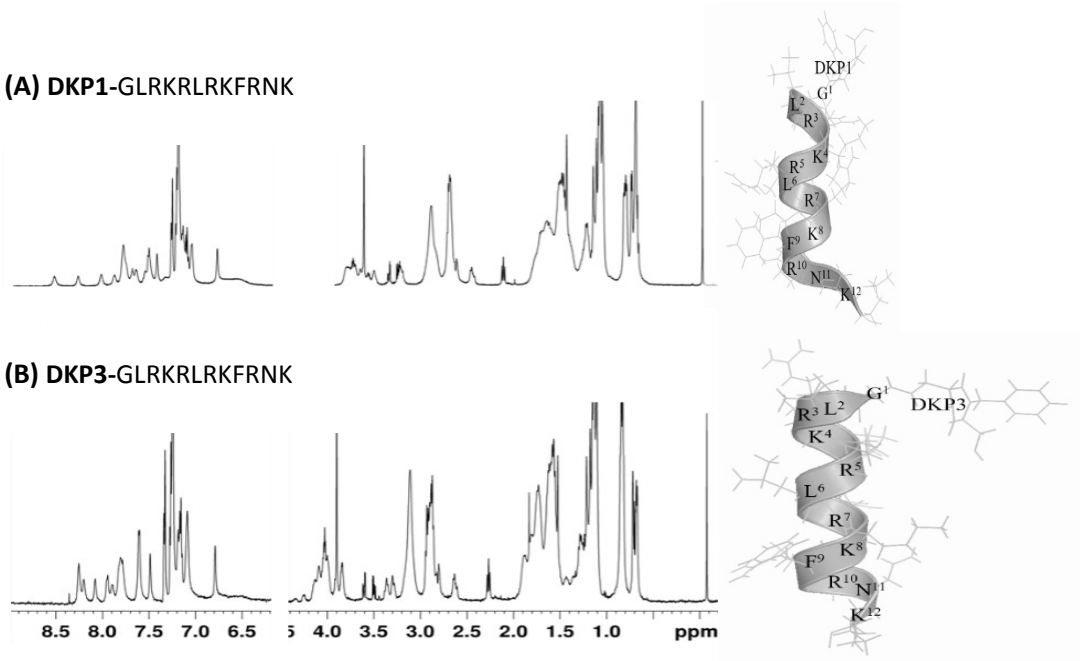


Figure S9. 1D ^{1}H NMR spectra of linear peptides in the presence of d_{25}-SDS micelles (A) **1**, **DKP1-sC18*** and (B) **2**, **DKP3-sC18*** (peptide concentration ca. 1.3 mM, peptide:SDS 1:80, 50 mM phosphate buffer, pH 6.08, water:D_2O 9:1, 298 K, 600 MHz) (*left*). Ribbon drawing of one representative conformer of the NMR solution structure of the peptides (*right*).

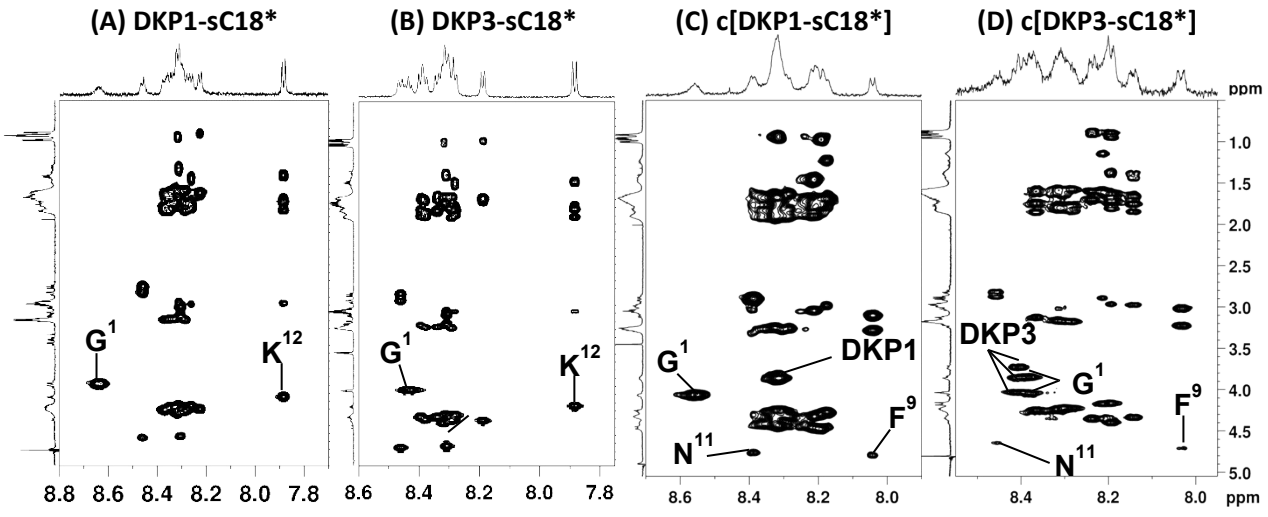


Figure S10. Excerpts of the fingerprint region (i.e., amide-aliphatic region) of the 2D ^{1}H-^{1}H-TOCSY spectra of linear peptides **1** (A) and **2** (B) just as cyclic peptides **3** (C) and **4** (D) in solution at r.t. (50mM phosphate buffer, pH 6.08, water:D_2O 9:1, mixing time 200 ms, 298K, 600 MHz).

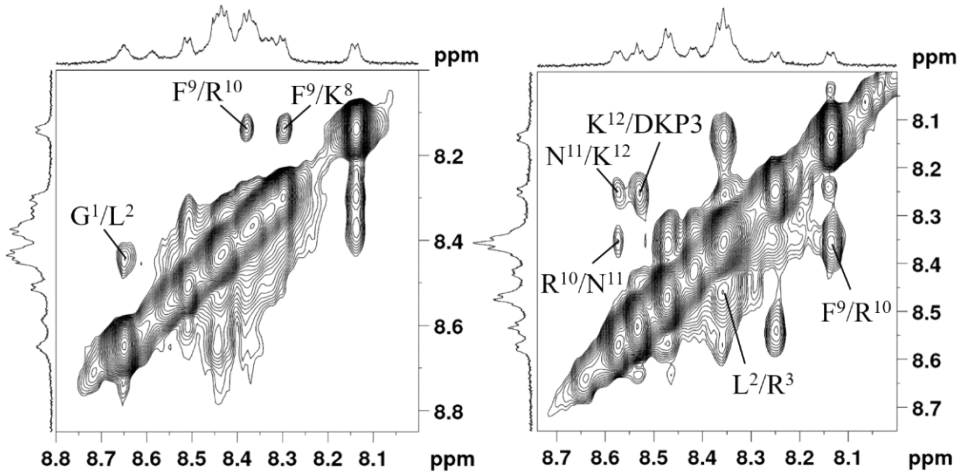


Figure S11. Excerpts of the amide-amide region of the 2D 1H,1H-NOESY spectra of 3 (A) and **4** (B) at 283 K in aqueous medium (50mm phosphate buffer, pH 6.08, water:D_2O 9:1, mixing time 200ms, 600 MHz).

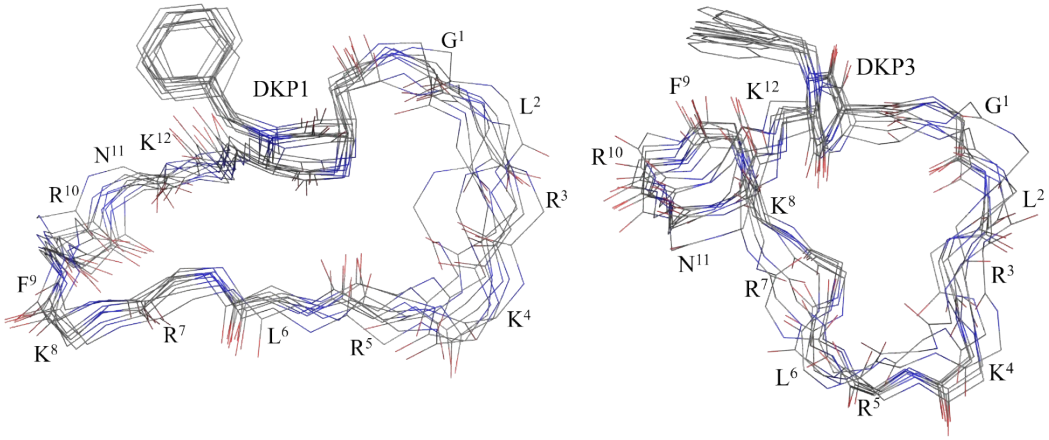


Figure S12. Overlay of the ensemble of 10 final energy-minimized CYANA structures of peptide **3** (left) and **4** (right) in solution.

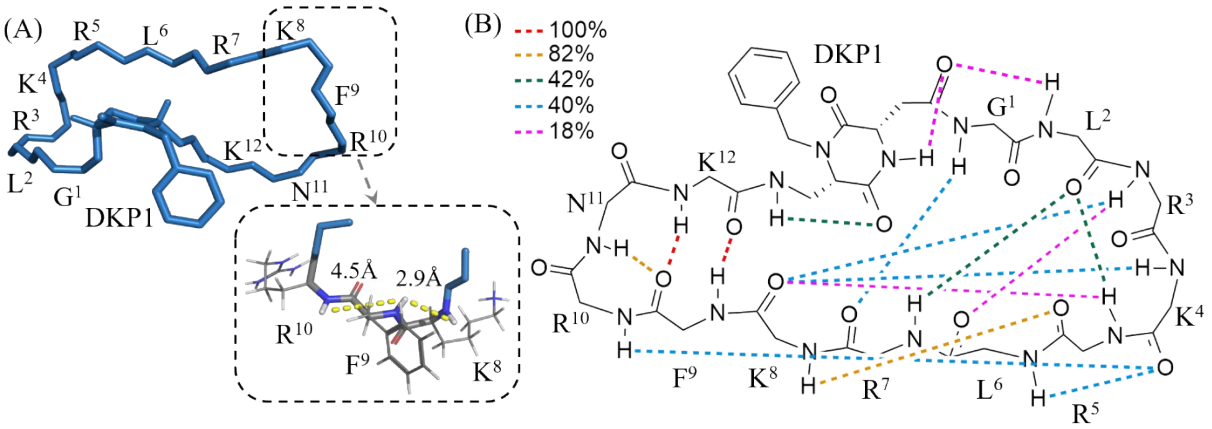


Figure S13. (A) a representative NMR derived structure of **3** in water at 283 K and a closer view (zoom) of the K^8-K^{12} fragment. According to the NMR experimental data the (volume amide-amide cross peaks NOE and correlated) internuclear amide-amide distances are indicated. (B) Preferred intramolecular hydrogen-bonded pattern proposed for calculated structures of **3**. The probability of occurrence of the hydrogen bonds (dotted lines) is color-coded as indicated in the legend (e.g. in 100% of structures hydrogen bonding between C^2O-K^{12}/NH-F^9 and C^2O-F^9/NH-K^{12} was observed).

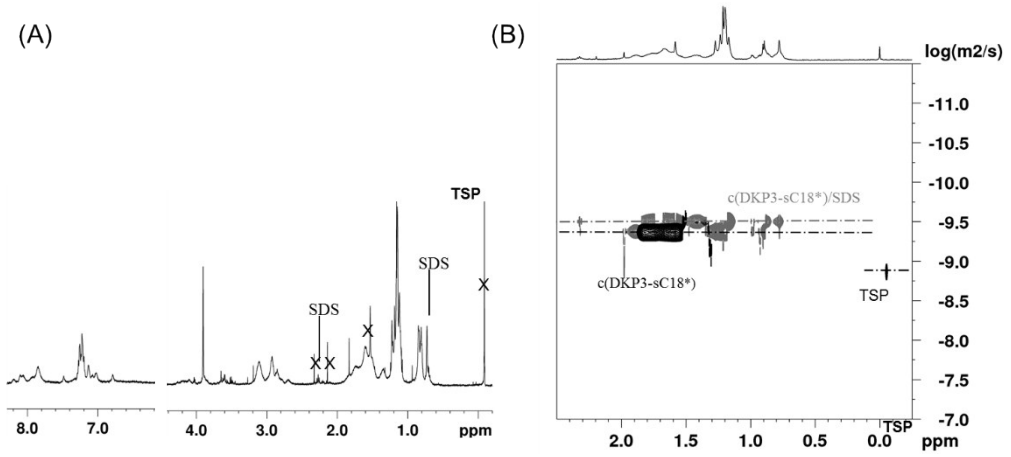


Figure S14. (**A**)1D ^{1}H NMR spectra of **c[DKP3-sC18*]** in the presence of SDS micelles. (**B**) Overlapped DOSY NMR spectra of the peptide free (black) and in the presence of SDS micelles (grey) (peptide:SDS 1:80, PBS, pH 6.08, water:D_2O 9:1, 298 K, 600 MHz).

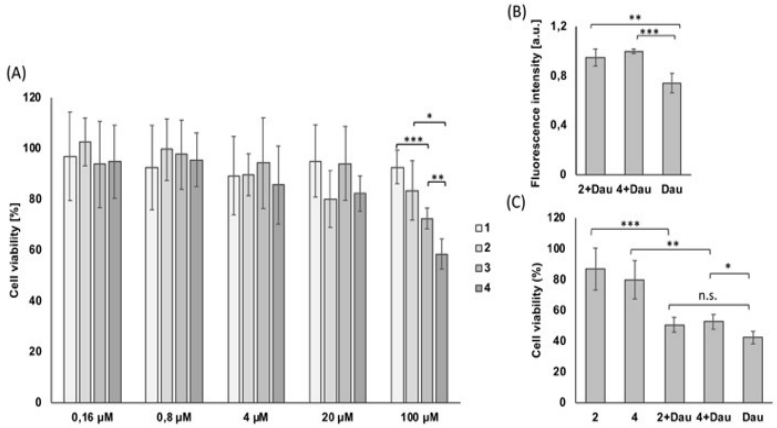


Figure S15. (A) HeLa cell were incubated with various concentrations of **1**-**4** for 24h incubation followed by washout of the medium and further incubation for 48h with fresh medium at 37 °C. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$. (B) Cellular uptake in HeLa cells of daunorubicin alone (1 µM) and complexed with **2** or **4** (10 µM), respectively. Cells were incubated for 30 min. **2+Dau**: 95%; **4+Dau** was set to 100%; **Dau**: 74%. (C) Viability assays carried out with **2** and **4** (10 µM, with or w/o daunorubicin) and daunorubicin (160 nm).

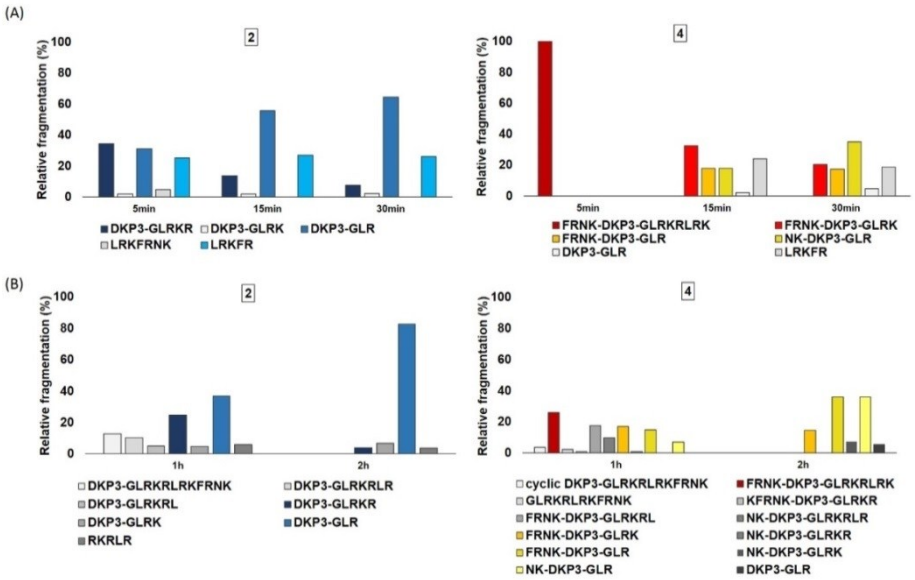


Figure S16. Degradation products of peptides **2** and **4** after treatment with (A) trypsin and (B) cell culture supernatant.

cycDKP3
cell culture supernatant

Incubation time	rt	Fragment sequence	Fragments MW (g/mol)
0 min	5.87	DKP3-GLRKRLRKFRNK	1827.26
1h	5.32	GLRKRLRKFRNK	1571.8
	5.72	DKP3-GLRKRLRKFRNK	1845.26
	5.83	NK-DKP3-GLRKRLR	1414.36
	6.11	FRNK-DKP3-GLRK FRNK-DKP3-GLRKRL	1291.58 1559,52
	6.39	FRNK-DKP3-GLR	1163.88
		NK-DKP3-GLR	860.16
2h	5.94	NK-DKP3-GLRK	988.47
	6.18	FRNK-DKP3-GLRK	1291.58
	6.49	FRNK-DKP3-GLR	1163.88
		NK-DKP3-GLR	860.16
	6.9	DKP3-GLR	617.56
24h	6.18	FRNK-DKP3-GLRK	1291.58
	6.51	FRNK-DKP3-GLR	1163.88
		NK-DKP3-GLR	860.16
	6.92	DKP3-GLR	617.56
	7.38	NK-DKP3-GL	703.86

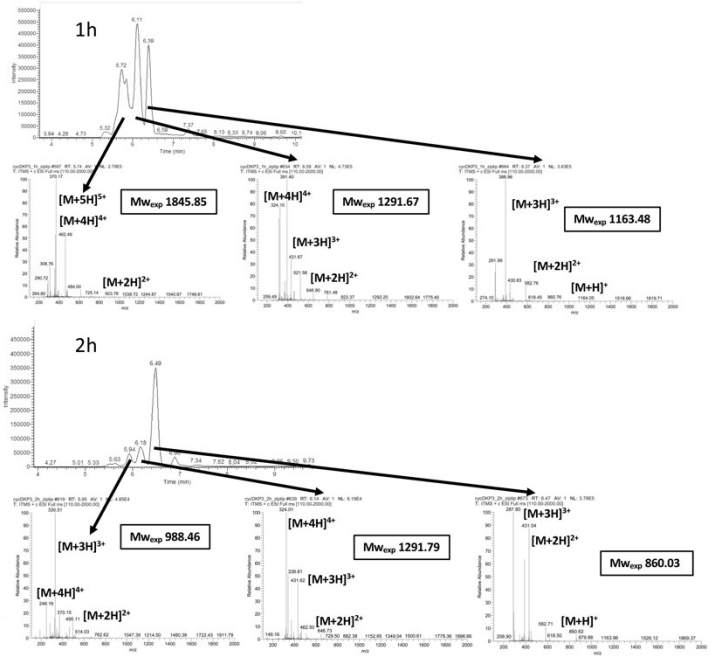


Figure S17. Degradation products were identified by using HPLC-MS. Table shows MW's of expected fragments. Chromatogram and MS spectra are exemplarily shown for degradation of cyclic DKP3 in cell culture supernatant.

ACRONYMS

ACN=acetonitrile; **Boc**= tert-butyloxycarbonyl; **BOP**= benzotriazol-1-yloxytris (dimethylamino)phosphonium hexafluorophosphate; **DCM**=dichloromethane; **DIC=** diisopropylcarbodiimide; **DIPEA**= N,N-Diisopropiletilamine; **DKP=**2,5-diketopiperazine; **DMF**= dimethyl formamide; **DOSY**= Diffusion Ordered SpectroscopY; **DTT**= Dithiothreitol; **EDT**= 1,2-ethanedithiol; **FA**=formic acid; **HOBt**= Hydroxybenzotriazole; **MS**=mass spectrometry; **NMP**=*N*-methyl-2-pyrrolidone; **NOESY**=Nuclear Overhauser Effect SpectroscopY; **Pbf**= 2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulfonyl; **d_{25}-SDS**=perdeuterated sodium dodecyl sulfate; **SPPS**= solid-phase peptide synthesis; ***t*Bu**= tert-Butyl; **TFA**=trifluoroacetic acid; **TFE**=trifluoroethanol; **TIS**= triisopropylsilane; **TOCSY**=TOtal Correlation SpectroscopY; **Trt**= triphenylmethyl; **TSP**= Trimethylsilyl)propionic- acid sodium salt;

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