Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

pH-Dependent Transport of Amino Acids across Lipid Bilayers by Simple Monotopic Anion Carriers

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Contents

1 General Information

1.1 Abbreviations

Gly: glycine;

Ala: alanine;

Val: valine;

Ile: isoleucine;

Leu: leucine;

Pro: proline;

Ser: serine;

Tyr: tyrosine;

Met: methionine;

Phe: phenylalanine;

Gln: glutamine;

His: histidine;

Cys: cysteine;

Trp: tryptophan;

Asp: aspartic acid;

Glu: glutaric acid;

Lys: lysine;

Arg: arginine;

LUV: Large Unilamellar Vesicle;

POPC: 1-palmitoyl-2-oleoylphosphatidylcholine;

HEPES: 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid;

HPTS: 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt;

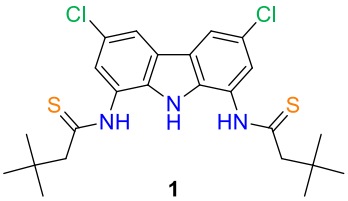
SPQ: 6-methoxy-N-(3-sulfopropyl)quinolinium;

DMSO: dimethyl sulfoxide.

1.2 Materials

Transporters **1** and **2** were obtained as described previously.[1]

Transporters **3**-**6** were obtained according to literature procedures.[2]



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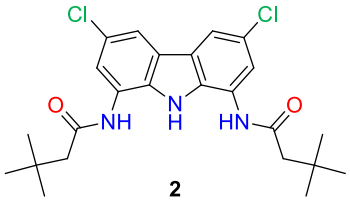
$C_{24}H_{29}Cl_2N_3S_2$ = 494.54



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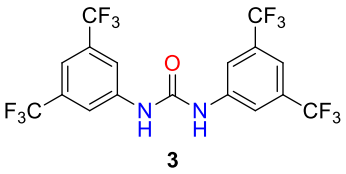
$C_{24}H_{29}Cl_2N_3O_2$ = 462.41



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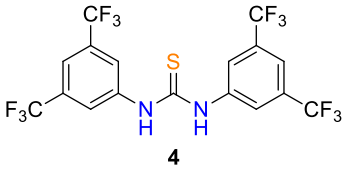
$C_{17}H_8F_{12}N_2O$ = 484.24



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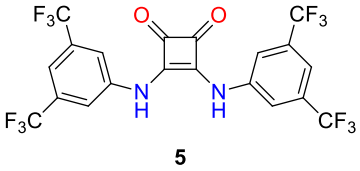
$C_{17}H_8F_{12}N_2S$ = 500.31



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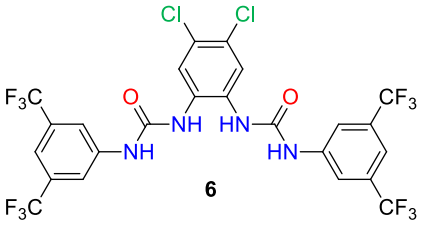
$C_{20}H_8F_{12}N_2O_2$ = 536.27



6

$C_{24}H_{12}Cl_2F_{12}N_4O_2$ = 687.27

Sigma-Aldrich: cholesterol (C8667, >99.5%), POPC: 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (42773, 500 mg, >99.0%), aluminum oxide (199443, activated, basic), chloroform (for HPLC, stab. with amylene, 34854-1L-M, ≥99.8%), sodium nitrate (221341, ≥99.0%), Sephadex G-25 fine (G2580), HEPES (H3375, ≥99.5%), HPTS (H1529, ≥96%), calcein (C0875), L-alanine-1-^{13}C (489867), L-serine (S4500, ≥99%), L-proline (81709, ≥99.5%), glycine (G7126, ≥99%), L-tyrosine (T3754, ≥98%), L-methionine-1-^{13}C (490083), manganese(II) sulfate monohydrate (M7634, ≥99%).

Merck: sodium hydroxide solution (2 N, Titripur, 1091361000), L-valine (8495, >99%).

TCI: L-methionine (M0099, >99%), H-Phe-OH (4354, >99%), H-Leu-OH (4346, >99%), H-Ala-OH (4343, >99%), L-glutamine (G0063, >99%), L-histidine (H0149, >99%), L-cysteine (C0515, ≥98.0%), L-tryptophan (T0541, ≥98.5%), L-aspartic acid (A0546, >99.0%), glutaric acid (G0069, >99.0%).

FluoroChem: H-Ile-OH (M03002, 98%), L-lysine (044909, 97%).

Labomics: SPQ (6-methoxy-*N*-(3-sulfopropyl)quinolinium) (C274).

GE Healthcare: Sephadex G-50 superfine (17004101).

Chempur: methanol (116219904, >99.8%).

Reanal: L-arginine (7110529).

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[1] Thioamide **1**: K. M. Bąk, B. van Kolck, K. Maslowska-Jarzyna, P. Papadopoulou, A. Kros, M. J. Chmielewski, Oxyanion Transport across Lipid Bilayers: Direct Measurements in Large and Giant Unilamellar Vesicles. *Chem. Commun.* **2020**, *56,* 4910–4913.
Amide **2**: K. M. Bąk, K. Chabuda, H. Montes, R. Quesada, M. J. Chmielewski, 1,8-Diamidocarbazoles: An Easily Tuneable Family of Fluorescent Anion Sensors and Transporters. *Org. Biomol. Chem.* **2018**, *16,* 5188–5196.

[2] Urea **3**, thiourea **4**, squaramide **5**: N. Busschaert, I. L. Kirby, S. Young, S. J. Coles, P. N. Horton, M. E. Light, P. A. Gale, Squaramides as Potent Transmembrane Anion Transporter. *Angew. Chem. Int. Ed*. **2012**, *51*, 4426–4430.
Diurea **6**: C. M. Dias, H. Li, H. Valkenier, L. E. Karagiannidis, P. A. Gale, D. N. Sheppard, A. P. Davis, Anion transport by ortho-phenylene bis-ureas across cell and vesicle membranes. *Org. Biomol. Chem.,* **2018**, *16*, 1083–1087.

All solvents and reagents were commercially available and used as received unless otherwise stated.

Water was taken from Milli-Q purification system.

Sodium salts of amino acids were prepared as follows: to a 5 mL volumetric flask 2.5 mmol of an amino acid was weighed. Then, sodium nitrate (2.5 mL, 450 mM) and stoichiometric amount of aqueous solution of NaOH (1.25 mL, 2.000 M) were added with a gas-tight syringe. Next, the volumetric flask was filled with water to 5 mL, to obtain 0.5 M solution of sodium salt. pH was measured for each salt solution after every preparation using a pH-meter.

1.3 Instruments and Methods

Weighing
Mettler Toledo Excellence XA105DU analytical balance was used for weighing all the samples.

UV-Vis spectroscopy
UV spectra were obtained on Thermo Scientific Evolution 300 UV-vis spectrometer.

Fluorescence spectroscopy
Fluorescence spectra were acquired using Hitachi F-7000 spectrophotometer equipped with Peltier temperature controller. Screw-capped SUPRASIL quartz fluorescence cuvettes (optical path length: 10 mm) were used.

NMR spectroscopy
NMR spectra were recorded using an Agilent NMR (^{13}C: 100 MHz) spectrometer (Agilent Technologies, Santa Clara, CA, USA).

Vortexing
IKA VORTEX 4 basic, model V 4 B S000 was used for vortexing during LUVs preparation.

Extrusion
AVESTIN LiposoFast-Basic with polycarbonate membranes with pore sizes of 200 nm was used for extrusion during LUVs preparation.

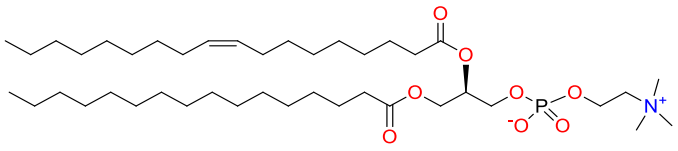
Measurements of pH
Measurements of pH were carried out with SevenExcellence pH/Cond meter S470 equipped with pH electrode InLab Expert Pro.

2 Amino acids transport studies using calcein assay

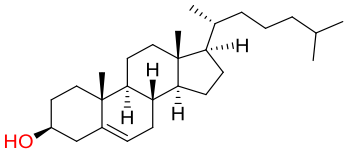
2.1 General procedure for amino acid transport measurements in LUVs at pH 7.4 using calcein assay

The general procedure is a slightly modified version of the previously published protocol.[3]

LUVs (mean diameter 200 nm), made of POPC and cholesterol in a 7:3 molar ratio, were prepared as follows. In a 5 mL round bottom flask a solution of lipids (POPC and cholesterol in a 7:3 molar ratio, total lipid concentration 6 mM) in chloroform (freshly deacidified by passing through a pad of activated basic alumina) was prepared.

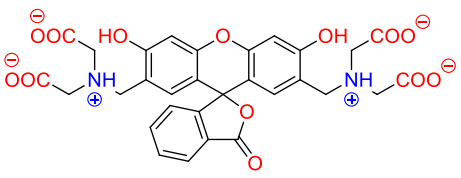


POPC
$C_{42}H_{82}NO_8P$ = 760.09

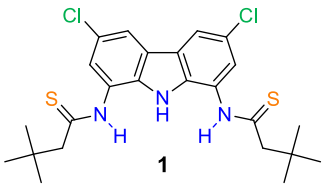


cholesterol
$C_{27}H_{46}O$ = 386.66

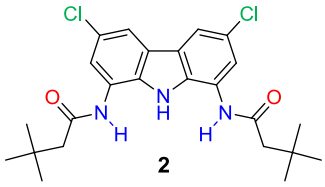
The solution of lipids in chloroform was evaporated in a round-bottom flask on a rotary evaporator and the formed lipid film was further dried under vacuum for at least 2 h. The lipid film was hydrated with 500 µl of a buffered aqueous solution containing Cu^{2+} and calcein (pH 7.4, 20 mM HEPES, 100 mM Na_2SO_4, 0.2 mM $CuSO_4$, 0.2 mM calcein), kept for 30 s in an ultrasonic bath and vortexed for 1 h. The lipid suspension was subjected to 10 freeze/thaw cycles, diluted to 1 mL by the addition of a buffered aqueous solution containing Cu^{2+} and calcein (pH 7.4, 20 mM HEPES, 100 mM Na_2SO_4, 0.2 mM $CuSO_4$, 0.2 mM calcein) and then extruded 29 times through a 200 nm polycarbonate membrane. The unentrapped calcein was removed by size exclusion chromatography on a Sephadex G25 column using buffer solution as eluent (pH 7.4, 20 mM HEPES, 100 mM Na_2SO_4).



calcein



1
$C_{24}H_{29}Cl_2N_3S_2$ = 494.54



2
$C_{24}H_{29}Cl_2N_3O_2$ = 462.41

The collected vesicles were diluted with buffered solution of Na_2SO_4 and $CuSO_4$ (pH 7.4, 20 mM HEPES, 100 mM Na_2SO_4, 0.2 mM $CuSO_4$) to 10.5 ml (lipid concentration ≈ 1.14 mM). The suspension of liposomes (1.76 mL) was placed into a quartz cuvette and stirred and thermostated at 25°C for 2 minutes. Next, 0.240 mL of buffered aqueous solution containing Cu^{2+} and amino acid (pH 7.4, 20 mM HEPES, 100 mM Na_2SO_4, 0.2 mM $CuSO_4$, 250 mM amino acid) were added to obtain total concentration of lipids ≈ 1 mM and concentration of amino acid = 30 mM.
DMSO solution of transporter **1** or **2** (20 µL, 10^{-3} M, 1 mol% of **1** or **2** with respect to the total amount of lipids) or DMSO alone (20 µL, blank) was added after 5 min to the vesicle suspension to initiate the amino acid transport, and fluorescence emission (λ_{ex} = 495 nm, λ_{em} = 515 nm) was recorded over 40 min.

[3] X. Wu, N. Busschaert, N. J. Wells, Y.-B. Jiang, P. A. Gale, Dynamic Covalent Transport of Amino Acids across Lipid Bilayers. *J. Am. Chem. Soc.*, **2015**, *137*, 1476–1484.

Blank experiments related to amino acid transport in LUVs at pH 7.4 by calcein assay

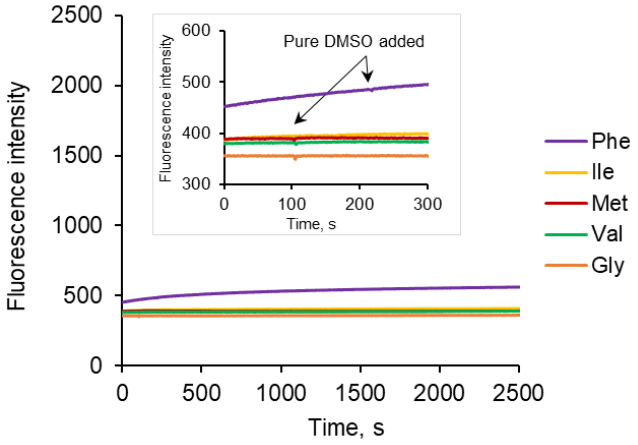


Figure S2.1.1 To the suspension of LUVs (1.76 ml, POPC:cholesterol 7:3, mean diameter 200 nm), loaded with solution of Cu^{2+} and calcein (pH 7.4, 20 mM HEPES, 100 mM Na_2SO_4, 0.2 mM $CuSO_4$, 0.2 mM calcein), a solution containing Cu^{2+} and amino acid (0.24 mL, pH 7.4, 20 mM HEPES, 100 mM Na_2SO_4, 0.2 mM $CuSO_4$, 250 mM amino acid) was added followed by the addition of pure DMSO (20 µL). The fluorescence intensity (λ_{ex} = 495 nm, λ_{em} =515 nm) was recorded for 2500 s.

Normalization of fluorescence intensity

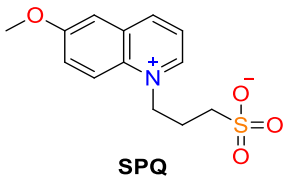
1 mol% (transporter to lipid) of **1** leads to saturation of the fluorescence intensity within 15 min for Phe, and thus fractional fluorescence intensity of all measurements was normalized according to the following equation:

$$I_f = \frac{I_t - I_0}{I_{max} - I_0}$$

where I_t is the fluorescence intensity at time t, I_0 is the fluorescence intensity at time t = 0, and I_{max} is the maximum fluorescence intensity obtained for Phe.

3 The development of the SPQ assay

3.1 Stability of SPQ in the presence of sodium salts of amino acids



SPQ

$C_{13}H_{14}O_4NS$ = 281.33

To an aqueous solution of SPQ (3 ml, 0.1 mM in 225 mM $NaNO_3$) in a septum-sealed, screw-capped quartz cuvette with optical path length of 10 mm, aliquots of an appropriate amino acid sodium salt solution (500 mM in 225 mM $NaNO_3$) were added with a gas-tight microsyringe. The addition of the amino acids sodium salts was followed by the addition of the same aliquots of more concentrated SPQ solution (0.2 mM in 225 mM $NaNO_3$) to compensate for the dilution of SPQ during the titration. UV-Vis spectrum was recorded after each addition. Only negligible changes in absorbance were observed, which shows that SPQ does not react with sodium salts of amino acids.

Additionally, the sample containing the highest concentration of amino acid sodium salt (30 mM) was measured after 5, 10, 15 and 30 min. No changes were observed, which demonstrates that SPQ is stable in the presence of sodium salts of amino acids.

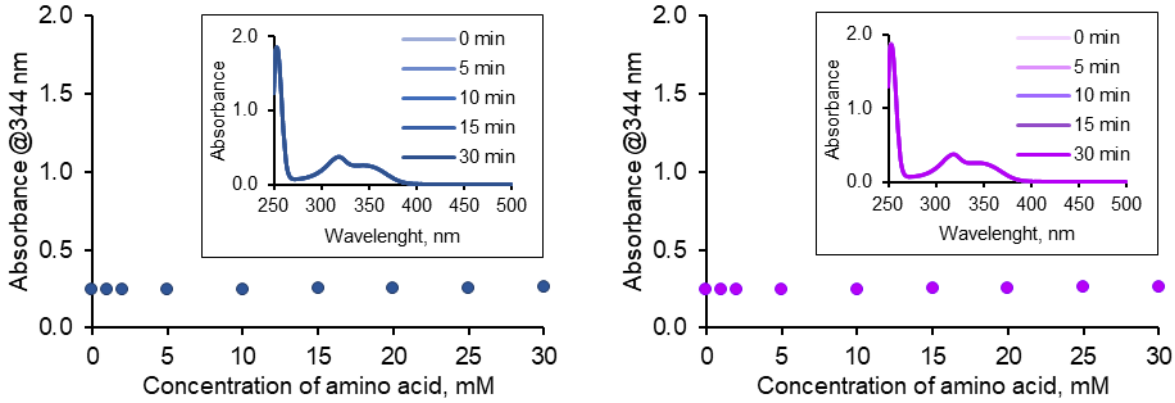


Figure S3.1.1 Left: UV-Vis titration of SPQ (10^{-4} M in 225 mM $NaNO_3$) with serine-Na (0.5 M) at 344 nm (SPQ excitation wavelength used in transport experiments). Inset: UV-Vis spectra of SPQ (10^{-4} M in 225 mM $NaNO_3$) in the presence of Ser-Na (30 mM) at time intervals. Right: UV-Vis titration of SPQ (10^{-4} M in 225 mM $NaNO_3$) with threonine-Na (0.5 M) at 344 nm (SPQ excitation wavelength used in transport experiments). Inset: UV-Vis spectra of SPQ (10^{-4} M in 225 mM $NaNO_3$) in the presence of Thr-Na (30 mM) at time intervals.

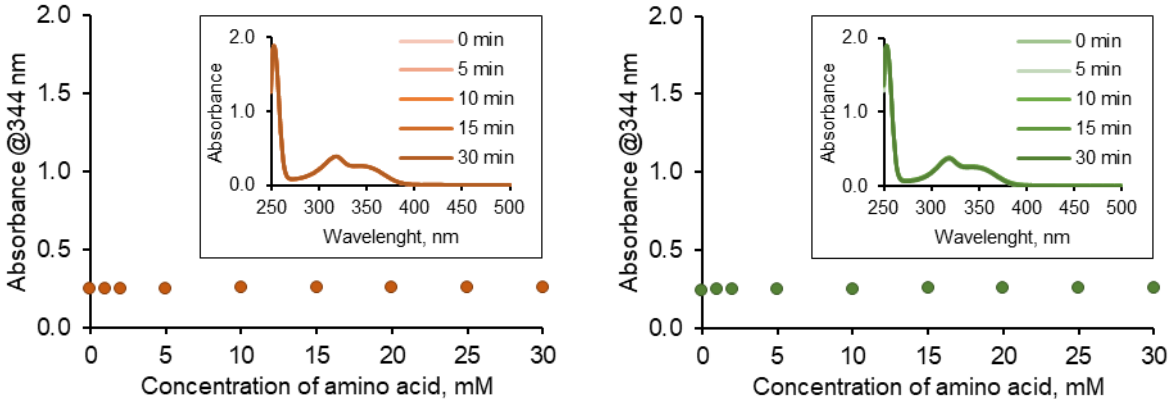


Figure S3.1.2 Left: UV-Vis titration of SPQ (10^{-4} M in 225 mM $NaNO_3$) with glycine-Na (0.5 M) at 344 nm (SPQ excitation wavelength used in transport experiments). Inset: UV-Vis spectra of SPQ (10^{-4} M in 225 mM $NaNO_3$) in the presence of Gly-Na (30 mM) at time intervals. Right: UV-Vis titration of SPQ (10^{-4} M in 225 mM $NaNO_3$) with alanine-Na (0.5 M) at 344 nm (SPQ excitation wavelength used in transport experiments). Inset: UV-Vis spectra of SPQ (10^{-4} M in 225 mM $NaNO_3$) in the presence of Ala-Na (30 mM) at time intervals.

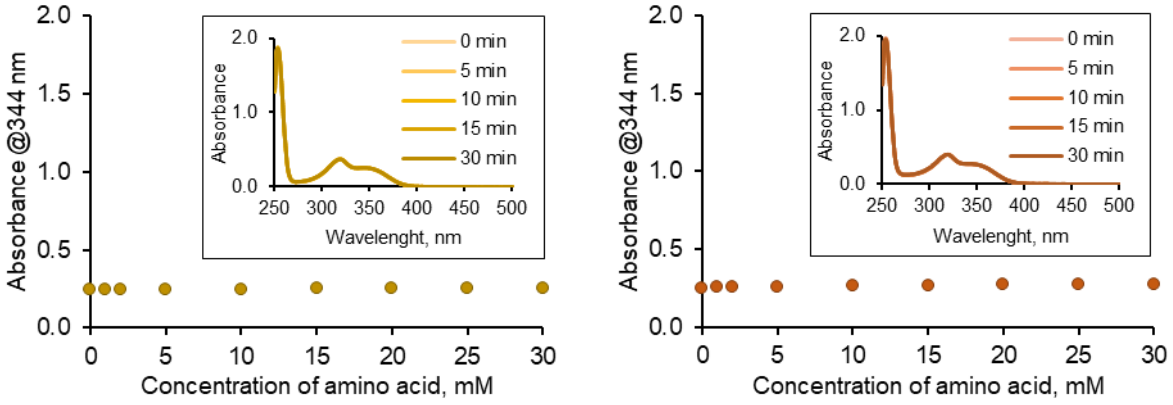


Figure S3.1.3 Left: UV-Vis titration of SPQ (10^{-4} M in 225 mM $NaNO_3$) with valine-Na (0.5 M) at 344 nm (SPQ excitation wavelength used in transport experiments). Inset: UV-Vis spectra of SPQ (10^{-4} M in 225 mM $NaNO_3$) in the presence of Val-Na (30 mM) at time intervals. Right: UV-Vis titration of SPQ (10^{-4} M in 225 mM $NaNO_3$) with isoleucine-Na (0.5 M) at 344 nm (SPQ excitation wavelength used in transport experiments). Inset: UV-Vis spectra of SPQ (10^{-4} M in 225 mM $NaNO_3$) in the presence of Ile-Na (30 mM) at time intervals.

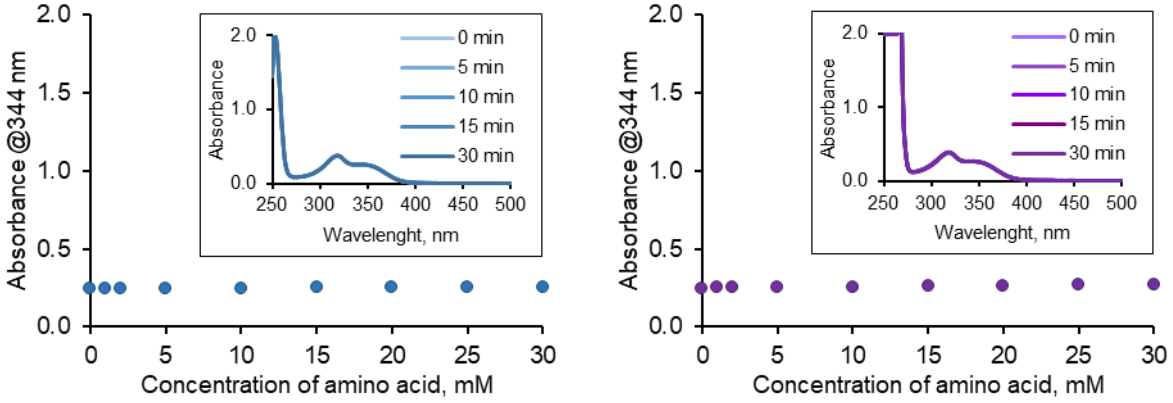


Figure S3.1.4 Left: UV-Vis titration of SPQ (10^{-4} M in 225 mM $NaNO_3$) with methionine-Na (0.5 M) at 344 nm (SPQ excitation wavelength used in transport experiments). Inset: UV-Vis spectra of SPQ (10^{-4} M in 225 mM $NaNO_3$) in the presence of Met-Na (30 mM) at time intervals. Right: UV-Vis titration of SPQ (10^{-4} M in 225 mM $NaNO_3$) with phenylalanine-Na (0.5 M) at 344 nm (SPQ excitation wavelength used in transport experiments). Inset: UV-Vis spectra of SPQ (10^{-4} M in 225 mM $NaNO_3$) in the presence of Phe-Na (30 mM) at time intervals.

3.2 Effect of NaOH on SPQ fluorescence

To an aqueous solution of SPQ (3 mL, 0.01 mM in 225 mM $NaNO_3$) in a septum-sealed, screw-capped fluorescence cuvette with optical path length of 10 mm, aliquots of NaOH (0.1 M) were added with a gas-tight microsyringe. The addition of NaOH to the SPQ solution was followed by the addition of the same aliquots of SPQ solution (0.02 mM) to avoid dilution of SPQ. Fluorescence spectrum was recorded after each addition (excitation wavelength: 344 nm, scan speed: 240 nm/min, temperature: 25°C). Titration curves were obtained by measuring the emission at 440 nm (excitation wavelength: 344 nm). The measured signal was normalized against the initial intensity.

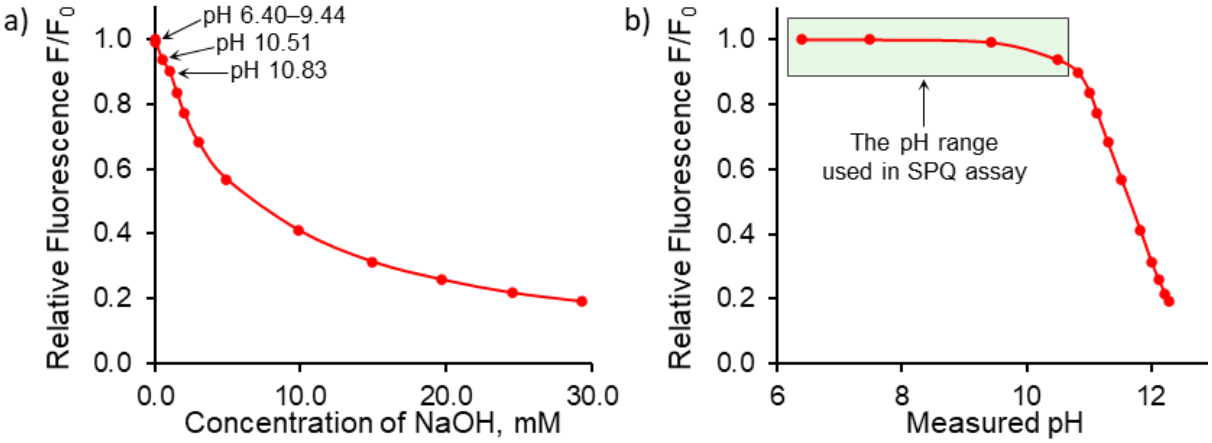


Figure S3.2.1 Fluorescence titration curves of an aqueous solution of SPQ (10^{-5} M in 225 mM $NaNO_3$) with NaOH (0.1 M). Chart (b) was obtained by replacing the horizontal scale of chart (a) with measured pH values. Excitation wavelength: 344 nm, emission wavelength: 440 nm.

3.3 Quenching of SPQ fluorescence by sodium salts of amino acids

Sodium salts of amino acids were prepared as described in Section 1.2.

To a solution of SPQ (3 mL, 10^{-5} M in 0.225 M aqueous $NaNO_3$) placed in a septum-sealed, screw-capped SUPRASIL quartz fluorescence cuvette with optical path length of 10 mm, aliquots of an appropriate amino acid salt (0.5 M, in 0.225 M aqueous $NaNO_3$) were added with a gas-tight microsyringe. The addition of each aliquot of the amino acid salt to the SPQ solution was followed by the addition of the same aliquot of the more concentrated SPQ solution ($2 \cdot 10^{-5}$ M in 0.225 M aqueous $NaNO_3$), to compensate for the dilution of SPQ. Fluorescence spectrum was recorded after each addition (excitation wavelength: 344 nm, scan speed: 240 nm/min, temperature: 25°C). The pH of the solution in the cuvette was measured by pH-meter after each fluorescent titration and never exceeded 10.7.

Titration curves were obtained by measuring emission at 440 nm; the signal was normalized against the initial intensity.

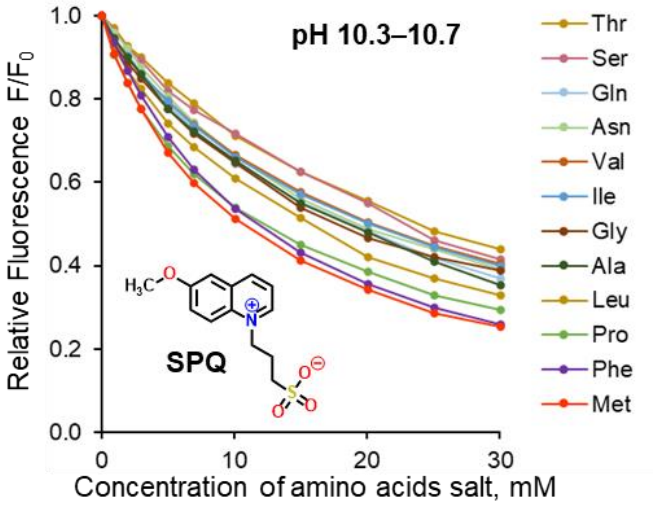


Figure S3.3.1 Fluorescence titration curves of SPQ (10^{-5} M in 225 mM $NaNO_3$) with 0.5M sodium salts of amino acids. Excitation: 344 nm, emission: 440 nm.

3.4 Quenching of SPQ fluorescence by amino acids

To a solution of SPQ (1.5 mL, 10^{-5} M in 225 mM aqueous $NaNO_3$) placed in a septum-sealed, screw-capped SUPRASIL quartz fluorescence cuvette with optical path length of 10 mm, aliquots of an appropriate amino acid (0.05 M in 225 mM aqueous $NaNO_3$) were added with a gas-tight microsyringe. The addition of each aliquot of the amino acid to the SPQ solution was followed by the addition of the same aliquot of the more concentrated SPQ solution ($2 \cdot 10^{-5}$ M in 0.225 M aqueous $NaNO_3$) to compensate for the dilution of SPQ. Fluorescence spectrum was recorded after each addition (excitation wavelength: 344 nm, scan speed: 240 nm/min, temperature: 25°C).

Titration curves were obtained by measuring emission at 440 nm; the signal was normalized against the initial intensity.

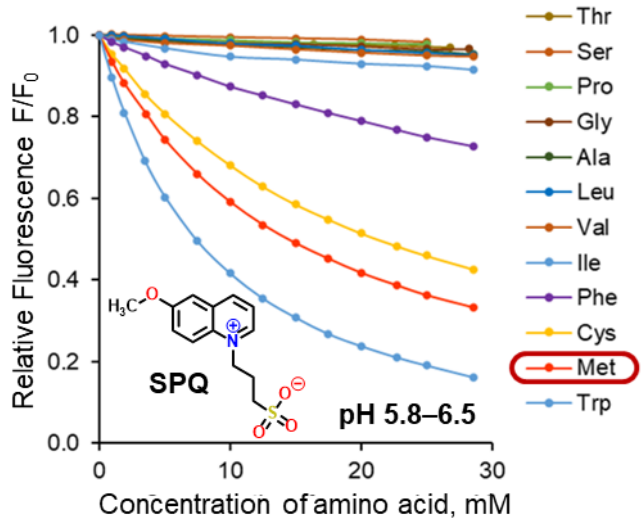


Figure S3.4.1 Fluorescence titration curves of SPQ (10^{-5} M in 225 mM $NaNO_3$) with 0.05 M amino acids. Excitation: 344 nm, emission: 440 nm.

Comparison of fluorescence quenching of SPQ by Met (pH 5.8) and Met-Na (pH 10.6)

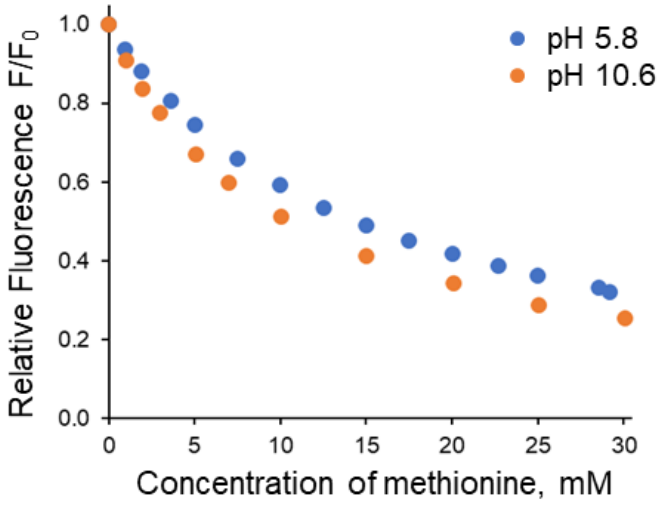


Figure S3.4.2 The fluorescence of SPQ (10^{-5} M in 225 mM $NaNO_3$) is quenched to a similar extent by methionine (at pH 5.8) and its sodium salt (at pH 10.6). Excitation wavelength: 344 nm, emission wavelength: 440 nm.

3.5 Determination of Stern-Volmer constants

To check the compliance with the linear Stern-Volmer relationship, the F_0/F values at 440 nm were plotted against the amino acids or amino acid salts concentration and fitted with the following equation:

$$\frac{F_0}{F} = 1 + K_{SV} \cdot [AA]$$

where K_{SV} is the Stern-Volmer constant and $[AA]$ is the concentration of a quencher – amino acid or its anion.

All of the studied amino acids and amino acids salts gave good linear correlations with $R^2 \geq 0.994$.

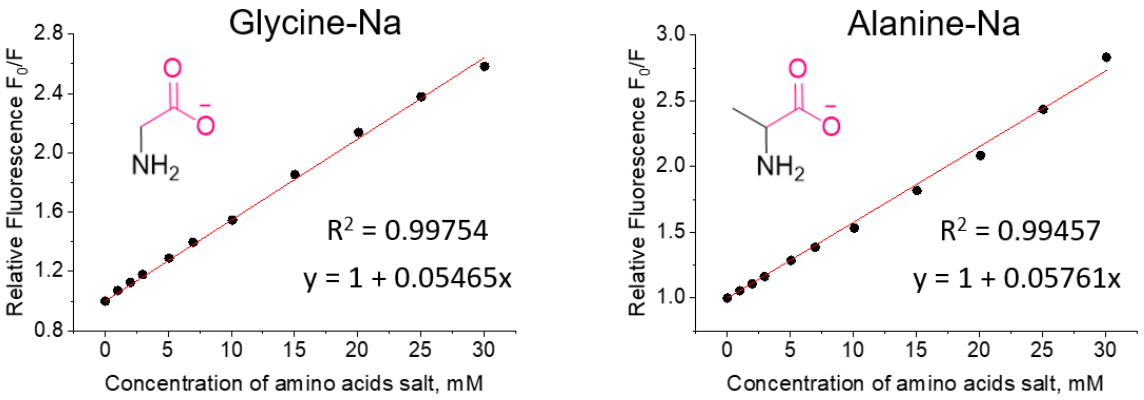


Figure S3.5.1 Stern-Volmer relationship between SPQ fluorescence and concentration of glycine-Na (left, pH 10.6) or alanine-Na (right, pH 10.6). Excitation: 344 nm, emission: 440 nm.

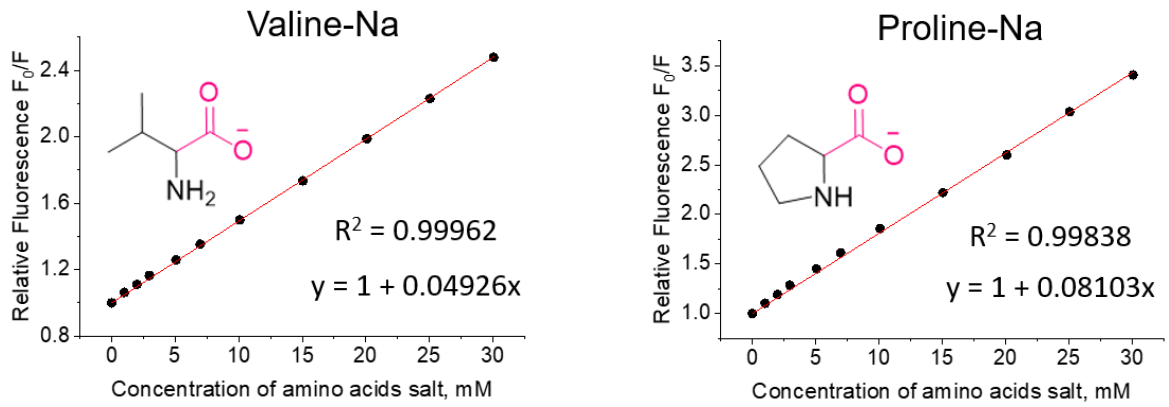


Figure S3.5.2 Stern-Volmer relationship between SPQ fluorescence and concentration of valine-Na (left, pH 10.6) or proline-Na (right, pH 10.7). Excitation: 344 nm, emission: 440 nm.

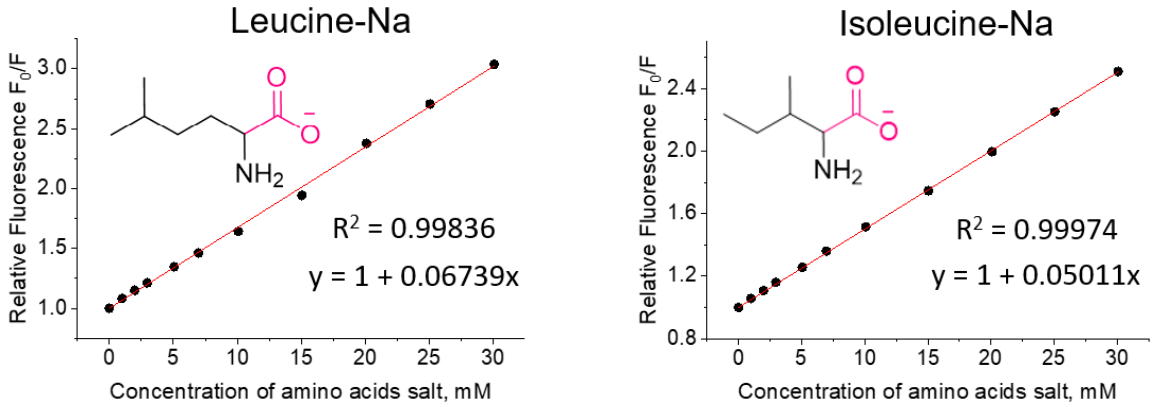


Figure S3.5.3 Stern-Volmer relationship between SPQ fluorescence and concentration of leucine-Na (left, pH 10.7) or isoleucine-Na (right, pH 10.6). Excitation: 344 nm, emission: 440 nm.

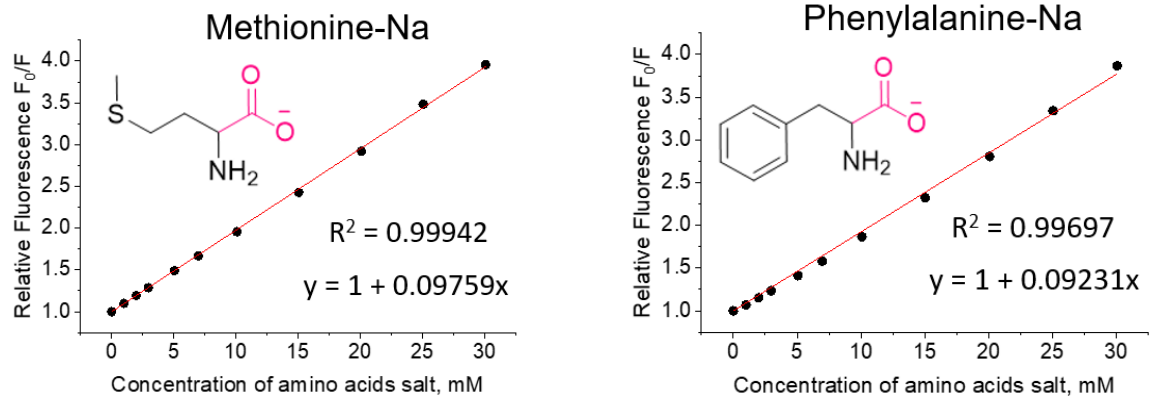


Figure S3.5.4 Stern-Volmer relationship between SPQ fluorescence and concentration of methionine-Na (left, pH 10.6) or phenylalanine-Na (right, pH 10.6). Excitation: 344 nm, emission: 440 nm.

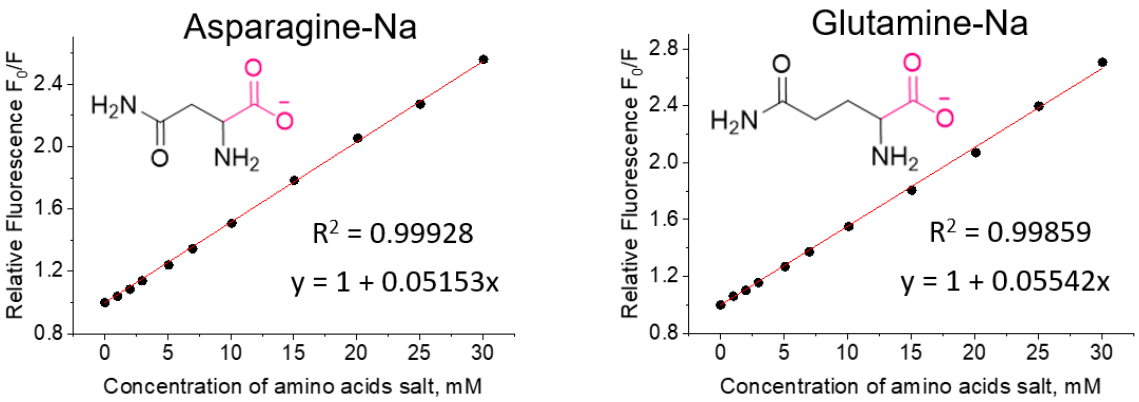


Figure S3.5.5 Stern-Volmer relationship between SPQ fluorescence and concentration of asparagine-Na (left, pH 10.6) or glutamine-Na (right, pH 10.6). Excitation: 344 nm, emission: 440 nm.

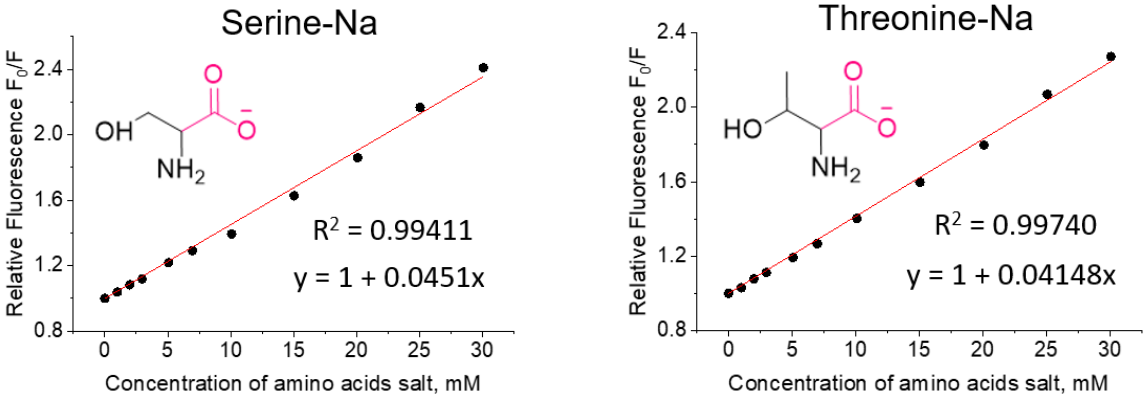


Figure S3.5.6 Stern-Volmer relationship between SPQ fluorescence and concentration of serine-Na (left, pH 10.6) or threonine-Na (right, pH 10.6). Excitation: 344 nm, emission: 440 nm.

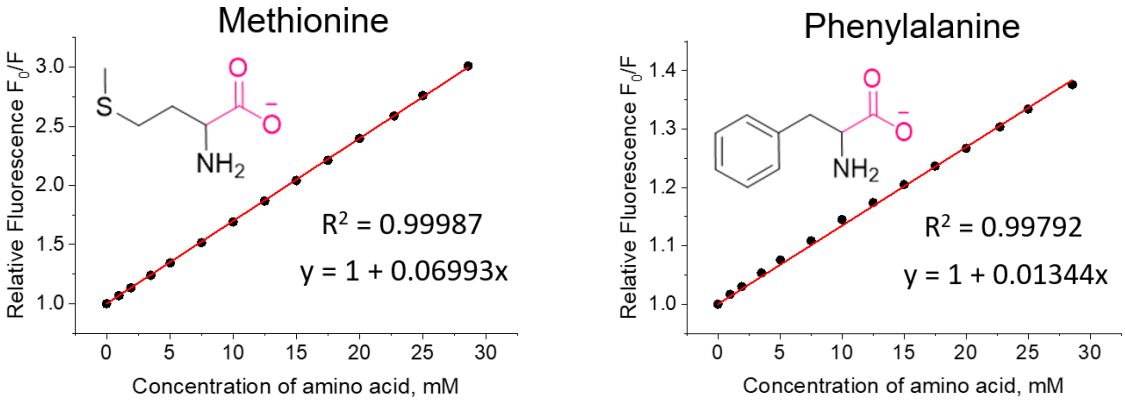


Figure S3.5.7 Stern-Volmer relationship between SPQ fluorescence and concentration of methionine (left, pH 5.8) or phenylalanine (right, pH 5.6). Excitation: 344 nm, emission: 440 nm.

4 Amino acids transport studies using SPQ assay

4.1 General procedure for AA^- and Cl^- transport studies using SPQ assay

The general procedure is a modified version of the chloride transport protocol published previously.[4]

In a 5 mL round bottom flask a solution of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol (total lipid concentration 6 mM, POPC to cholesterol molar ratio 7:3) in chloroform (freshly deacidified by passing through a pad of activated basic alumina) was prepared. The organic solvent was evaporated on a rotary evaporator and the residue was dried under high vacuum for 1 h. The lipid film was hydrated with 0.50 mL of aqueous solution containing SPQ (1 mM) and $NaNO_3$ (225 mM) or Na_2SO_4 (225 mM), sonicated for 30 s and vortexed for 1 h resulting in vesicle formation. Next, the mixture was subjected to 10 freeze-thaw cycles, diluted to 1 mL by the addition of an aqueous solution containing SPQ (1 mM) and $NaNO_3$ (225 mM) or Na_2SO_4 (225 mM), and extruded 29 times through a polycarbonate membrane (200 nm pore size). Unencapsulated SPQ was removed by passing the mixture through a column with Sephadex 50G (ca. 2 g, superfine) using a 225 mM aqueous $NaNO_3$ or Na_2SO_4 as eluent. The collected vesicles were diluted with 225 mM $NaNO_3$ or Na_2SO_4 to 15 mL (lipid concentration ≈ 0.4 mM).

Sodium salts of amino acids were prepared as described in Section 1.2 except that Na_2SO_4 was used instead of $NaNO_3$ when necessary.

3 mL of the vesicle suspension was placed in a quartz cuvette with a small stirring bar and its fluorescence was measured as a function of time (excitation: 344 nm, emission: 440 nm). MeOH solution of transporters **1**-**6** (10 µL, $1.2\cdot10^{-6}$ M, $2.4\cdot10^{-6}$ M, $6\cdot10^{-6}$ M, $1.2\cdot10^{-5}$ M, $2.4\cdot10^{-5}$ M, $6\cdot10^{-5}$ M, $1.2\cdot10^{-4}$ M and $6\cdot10^{-4}$ M; 0.001 mol%, 0.002 mol%, 0.005 mol%, 0.01 mol%, 0.02 mol%, 0.05 mol%, 0.1 mol% and 0.5 mol% with respect to the total amount of lipids) or MeOH alone (10 µL, blank) was added to the vesicle suspension followed by the addition of aqueous solution of sodium salt of an appropriate amino acid or NaCl (0.150 mL, 0.5 M) and the fluorescence intensity was measured for 20 minutes. The pH of liposome suspension was measured with a pH meter after every experiment and these measured pH values are given as conditions of each experiment.

To study AA^-/K^+ co-transport the same procedure was applied, except that K_2SO_4 (225 mM) was used instead of Na_2SO_4 (225 mM). An aliquot of cationophore valinomycin was added in MeOH (10 µL, 0.1 mol% with respect to the total amount of lipids) to the vesicle suspension in cuvette, to initiate AA^-/K^+ co-transport (Figure 5c).

Data processing

Fluorescence data were collected for at least two runs. The plateau (before addition of anions) and the vertical drop (the first 0.5-2 seconds after the addition of anions) due to quenching of residual external SPQ were removed. Next, the data were normalized: all fluorescence values (F) were divided by the fluorescence value measured before the addition of anion (F_0). These normalized traces were averaged and plotted together with the area between the extreme measured values in Figures 3-5 and 7 in the manuscript and Figures S4.2.1-S4.2.6, S4.4.2, S4.11.1-S4.11.2 and S4.12.1 in the ESI.

[4] H. Valkenier, L. W. Judd, H. Li, S. Hussain, D. N. Sheppard, A. P. Davis, Preorganized Bis-Thioureas as Powerful Anion Carriers: Chloride Transport by Single Molecules in Large Unilamellar Vesicles. *J. Am. Chem. Soc.*, **2014**, *136*, 12507–12512.

Hill plot analyses were performed using Origin 2022 to obtain Hill coefficients n, which pertain to the stoichiometry of the species mediating amino acid transport, and parameters $EC_{50,\ 270}$, which are used as a measure of amino acid transport activity. The $EC_{50,\ 270}$ is defined as the effective carrier concentration necessary to reach 50% of the maximum transport within 270 s.

4.2 The transport of deprotonated amino acids into LUVs facilitated by **1**

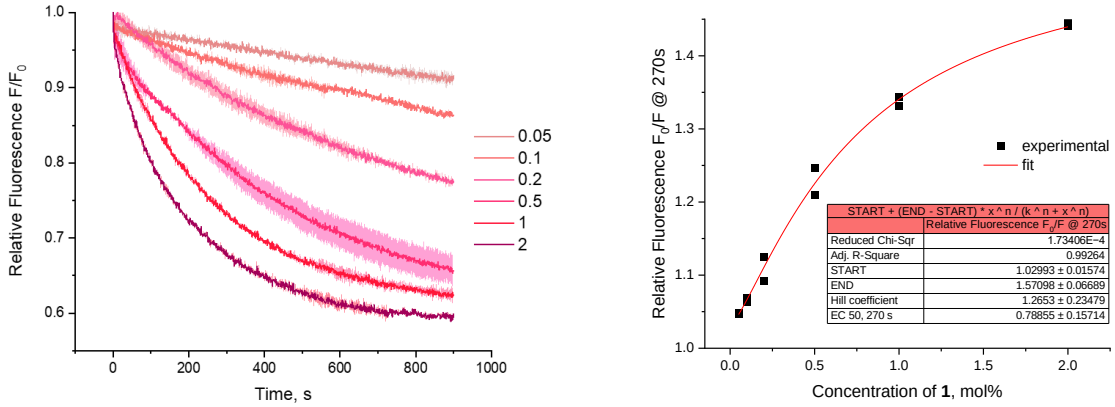


Figure S4.2.1 (left) Concentration-dependent transport of glycine-Na (measured by the SPQ assay) facilitated by **1**. LUVs (POPC:cholesterol 7:3, mean diameter 200 nm) loaded with $NaNO_3$ (225 mM) and SPQ (1 mM) were suspended in an external solution containing Gly-Na (25 mM, pH 10.6) and $NaNO_3$ (225 mM). At t = 0 s, MeOH solution of **1** was added, and the fluorescence intensity (λ_{ex} = 344 nm, λ_{em} = 440 nm) was recorded. (right) Hill plot for Gly-Na transport facilitated by **1**. Transporter loadings are expressed as transporter to lipid molar ratios.

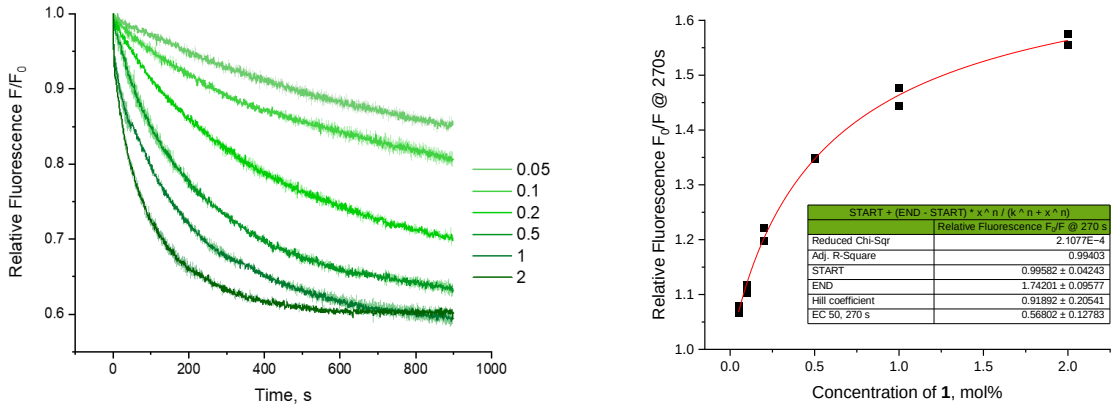


Figure S4.2.2 (left) Concentration-dependent transport of L-alanine-Na (measured by the SPQ assay) facilitated by **1**. LUVs (POPC:cholesterol 7:3, mean diameter 200 nm) loaded with $NaNO_3$ (225 mM) and SPQ (1 mM) were suspended in an external solution containing Ala-Na (25 mM, pH 10.62) and $NaNO_3$ (225 mM). At t = 0 s, MeOH solution of **1** was added, and the fluorescence intensity (λ_{ex} = 344 nm, λ_{em} = 440 nm) was recorded. (right) Hill plot for Ala-Na transport facilitated by **1**. Transporter loadings are expressed as transporter to lipid molar ratios.

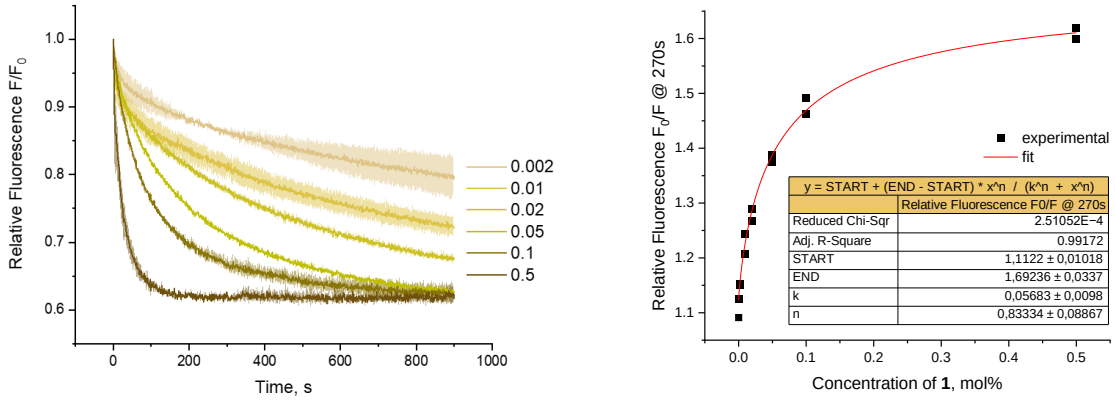


Figure S4.2.3 (left) Concentration-dependent transport of L-valine-Na (measured by the SPQ assay) facilitated by **1**. LUVs (POPC:cholesterol 7:3, mean diameter 200 nm) loaded with $NaNO_3$ (225 mM) and SPQ (1 mM) were suspended in an external solution containing Val-Na (25 mM, pH 10.6) and $NaNO_3$ (225 mM). At t = 0 s, MeOH solution of **1** was added, and the fluorescence intensity (λ_{ex} = 344 nm, λ_{em} = 440 nm) was recorded. (right) Hill plot for Val-Na transport facilitated by **1**. Transporter loadings are expressed as transporter to lipid molar ratios.

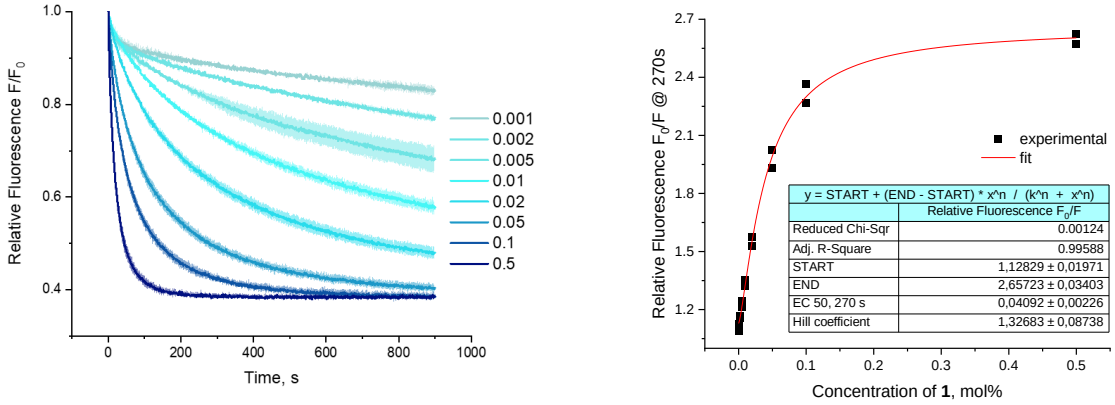


Figure S4.2.4 (left) Concentration-dependent transport of L-methionine-Na (measured by the SPQ assay) facilitated by **1**. LUVs (POPC:cholesterol 7:3, mean diameter 200 nm) loaded with $NaNO_3$ (225 mM) and SPQ (1 mM) were suspended in an external solution containing Met-Na (25 mM, pH 10.6) and $NaNO_3$ (225 mM). At t = 0 s, MeOH solution of **1** was added, and the fluorescence intensity (λ_{ex} = 344 nm, λ_{em} = 440 nm) was recorded. (right) Hill plot for Met-Na transport facilitated by **1**. Transporter loadings are expressed as transporter to lipid molar ratios.

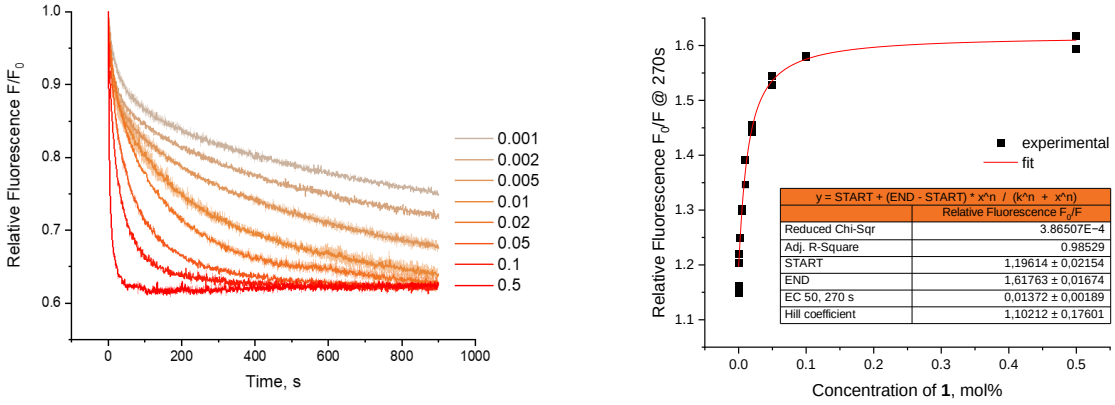


Figure S4.2.5 (left) Concentration-dependent transport of L-isoleucine-Na (measured by the SPQ assay) facilitated by **1**. LUVs (POPC:cholesterol 7:3, mean diameter 200 nm) loaded with $NaNO_3$ (225 mM) and SPQ (1 mM) were suspended in an external solution containing Ile-Na (25 mM, pH 10.68) and $NaNO_3$ (225 mM). At t = 0 s, MeOH solution of **1** was added, and the fluorescence intensity (λ_{ex} = 344 nm, λ_{em} = 440 nm) was recorded. (right) Hill plot for Ile-Na transport facilitated by **1**. Transporter loadings are expressed as transporter to lipid molar ratios.

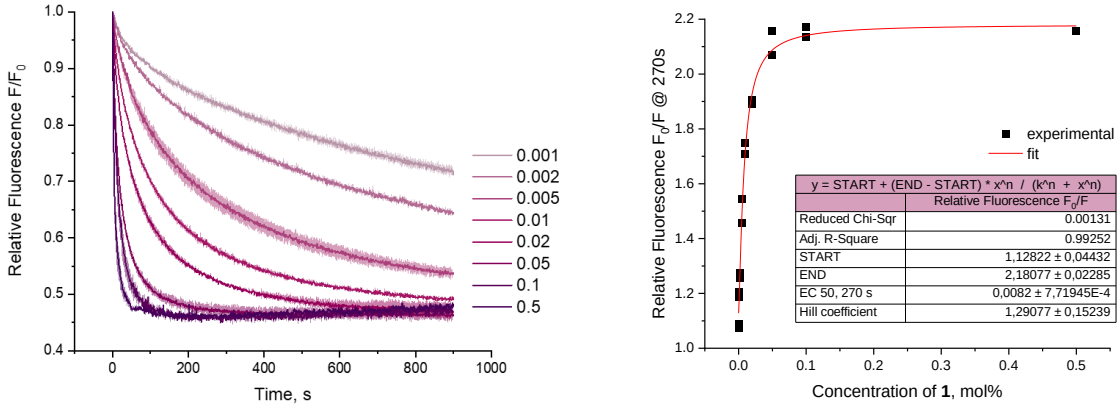


Figure S4.2.6 (left) Concentration-dependent transport of L-phenylalanine-Na (measured by the SPQ assay) facilitated by **1**. LUVs (POPC:cholesterol 7:3, mean diameter 200 nm) loaded with $NaNO_3$ (225 mM) and SPQ (1 mM) were suspended in an external solution containing Phe-Na (25 mM, pH 10.6) and $NaNO_3$ (225 mM). At t = 0 s, MeOH solution of **1** was added, and the fluorescence intensity (λ_{ex} = 344 nm, λ_{em} = 440 nm) was recorded. (right) Hill plot for Phe-Na transport facilitated by **1**. Transporter loadings are expressed as transporter to lipid molar ratios.

4.3 The transport of deprotonated amino acids by **1** and **2** pre-incorporated in LUVs

In a 5 mL round bottom flask a solution of POPC and cholesterol (total lipid concentration 6 mM, POPC to cholesterol ratio 7:3) in chloroform (freshly deacidified by passing through a pad of activated basic alumina) was prepared. Anionophore **1** (0.01 M in $CHCl_3$:MeOH 1:1 v/v) was added to the lipid solution at 0, 0.1 or 0.5 mol% ratio relative to the total amount of lipids.

The organic solvents were evaporated on a rotary evaporator and the residue was dried under high vacuum for 1 h. The lipid film was hydrated with 0.50 mL of aqueous solution containing SPQ (1 mM in 225 mM $NaNO_3$), sonicated for 30 s and vortexed for 1 h resulting in vesicle formation. Next, the mixture was subjected to 10 freeze-thaw cycles, diluted to 1 mL by the addition of an aqueous solution containing SPQ (1 mM in 225 mM $NaNO_3$) and extruded 29 times through a polycarbonate membrane (200 nm pore size). Unencapsulated SPQ was removed by passing the mixture through a column with Sephadex 50G (*ca.* 2 g, superfine) using a 225 mM aqueous $NaNO_3$ as an eluent. The collected vesicles were diluted with 225 mM $NaNO_3$ to 15 mL (lipid concentration ≈ 0.4 mM).

Sodium salts of amino acids were prepared as described in Section 1.2.

3 mL of the vesicle suspension was placed in a quartz cuvette with a small stirring bar and its fluorescence was measured as a function of time (excitation: 344 nm, emission: 440 nm). After 60 seconds, an aqueous solution of sodium salt of an appropriate amino acid (0.150 mL, 0.5 M) was added and the fluorescence intensity was measured for 20 minutes. The pH of liposome suspension was measured with a pH meter after every experiment.

Fluorescence data were collected for at least two runs. The plateau (before the addition of anions) and the vertical drop (the first 0.5-2 seconds after the addition of anions) due to quenching of residual external SPQ were removed. Next, the data were normalized: all fluorescence values (F) were divided by the fluorescence value measured before the addition of anion (F_0). These normalized traces were averaged and are plotted in Figure S4.4.1 together with the area between measured data plots.

4.4 Comparison of transport of deprotonated amino acids by **1** and **2** pre- and post-incorporated in LUVs

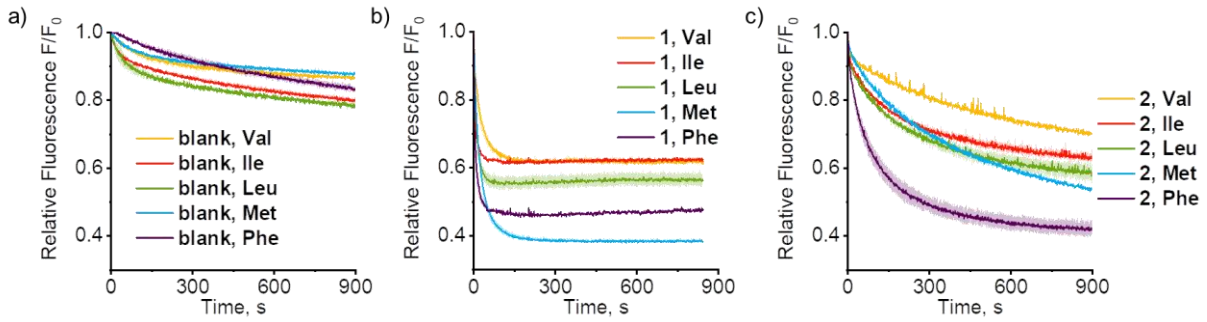


Figure S4.4.1 The comparison of unfacilitated diffusion of amino acids' anions (a) and the transport facilitated by 0.5 mol% of transporter **1** (b) or **2** (c) preincorporated in the lipid membrane of LUVs (POPC/cholesterol 7:3, 200 nm).

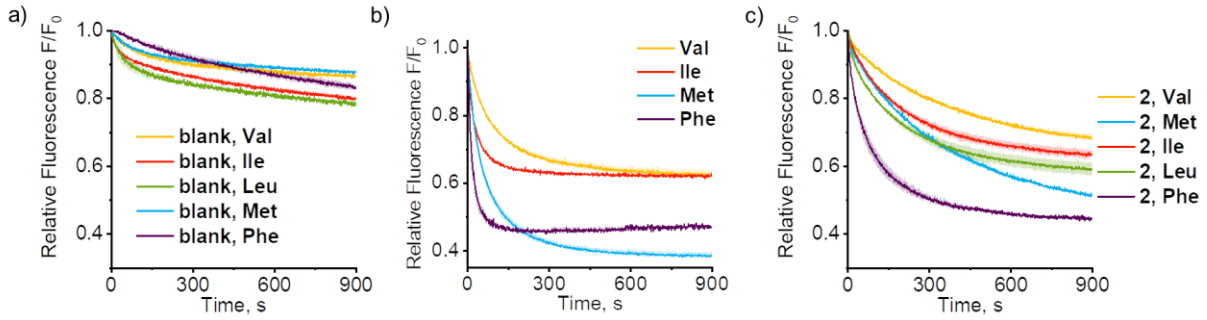


Figure S4.4.2 The comparison of unfacilitated diffusion of amino acids' anions (a) and the transport facilitated by 0.5 mol% of transporter **1** (b) or **2** (c) postincorporated in the lipid membrane of LUVs (POPC/cholesterol 7:3, 200 nm).

4.5 Quantification of transport rates

As a first approximation, we can assume that the rate of anion transport (i.e. concentration change $d[A]/dt$ inside the vesicles) is proportional to the transmembrane gradient of anion concentration. Since the extravesicular anion concentration $[A]_0$ remains practically constant, the rate is proportional to the anion concentration inside the vesicle:

$$\frac{d[A]}{dt} = k([A]_0 - [A])$$

We can assume also that free diffusion and carrier-mediated transport are independent of each other, so that:

$$\frac{d[A]}{dt} = k_{diffusion}([A]_0 - [A]) + k_{transport}([A]_0 - [A])$$

and

$$k = k_{diffusion} + k_{transport}$$

The solution of this equation is the exponential decay:

$$[A] = [A]_0(1 - \exp(-kt))$$

In earlier work[1] we have plotted the decay of fluorescence F as the ratio F/F_0 (F_0 = initial fluorescence). Here we use F/F_0 for illustrative purposes but employ the reciprocal F_0/F for quantification. The use of F_0/F instead F/F_0, was also justified in previous publication.[3] According to the Stern−Volmer equation:

$$\frac{F_0}{F} = 1 + k_q\tau_0[Q]$$

(where k_q is the rate constant of the fluorescence quenching process and τ_0 is the lifetime of the emissive excited state of fluorophore in the absence of quencher Q) it is the F_0/F ratio which is proportional to the concentration of the quencher Q (in our case – to the concentration of anions of amino acids inside the vesicles, i.e. to $[A]$). Thus, plots of F_0/F are directly related to the increase of the anion concentration inside the vesicles, and the derivatives of these plots are proportional to the transport rates. The first 900 s of the traces were fitted therefore to a single exponential decay function $F_0/F = \mathrm{y} - \mathrm{a} \cdot \exp(-x \cdot k)$, where y, a and k were treated as fitting parameters. The rate constants thus derived give approximate half-times according to the equation:

$$t_{1/2} = ln(2)/k$$

4.6 Determination of transport rate constants and half-times in the transport of deprotonated amino acids by **1**

Data analysis, including curve fitting, was performed using Origin 2019.

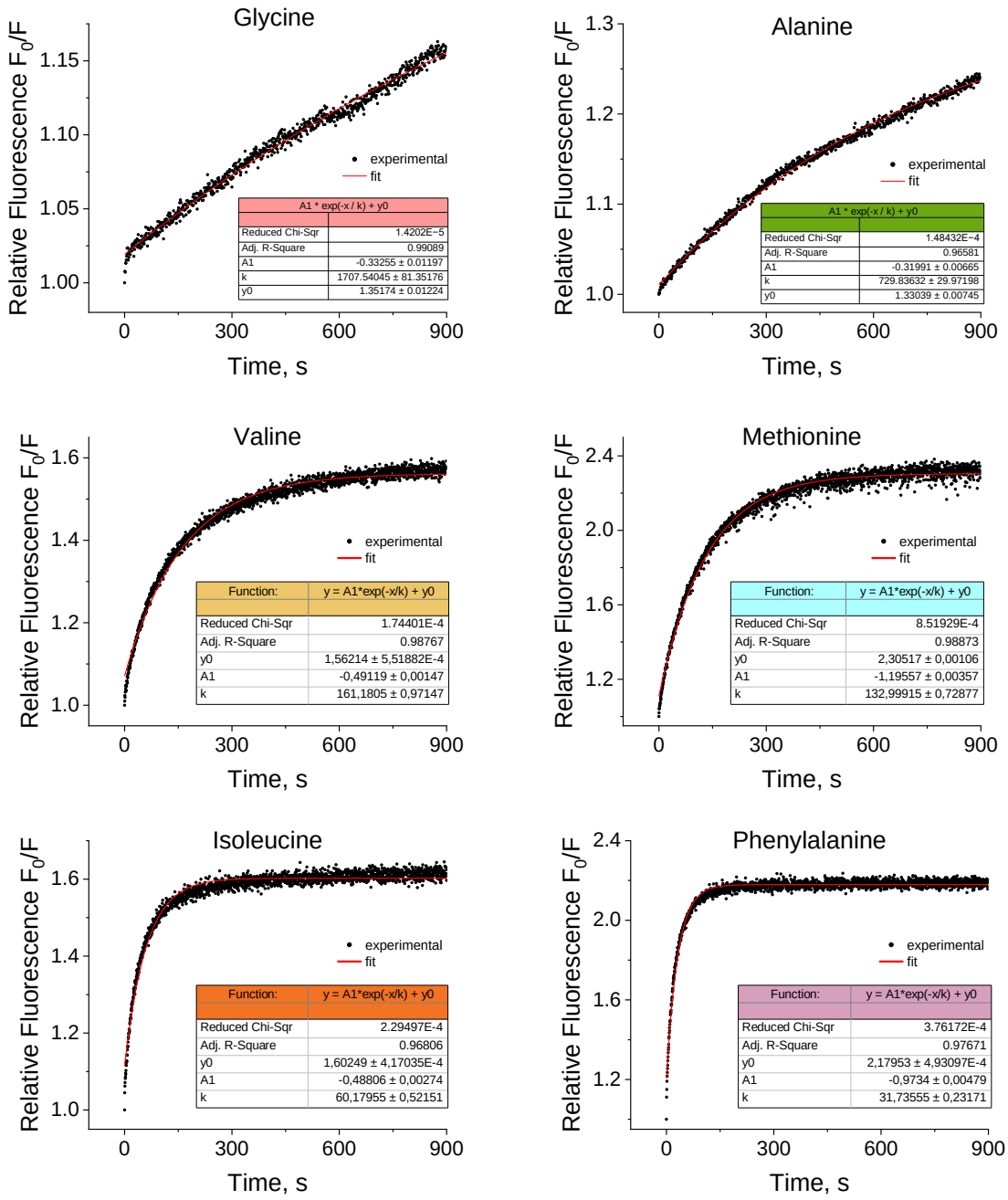


Figure S4.6.1 Relative fluorescence F_0/F and single exponential fits of F_0/F for the transport of sodium salts of various amino acids into 200 nm LUVs with transporter **1** (0.1 mol%).

	Gly	Ala	Val	Met	Ile	Phe
Rate constant k, s	1707.54 ± 81.35	729.84 ± 29.97	161.18 ± 0.97	133.00 ± 0.73	60.18 ± 0.52	31.74 ± 0.23
Half- time $t_{1/2}$, s	1183.58 ± 56.39	505.89 ± 20.77	111.72 ± 0.67	92.19 ± 0.51	41.71 ± 0.36	22.00 ± 0.16

4.7 General procedure for methionine transport studies at various pHs using the SPQ assay

In a 5 mL round bottom flask a solution of lipids (POPC and cholesterol in a 7:3 molar ratio, total lipid concentration 6 mM) in chloroform (freshly deacidified by passing through a pad of activated basic alumina) was prepared. Anionophore **1** (1.5 mM in $CHCl_3$:MeOH 1:1 v/v) was added to the lipid solution at 0.1 mol% ratio relative to the total amount of lipids.

The organic solvents were removed on a rotary evaporator and the residue was dried under high vacuum for 1 h. The lipid film was hydrated with 0.50 mL of aqueous solution containing SPQ (1 mM) and $NaNO_3$ (225 mM) or Na_2SO_4 (225 mM), sonicated for 30 s and stirred for 1 h resulting in vesicle formation. Next, the mixture was subjected to 10 freeze-thaw cycles, diluted to 1 mL by the addition of an aqueous solution containing SPQ (1 mM) and $NaNO_3$ (225 mM) or Na_2SO_4 (225 mM) and extruded 29 times through a polycarbonate membrane (200 nm pore size). Unencapsulated SPQ was removed by passing the mixture through a column with Sephadex 50G (ca. 2 g, superfine) using 225 mM $NaNO_3$ or 225 mM Na_2SO_4 as eluent. The collected vesicles were diluted with 225 mM $NaNO_3$ or 225 mM Na_2SO_4 to 15 mL (total lipid concentration ≈ 0.4 mM).

3 mL of the vesicle solution was placed in a quartz cuvette with a small stirring bar and its fluorescence was measured as a function of time (excitation: 344 nm, emission: 440 nm). After 60 seconds 0.25 M solution of methionine (0.320 mL) in 225 mM $NaNO_3$ or 225 mM Na_2SO_4 at various pH (adjusted by adding NaOH) was added and the fluorescence intensity was measured for 20 minutes. The pH of liposome suspension was measured with a pH meter after every experiment and these measured pH values are given as conditions of each experiment.

Fluorescence data were collected for at least two runs. The plateau (before the addition of methionine) and the vertical drop (the first 0.5-2 seconds after the addition of methionine) due to quenching of external SPQ were removed. Next, the data were normalized: all fluorescence values (F) were divided by the fluorescence value measured before the addition of methionine (F_0). These normalized traces were averaged and together with the area between the measured data plots are plotted in Figure 6 in the main text and in Figures S4.8 and S4.10 in ESI.

The first 900 s of F_0/F traces were fitted with a single exponential decay function to calculate the transport rate constants (Figure S4.9.1). Half-times of methionine transport by **1** at different pH values were calculated according to the procedure described in chapter 4.5 of this ESI.

4.8 The transport of methionine into LUVs by **1** at various pHs in nitrate medium

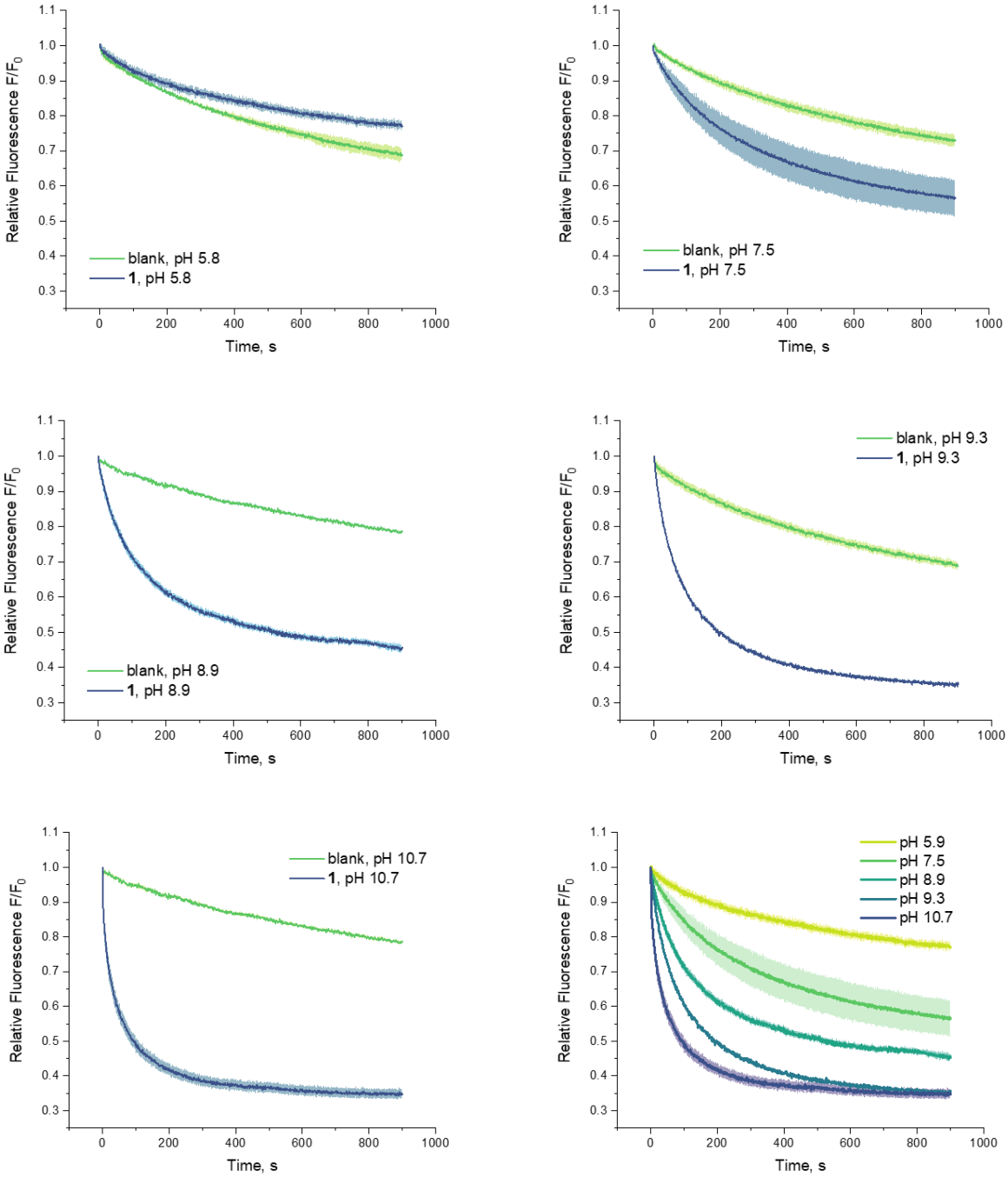


Figure S4.8.1 Effect of pH on methionine transport across lipid bilayer of LUVs facilitated by **1** (0.1 mol%) or non-facilitated (blank). POPC/cholesterol 7:3 LUVs (200 nm) were suspended in 225 mM $NaNO_3$. pH was measured by pH-meter after transport experiment. Excitation wavelength: 344 nm, emission wavelength: 440 nm.

4.9 Determination of half-times for the transport of methionine into LUVs by **1** at various pHs in nitrate medium

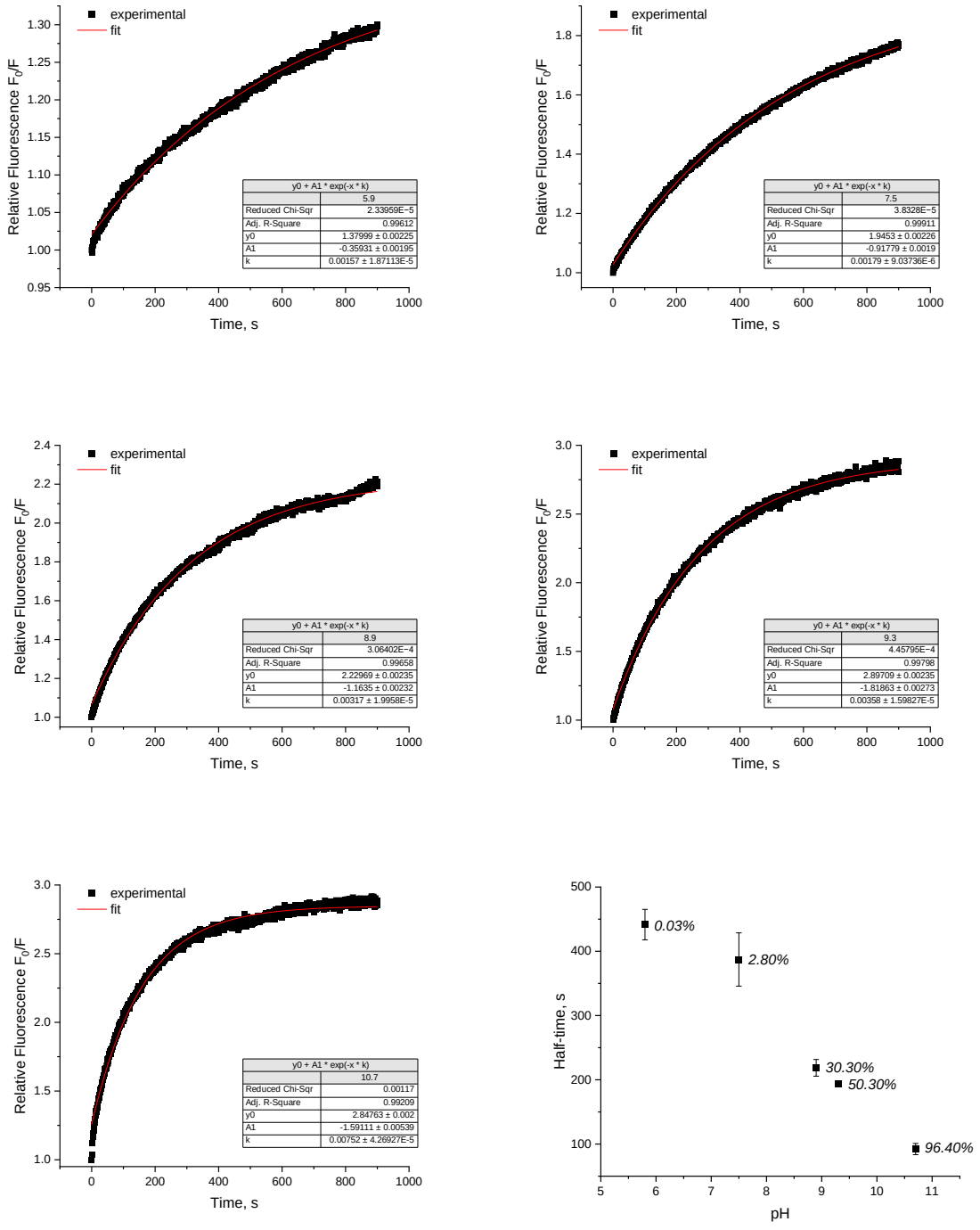


Figure S4.9.1 Relative fluorescence F_0/F and single exponential fits of F_0/F for the transport of methionine into LUVs with transporter **1** (0.1 mol%) at different pH values. LUVs (POPC/cholesterol 7:3, 200 nm) were suspended in 225 mM $NaNO_3$. pH was measured by pH-meter after transport experiment. Excitation wavelength: 344 nm, emission wavelength: 440 nm. Bottom, right: plot of the half times of methionine transport by **1** (0.1 mol%) as a function of pH. Estimated percentage of anionic form of methionine is given near the data points.

4.10 The transport of methionine into LUVs by **1** at various pHs in sulfate medium

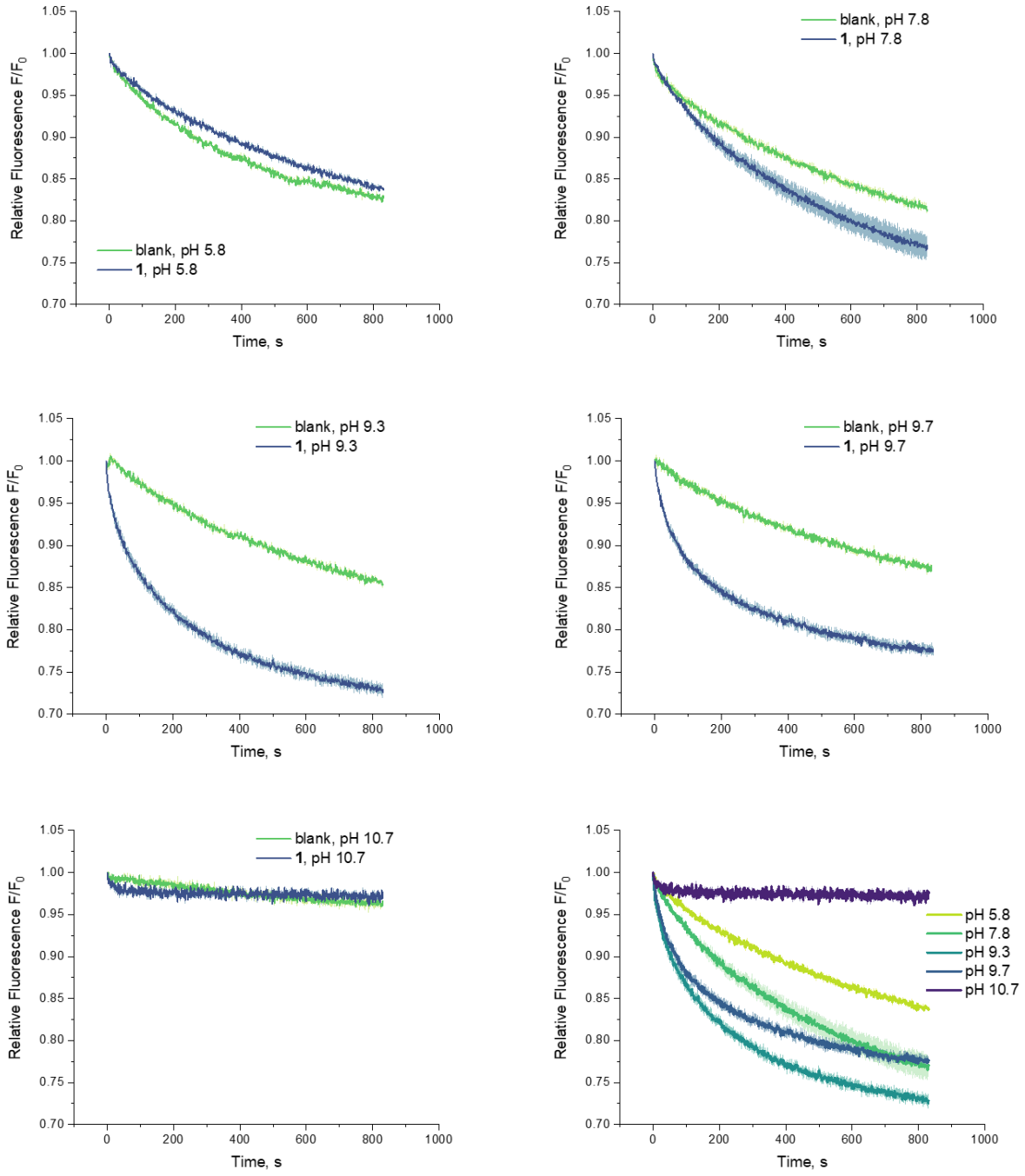


Figure S4.10.1 Effect of pH on methionine transport through lipid bilayers of LUVs facilitated by **1** (0.1 mol%) or non-facilitated (blank). POPC/cholesterol 7:3 LUVs (200 nm) were suspended in 225 mM Na_2SO_4. pH was measured by pH-meter after transport experiment. Excitation wavelength: 344 nm, emission wavelength: 440 nm.

4.11 The transport of methionine into LUVs by **1**-**4** at pH 7.4

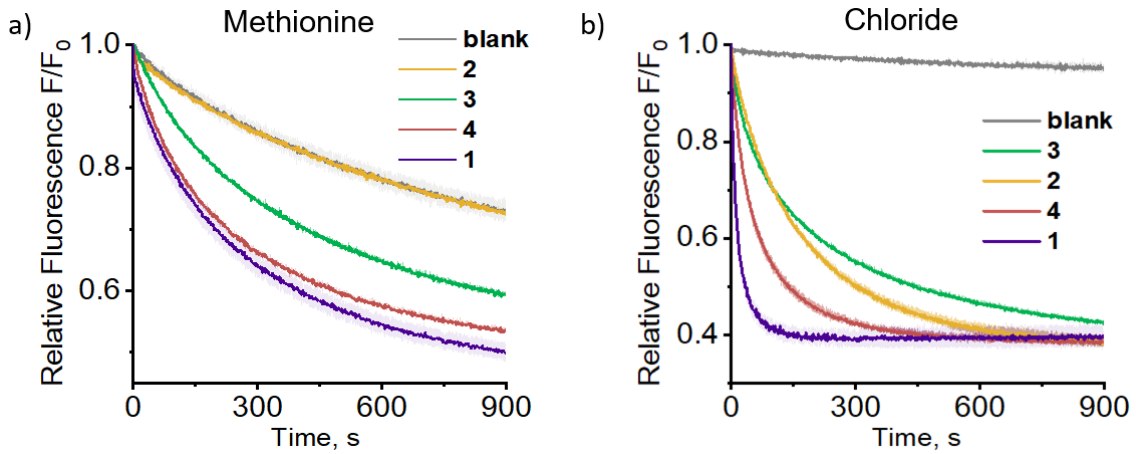


Figure S4.11.1 a) Comparison of normalized fluorescence intensity over time measured for LUVs suspension with 1 mol% and without (blank) transporters **1**-**4** after the addition of methionine, pH 7.4 in nitrate medium. b) Comparison of normalized fluorescence intensity over time measured for LUVs suspension with 0.1 mol% and without (blank) transporters **1**-**4** after the addition of NaCl, pH 7.4 in nitrate medium. Excitation wavelength: 344 nm, emission wavelength: 440 nm.

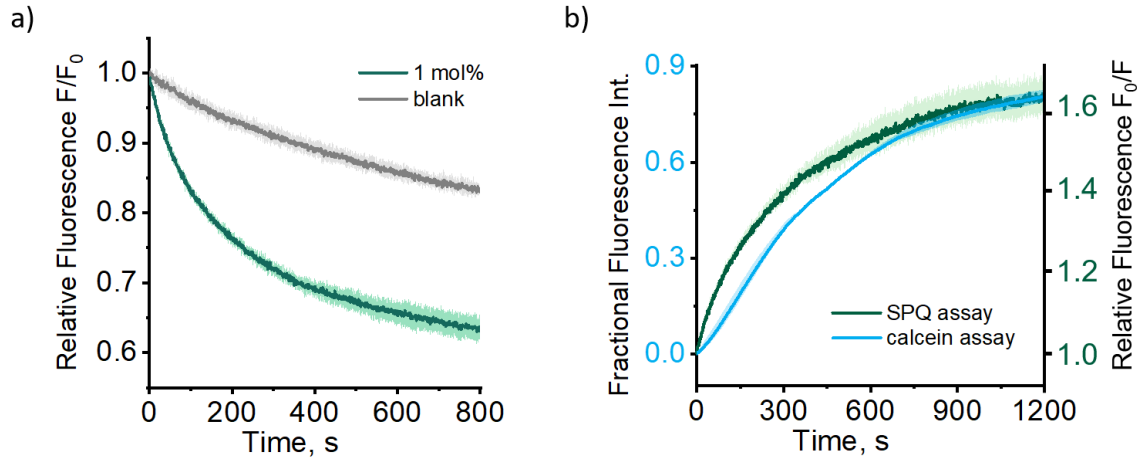
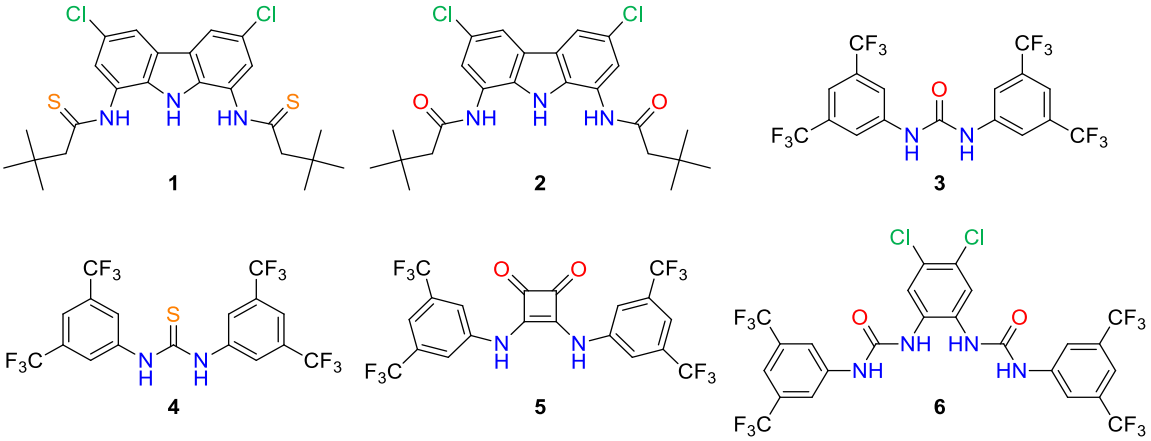


Figure S4.11.2 a) Comparison of normalized fluorescence intensity over time measured for LUVs suspension with 1 mol% and without (blank) transporter **1** after the addition of methionine, pH 7.4 in sulfate medium. Excitation wavelength: 344 nm, emission wavelength: 440 nm. b) Comparison of methionine transport at pH 7.4 by **1** (1 mol%) measured by two independent methods: SPQ assay and calcein assay.

4.12 Comparison of the rates of transport of methionine anion and chloride anion by 1-6 at pH 10.7

In order to study the selectivity of receptors **1**-**6**, we measured their transport rates with methionine-Na and chloride anions under conditions, where the concentrations of both anions could be made identical, *i.e.* at pH 10.7. At this pH methionine is nearly completely deprotonated and the concentration of its anionic form may be assumed equal to the total concentration of the amino acid. Under these conditions, receptors **4** and **5** show no activity (Figure 8 in the manuscript) due to their deprotonation at high pH. Of the remaining four receptors, **1** and **2** show selectivity for chloride (Figure S4.3.1, left), **6** displays no noticeable selectivity at all, and receptor **3** was found to transport deprotonated methionine faster than chloride (Figure S4.3.1, right). The latter result is particularly interesting because it demonstrates that even very simple monotopic anionophores may show some preference for amino acids with respect to chloride.



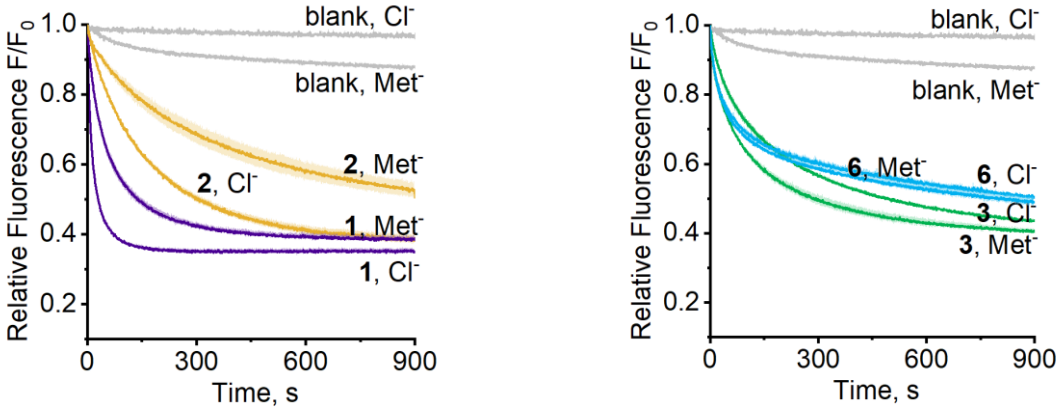


Figure S4.12.1 Comparison of normalized fluorescence intensity over time measured for LUVs suspension containing 0.1 mol% of transporters **1** & **2** (left) and **3** & **6** (right), after the addition of Met-Na or NaCl, at pH 10.7. Excitation wavelength: 344 nm, emission wavelength: 440 nm.

5 Equilibration of pH gradients by **1**: studies using SPQ and HPTS assays

5.1 pH gradient equilibration by **1** in nitrate and sulfate media: results from the SPQ assay

SPQ fluorescence is only slightly quenched by OH^- at pH < 10.7 (Figure S3.2.1), so the increase of pH inside the vesicles, which accompanies the transport of amino acids' anions, does not contribute significantly to the fluorescence drop in amino acid transport studies. In blank experiments, however, when there is no transporter in the membrane, the non-facilitated diffusion of amino acids is so minor, that the contribution from OH^- might be significant. In fact, in all blank experiments a slight (approx. 10%) and relatively fast decrease of fluorescence intensity was observed immediately after the addition of amino acid salts. We hypothesized that this might be due to the pH equalization on both sides of the membrane (for example by flip-flop of fatty acid impurities), rather than due to spontaneous diffusion of amino acids.[5] Indeed, very similar decrease of fluorescence was observed upon addition of NaOH (to pH 10.7), instead of amino acids' salts, to the vesicle suspension (Figure 4 in the main manuscript, shown also below as Figure S5.1.1). However, similar decrease of fluorescence upon addition of NaOH was not observed if there was a transporter in the membrane (even when its concentration was as low as 0.1 mol%, see Figure S5.1.1b below). In such a case, the relative SPQ fluorescence is lower than in the blank experiment from the very beginning, and does not change significantly during the first 100 s. This suggests that **1** equalizes the pH on both sides of the membrane instantaneously, and that the resulting ca. 10% drop in fluorescence merges with the initial vertical drop due to quenching of the residual external SPQ, and is subtracted during routine data processing. To further investigate this hypothesis, we studied the pH gradient equilibration by **1** using HPTS – a much more sensitive pH indicator than SPQ (see next paragraph).

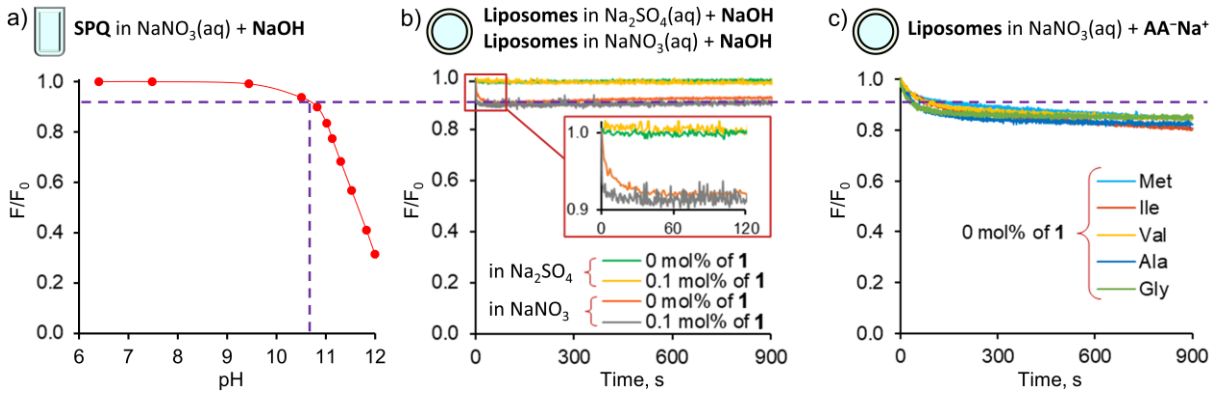


Figure S5.1.1 Effect of pH on SPQ fluorescence in solution and in LUVs: a) effect of pH on the fluorescence of aqueous solution of SPQ (10^{-5} M in 225 mM $NaNO_3$); b) fluorescence changes of SPQ encapsulated in POPC/cholesterol LUVs, having 0 or 0.1 mol% of the transporter **1** in their membrane, upon basification to pH 10.7 by a pulse of NaOH; c) fluorescence changes of SPQ encapsulated in POPC/cholesterol LUVs, having 0 mol% of the transporter **1** in their membrane, upon addition of sodium salts of various amino acids.

[5] X. Wu, P. A. Gale, Small-Molecule Uncoupling Protein Mimics: Synthetic Anion Receptors as Fatty Acid-Activated Proton Transporters. *J. Am. Chem. Soc.* **2016**, *138*, 16508–16514

5.2 pH gradient equilibration by **1** in nitrate and sulfate media: results from the HPTS assay

In a 5 mL round bottom flask a solution of lipids (POPC and cholesterol in a 7:3 molar ratio, total lipid concentration 6 mM) in chloroform (freshly deacidified by passing through a pad of activated basic alumina) was prepared. The organic solvent was evaporated on a rotary evaporator and the residue was dried under high vacuum for 1 h. The lipid film was hydrated with 0.50 mL of aqueous solution containing HPTS (1 mM) and $NaNO_3$ (225 mM) or Na_2SO_4 (225 mM), sonicated for 30 s and stirred for 1 h resulting in vesicle formation. Next, the mixture was subjected to 10 freeze-thaw cycles, diluted to 1 mL by the addition of an aqueous solution containing HPTS (1 mM) and $NaNO_3$ (225 mM) or Na_2SO_4 (225 mM), and extruded 29 times through a polycarbonate membrane (200 nm pore size). The unencapsulated HPTS was removed by passing the mixture through a column with Sephadex 50G (ca. 2 g, superfine) using a 225 mM aqueous $NaNO_3$ or Na_2SO_4 as eluent. The collected vesicles were diluted with 225 mM $NaNO_3$ or Na_2SO_4 to 15 mL (total lipid concentration ≈ 0.4 mM).

0.75 mL of the vesicle suspension was placed in a quartz cuvette with a small stirring bar and diluted to 3 ml with $NaNO_3$ (225 mM) or Na_2SO_4 (225 mM) to afford a lipid concentration of 0.1 mM. MeOH solution of transporter **1** (10 µL, $3 \cdot 10^{-5}$ M, 0.1 mol% with respect to the total amount of lipids) or MeOH alone (10 µL, blank) was added to the vesicle suspension, followed by the addition of NaOH (30 µl, 0.08 M). The ratio R of the fluorescent emission intensity I_{460} (λ_{ex} = 460 nm, λ_{em} = 510 nm, i.e. emission from the basic form of HPTS) to I_{403} (λ_{ex} = 403 nm, λ_{em} = 510 nm, i.e. emission from the acidic form of HPTS) was followed in time to investigate changes of pH inside vesicles. The final pH in cuvette was 10.7 (measured by pH-meter).

The R values at different times were converted to fractional fluorescence values (I_f) using equation:

$$I_f = \frac{R_t - R_0}{R_f - R_0}$$

where R_t is the fluorescence ratio at time t, R_0 is the fluorescence ratio at time t = 0, and R_f is the final fluorescence ratio (after lysing the vesicles exposing 100% of the encapsulated HPTS).

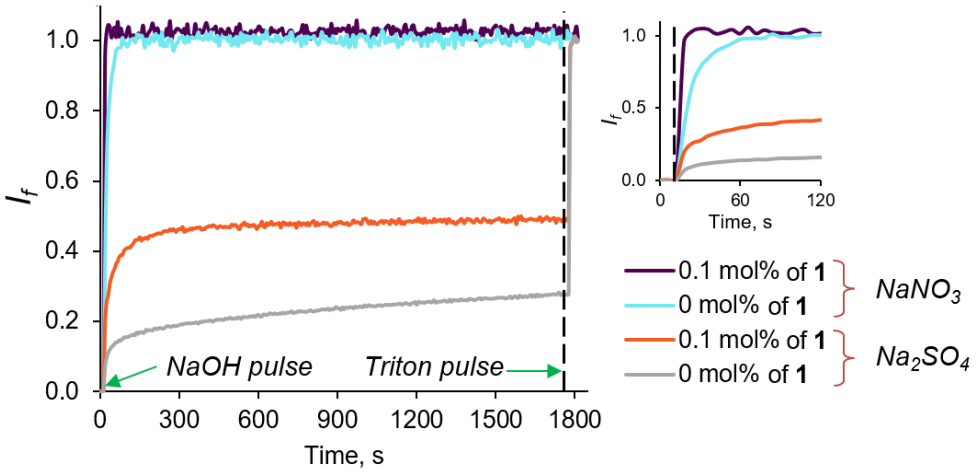


Figure S5.2.1 pH changes inside LUVs (POPC/cholesterol 7:3, 200 nm, suspended in $NaNO_3$ (225 mM) or Na_2SO_4 (225 mM)) upon addition of an external pulse of NaOH (30 µl, 0.08 M in 225 M $NaNO_3$ or Na_2SO_4), expressed as the normalised ratio $R_{460/403}$ of the fluorescent emission intensity (λ_{em} = 510 nm) from the basic (λ_{ex} = 460 nm) and acidic (λ_{ex} = 403 nm) form of HPTS. HPTS concentration inside LUVs: 1 mM, transporter loading: 0.1 mol% or 0 mol% (blank).

Titration of HPTS ($4 \cdot 10^{-7}$ mM) with NaOH (10 mM) in Na_2SO_4 (225 mM) was performed using Hitachi F-7000 spectrophotometer and pH electrode InLab Expert Pro. Next, the obtained data (Figure S5.2.2a) were fitted with the following equation:

$$y = \frac{a}{(1 + e^{-k \cdot (x - x_c)})}$$

and reconstructed changes of pH inside liposomes during the transport experiment with HPTS in Na_2SO_4 are presented in Figure S5.2.2b.

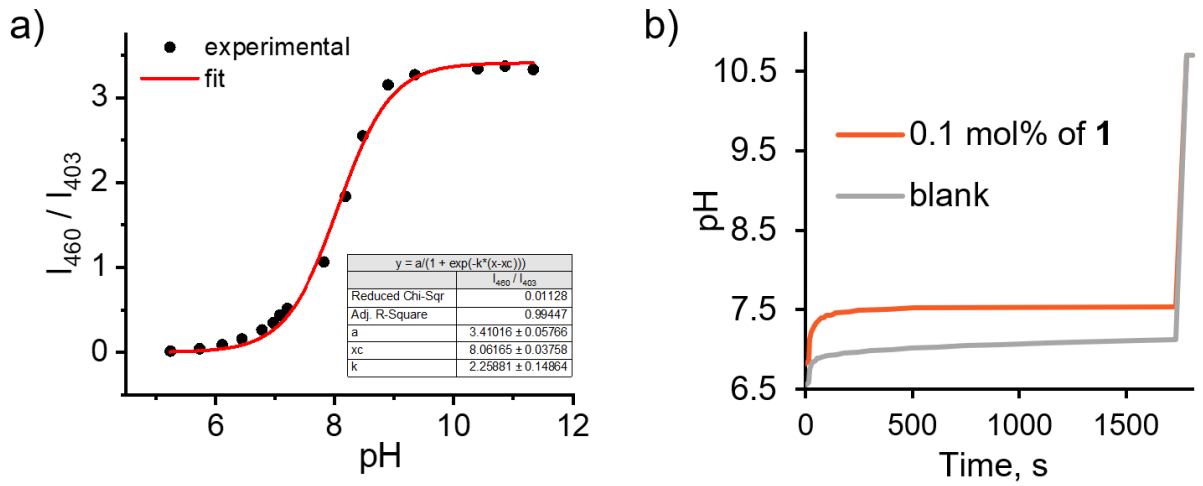


Figure S5.2.2 a) Titration of HPTS ($4 \cdot 10^{-7}$ mM in 225 mM Na_2SO_4) with NaOH (10 mM in 225 mM Na_2SO_4); b) reconstructed changes of pH inside liposomes during the transport experiment shown in Figure S5.2.1.

It can be estimated that in the experimental conditions applied above it is enough to transport just 0.72 (!) OH^- ions into a statistical liposome in order to rise the internal pH by one unit (from 6.5 to 7.5).

$$V_{liposome} = \frac{4}{3}\pi r^3 = \frac{4}{3}\pi \cdot 100^3 = 4.19^{-18} \text{ L}$$

$$\Delta n_{OH^-} = ([OH]_{pH\ 7.5} - [OH]_{pH\ 6.5}) * V_{liposome} = 3.16^{-8} - 3.16^{-7} * 4.19^{-18} = 1.19^{-24} \text{ mol}$$

$$N = n \cdot N_{Avogadro} = 1.19^{-24} \cdot 6.02 \cdot 10^{23} = 0.72 \text{ } OH^- \text{ ions.}$$

Since the HPTS assay shows that the internal pH never rises by more than 1 unit, we conclude that in practice no H^+ or OH^- transport takes place in Na_2SO_4 medium.

6 Amino acids transport studies using ^{13}C NMR assay

6.1 A general procedure for studies of amino acid transport by **1** using ^{13}C NMR assay at pH 7.4

The general procedure is a slightly modified version of the previously published protocol.[6]

In a 5 mL round bottom flask a solution of POPC (28 µmol) and cholesterol (12 µmol) in chloroform (freshly deacidified by passing through a pad of activated basic alumina) was prepared. The organic solvent was evaporated on a rotary evaporator and the residue was dried under high vacuum overnight. The lipid film was hydrated with 0.90 mL of a buffered solution (225 mM $NaNO_3$, 10 mM HEPES, pH 7.4) by sonication for 30 s and vortexing for 5 min to facilitate vesicles formation. The resulting suspension was subjected to 25 freeze-thaw cycles, extruded 35 times using a polycarbonate membrane with 1 µm pore diameter and diluted with buffered solution (225 mM $NaNO_3$, 10 mM HEPES, pH 7.4) to 1.130 mL (total lipids concentration ≈ 33.6 mM).

In an NMR tube, 250 µL of liposomes stock suspension was mixed with 250 µL of buffered amino acid solution (200 mM $^{13}C(1)$-L-Gly, $^{13}C(1)$-L-Ala or $^{13}C(1)$-L-Met, 10 mM HEPES, pH 7.4) and 55 µL of D_2O. At t = 0 min, 10 µL of pure MeOH (blank) or MeOH solution of **1** ($8.4 \cdot 10^{-3}$ M, 1 mol% with respect to the lipids) were added, and the first ^{13}C NMR spectrum was registered. Acquisition parameters: frequency – 100.6 MHz; relaxation delay – 2 s; number of scans – 512; temperature – 298 K, total acquisition time – ca. 30 min. At t = 60 min, 25 µL of aqueous $MnSO_4$ (20 mM, 1% to total AA* concentration) were added and second ^{13}C NMR spectrum was registered. Finally, at t = 90 min, 100 µL of Triton X-100 (10% in water v/v) were added to lyse the liposomes and third ^{13}C NMR spectrum was recorded. The results thus obtained are presented in Figure 7 in the main text.

6.2 Visualization of intravesicular amino acids by NMR shifts reagent

Paramagnetic Mn^{2+} ions broaden the ^{13}C signal of the extravesicular amino acids (OUT), but do not shift them considerably. Therefore, in case of slow transport, it is difficult to see the growing signal from intravesicular amino acid (IN), because it overlaps with the much larger (albeit broad) signal of extravesicular amino acid. To make the signal of intravesicular amino acid more easily visible, we performed additional experiments, in which NMR shift reagent $Eu(NO_3)_3$ was used instead of $MnSO_4$.[7]

Liposomes were prepared as described above (chapter 6.1), except that instead of 25 µL of aqueous $MnSO_4$, 25 µL of aqueous $Eu(NO_3)_3$ (0.2 M, 10% with respect to the total AA* concentration) was added and ^{13}C NMR spectrum was registered. The obtained results are presented in Figure S6.2.1.

[6] L. Martinez-Crespo, J. Liang Sun-Wang, A. Felipe Sierra, G. Aragay, E. Errasti-Murugarren, P. Bartoccioni, M. Palacin, P. Ballester, Facilitated Diffusion of Proline across Membranes of Liposomes and Living Cells by a Calix[4]pyrrole Cavitand, *Chem*, **2020**, *6*, 3054–3070.
[7] L. Chen, W. Si, L. Zhang, G. Tang, Z.-T. Li and J.-L. Hou, Chiral Selective Transmembrane Transport of Amino Acids through Artificial Channels, *J. Am. Chem. Soc.*, **2013**, *135*, 2152–2155.

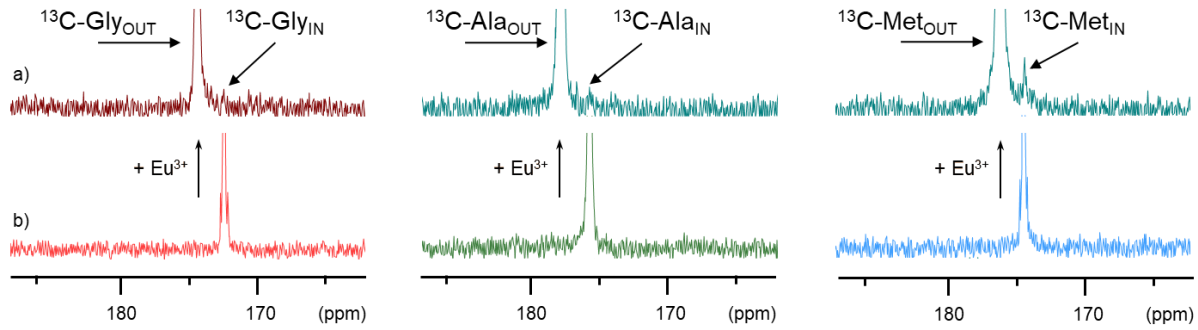


Figure S6.2.1 ^{13}C NMR spectra of isotopically labelled amino acids Gly, Ala, and Met in the ^{13}C NMR transport assay after the addition of **1** (1 mol%) (bottom), followed, after 60 min, by the addition of Mn^{2+} (top).

To additionally broaden the ^{13}C signals of extravesicular amino acids, we performed also yet another experiment, where the NMR shift reagent $Eu(NO_3)_3$ was used together with $MnSO_4$. Liposomes were prepared as described in chapter 6.1, except that after adding 25 µL of aqueous $MnSO_4$ (20 mM, 1% to total AA* concentration), 25 µL of aqueous $Eu(NO_3)_3$ (0.2 M, 10% to total AA* concentration) was also added and the ^{13}C NMR spectrum was registered. The results are presented in Figure S6.2.2.

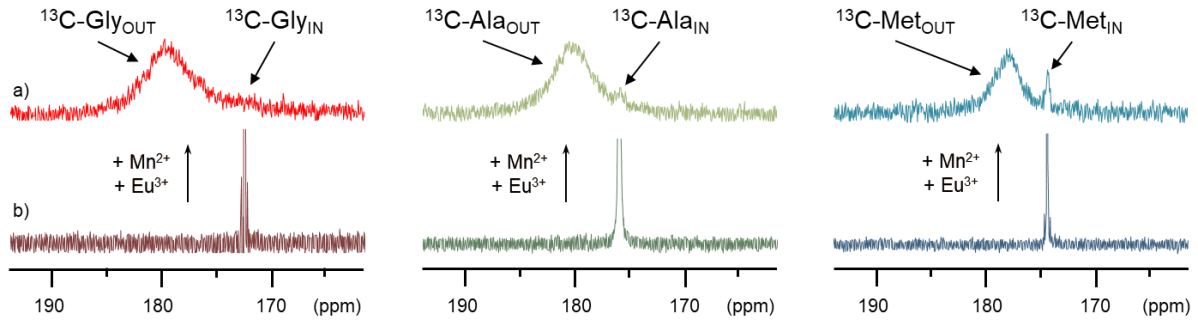


Figure S6.2.2 ^{13}C NMR spectra of isotopically labelled amino acids Gly, Ala, and Met in the ^{13}C NMR transport assay after the addition of **1** (1 mol%) (bottom), followed, after 60 min, by the addition of Mn^{2+} + Eu^{3+} (top).