Supporting Information

Passive Membrane Permeability of Sizeable Acyclic β-Hairpin Peptides

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I. General Information

Reagents and Method. All reagents, Fmoc-amino acids and resins used in the present paper were purchased from Chemimpex and Millipore Sigma. All bulk solvents were acquired from Fischer Scientific. Peptides, **1a-f**, **1j-l**, **1n-r**, **2a-b**, **3a-b**, **5a** and **7a** were synthesized using a standard automatized Fmoc-SPPS technique (solid-phase peptide synthesis) on a Protein Technologies PS-3 peptide synthesizer. Syntheses were accomplished on a Fmoc-Glu-Wang resin (0.4 meq/g). No unexpected or unusually high safety hazards were encountered during the synthesis of these peptides and their purification. Peptides **1g, 1h, 1i, 1m, 1s, 4a, 4b** and **6a** were purchased from Peptide 2.0 Inc. The purity of synthetic peptides was quantified by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) on a Hitachi L-7000 series equipped with a XBridge BEH C_{18} column (130 Å, 10 µm, 4.6 mm x 250 mm) and their molecular weight determined by mass spectrometry using a Bruker Microflex LRF matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). Peptide solutions for circular dichroism (CD) were prepared at 20-100 µM concentration range in phosphate buffer (15 mM, pH 6.5) with addition of MeOH (up to 50% v/v) if required to increase solubility. Each peptide concentration was determined accurately by measuring the UV-absorbance of the solution using a JASCO V-670 spectrophotometer based on the combined molar absorptivity of Trp (ε_{280} = 5580 $M^{-1}.cm^{-1}$ per Trp) and Tyr (ε_{280} = 1280 $M^{-1}.cm^{-1}$ per Trp) within the peptide. CD spectra were recorded on a JASCO J-810 Spectropolarimeter with a temperature controller module JASCO PFD-425S. Raw CD melting curves were implemented in OriginPro 9.0 (Originlab Corporation, U.S.A.) and fitted to two-state model (folded/unfolded) using a nonlinear fitting square routine protocol derived from the thermodynamic Gibbs-Helmholtz equation which was described elsewhere.[SI-1] Passive membrane permeation assays were run using a Corning® GentestTM precoated PAMPA plate system (Corning, NY, USA), using a 96-well filter plate with a permeable support system of structured phospholipids layers. Peptide concentrations were accurately measured by UV-absorbance at 280 nm using DeNovix DS-11 microvolume spectrophotometer (Wilmington, DE, USA).

[SI-1] Greenfield, N. J. Using Circular Dichroism Collected as a Function of Temperature to Determine the Thermodynamics of Protein Unfolding and Binding Interactions. *Nat. Protoc.* **2006**, *1*, 2527–2535.

II. Peptide Synthesis and Characterization.

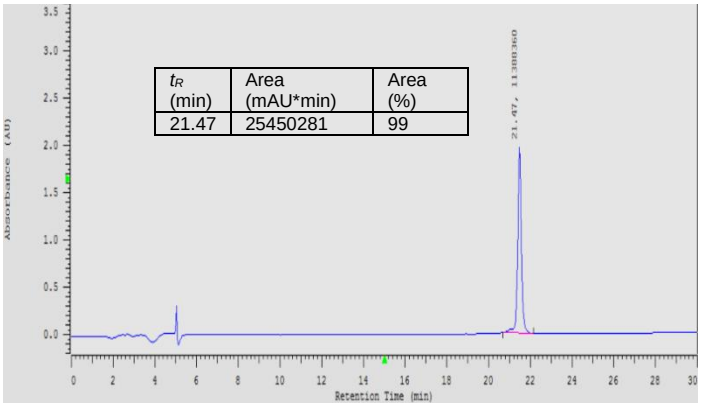
General procedure for the synthesis via Fmoc-SPPS: Syntheses were carried out at room temperature in anhydrous DMF using Wang resin (300 mg, 1.0 equiv. for each peptide) by successive iterations of deprotection/coupling. Vials of each Fmoc-protected α-amino acid (4.0 eq.) were prepared with HBTU (4.4 equiv.) and HOBt (4.4 equiv.) neat. For each iteration, the deprotection/coupling sequence entails: (**1**) Wash of the resin with DMF (3 X 5.0 mL) for 0.5 min each, (**2**) Fmoc-deprotection run twice using an excess of piperidine in DMF (20% v/v, 5.0 mL) for 5 mins each, (**3**) Wash with DMF (6 X 5.0 mL) for 0.5 min each, (**4**) The cocktail from the entire vial (described above) was dissolved with N-methylmorpholine in DMF (3.0 mL, 4.0 M) and added for a 40-min coupling, (**5**) Wash with DMF (3 X 5.0 mL) for 0.5 min each, finalized the sequence. After the final N-terminal coupling a final Fmoc-deprotection was achieved (step 2). Resulting peptides attached to the resin were washed with CH_2Cl_2 (2 X 10 mL), and the resin was dried under vacuum before storage under argon at -78 °C until cleavage.

General procedure for the peptide cleavage off the resins: The dried resin was suspended in a cleavage cocktail of TFA/thioanisole/EDT/anisole (90:5:3:2 v/v, 1.0 mL per 20 mg of resin) and shaken for 1.5 h at RT. The mixture was filtered to remove the resin and the TFA and other volatiles were removed on a rotary evaporator. The crude peptides (~250 mg) were precipitated in cold ether (40 mL), then centrifuged and washed with cold ether (3 X 40 mL). The resulting crude peptides were solubilized in water and lyophilized before being stored as dry powders under argon at -78 °C.

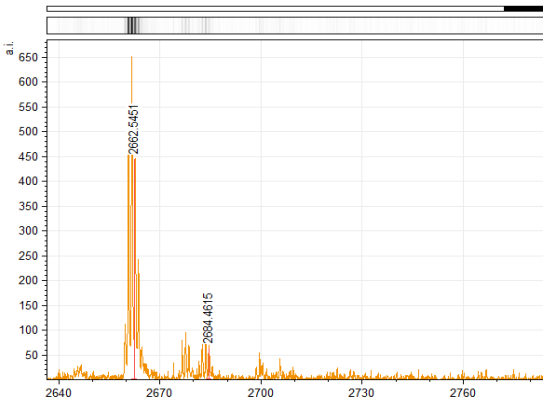
Peptide characterization by MALDI-TOF MS and RP-HPLC (summary of results in **Table S1**). All lyophilized crude peptides were analyzed by analytical RP-HPLC on a XBridge BEH C18 column. HPLC grade acetonitrile and deionized water, each containing 0.1% trifluoroacetic acid, were used as mobile phases for analytical and semi-preparative RP-HPLC. Each peptide was analyzed using a gradient from 10% to 50% of acetonitrile over 30 mins with a flow rate of 1.0 mL/min at room temperature and a detection at 220 nm. Selected data shown below in **Figure S1** and the retention times (T_R) for all the peptides studied are reported in **Table S1**. Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. All peptides were purified on a semi-preparative XBridge BEH C18 stationary phase (130 Å, 10μm, 10 mm x 250 mm) by scaling up the analytical RP-HPLC conditions. Pure fractions of peptides were collected and analyzed by RP-HPLC and mass spectrometry. Purified samples were resuspended in acetonitrile and water (1:1 v/v) for mass determination. A volume of 0.3 μL from each re-suspended fraction was layered on the wells of a Bruker MSP 96-well polished steel plate and allowed to dry over a previous coat of 0.3 μL α-cyano-4-hydroxycinnamic acid matrix (CHCA, 25 mg/mL in acetonitrile/methanol (54:31 v/v), ACROS Organics, NJ, USA).

Figure S1. Selected HPLC and MALDI data of **1f**, **1p**, and **7a**.

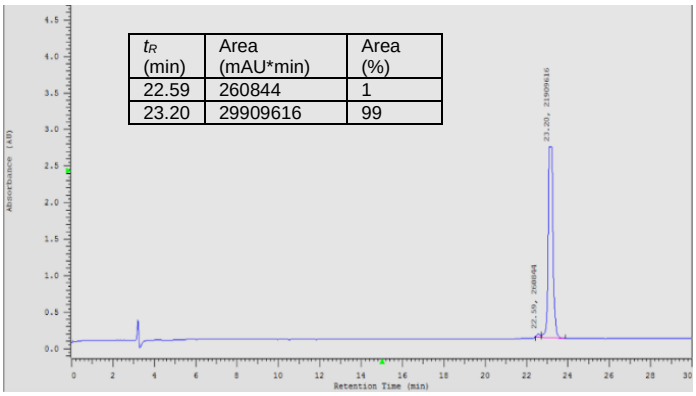
Peptide **1f**: RWVA-RRDYRGDMGFDY-WVWE
HPLC: Peptide **1f** retention time t_R = 21.47 min with a purity of 99%.
PAMPA acceptor plate MALDI: Peptide **1f** with *m/z* $[M+H]^+$ of 2662.55 and $[M+Na]^+$ of 2684.46.



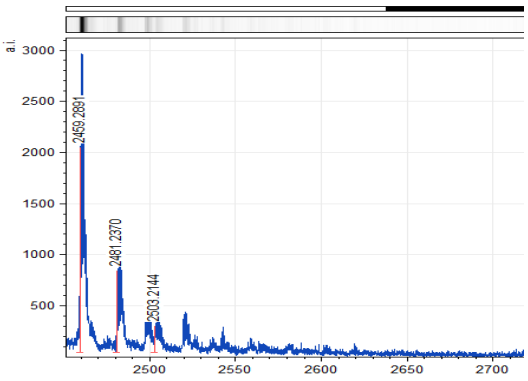
t_R (min)	Area (mAU*min)	Area (%)
21.47	25450281	99



Peptide **1p:** RWVW-RDYRGDMGFD-WVWE
HPLC: Peptide **1p** retention time t_R = 23.20 min with a purity of 99%.
MALDI: Peptide **1p** *m/z* $[M+H]^+$ of 2459.29, $[M+Na]^+$ of 2481.24, and $[M+2Na]^+$ of 2503.21.

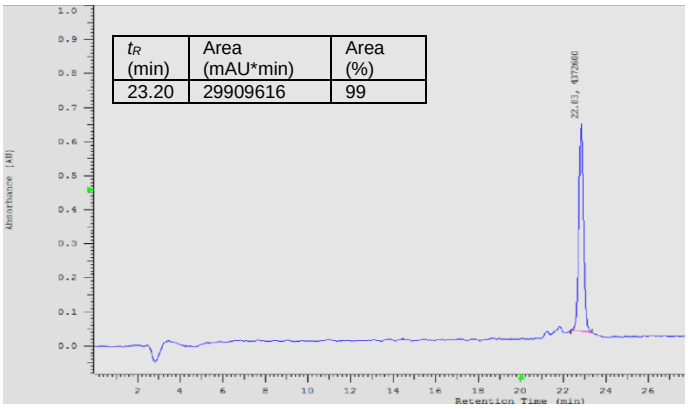


t_R (min)	Area (mAU*min)	Area (%)
22.59	260844	1
23.20	29909616	99



Peptide **7a**: RWVW-SPDSSGVAY-WVWE

HPLC: Peptide **7a** retention time t_R = 22.83 min with a purity of 99%.
PAMPA acceptor plate MALDI: Peptide **7a** with *m/z* $[M+H]^+$ of 2009.79 and $[M+Na]^+$ of 2131.74.



t_R (min)	Area (mAU*min)	Area (%)
23.20	29909616	99

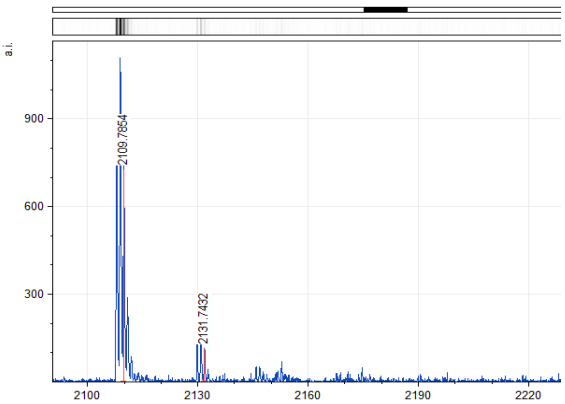


Table S1. Summary of peptides characterization.

ID	Peptide Sequences [a] (net charge)	Molecular weight (Da)	%-Charge/ %-Lipo [b]	Solubility *S* (mM) [c]	%-fold χ_F at 291K [d]	HPLC T_R (mins)[e] [%-purity]	log$D_{7.4}$[f]	P_{app} (nm/s)[g]
1a	RWVAR-$G_4K_2G_2$FD-YWVWE (+2)	2311.6	30/35	0.54 ± 0.15	87 ± 1%	19.4 [99]	-1.7 ± 0.4	5.3 ± 2.3
1b	RWVW-$G_4K_2G_4$-WVWE (+2)	1959.2	22/33	0.66 ± 0.12	88 ± 1%	17.2 [92]	-2.2 ± 0.3	5.3 ± 1.3
1c	RGVAR-RDYRGDPGFD-YWHWE (+1)	2538.7	45/30	17.85 ± 0.89	*RC* 0%	18.8 [98]	-1.9 ± 0.4	2.6 ± 1.8
1d	RWCAR-RDYRFDMGFD-YWGWE (0)	2716.0	40/35	0.03 ± 0.01	*Native* 0%	23.7 [90]	-0.4 ± 0.4	11.8 ± 30.9
1e	RWVAR-RDYRFDMGFD-YWVWE (0)	2754.1	40/**45**	0.01 ± 0.02	56 ± 1%	23.1 [99]	0.2 ± 0.1	**21.8** ± 12.3
1f	RWVAR-RDYRGDMGFD-YWVWE (0)	2664.0	40/40	0.02 ±0.03	92 ± 1%	21.5 [99]	0.6 ± 0.2	**15.9** ± 6.0
1g	RWVVR-RDYRFDMGFD-YWVWE (0)	2782.1	40/45	0.02 ± 0.04	49 ± 2%	23.0 [97]	-1.1 ± 0.8	**26.1** ± 7.4
1h	RWVVR-RDYRGDMGFD-YWVWE(0)	2692.0	40/40	0.03 ± 0.01	89 ± 1%	20.6 [99]	-0.9 ± 0.5	0.4 ± 0.9
1i	RWVKR-RDYRFDMGFD-YWVWE (+1)	2811.2	45/40	0.02 ± 0.04	37 ± 1%	21.9 [99]	-0.4 ± 0.4	1.4 ± 2.3
1j	RWVKR-RDYRGDMGFD-YWVWE (+1)	2721.1	45/35	1.80 ± 0.16	92 ± 1%	19.5 [97]	-1.3 ± 0.3	0.2 ± 0.2
1k	RWVAR-RDYRFDMGFD-YWHWE (+1)	2792.1	45/**45**	0.01 ± 0.02	48 ± 1%	21.8 [97]	-0.5 ± 0.4	**25.5** ± 24.3
1l	RWVAR-RDYRGDMGFD-YWHWE (+1)	2702.0	45/40	0.04 ± 0.03	97 ± 1%	20.3 [95]	0.7 ± 0.5	0.7 ± 0.5
1m	RWVAR-RDYRFDMGFN-YWVWE (+1)	2753.1	35/**45**	0.05 ± 0.06	50 ± 1%	22.6 [96]	0.7 ± 0.4	**40.4** ± 26.2
1n	RWVAR-RDYRGDMGFN-YWVWE (+1)	2663.0	35/40	0.06 ± 0.03	97 ± 1%	20.9 [99]	-0.8 ± 0.4	0.2 ± 0.6
1o	RWVW-RDYRFDMGFD-WVWE (-1)	2550.0	39/**50**	0.02 ± 0.03	40 ± 1%	25.2 [86]	0.1 ± 0.4	**52.8** ± 45.0
1p	RWVW-RDYRGDMGFD-WVWE (-1)	2459.7	39/44	0.03 ± 0.03	81 ± 1%	23.3 [99]	0.9 ± 0.4	0.1 ± 0.2
1q	RWVW-RRDYRFDMGFDY-WVWE (0)	2869.2	42/47	0.01 ± 0.03	48 ± 1%	23.7 [90]	0.9 ± 0.4	0.2 ± 1.9
1r	*cyclo*RW[CAR-RDYRFDMGFD-YWC]WE (0)	2760.1	40/35	0.01 ± 0.02	82 ± 1%	22.0 [99]	-0.6 ± 0.1	**37.1** ± 16.3
1s	RWVWR-RDYRGDMGFD-YWVWE (0)	2779.1	40/40	0.02 ± 0.06	45 ± 1%	21.6 [97]	-1.0 ± 0.4	0.4 ± 9.1
2a	RWVAR-EGMNTDWYFD-YWHWE (-1)	2748.0	35/40	0.02 ± 0.05	36 ± 2%	23.3 [94]	1.2 ± 0.4	4.2 ± 2.6
2b	RWVAR-EGMNTDGYFD-YWHWE (-1)	2618.8	35/35	0.69 ± 0.05	36 ± 1%	21.1 [98]	0.3 ± 0.6	0.2 ± 0.02
3a	RWVAR-AYGNYWYID-VWHWE (+1)	2570.9	26/**47**	0.002 ± 0.008	β-sheet	24.1 [99]	0.6 ± 0.1	**41.5** ± 18.7
3b	RWVAR-AYGNYGYID-VWHWE (+1)	2441.7	26/42	0.07 ± 0.24	91 ± 1%	21.9 [98]	-1.3 ± 0.3	3.8 ± 6.9
4a	RWVW-QYGSLPWT-WVWE (0)	2179.5	12/56	0.02 ± 0.03	91 ± 1%	28.1 [97]	-0.6 ± 0.5	2.4 ± 4.4
4b	RWVAR-EGGWFGELAFN-YWVWE (-1)	2659.0	24/**52**	0.26 ± 0.04	β-sheet	21.2 [97]	-0.5± 0.1	**20.5** ± 4.3
5a	RWVW-RDLDIATTRDY-WVWE (-1)	2566.9	37/47	0.14 ± 0.12	78 ± 1%	23.2 [93]	0.8 ± 0.6	2.0 ± 1.1
6a	RW-VISPYGGSTV-WE (0)	1636.8	14/43	3.00 ± 0.17	57± 1%	19.5 [99]	-2.0 ± 0.4	2.9 ± 1.0
7a	RWVW-SPDSSGVAY-WVWE (-1)	2110.3	18/53	0.53 ± 0.14	85 ± 1%	22.8 [99]	0.9 ± 0.6	0.9 ± 0.3

[a] Loop residues are presented in green and bulge motif residues edging the loop in blue based on straps A & B. [b] Charged and lipophilic residues considered are H, K, R, E, D, and A, P, W, F, V, I, L, M respectively. A high peptide solubility is typically associated with ~25% charge and <50% lipophilicity. [c] Kinetic solubility measured in a phosphate buffer (PB, 50 mM) at pH 7.4. [d] Fractions of folding determined from the thermal denaturation melting curves recorded by CD spectroscopy. [e] retention times determined by analytical RP-HPLC from peak integration detected by UV-absorbance at 220 nm on a XBridge BEH C_{18} column using a 10-50% gradient of acetonitrile in water (0.1% TFA in both mobile phases) over 30 mins with a flow rate of 1.0 mL/min. [f] Measured by shake flask assay in triplicate ($n = 3$) with the corresponding standard deviation values. [g] Apparent passive membrane permeability values (P_{app}) determined by PAMPA ($n \geq 3$).

Table S2. Analysis of peptides by MALDI-TOF mass spectrometry.

ID	Molecular formula	m/z $[M+H]^+$or $[M+Na]^+$	
		Calcd	Found
1a	$C_{109}H_{151}N_{31}O_{26}$	2310.14	2310.77
1b	$C_{93}H_{127}N_{27}O_{21}$	1957.97	1958.41
1c	$C_{115}H_{156}N_{36}O_{31}$	2538.18	2538.17
1d	$C_{125}H_{163}N_{35}O_{31}S_2$	2715.18	2715.31
1e	$C_{130}H_{173}N_{35}O_{31}S$	2753.28	2753.87
1f	$C_{123}H_{167}N_{35}O_{31}S$	2663.24	2664.98
1g	$C_{132}H_{177}N_{35}O_{31}S$	2781.31	2781.13
1h	$C_{125}H_{171}N_{35}O_{31}S$	2691.26	2691.35
1i	$C_{133}H_{180}N_{36}O_{31}S$	2810.34	2811.21
1j	$C_{126}H_{174}N_{36}O_{31}S$	2720.29	2720.61
1k	$C_{131}H_{171}N_{37}O_{31}S$	2791.27	2791.74
1l	$C_{124}H_{165}N_{37}O_{31}S$	2701.23	2701.94
1m	$C_{130}H_{174}N_{36}O_{30}S$	2752.30	2752.28
1n	$C_{123}H_{168}N_{36}O_{30}S$	2662.25	2663.15
1o	$C_{123}H_{157}N_{31}O_{28}S$	2549.16	2549.60
1p	$C_{116}H_{151}N_{31}O_{28}S$	2459.11	2459.39
1q	$C_{138}H_{178}N_{36}O_{31}S$	2868.33	2868.26
1r	$C_{126}H_{163}N_{35}O_{31}S_3$	2759.14	2759.63
1s	$C_{131}H_{172}N_{36}O_{31}S$	2778.28	2779.40
2a	$C_{130}H_{163}N_{33}O_{33}S$	2746.18	2747.46
2b	$C_{121}H_{156}N_{32}O_{33}S$	2617.12	2617.96
3a	$C_{126}H_{160}N_{32}O_{28}$	2570.21	2570.07
3b	$C_{117}H_{153}N_{31}O_{28}$	2441.18	2441.51
4a	$C_{110}H_{139}N_{25}O_{23}$	2179.05	2179.32
4b	$C_{130}H_{168}N_{32}O_{30}$	2658.27	2658.56
5a	$C_{121}H_{168}N_{32}O_{31}$	2566.26	2566.88
6a	$C_{77}H_{109}N_{19}O_{21}$	1636.81	1637.36
7a	$C_{102}H_{132}N_{24}O_{26}$	2109.98	2110.69

II. Peptide Folding Studies

i. Circular Dichroism (CD). Raw CD data were recorded in mdeg from 190 nm to 270 nm and the CD spectra of the blank was subtracted. Spectra were then smoothed with the baseline set up to zero between 260 nm to 270 nm using SpectraGryph 1.2,[SI-2] and the ellipticity scale was converted into molar ellipticity (deg.cm^2.dmol^{-1}). Melting curves representing the unfolding transitions were obtained for each peptide by the following method: An automatized temperature ramp was set on a JASCO Spectropolarimeter from 0 to 95 °C with a rate of temperature increase of 45 °C per hour. Intensities at 230 ± 2 nm (hairpin) were recorded automatically by the instrument every 0.1 °C to generate the melting curves. The molar ellipticity values from the blank were also recorded at 230 ± 2 nm on the same range of temperatures and subtracted to the sample melting curve to afford the raw experimental melting curve. Peptide **1c** was used as random coil.

ii. Melting curves best-fitting protocol. To determine the thermodynamic parameters characterizing the folding of each peptide construct based on their secondary structures, raw data of the molar ellipticity [θ(T)] obtained at 230 ± 2 nm were fitted to Eq. S1 using the Gibbs-Helmholtz equation (Eq. S2) for a two-state model (folded/unfolded transition) accordingly to the reported procedure by N. J. Greenfield. Raw melting curves of molar ellipticity as a function of temperature $[\theta(T)]_{228}$ were fitted following fitting protocol previously described[SI-3] using the software OriginPro 9.0, to the following equation:

$$\theta(T) = \theta_U + (\theta_F - \theta_U)\frac{e^{-\frac{\Delta G(T)}{RT}}}{1+e^{-\frac{\Delta G(T)}{RT}}} \qquad \text{Eq. S1}$$

with

$$\Delta G(T) = \Delta H_m * \left(1 - \frac{T}{T_m}\right) - \Delta Cp * ((T_m - T) + T * \ln\left(\frac{T}{T_m}\right)) \qquad \text{Eq. S2}$$

$[\theta]_U$ and $[\theta]_F$ being the molar ellipticity values for the 0%- and 100%-folded states respectively, R being the ideal gas constant (R = 1.987 cal.mol^{-1}.K^{-1}), ΔH_m the enthalpy at the melting temperature T_m, and ΔC_p the specific heat capacity at constant pressure ($\Delta C_p < 0$ for a folding transition). $[\theta]_U$ was obtained from the synthetic random coil **1c**, while $[\theta]_F$ was let to vary in the fitting protocol.

A melting curve representing the evolution of the fraction of folding with the temperature (thermodynamic structural transition) was also plotted accordingly to the following equation:

$$x_F(T) = \frac{\theta(T)-\theta_U}{\theta_F-\theta_U} \qquad \text{Eq. S3}$$

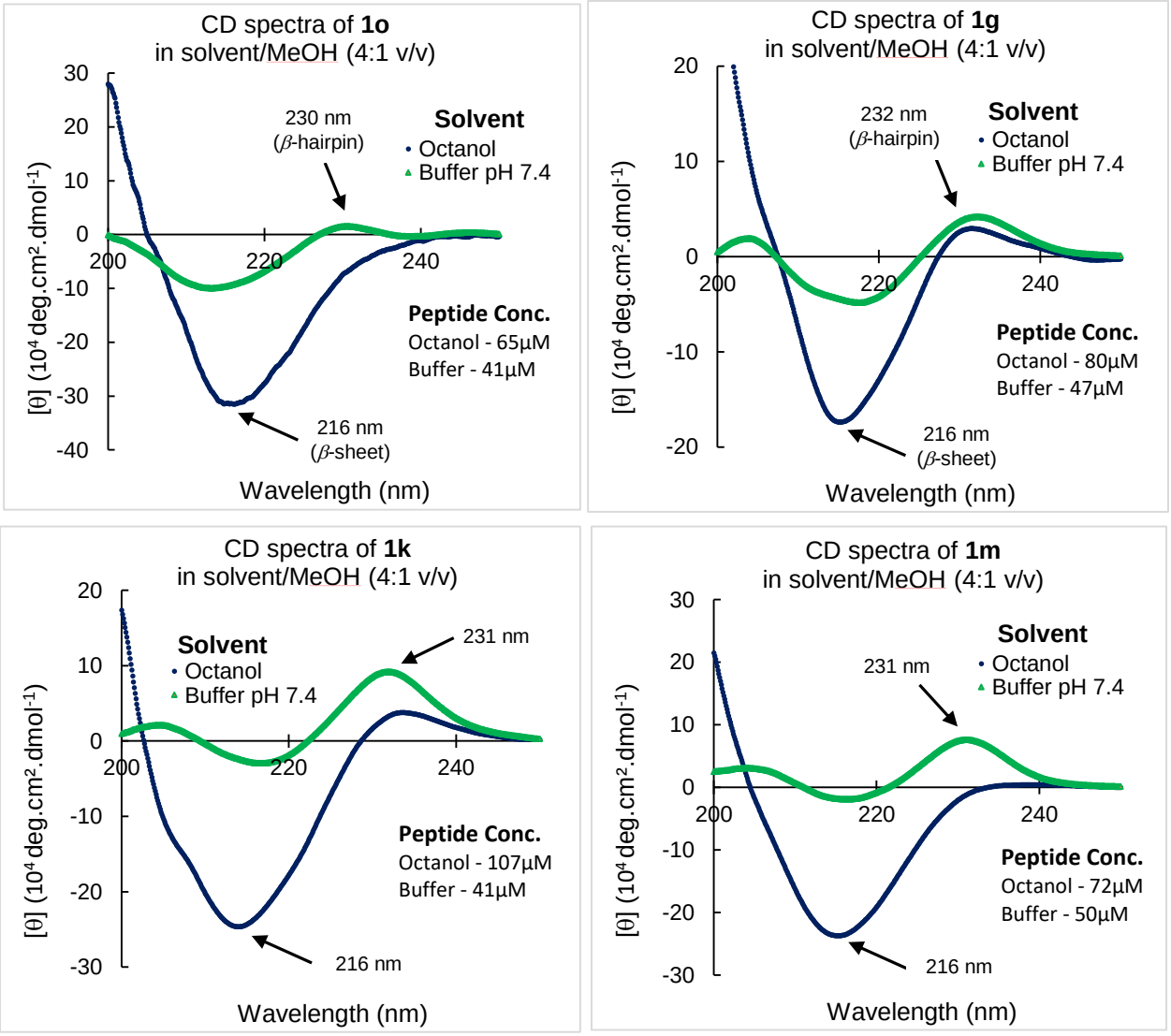
With $\mathcal{X}_F$ representing the fraction of folding at a temperature *T*. Results are tabulated in **Table S1**.

[SI-2] Menges, F. Spectragryph - optical spectroscopy software, Version 1.2.16.1, 2022, http://www.effemm2.de/spectragryph/

[SI-3] Richaud, A. D.; Zhao, G.; Hobloss, S.; Roche, S. P. Folding in Place: Design of β-Strap Motifs to Stabilize the Folding of Hairpins with Long Loops. *J. Org. Chem.* **2021**, *86*, 13535–13547.

iii. Octanol/Buffer CD comparison. Samples were prepared in a mixture of PB (15 mM, pH 7.4) and methanol (PB/MeOH 80:20 v/v) as well as in a mixture of octanol and methanol (Octanol/MeOH 80:20 v/v) at similar concentrations (40 to 100 µM range). CD spectra were recorded between 200 nm (octanol cutoff) and 270 nm at room temperature. Raw spectra were smoothed, and baseline corrected from 260 to 270 nm in SpectraGryph 1.2. Spectral comparison for the most membrane permeable peptides is shown in **Figure S2**.

Figure S2. CD spectra of peptides **1g**, **1k**, **1m**, and **1o** in octanol and buffer showing the drastic changes in conformation.



III. Physiochemical Property Measurements

i. Solubility Measurements. For kinetic solubility measurements, 1.5 mg of lyophilized peptide was weighed in a 1.5 mL conical flask. A solution of potassium phosphate buffer (50 mM, pH 7.4, 1 mL) was added to solubilize the maximum quantity of peptide. If the solubility was greater than 1.5 mg/ml, an additional 0.5 mg of peptide was added to obtain a saturated solution with some observable peptide powder in suspension. The heterogeneous mixture was successively sonicated (3 X 5 mins), then centrifuged at 6000 rpm for 5 mins to allow any undissolved peptide to separate from the supernatant. The precise concentration in peptide of the saturated solution was measured in triplicate by UV-absorbance at 280 nm. The maximum kinetic solubility (*S*) was calculated from the Beer law: $S = \frac{A}{\varepsilon * l}$, with *A* the measured absorbance, ε the molar absorptivity, and *l'* the path-length of the cuvette. The resulting supernatant was then collected for further log$D_{7.4}$ measurements.

ii. Partition Coefficients Measured by Shake Flask Assay (log $D_{7.4}$). A reagent-grade octanol was saturated with water prior to use. The saturated solution of peptide in a PB solution obtained from the solubility measurement experiment above (50 mM, pH 7.4, 1.0 mL) was separated into three portions and placed in conical flasks (1.5 mL). Each flask was charged with the adequate volume of octanol: **1.** octanol/PB (1:1 v/v), **2.** octanol/PB (1:6 v/v), and **3.** octanol/PB (10:1 v/v). Each tube was thoroughly shaken every 20 mins (3 X 1 min) over one hour, then the biphasic system was allowed to reach equilibrium for 24 hours at room temperature. The octanol/water phases were then separated and aliquots (2.0 µL) of each phase were analyzed in triplicate by UV-absorbance at 280 nm to obtain the peptide concentration in both phases. As a control, the sum of final peptide concentrations in PB (C_{pb}) and in octanol (C_{oct}) should be similar to the starting concentration (*S*) ± 10%. The partition coefficients, log$D_{7.4}$, were calculated using equation S1:

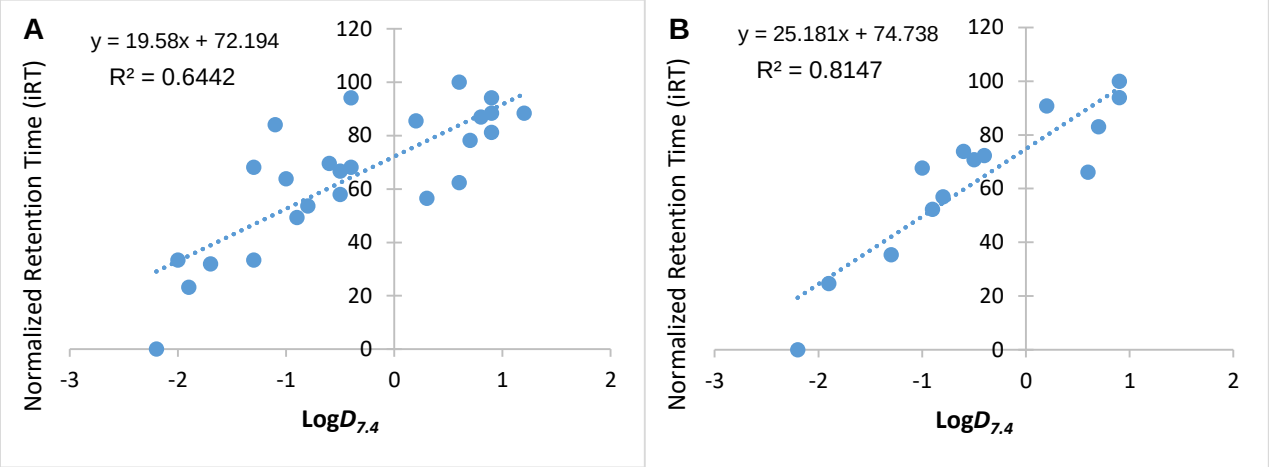
$$logD_{7.4} = \log\left(\left(\frac{S}{C_{pb}-1}\right) * x_{pb}\right) \qquad \text{(Eq. S1)}$$

With *S* as the initial concentration of peptide in the buffer solution, C_{pb} the resulting concentration of peptide in the buffer phase, and χ_{pb} the ratio PB/octanol. The averaged log $D_{7.4}$ values and the corresponding standard deviations from triplicates are reported in the **Table S1**.

To compare the experimental log $D_{7.4}$ values with the retention times obtained for each hairpin on the C18-BEH RP-HPLC column, a method of retention times (RT) normalization was developed[SI-4] using the equation $iRT_x = [(RT_x - RT_b)/(RT_i - RT_b)] \times 100$, where RT_b is the retention time for our reference peptide (**1b**), RT_i is the latest retention time (less polar hairpin), and RT_x is any of the peptides within the library. The iRT_x were thus normalized for each peptide on a scale from 0-100 using the benchmark hairpin **1b** with a [$G_4K_2G_4$] loop to remove the intrinsic polarity given by the hairpin strap. This approach allows to more accurately compare the changes of polarity imparted by the different loops on both log$D_{7.4}$ and iRT. Plots of the iRT linear regressions with R^2 values (**Figure S3**) show that a trend between log$D_{7.4}$ and iRT exist within the entire library and that β-hairpins with higher sequence similarity (pembrolizumab analogs) have a strong correlation between normlized retention times and partition coefficients.

[SI-4] Escher, C.; Reiter, L.; MacLean, B.; Ossola, R.; Herzog, F.; Chilton, J.; MacCoss, M. J.; Rinner, O. iRT, a normalized retention time for more targeted measurement of peptides. *Proteomics* **2012**, *12*, 1111.

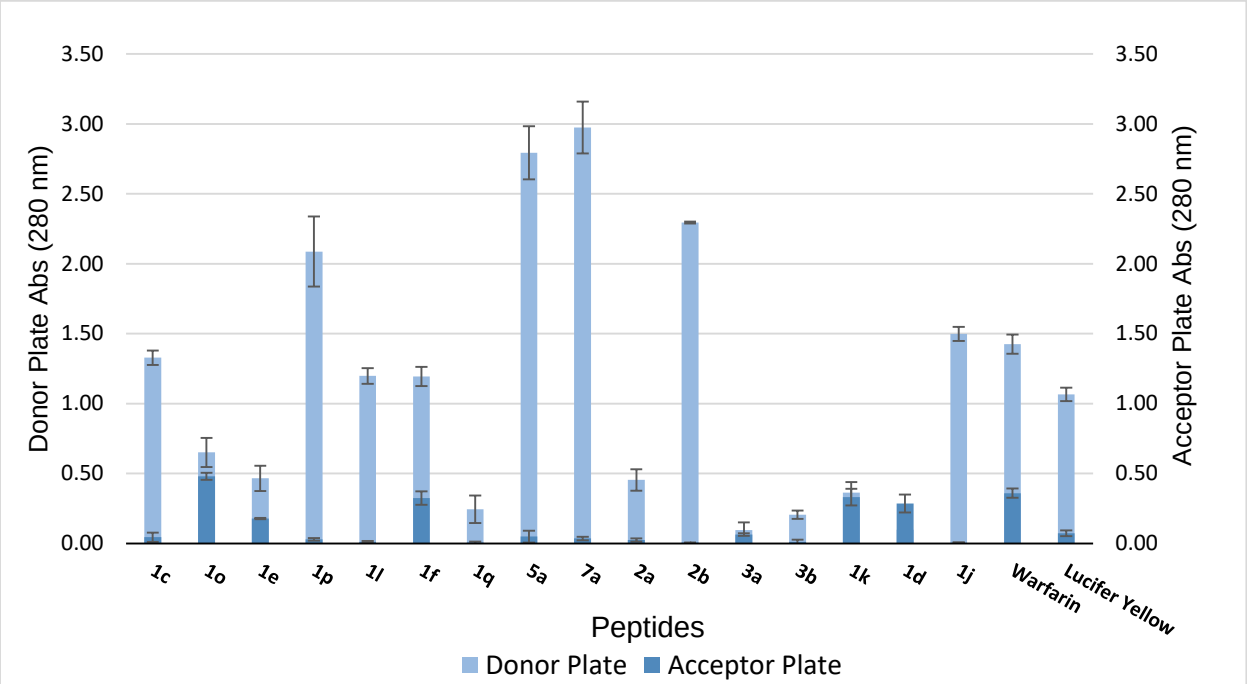
Figure S3. Graphs of normalized retention times (iRT) versus log$D_{7.4}$ for **A.** all hairpins within the library and **B.** all pembrolizumab hairpin mimics **1e-s**.

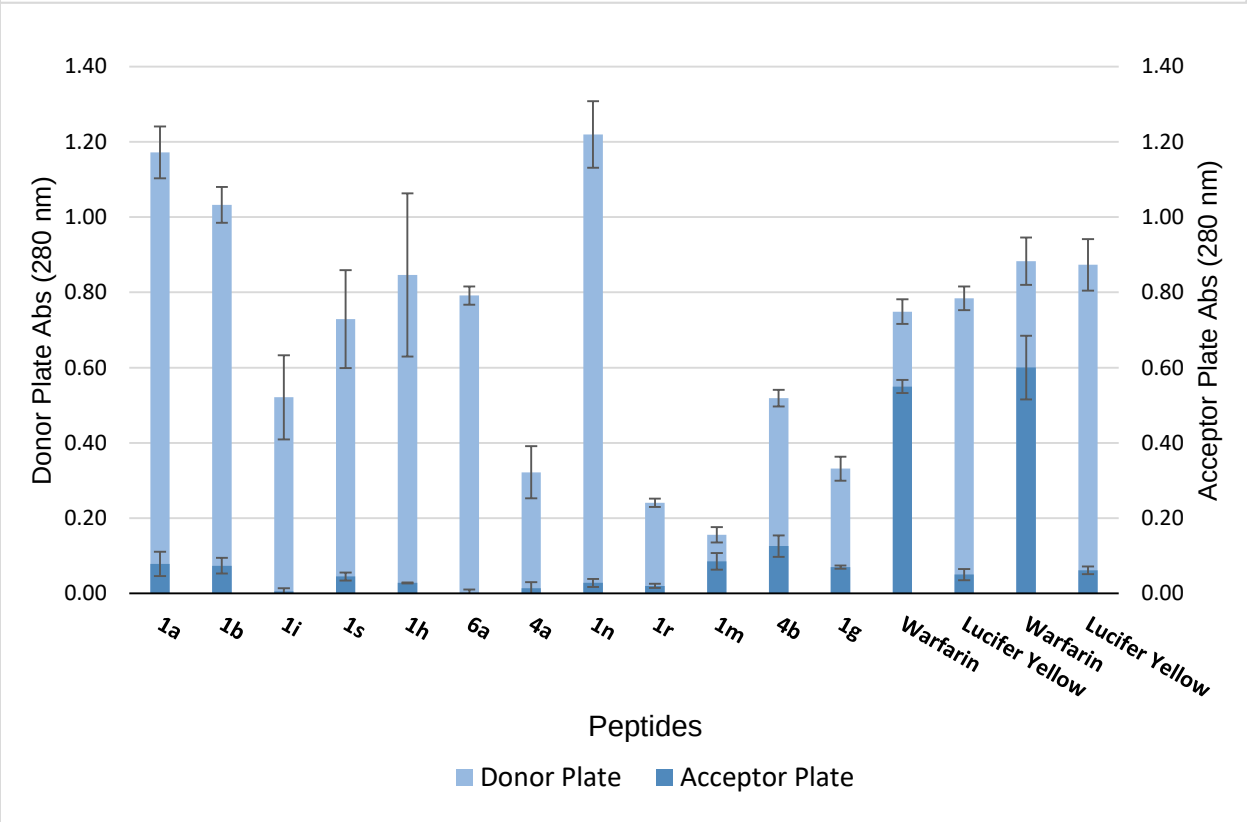


iii. PAMPA. Passive membrane permeation assays were run using a Corning® GentestTM precoated PAMPA plate system (Corning, NY, USA), using a 96-well filter plate with a permeable support system of structured phospholipids layers. Individual peptides were solubilized at their maximum concentrations in a phosphate buffer saline solution (PBS, 50 mM, pH 7.4). The stock solution was then diluted to obtain PBS/DMSO solution (95:05 vol/vol) with a ~100 µM final concentration in peptide. Sonication was used to ensure maximum peptide solubility. The peptide solutions were centrifuged, and their concentrations were accurately measured by UV-absorbance at 280 nm (DeNovix DS-11 microvolume spectrophotometer, Wilmington, DE, USA) prior to starting the experiment. Peptide solutions were then loaded, in triplicate, onto the donor plate (300 µL/well), and the acceptor plate was filled with PBS (200 µL/well). Warfarin (300 µL, 100 µM) and Lucifer yellow (300 µL, 100 µM) were also loaded in triplicates across the donor plate as positive and negative controls, respectively. The acceptor plate was coupled to the donor plate and incubated for 24 hours at room temperature without agitation. After 24 hours, the donor and acceptor plates were separated, and the final concentration for each peptide was measured in triplicate from the acceptor and donor wells at 280 nm. Apparent membrane permeability values, P_{app}, were determined for each peptide from the average concentration of the donor and acceptor triplicates in the same experimental assay plate. The identity of the peptides in the donor and acceptor plates were confirmed by mass spectrometry (*e.g.,* hairpin **1f** and **7a** m/z $[M+H]^+$ of 2662.55 and 2109.79 g/mol respectively). The molecular masses of peptides were ascertained by sampling 100 µL from the acceptor plate of the PAMPA system, which were immediately lyophilized and later analyzed by positive ion MALDI-TOF (reflector mode) after resuspension in acetonitrile and water (50:50 v/v) as described in the method above for purified peptides. P_{app} values were calculated according to the following equation: P_{app} (cm/s) = $\{-\ln[1-C_A(t)/C_{eq}]\}/[A\bullet(1/V_D+1/V_A)\bullet t]$, with A= filter area (0.3 cm^2), V_D = donor well volume (300 µL), V_A = acceptor well volume (200 µL), t = incubation time (seconds), $C_A(t)$ and $C_D(t)$ are the compound concentrations in acceptor and donor wells respectively measured at 24 h, and $C_{eq} = [C_D(t)\bullet V_D+C_A(t)\bullet V_A]/(V_A+V_D)$.

Averaged P_{app} values and the corresponding standard deviations were calculated from the triplicates shown in **Figure S4**.

Figure S4. PAMPA permeability results of all peptides in the study with concentrations obtained after 24 hours in the donor and acceptor wells. Lucifer yellow and warfarin were used as negative and positive controls respectively on each 96-well plate in triplicate.





To confirm the results for the most permeable peptides, PAMPA were repeated three times (on different plates and days). P_{app} values obtained from the three separate experiments were averaged to determine the final P_{app} and the corresponding standard deviations and shown in **Figure S5**. In addition, for Lucifer yellow and warfarin used as negative and positive controls, P_{app} values of 5.4 ± 0.6 and 70.6 ± 5.4 were obtained respectively. To determine uniformity amongst PAMPA plates, coefficients of variation (CV) for the positive (warfarin) and negative (Lucifer yellow) controls were calculated and used as a measure of dispersion around the mean P_{app} value. CV = $\frac{\sigma}{\mu}$, where σ = averaged standard deviation and μ = mean P_{app}. A CV of 8% for warfarin and 11% for Lucifer yellow were obtained, representing a strong correlation of measurements within all PAMPA plates used in this study.

Figure S5. PAMPA permeability results for peptides **1e, 1f, 1g, 1k, 1m, 1o, 1r,** and **3a** from triplicates obtained on different plates at 24 hours.