Supplementary Information

Label-free quantification of passive membrane permeability of cyclic peptides across lipid bilayers: Penetration speed of cyclosporin A across lipid bilayers

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Materials and methods

Abbreviation

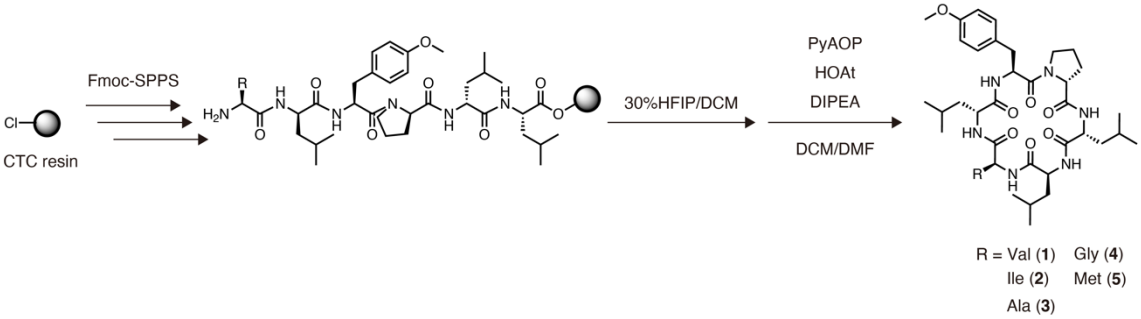
ACN, acetonitrile; COMU, ethyl 2-cyano-2-((dimethyliminio)(morpholino)methyloxyimino)acetate hexafluorophosphate; CTC resin, 2-chlorotrityl chloride resin; DCM, dichloromethane; DIC, *N*,*N*'-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Fmoc, 9-fluorenylmethyloxycarbonyl; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HOAt, 3*H*-1,2,3-triazolo[4,5-*b*]pyridin-3-ol; HPLC, high performance liquid chromatography; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid; HRMS, high resolution mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; MeOH, methanol; Oxyma, ethyl cyano(hydroxyimino)acetate; PAMPA, parallel artificial membrane permeability assay; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; PyAOP, (7-Azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; UPLC, ultra performance liquid chromatography.

General

Chemicals and solvents used in this study were purchased from commercial suppliers and used without further purification. CsA was purchased from Tokyo Chemical Industry Co., Ltd.. Preparative HPLC was performed on a Prominence HPLC system (Shimadzu) with a 5C_{18}-AR-II column (Nacalai tesque, 10 mm I.D.×150 mm, 34350-41). All the HPLC was performed using two solvents (solvent A: H_2O containing 0.1% TFA; Solvent B: acetonitrile containing 0.1% TFA). UPLC for purity check of peptides was performed on LC-2040C 3D Plus (Shimadzu) with Shim-pack Velox C_{18} 1.8 mm Column (Shimadzu, 2.1 I.D. × 50 mm). LC-MS was performed on an ACQUITY UPLC H-Class/SQD2 (Waters) using ACQUITY UPLC® BEH C_{18} 1.7 mm Column (Waters, 2.1 I.D. × 50 mm). The current was measured with CEZ2400 amplifier (Nihon Kohden). The observation of lipid bilayers was performed with IX-71 microscope (Olympus). Fluorescence was measured using a plate reader, Infinite M200 PRO (TECAN). HRMS data were obtained using micrOTOF II (Bruker Daltonics). As permeability coefficients, P_e or P_{app} was used in this paper. P_{app} is used only when the concentration of analytes in the donor chamber can be approximated by the initial concentration. In this research, P_{app} was used in Horizon-LBA, and P_e was used in PAMPA as permeability coefficients.

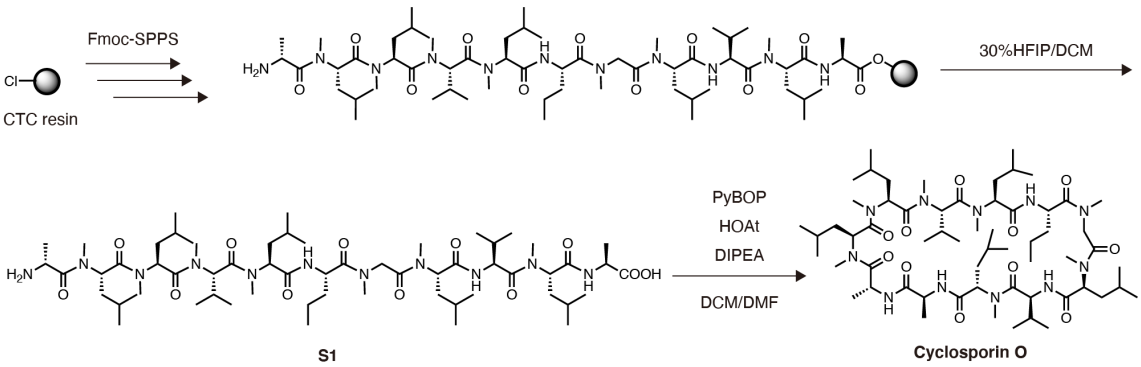
Synthesis

Cyclic peptide 1–5



1, **2**, **3**, **4**, and **5** were synthesized on CTC resin (1.34 mmol/g). Resin (475 mg, 0.64 mmol) was first swelled in DCM in a 6 mL fritted syringe with continuous shaking. DIPEA (433 μL, 2.56 mmol, 4 equiv.) and Fmoc-L-Leu-OH (450 mg, 1.28 mmol, 2 equiv.) were dissolved in 6.4 mL DCM, and the solution was applied to the resin. The resin was incubated for 2 h at room temperature with continuous shaking. After the reaction, the resin was washed with DCM, DCM/methanol/DIPEA = 17/2/1, and DCM three times each. The loading of the beads was quantified according to the previous report.[1] The loading percentage was determined as 93%. 169 mg of the resin (0.15 mmol) was applied to further peptide synthesis. Fmoc deprotection was performed by incubating the resin with 20% piperidine in DMF twice (3 min and 12 min). After the reaction, the resin was washed with DMF three times. A coupling reaction of amino acids was performed using Fmoc-protected amino acid (3 equiv.), Oxyma (3 equiv.) and DIC (3 equiv.) in DMF (0.2 M with respect to Fmoc-protected amino acid) for 1–14 h. Before the coupling reaction, pre-activation of the reaction mixture was conducted at room temperature for 10 min. After the reaction, the resin was washed with DMF three times. Before the coupling reaction of the 6th residue from the C-terminus, the resin was split for each sequence. The coupling and deprotection were repeated until the 6th residue. The synthesized peptides were cleaved from the resin by incubating the resin with 30% HFIP in DCM for 1 h once and 15 min twice. The resin was washed with DCM and MeOH. The filtrate was collected in a recovery flask. After all the filtrates were combined, the solution was evaporated under reduced pressure. To the residue, PyAOP (31 mg, 2 equiv.), HOAt (8 mg, 2 equiv.), DIPEA (25 μL, 5 equiv.) and anhydrous DMF/DCM (=1/1, 5.9 mL) were added, and the reaction mixture was shaken for 3 h. The solvent was removed under reduced pressure, and the residue was purified by a reversed phase column on HPLC to give **1** (12 mg, 16 μmol, 55%), **2** (16 mg, 23 μmol, 76%), **3** (13 mg, 19 μmol, 64%), **4** (1.0 mg, 1.3 μmol, 4%) and **5** (14 mg, 19 μmol, 62%).

Cyclosporin O (CsO)



CsO was synthesized on CTC resin (1.36 mmol/g). Resin (246 mg, 0.33 mmol) was first swelled in DCM in a 6 mL fritted syringe with continuous shaking. DIPEA (227 μL, 1.34 mmol, 4 equiv.) and Fmoc-L-Ala-OH (208 mg, 0.66 mmol, 2 equiv.) were dissolved in 3.3 mL anhydrous DCM, and the solution was applied to the resin. The resin was incubated for 2 h at room temperature with continuous shaking. After the reaction, the resin was washed with DCM, DCM/methanol/DIPEA = 17/2/1, and DCM three times each. The loading of the beads was quantified according to the previous report.[1] The loading percentage was determined as 85%. 87 mg of the resin (71 μmol) was applied to further peptide synthesis. Fmoc deprotection was performed by incubating the resin with 20% piperidine in DMF twice (3 min and 12 min). After the reaction, the resin was washed with DMF three times. A coupling reaction of amino acids was performed using Fmoc-protected amino acid (4 equiv.), COMU (4 equiv.) and DIPEA (8 equiv.) in DMF (0.2 M for Fmoc-protected amino acid and COMU, and 0.4 M for DIPEA) for 1 h. Double coupling was conducted in the coupling reaction of 3rd L-Val, 8th L-MeVal, 9th L-MeLeu, and 10th L-MeLeu. After the reaction, the resin was washed with DMF three times. The coupling and deprotection were repeated until the 11th residue. The synthesized peptides were cleaved from the resin by incubating the resin with 30% HFIP in DCM for 20 min three times. The resin was washed with DCM and MeOH. The filtrate was collected in a recovery flask. After all the filtrates were combined, the solution was evaporated under reduced pressure. The residue was purified using a reversed phase column on HPLC to give **S1** (46 mg, 39 μmol, 55%). To **S1**, PyBOP (41 mg, 78 μmol, 2 equiv.), HOAt (11 mg, 78 μmol, 2 equiv.), DIPEA (66 μL, 0.39 mmol, 10 equiv.) and DMF/DCM (=1/1, 39 mL) were added, and the reaction mixture was shaken for 3 h at room temperature. The solvent was removed under reduced pressure, and the residue was purified by a reversed phase column on HPLC to give **CsO** (21 mg, 18 μmol, 25% from the 1st residue on the resin). MS (LC-ESI-MS. *m/z*) for $C_{60}H_{110}N_{11}O_{11}$ $[M+H]^+$: calcd. 1160.8, found 1161.5.

Assay

Measurement of the membrane thickness of PAMPA and Horizon-LBA

The thickness of the membranes of PAMPA and Horizon-LBA was calculated based on the capacitance value. The current value was monitored using a patch clamp amplifier (Nihon Kohden patch/whole cell clamp amplifier CEZ-2400). The current flowing with purchased capacitors under a rectangular voltage (V = ± 1.55 mV) generated by a voltage waveform generator (KENWOOD oscillator generator AG-203D) was measured. At each capacitor, the difference between the current value when the voltage shifted from positive to negative and the one from positive to negative was recorded in triplicate. A calibration curve was produced using the average of these current values and the electrostatic capacity.

A 96-well PAMPA donor plate was disassembled into a chamber, and a glass tube was attached to the chamber to connect the chamber to the patch clamp amplifier. The PVDF support in the chamber was first wet with methanol for 10 min and was wet with 1% DMSO/PBS. Next, 1% DMSO/PBS was added to the donor/acceptor chamber. The voltage between the donor and acceptor chamber was set as 0 mV when the chambers and PVDF support were fulfilled with 1% DMSO/PBS. After the chamber was washed with water and ethanol, it was dried. 5 μL of 10 mg/mL DOPC/decane was added to the PAMPA membrane, and 1% DMSO/PBS was added to the acceptor and donor well. The current was measured under the same rectangular voltage as the measurement of purchased capacitors in n = 3.

In measuring the lipid bilayers of the Horizon-LBA system, 5 μL 10 mg/mL DOPC/decane was added to the bottom of the acceptor chamber, and 1% DMSO/PBS was added to the acceptor and donor chambers. The chamber was lowered until the upper chamber contacted the water surface to make lipid bilayers. The current was measured under the same rectangular voltage as the measurement of the purchased capacitors in n = 3.

The capacitance of the artificial membranes and the lipid bilayers was calculated using the calibration curve. The thickness *d* of the membranes was calculated from the capacitance following equation.

$$d = \frac{\varepsilon S}{C}$$

Where S is the filter area in PAMPA (0.3 cm^2) and the pore area in Horizon-LBA. The pore area was calculated from the picture of the lipid bilayers. C is the capacitance. $\varepsilon = \varepsilon_0 \times \varepsilon_s$, where ε_0 is the electric constant and ε_s is the dielectric constant in lipids ($\varepsilon_s = 2$).[2]

PAMPA

Peptide permeability was measured by PAMPA.[3] On PAMPA, 300 μL of 1% DMSO in PBS was added to each well of the acceptor plate (MultiScreen 96-well Transport Receiver Plate, Merck). 150 μL of peptide solution (10 μM **1**, **2**, **3**, **4**, and **5**) in 1% DMSO/PBS was added to each well of the donor plate (MultiScreen-IP Filter Plate, 0.45 μm, Merck). In the donor solution, propranolol and norfloxacin were added as control compounds. 5 μL of 10 mg/mL DOPC/decane was applied to the membrane support (PVDF) on each well of the donor plate. The donor plate was put on the acceptor plate. In an incubator, the plate was left for 7 h at 25 °C. The concentration of peptides was determined using LC/MS. The experiment was performed in triplicate. The permeability value (P_e) was calculated using the following equations:

$$P_e = -\frac{\ln\left[1 - C_A(t)/C_{equilibrium}\right]}{A \times (1/V_D + 1/V_A) \times t}$$

$$C_{equilibrium} = \frac{C_D(t) \times V_D + C_A(t) \times V_A}{V_D + V_A}$$

Where:

A = filter area (0.3 cm^2)

V_D = donor well volume (0.15 cm^3)

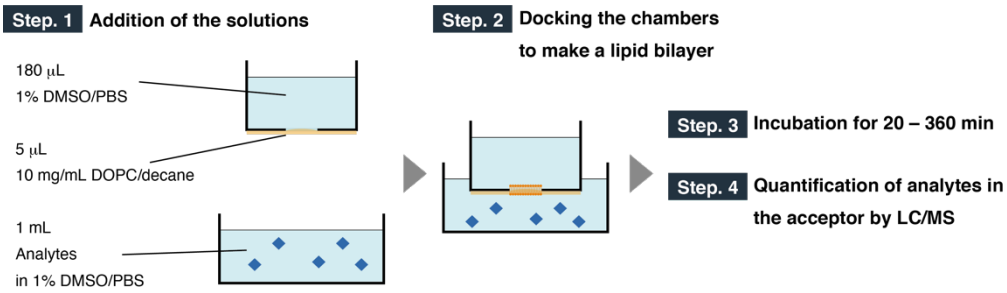
V_A = acceptor well volume (0.3 cm^3)

t = incubation time (s)

$C_D(t)$ = compound concentration in donor well at time t

$C_A(t)$ = compound concentration in acceptor well at time t

General scheme of permeability measurements by Horizon-LBA



Scheme S1. Experimental procedure of Horizon-LBA

The apparatus consists of two chambers. A thin plastic film with a small pore (~120 μm) was attached to the bottom of the upper chamber. 5 μL of 10 mg/mL DOPC/decane was added and spread on the bottom of the upper chamber. Immediately, 1 mL of analytes in 1% DMSO/PBS was added to the lower chamber (donor chamber), and 180 μL of 1% DMSO/PBS was added to the upper chamber (acceptor chamber). Propranolol and HPTS were included in the donor solution as controls. HPTS was used to confirm the integrity of lipid bilayers during the permeability assay. The upper chamber was lowered until it touched the water surface in the lower chamber, which resulted in the generation of a lipid bilayer. The lipid bilayer formation was confirmed by a visual inspection under the microscope and from the current value through the membrane. The success rate of constructing lipid bilayers was over 90%. The system was incubated for 20–360 min, and the solution in the acceptor chamber was collected. Quantification of compounds was conducted by LC/MS. In this assay, the amount of the analytes coming to the acceptor well is less than 0.2% of the initial amount in the donor well, even when the assay is conducted using highly permeable propranolol in 6 h incubation. Therefore, it is reasonable to interpret that the analytes permeate the membrane at the initial speed at least during ~6 h incubation in our permeability assay because the concentration of the donor well can be regarded unchanged during the permeability experiments. Therefore, as a permeability coefficient, P_{app} was used and was calculated using the following equation:

$$P_{app} = \frac{Q}{A \times t \times C_0}$$

Where Q (mol) is the amount of analytes in the acceptor well measured in LC/MS. t (s) is the incubation time. C_0 (mol cm^{-3}) is the initial concentration of the analyte in the lower (donor) chamber. A is the hole area (cm^2). A was calculated based on a picture taken at t = 1 min.

Permeability measurement of small molecules by Horizon-LBA

The permeability of small molecules across the lipid bilayer was measured by Horizon-LBA. Small molecules (100 μM propranolol, memantine, metoprolol, caffeine, phenytoin, antipyrine, carbamazepine, *N*-acetylated tryptophan, acebutolol, HPTS, and 400 μM cephalexin) in 1% DMSO/PBS was used as the donor solution. After the system was incubated for 20 min at room temperature, the acceptor solution was collected. The small molecules in the acceptor well were quantified by LC/MS. The P_{app} of phenytoin was under the quantification limit. The experiment was conducted in quintuplicate.

Permeability measurement of peptides 1–5 by Horizon-LBA

The permeability of peptides across the lipid bilayer was measured by Horizon-LBA. 10 μM **1**, **2**, **3**, **4**, **5**, propranolol, and HPTS in 1% DMSO/PBS was used as the donor solution. After the system was incubated for 1 h at room temperature, the acceptor solution was collected. The analytes in the acceptor well were quantified by LC/MS. The experiment was conducted in quintuplicate.

Permeability measurement of CsA and CsO by Horizon-LBA

The permeability of CsA and CsO across the lipid bilayer was measured by Horizon-LBA. 6 μM CsA, 6 μM CsO, 10 μM propranolol, and 40 μM HPTS in 1% DMSO/PBS were used as the donor solution. After the system was incubated for 2, 4, and 6 h at room temperature, the acceptor solution was collected. The experiment was conducted in triplicate at each incubation time. The peptides in the acceptor well were quantified by LC/MS. Significant differences between the apparent permeability values (P_{app}) of CsA at different incubation times were not observed (Figure S1). The independence of P_{app} on incubation time suggests that the initial speed of permeation is measured by Horizon-LBA under our experimental conditions.

Supporting figures & Tables

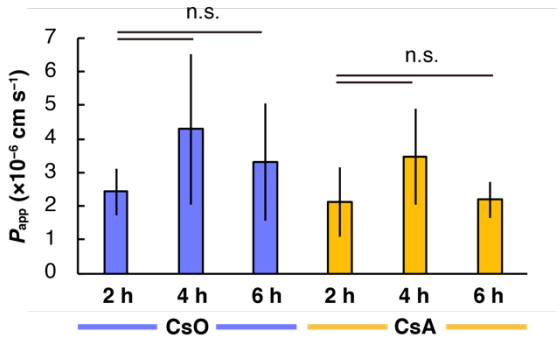


Figure S1. Lipid bilayer permeability of CsA and CsO at 2, 4, and 6 h. n.s. denotes not significant. Data are presented as mean ± standard deviation.

All the peptides after purification were analyzed using UPLC. The purity of each peptide was checked by running UPLC at a flow rate of 0.4 mL/min using mobile phase A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) over 14 min gradient. The column temperature was 40 °C.

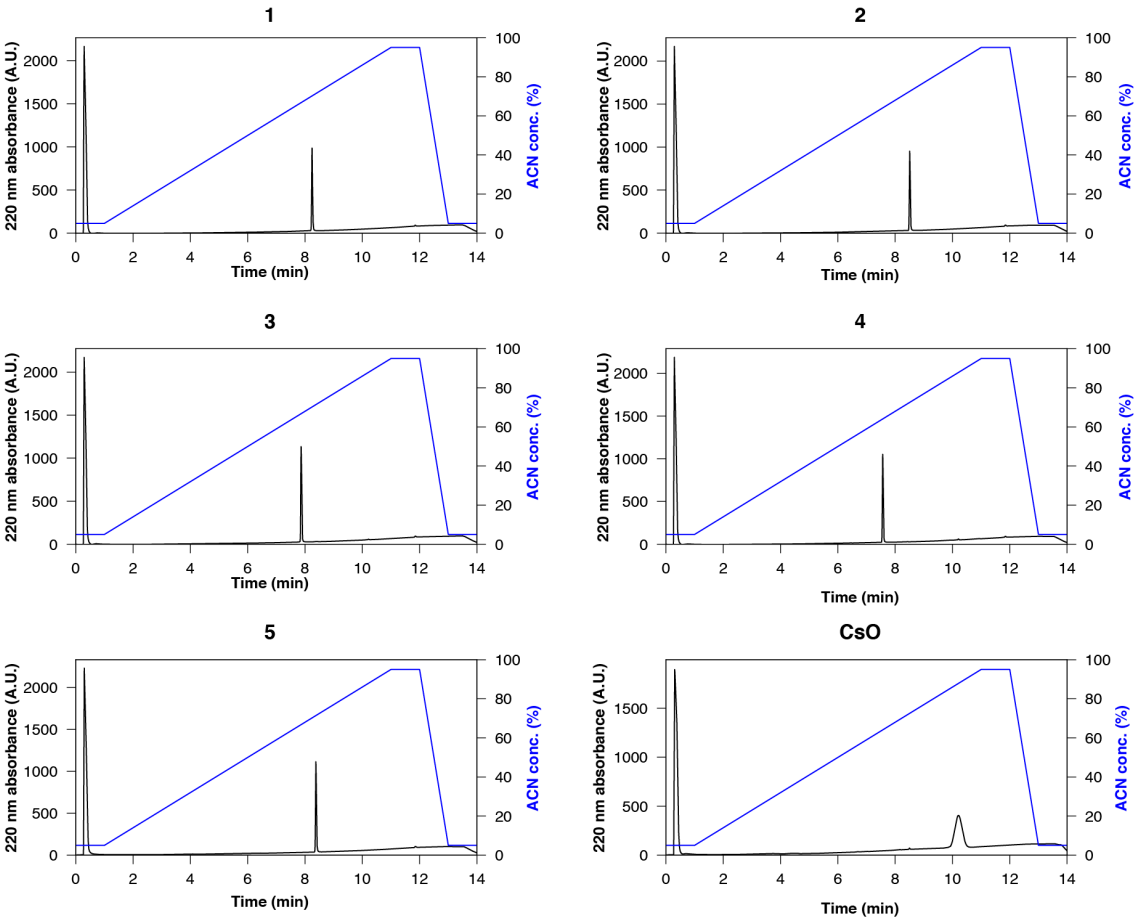


Figure S2. UV chromatogram of all the peptides analyzed in this study.

Table S1. The thickness of lipid bilayers in Horizon-LBA and artificial membranes in PAMPA. Data are presented as means ± standard deviation.

	Horizon-LBA Lipid bilayer	**PAMPA Artificial membrane**
Capacitance (pF)	30 ± 10	13 ± 2
Area (cm^2)	$15 \pm 3 \times 10^{-5}$	0.3
Thickness	8 ± 1 nm	43 ± 7 μm

Table S2. Permeability coefficients of small molecules measured by Horizon-LBA, PAMPA, and Caco-2.[4,5] Data are presented as means ± standard deviation.

Entry	**Compound**	**Horizon-LBA** P_{app} **(×10^{-4} cm s^{-1})**	**PAMPA** [a] P_e **(×10^{-6} cm s^{-1})**	**Caco-2** [b] P_{app} **(×10^{-4} cm s^{-1})**
1	Acebutolol	0.04 ± 0.01	0.03 ± 0.01	0.0380 ± 0.0026
2	Cephalexin	0.04 ± 0.01		0.0027 ± 0.0008
3	NAc-Trp	0.17 ± 0.01		
4	Propranolol	1.7 ± 0.3	14.30 ± 0.10	1.10 ± 0.13
5	Carbamazepine	2.5 ± 0.4	6.40 ± 0.20	
6	Memantine	2.5 ± 0.2		
7	Metoprolol	2.5 ± 0.2	0.41 ± 0.05	1.4± 0.1
8	Antipyrine	2.8 ± 0.4	0.74 ± 0.06	1.50 ± 0.12
9	Caffeine	3.0 ± 0.6	1.20 ± 0.10	
10	Phenytoin	N. D. [c]	0.38 ± 0.10	1.60 ± 0.18

[a] The values are from reference [4]. [b] The values are from reference [5]. [c] N. D. means not determined.

Table S3. Permeability coefficients of cyclic peptide **1**–**5** by Horizon-LBA and PAMPA. Data are presented as means ± standard deviation.

Peptide	**Horizon-LBA** P_{app} **(×10^{-4} cm s^{-1})**	**PAMPA** P_e **(×10^{-6} cm s^{-1})**
1	3 ± 1	16.5 ± 0.9
2	3 ± 1	11.9 ± 0.9
3	2 ± 1	19 ± 1
4	1.4 ± 0.5	9.1 ± 0.3
5	0.4 ± 0.2	13 ± 1

Table S4. Calculated and observed mass of cyclic hexapeptides.

			Calculated		
Peptide	**Sequence**	**Formula**	**Exact MS**	**$[M+Na]^+$**	**Observed**
1	cyclo[VlPhe(*p*-OMe)plL]	$C_{38}H_{60}N_6O_7$	712.4523	735.4416	735.4424
2	cyclo[IlPhe(*p*-OMe)plL]	$C_{39}H_{62}N_6O_7$	726.4680	749.4572	749.4583
3	cyclo[AlPhe(*p*-OMe)plL]	$C_{36}H_{56}N_6O_7$	684.4210	707.4103	707.4104
4	cyclo[GlPhe(*p*-OMe)plL]	$C_{35}H_{54}N_6O_7$	670.4054	693.3946	693.3946
5	cyclo[MlPhe(*p*-OMe)plL]	$C_{38}H_{60}N_6O_7S$	744.4244	767.4136	767.4138

Reference

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