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Active Transport of Amino Acids by a Guanidiniocarbonyl–Pyrrole Receptor

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Abstract: Herein we report the synthesis and characterization of a transporter **9** for N-acetylated amino acids. Transporter **9** is a conjugate of a guanidiniocarbonyl pyrrole cation, one of the most efficient carboxylate binding motifs reported so far, and a hydrophobic tris(dodecylbenzyl) group, which ensures solubility in organic solvents. In its protonated form, **9** binds N-acetylated amino acid carboxylates in wet organic solvents with association constants in the range of $10^4\,\text{M}^{-1}$ as estimated by extraction experiments. Aromatic amino acids are preferred due to additional cation-π-interactions of the amino acid side chain with the guanidiniocarbonyl pyrrole moiety. U-tube experiments established efficient transport across a bulk liquid chloroform phase with fluxes approaching $10^{-6}\,\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$. In experiments with single substrates, the release rate of the amino acid from the receptor–substrate complex at the interface with the receiving phase is rate determining. In contrast to this, in competition experiments with several substrates, the thermodynamic affinity to **9** becomes decisive. As **9** can only transport anions in its protonated form and has a pK_a value of approximately 7, pH-driven active transport of amino acids is also possible. Transport occurs as a symport of the amino acid carboxylate and a proton.

Keywords: active transport · amino acids · guanidinium cations · membrane proteins · molecular recognition

Introduction

The transport of amino acids and amino acid derivatives across a lipid membrane is a crucial part of metabolism in biological systems. As polar molecules they are not capable of crossing the membrane by passive diffusion and thus require a membrane transporter to do so. Accordingly, the development of artificial transporters has become an active area of research since the advent of supramolecular chemistry about 30 years ago.[1] Transport of any compound from one aqueous phase (source phase) across a membrane or a bulk liquid organic phase to another aqueous phase (receiving phase) requires a host molecule (an artificial receptor) capable of efficiently binding the substrate under aqueous conditions (or at least at the water/organic interface). Therefore, binding of the amino acid to the receptor must be energetically similar to solvation by water molecules, otherwise the substrate cannot be pulled out of the aqueous phase by complex formation. Thus the strongest noncovalent interactions are required, such as electrostatic interactions or metal–ligand binding.[2] Even though a variety of efficient host molecules for amino acids have been developed over the last years,[3] efficient artificial transporters are still rare. One problem is that very often receptors which bind amino acids in aqueous solvents are charged, containing, for example, ammonium or guanidinium cations or metal ions.[4] Hence, they possess limited solubility in a lipid membrane or the nonpolar bulk organic phase used for transport experiments. In transporters realized so far, such binding motifs have, therefore, been combined with nonpolar hydrophobic groups (e.g., such as steroids or aromatic units) to increase their solubility in the organic phase.

One of the first examples for amino acid transport was reported by Lehn who showed in 1973 that amino acids and dipeptides can be transported from one aqueous phase through a chloroform phase into another aqueous phase by lipophilic quaternary ammonium compounds, such as Aliquat 336 (methyltricapryl ammonium chloride, also used as a phase-transfer catalyst).[5] Substrate binding in this case was simply due to unspecific electrostatic interactions and transport efficiency was thus determined by the lipophilicity of the amino acid side chain: Phenylalanine and tryptophan were transported faster than, for example, alanine or glycine. Tsukube then introduced crown ethers and their open chain analogues, lariat ethers, as amino acid transporters, which have been used extensively in this context either

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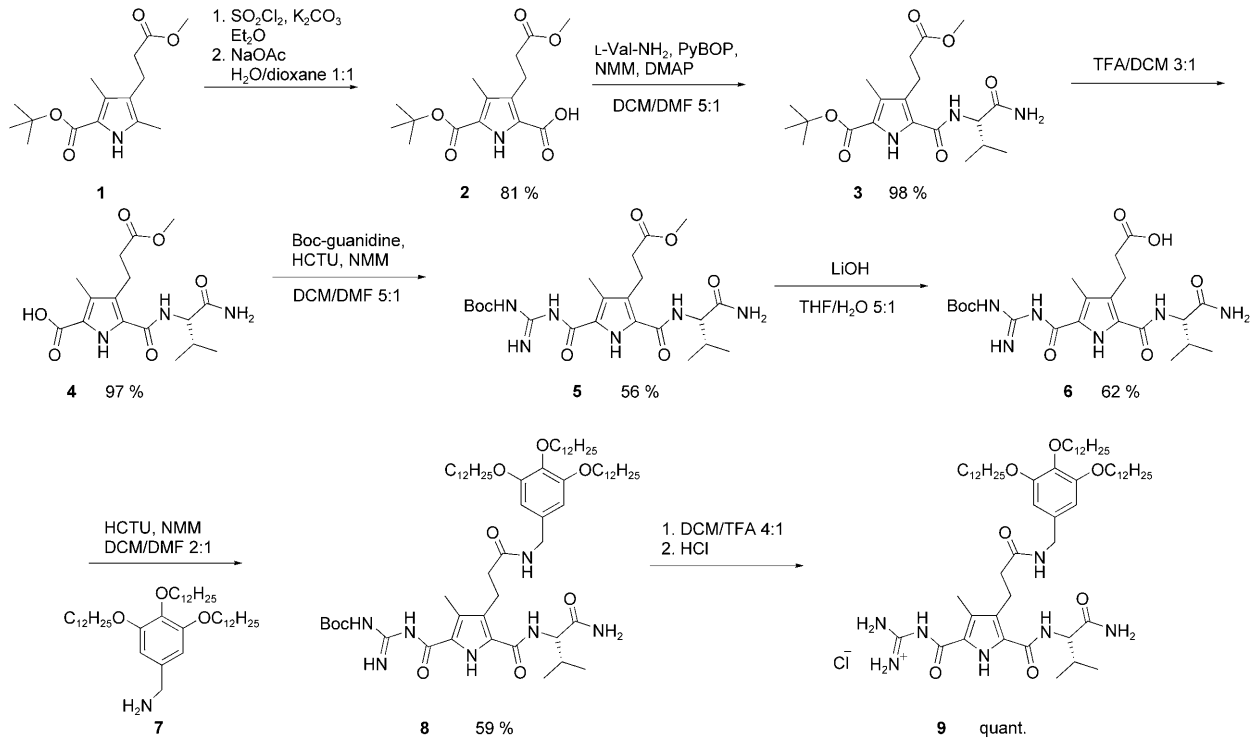
alone or in combination with additional binding sites ever since (e.g., also by Zinic and Gokel, Voyer, Reetz, or more recently by Hosgören).[6] Enantioselective transport of amino acid derivatives was accomplished with chiral crown ether derivatives. By using the high affinity of crown ethers for potassium ions, active transport of amino acids can also be achieved when the transport of the amino acid is coupled to a counter-transport of the alkali metal cation. Charged hosts have also been used for amino acid transport. For example, in early work Rebek used a Kemp's triacid derived acridinium–carboxylate zwitterionic host to extract free zwitterionic amino acids from aqueous solutions into chloroform.[7] Transport was also reported, but suffered from the hydrolytic instability of the host. Another zwitterionic transporter based on a sapphyrin–lasalocid conjugate was reported by Sessler.[8] This system preferentially binds aromatic amino acids in their zwitterionic form by double-ion-pair formation and transports them over a bulk liquid membrane. Negatively charged N-protected amino acid carboxylates can be transported by cationic transporters. For example, Davis developed a steroidal guanidinium receptor, which enantioselectively transported N-acetylated amino acids through a bulk liquid membrane in a U-tube experiment.[9] Another elegant guanidinium cation based transporter is a crown ether conjugate developed by de Mendoza which facilitates passive transport of underivatized zwitterionic amino acids under neutral conditions.[10] The disadvantage of classical guanidinium cation based transporters is the high basicity of the guanidinium group, which prevents deprotonation under ambient conditions (the pK_a of the guanidinium cation is ca. 13.5). Hence, the transporter is always protonated. Transport of N-acylated amino acid carboxylates by guanidinium-based receptors thus requires the counter-transport of another anion (most often halide) from the receiving phase back into the starting phase. Using a corresponding halide gradient in the back direction allows active transport of the amino acid to the receiving phase, but the overall transport always is a counter-transport. In this context we present here the first example, to the best of our knowledge, of an artificial symporter **9**, which allows for active transport of N-acylated amino acid carboxylates by using a pH gradient as the driving force.

Results and Discussion

Receptor design and synthesis: A few years ago we introduced the guanidiniocarbonyl pyrrole cation as a highly efficient oxoanion binding site, which also allows complex formation even in aqueous solutions.[11] Substrate binding is due to the formation of a hydrogen bond directed ion pair with the anion. One reason for the improved anion binding efficiency of the guanidiniocarbonyl cation relative to simple guanidinium cations is its lower pK_a value of approximately 6–7.[12] Hence, the guanidinium NHs are significantly more acidic and thus better hydrogen bond donors. We observed side-chain selective binding of anionic N-acetylated amino acids by this class of cationic receptors. Also stereoselective complexation of small oligopeptides in aqueous solution was achieved.[13] We, therefore, reasoned that this highly efficient binding motif could also be used for the transport of N-acetylated amino acids across a bulk liquid membrane. However, this was so far hampered by the insolubility of this receptor class in nonpolar organic solvents. We have, therefore, now developed an amphiphilic derivative **9**, which efficiently transports N-acylated amino acid carboxylates across a bulk liquid membrane of chloroform and also allows an active symport of the carboxylate and a proton by using a pH gradient as the driving force.

To increase the solubility of the guanidiniocarbonyl pyrrole receptor in organic solvents, a lipophilic tris(dodecylbenzyl) group was attached.[14] It contains three long alkyl chains, which should ensure sufficient solubility in nonpolar organic solvents, while also preventing the receptor from entering the aqueous phase. To connect this moiety to the guanidiniocarbonyl pyrrole cation moiety without affecting its anion binding properties, we used an orthogonally protected pyrrole triester scaffold recently developed by us.[15] The synthesis of the transporter **9** thus started with the triacid derivative **2** (Scheme 1). Relative to the literature protocol, we could further improve the yield of **2** by adjusting the conditions for the oxidation of **1** by excluding light and limiting the use of diethyl ether, thus reducing unwanted radical side reactions. By using our initially reported amino acid receptor[11] as a blueprint, we then attached L-Val-NH_2 to the pyrrole before introducing the protected guanidine. The ester functionality in the propionic acid side chain can be selectively hydrolyzed by using LiOH in THF/water mixtures. To this free carboxylic acid group, the lipophilic amine **7** was coupled by using HCTU as the coupling reagent. Amine **7** was synthesized by starting from the corresponding hydroxyl compound, which can be obtained in two steps from commercially available gallic acid ethyl ester according to a literature procedure.[16] The hydroxyl compound was first transformed into the bromide with phosphorous tribromide. Subsequently the bromide was reacted with sodium azide and the resulting azide was reduced with lithium aluminum hydride to yield the desired amine **7** in 71% over all steps. Finally all protecting groups were cleaved off by using TFA in CH_2Cl_2. Lyophilization with aqueous HCl then yielded transporter **9** as the chloride salt.

Extraction experiments: To examine the ability of **9** to transfer anionic N-acylated amino acids into an organic phase, first extraction experiments were performed. Receptor **9** (1 mM) was dissolved in chloroform and used to extract the N-acetylated amino acids valine, tyrosine, and tryptophane (10 mM) from aqueous buffered solutions of pH 6. This pH was chosen to ensure that **9** will be protonated as needed for anion binding, while at the same time the amino acids will still be present as the carboxylate. This allows ion-pair formation between the receptor and the amino acid substrate in the organic phase. The amino acid uptake into the organic phase was quantified by using HPLC with an RP-8



Scheme 1. Synthesis of transporter **9**. DCM = dichloromethane, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, NMM = *N*-methylmorpholine, DMAP = 4-dimethylaminopyridine, TFA = trifluoroacetic acid, HCTU = *O*-(6-chloro-1-hydrocibenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

column and UV detection at 220 nm. All three amino acids were transferred into the organic phase in approximately equimolar amounts to **9** (Table 1).

The differences in the extraction efficiency and the relative order relate to the hydrophobicity of the amino acids[17]

Table 1. Distribution constants of N-acetylated amino acids between aqueous BIS-TRIS buffer at pH 6 and chloroform and calculated binding constants with receptor **9**.[a]

Substrate	K_{ex}[b]	Extraction efficiency [%][c]	K_{ass} [M^{-1}]
Ac-Val-OH	0.149	126	4.3×10^3
Ac-Tyr-OH	0.046	92	1.5×10^4
Ac-Trp-OH	0.071	106	1.4×10^4

[a] Conditions for the extraction: 1 mL of 100 mM BIS-TRIS buffer at pH 6 with a substrate concentration of 10 mM was extracted with 1 mL of a 1 mM solution of receptor **9** in chloroform. [b] Distribution constant $K_{ex} = c_{org}/c_{aq}$. [c] Concentration of substrate in the organic phase as a percentage of receptor concentration. Note that the efficiency can be more than 100%, because free substrate is also distributed between the two phases.

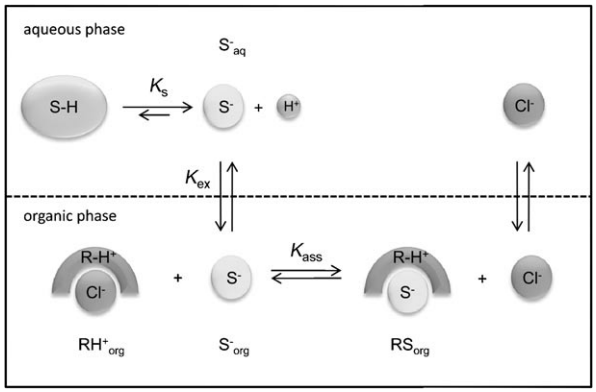


Figure 1. Extraction of an anionic substrate S^- by the cationic receptor **9** from an aqueous phase into an organic phase.

and their intrinsic solubility in chloroform, which were also determined in control experiments. The valine derivative was extracted the most, followed by tryptophane and then tyrosine. From the extraction experiments, also the binding constants of the amino acids to **9** were calculated based on the binding and exchange equilibria shown in Figure 1.

According to the law of mass action the association constant K_{ass} is defined as follows:

$$K_{ass} = \frac{c_{org}(RS)}{c_{org}(S^-)c_{org}(RH^+)}$$

$c_{org}(RS)$ is the concentration of receptor–substrate complex in the organic phase, $c_{org}(S^-)$ is the concentration of free amino acid (present as the carboxylate) in the organic phase and $c_{org}(RH^+)$ the concentration of the free cationic receptor in the organic phase. The concentration of free substrate in the organic phase depends on its distribution between the aqueous and organic phase according to the Nernst distribution law:

$$c_{org}(S^-) = K_{ex}c_{aq}(S^-)$$

K_{ex} is the distribution constant and was measured by extraction experiments of the N-acetylated amino acids in the absence of **9** (data given in Table 1) and $c_{aq}(S^-)$ is the concentration of the amino acid in the aqueous phase, which was determined experimentally by HPLC measurements. The amount of receptor–substrate complex in the organic phase could not be measured directly, only the overall amount of free and bound substrate $c_{org}(S^-{+}RS)$ could be determined by subtracting the amount of substrate in the aqueous phase $c_{aq}(S^-)$ from the total concentration $c_0(S^-)$.

$$c_{org}(S^- + RS) = c_{org}(S^-) + c_{org}(RS) = c_0(S^-) - c_{aq}(S^-)$$

Combining these formulas, the association constant K_{ass} can be calculated as follows:

$$K_{ass} = \frac{c_0(S^-) - c_{aq}(S^-) - K_{ex}c_{aq}(S^-)}{k_{ex}c_{aq}(S^-)[c_0(R) - c_0(S^-) + c_{aq}(S^-) + K_{ex}c_{aq}(S^-)]}$$

This formula is derived on the assumption that the receptor and the receptor–substrate complex are only present in the organic phase and not in the aqueous phase (Figure 1). That this is a valid assumption could be proven by UV measurements of the aqueous phase, which traced at most a minute amount of **9** (<1%) in the aqueous phase. Hence, compound **9** due to the large lipophilic tris(dodecylbenzyl) group is nearly insoluble in water despite its charge. The association constants calculated by using this formula are given in Table 1. The aromatic amino acids tryptophan and tyrosine have similar binding constants of $K_{ass} = 1.4 \times 10^4$ and $1.5 \times 10^4\,M^{-1}$, respectively. They are bound about three times stronger than the nonaromatic amino acid valine ($K_{ass} = 4.3 \times 10^3\,M^{-1}$). Similar association constants of approximatley 10^3–$10^4\,M^{-1}$ were obtained by using UV/Vis spectroscopy instead of HPLC to determine the concentration of the amino acids in the aqueous phase. Hence, the amino acid carboxylates are bound very efficiently by **9**. In accordance with the less-polar solvent (wet chloroform), the binding constants are approximatley one order of magnitude larger than previously reported data for amino acid binding by guanidiniocarbonyl pyrrole receptors in aqueous DMSO.[11b]

The preference for aromatic side chains corresponds to the binding properties of guanidiniocarbonyl pyrrole receptors in general, as already observed in earlier studies.[11a] Cation-π-stacking interactions[18] between the aromatic side chain and the planar guanidiniocarbonyl pyrrole cation further stabilize the complex in addition to the ion-pair formation. Experiments with the L- and D-enantiomers of the amino acids (data not shown) did not reveal any stereoselectivity; the binding constants were identical within the error margin of this experiment (±10%). A simple force-field calculation confirmed the idea of cation-π-interactions. A MonteCarlo conformational search (Macromodel V8,[19] amber* force field, GB/SA solvation model for chloroform, 50.000 steps) revealed an energy-mimimized structure (Figure 2), in which the aromatic indole ring indeed π-stacks with the planar guanidiniocarbonyl pyrrole cation. The top face is shielded by the tris(dodecylbenzyl) group, whereas the carboxylate forms an hydrogen bond enforced ion pair with the cation as known from related receptors.

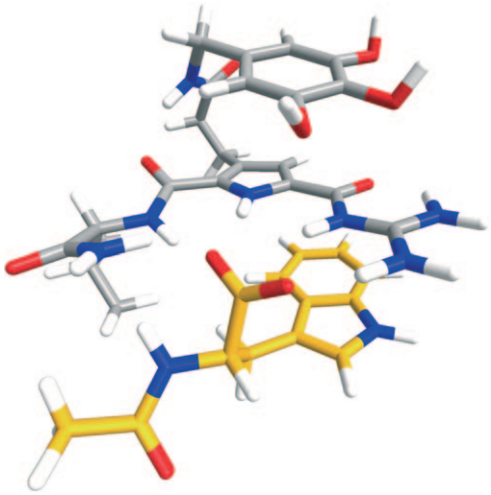


Figure 2. Calculated energy-minimized structure of the complex between L-*N*-Ac-Trp (yellow) and **9** (grey) (chloroform solvation model). The alkyl chains of the tris(dodecyl) benzyl group were omitted for clarity.

Transport experiments: After we successfully established that receptor **9** is soluble in organic solvents, but not in water and efficiently binds amino acid carboxylates even in the presence of water, we measured the transport capacity of **9** in a U-tube experiment. The experimental setup is shown in Figure 3.

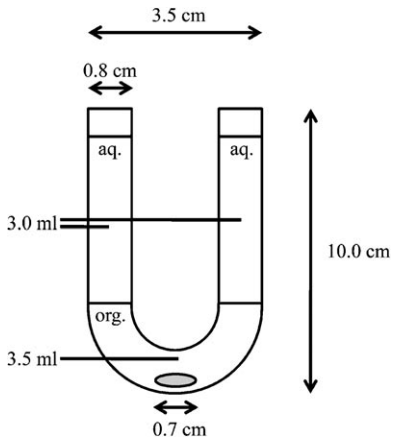


Figure 3. Setup of the U-tube experiment for the determination of transport rates.

As the source phase, 3.0 mL of a 50 mM solution of the N-acetylated amino acid in 100 mM BIS-TRIS buffer at pH 6 were used and as the transport layer 3.5 mL of a 1 mM solu-

tion of receptor **9** in chloroform were used. The receiving phase consisted of 3.0 mL of 100 mM BIS-TRIS buffer at pH 8. These conditions assured that the substrates were transported in the direction of the receiving phase, due to the different protonation states and hence anion binding properties of **9** at these two pH values. The receptor **9** has a pK_a of approximately 7 and is, therefore, protonated at pH 6 at the interface to the source phase, whereas **9** will be deprotonated at the interface with the receiving phase (pH 8). However, anion binding under these conditions occurs only by the cationic receptor and not by the deprotonated neutral one. As shown in Figure 4, the transport is a symport of the negatively charged amino acid carboxylate and a proton.

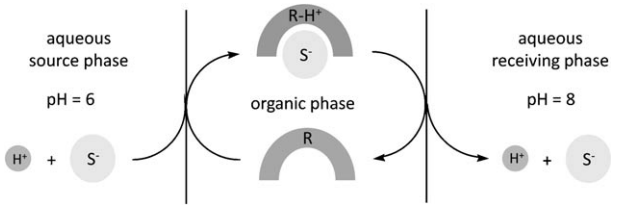


Figure 4. Transport of acetylated amino acid carboxylates (S) by receptor **9** (R). Only the protonated form RH^+ of the receptor can bind the anionic substrate S^- and transport it across the bulk liquid membrane.

The experiments were conducted with the N-acetylated L-amino acids of alanine, valine, tyrosine, phenylalanine, and tryptophan. At first transport was measured for each amino acid alone. Samples from the receiving phase were taken over a period of one to two weeks and were analyzed by HPLC analysis on a RP-8 column with UV detection at 220 nm. Prior to the experiments, the aqueous source phase and the organic phase were equilibrated by shaking the two phases together for 5 min. Otherwise an induction period was observed before the transport started. Each experiment was performed twice along with a control experiment. For the control experiments, the exact same setup and procedure, but without the receptor **9**, was used. The data given are the average values of both runs. To test for enantioselective transport, the experiments were also carried out with the D-enantiomers (data not shown), but as in the extraction experiments the differences were found to be within the error range, which was estimated to be in a range of $\pm 10\,\%$. The results are summarized in Table 2 and shown in Figure 5.

As the data show, receptor **9** is capable of efficiently transporting the amino acids studied across the chloroform phase. The efficiency (as, for example, expressed by the flux values), significantly depends on the amino acid and decreases in the order Val > Phe > Ala > Trp > Tyr (Figure 5, red columns). The valine derivative was transported fastest, with a very high flux of $1.11\times10^{-6}\,mol\,m^{-2}s^{-1}$. In comparison, the tyrosine derivative was transported 18 times slower with a flux of $6.24\times10^{-8}\,mol\,m^{-2}s^{-1}$ (Table 2). Whereas the flux determined in the control experiments reflects the hydrophobicity of the amino acids, the transport rates in the

Table 2. Results of the transport experiments.

Substrate	Flux (single) $[mol\,m^{-2}s^{-1}]$[a]	Flux (comp.) $[mol\,m^{-2}s^{-1}]$[b]	Flux (control) $[mol\,m^{-2}s^{-1}]$
Ac-Val-OH	1.11×10^{-6}	1.54×10^{-7}	1.23×10^{-7}
Ac-Phe-OH	4.67×10^{-7}	2.03×10^{-7}	4.54×10^{-8}
Ac-Ala-OH	1.92×10^{-7}	2.30×10^{-8}	n/a
Ac-Trp-OH	8.05×10^{-8}	2.10×10^{-7}	4.62×10^{-8}
Ac-Tyr-OH	6.24×10^{-8}	5.65×10^{-8}	2.20×10^{-9}

[a] Conditions: the source phase was 3 mL of 100 mM BIS-TRIS buffer at pH 6 with a substrate concentration of 50 mM, the organic phase was 3.5 mL of a 1 mM solution of receptor **9** in chloroform, the receiving phase was 3 mL of 100 mM BIS-TRIS buffer at pH 8. [b] Conditions: the source phase was 3 mL of 100 mM BIS-TRIS buffer at pH 6 with substrate concentrations of 2 mM each, the organic phase was 3.5 mL of a 1 mM solution of receptor **9** in chloroform, the receiving phase was 3 mL of 100 mM BIS-TRIS buffer at pH 8.

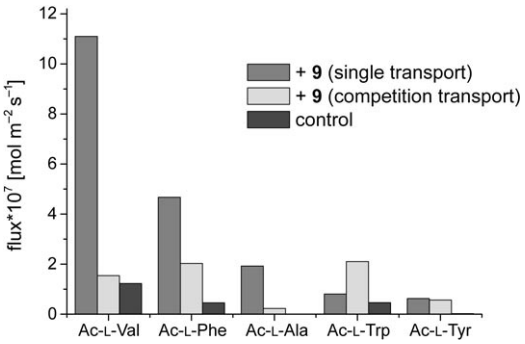


Figure 5. Comparison of the flux values of the single, competitive, and the control experiment.

presence of a receptor showed a different behavior.[17] The valine, phenylalanine, alanine, and tyrosine derivatives are roughly in the expected relative order, whereas tryptophan is transported slower than would be expected based on its hydrophobicity. Even more interesting is that these transport rates are not in accord with the relative order of association constants as determined in the extraction experiments (Table 1). Tryptophan and tyrosine are both bound significantly more strongly by **9** than valine, but are transported more than one order of magnitude slower. This shows that the transport rate under this experimental setup is not determined by the affinity of the substrate to the transporter. The transporter is present in only 2.3 mol% relative to the amount of amino acid in the source phase. The rate-determining step is, therefore, not the binding of the substrate but its release upon contact with the receiving phase. The stronger a substrate binds to **9**, the higher is the energy barrier for its release from the receptor–substrate complex, in accord with the Bell–Evans–Polanyi principle. Therefore the most stable complexes show the slowest exchange rates and thus the slowest overall transport rate.

We also performed a competition experiment, in which all five amino acids were present at the same time (Figure 5, light grey columns). In this experiment, quite different values for flux and selectivity were found. The individual

overall transport rates are slower than in the first experiment, due to the much smaller concentration of each amino acid in the source phase (2 mM instead of 50 mM, respectively). Furthermore, the relative transport rate decreases in the order Trp > Phe > Val > Tyr > Ala. Tryptophan is now transported the fastest and approximately nine times faster than the slowest substrate alanine. This change in the relative order of the transport efficiency shows that under competitive conditions the binding strength is now the determining factor for transport and not the release rate. With direct competition for the receptor, the substrates with higher binding constants are preferred. The substrates with weaker binding affinity cannot compete for the receptor at the interface of the source phase and accordingly cannot be transported across the organic phase. For example, tryptophan, which has the highest affinity for receptor **9**, consequently also has the highest transport rate in the competitive experiment, whereas in the single experiments it showed one of the slowest transport rates of all substrates. The opposite is true for valine, which due to its significantly weaker binding affinity has no chance in the competitive experiment, as it cannot beat the other substrates. But in the individual experiment its faster release from the complex at the interface with the receiving phase is decisive.

Active transport: By using equal concentrations of amino acid in both the source and the receiving phase, active transport against the concentration gradient was investigated by using phenyl alanine as the substrate. And indeed efficient transport with a concentration change of approximately 15 mM within 15 days was observed. The measured transport velocity of $4.91 \times 10^{-7}\,\mathrm{mol\,m^{-2}\,s^{-1}}$ is similar to the rate measured in the single experiment. The driving force for this transport against the concentration gradient of the amino acid stems from the proton gradient between the source (pH 6) and receiving phase (pH 8). As the transport scheme in Figure 4 shows, transport of the substrate is coupled to the transport of a proton in the same direction. Hence, symport of the amino acid carboxylate and a proton occurs and overall the uncharged N-acetylated protonated amino acid is thus transported. Thereby the thermodynamically favorable transport of the proton along the pH gradient drives the unfavorable transport of the substrate. This active transport can occur as long as the overall chemical potential for this symport is favorable. In general, any concentration gradient between the source and receiving phase creates a difference in the chemical potential $\Delta\mu$ and thus a thermodynamic driving force for transport as long as the concentrations are not equal and $\Delta\mu$ did not yet reach zero.

$$\Delta\mu = RT\ln\frac{c_2}{c_1}$$

As the concentration of H_3O^+ ions in both phases changes over the course of the experiment due to the symport of substrate and proton so does the difference in the chemical potential. Accordingly, the transport leads to an increase in the concentration of the substrate in the receiving phase relative to the source phase and thus creates a difference in chemical potential that thermodynamically favors the back transport of the substrate. pH gradient driven active transport of the amino acid can occur as long as the sum of the chemical potentials of both species is still negative. As shown in Figure 6, in our experimental setup active transport can occur until the concentration of the amino acid in the receiving phase has increased to 74.4 mM, thus approximately half of the amino acid has been transported from the source phase to the receiving phase.

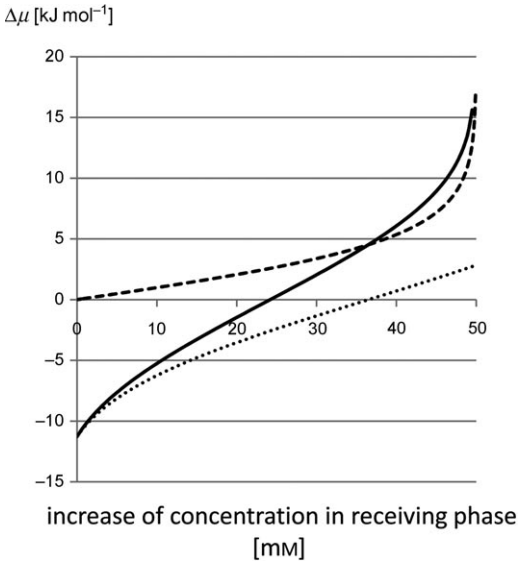


Figure 6. Change in the chemical potential during transport. ——= Coupled transport of proton and substrate, -----= 50 mM substrate in both phases, and •••••= 100 mM buffer (pH 6 to 8).

Conclusion

We here have presented a new transporter **9**, which allows pH gradient driven active transport of N-acetylated amino acids. Transporter **9** combines the efficient anion binding properties of a guanidiniocarbonyl pyrrole cation with the hydrophobic character of a tris(dodecylbenzyl) group, thus ensuring solubility of **9** in a nonpolar organic phase. Due to the lowered pK_a of **9** relative to a simple guanidinium cation, **9** can both be protonated and deprotonated around a neutral pH. However, only the protonated, cationic form of **9** binds anions. Hence, by using a pH gradient between the source and receiving phase, symport of the amino acid carboxylate and a proton occurs. In transport experiments of single amino acids (with an excess of substrate over the transporter), the transport rates are determined by the release rate of the substrate from the complex at the interface with the receiving phase. The substrates that are bound to the weakest are, therefore, transported the fastest and vice versa. When several substrates have to compete for the

transporter in a competition experiment, the thermodynamic stability of the receptor–substrate complex determines the transport rate. As receptor **9** prefers aromatic amino acids due to additional cation-π-interactions within the complex, tryptophan and phenylalanine show the fastest transport rates. In all experiments transport is very efficient with fluxes in the order of 10^{-6} mol m^{-2} s^{-1} (similar to the most efficient artificial transporters reported in the literature so far). The use of a pH gradient from pH 6 in the source phase to 8 in the receiving phase also enabled active transport of the amino acid carboxylate against a concentration gradient. The transport of the substrate is coupled to the simultaneous transport of a proton (symport), which provides the thermodynamic driving force.

Experimental Section

General: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance-400 spectrometer at 25 °C. Chemical shifts are reported relative to residual undeuterated solvent peaks. Melting points were determined on a Büchi melting point apparatus SMP-20. IR spectra were obtained from a Jasco FTIR 410 on a diamond ATR crystal. Flash column chromatography was carried out on MP Biomedicals GmbH silica gel MP-Silica 32–63, 60 Å. Reagents were purchased from Aldrich, Fluka, Lancaster, Iris Biotech, or Sigma and were used without further purification. All solvents were distilled prior to use; water for chromatography, extraction, and transport experiments was taken from a TKA MicroPure water system. HPLC spectra were recorded on a system consisting of a Shimadzu LP-6A Liquid Chromatograph pump with a SCL-6B System Controller, a Rheodyne 7125 with 20µl sample loop and a SPD-6A Spectrophotometric Detector. HPLC columns were Supelcosil LC-8 25 cm × 4.6 mm, 5 µm and Astec Chirobiotic T.

3-(2-Methoxycarbonylethyl)-4-methyl-1*H*-pyrrole-2,5-dicarboxylic acid-5-*tert*-butyl ester (2): Compound **1** (2.00 g, 7.11 mmol) and K_2CO_3 (4.00 g, 28.9 mmol) were suspended under Ar in anhydrous Et_2O (50 mL) under the exclusion of light and cooled down to 0 °C. Freshly distilled sulfuryl chloride (2.33 mL, 24.9 mmol) was added slowly through a septum with a syringe. The mixture was warmed slowly to room temperature and stirred for 2 h at room temperature. The solvent was removed at room temperature under reduced pressure and the oily residue was stirred for 1 h in a solution of NaOAc (5.00 g, 61.0 mmol) in water/dioxane (1:1, 100 mL) at 110 °C. The solution was cooled down to 0 °C and adjusted to pH 2 with concentrated hydrochloric acid. The solution was extracted with Et_2O (3 × 50 mL). The combined organic phases were extracted with a half-saturated aqueous solution of $NaHCO_3$ (3 × 50 mL) and the combined aqueous solutions were cooled down to 0 °C. This aqueous solution was then acidified slowly and under vigorous stirring with concentrated hydrochloric acid to pH 2. The precipitate was filtered off and washed with cold water (3 × 50 mL) to give **5** (1.79 g, 5.76 mmol, 81 %) as a white solid. M.p. 169 °C; ^{1}H NMR (400 MHz, DMSO): δ = 1.52 (s, 9H; C-(C*H*$_3$)$_3$), 2.17 (s, 3H; pyrrole-C*H*$_3$), 2.44 (t, $^3J_{H-H}$ = 8.10 Hz, 2H; pyrrole-CH$_2$C*H*$_2$), 2.90 (t, 2H, $^3J_{H-H}$ = 8.10 Hz; pyrrole-C*H*$_2$), 3.57 (s, 3H; OC*H*$_3$), 11.2 ppm (s, 1H; pyrrole-N*H*); ^{13}C NMR (100 MHz, CDCl$_3$): δ = 9.7 (pyrrole-*C*H$_3$), 19.7 (pyrrole-*C*H$_2$), 28.0 (C(*C*H$_3$)$_3$), 34.3 (pyrrole-CH$_2$*C*H$_2$), 51.2 (OC*H*$_3$), 80.5 (*C*(CH$_3$)$_3$), 119.3, 122.4, 125.0 (pyrrole-*C*), 159.9, 161.8, 172.7 ppm (carbonyl-*C*).

5-[(*R*)-1-Carbamoyl-2-methylpropylcarbamoyl]-4-[2-(methoxycarbonyl)-ethyl]-3-methylpyrrole-2-*tert*-butylester (3): A solution of compound **2** (500 mg, 1.61 mmol), L-Val-NH$_2$ (369 mg, 2.41 mmol), HCTU (797 mg, 1.92 mmol), and DMAP (30 mg, 0.245 mmol) in CH$_2$Cl$_2$/DMF 5:1 (25 mL) with NMM (1 mL) was stirred for 24 h at room temperature. The solvent was removed under reduced pressure to give an oily residue. Flash column chromatography (silica gel, CH$_2$Cl$_2$/MeOH 15:1 + 1 % HOAc) yielded a colorless solid (643 mg, 1.57 mmol, 98 %). M.p. 202 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ = 1.05 (t, $^3J_{H-H}$ = 8.28 Hz, 6H; CHCH-(C*H*$_3$)$_2$), 1.56 (s, 9H; C(C*H*$_3$)$_3$), 2.24 (s, 3H; pyrrole-C*H*$_3$), 2.26–2.31 (m, 1H; CHC*H*(CH$_3$)$_2$), 2.63 (t, $^3J_{H-H}$ = 7.26 Hz, 2H; pyrrole-CH$_2$C*H*$_2$), 3.05 (t, 2H, $^3J_{H-H}$ = 7.32 Hz; pyrrole-C*H*$_2$), 3.65 (s, 3H; OC*H*$_3$), 4.45 (t, 1H, $^3J_{H-H}$ = 8.72 Hz; C*H*CH(CH$_3$)$_2$), 6.20 (s, 1H), 8.40 (d, $^3J_{H-H}$ = 8.72 Hz, 1H; amide-N*H*), 10.8 ppm (s, 1H; pyrrole-N*H*); ^{13}C NMR (100 MHz, CDCl$_3$): δ = 10.5 (pyrrole-*C*H$_3$), 19.6 (CHCH(*C*H$_3$)$_2$), 20.6 (pyrrole-*C*H$_2$), 28.5 (C(*C*H$_3$)$_3$), 30.6 (CH*C*H(CH$_3$)$_2$), 34.6 (pyrrole-CH$_2$*C*H$_2$), 51.7 (OC*H*$_3$), 58.9 (*C*HCH(CH$_3$)$_2$), 82.0 (*C*(CH$_3$)$_3$), 122.3, 124.8, 126.0 (pyrrole-*C*), 161.0, 162.4, 171.0, 174.2 ppm (carbonyl-*C*); IR: (solid): $\tilde{\nu}$3316 (w), 3189 (w), 2961 (w), 1734 (w), 1673 (s), 1619 (s), 1534 (w), 1433 (m), 1368 (w), 1309 (m), 1247 (m), 1149 (s), 1099 (w), 975 (w), 837 (s), 781 (m), 664 (m), 619 cm^{-1} (w); HRMS (pos. ESI): *m*/*z*: calcd for C$_{20}$H$_{31}$N$_3$NaO$_6$: 432.2107 [*M*+Na]$^+$; found: 432.2105.

5-[(*R*)-1-Carbamoyl-2-methylpropylcarbamoyl]-4-[2-(methoxycarbonyl)-ethyl]-3-methylpyrrole-2-carboxylic acid (4): Compound **3** (367 mg, 0.896 mmol) was dissolved in TFA/CH$_2$Cl$_2$ 3:1 (20 mL) and was stirred at room temperature for 5 h. The solvent was removed under reduced pressure and the residue was suspended in water and lyophilized. Compound **4** was obtained (308 mg, 0.872 mmol, 97 %) as a colorless solid. M.p. 205 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ = 0.88 (d, $^3J_{H-H}$ = 6.76 Hz, 6H; CHCH(C*H*$_3$)$_2$), 2.02 (q, $^3J_{H-H}$ = 6.82 Hz, 1H; CHC*H*(CH$_3$)$_2$), 2.16 (s, 3H; pyrrole-C*H*$_3$), 2.41 (t, $^3J_{H-H}$ = 7.74 Hz, 2H; pyrrole-CH$_2$C*H*$_2$), 2.90 (t, 2H, $^3J_{H-H}$ = 7.76 Hz; pyrrole-C*H*$_2$), 3.52 (s, 3H; OC*H*$_3$), 4.24 (t, 1H, $^3J_{H-H}$ = 4.14 Hz; C*H*CH(CH$_3$)$_2$), 6.99 (s, 1H), 7.54 (s, 1H), 8.19 (d, $^3J_{H-H}$ = 8.32 Hz, 1H; amide-N*H*), 11.88 ppm (s, 1H; pyrrole-N*H*); ^{13}C NMR (100 MHz, CDCl$_3$): δ = 10.0 (pyrrole-*C*H$_3$), 18.8 (CHCH(*C*H$_3$)$_2$), 20.4 (pyrrole-*C*H$_2$), 30.6 (CH*C*H(CH$_3$)$_2$), 34.7 (pyrrole-CH$_2$*C*H$_2$), 51.8 (OC*H*$_3$), 58.3 (*C*HCH(CH$_3$)$_2$), 120.1, 124.7, 126.0, 128.6 (pyrrole-*C*), 160.7, 162.9, 173.8, 174.1 ppm (carbonyl-*C*); IR (solid): $\nu\ddot{U}$ = > = 3315 (m), 2959 (w), 1676 (s), 1615 (s), 1576 (w), 1540 (w), 1487 (m), 1436 (m), 1305 (m), 1272 (s), 1242 (m), 1196 (w), 1169 (s), 981 (w), 898 (w), 834 (w), 720 cm^{-1} (m); HRMS (pos. ESI): *m*/*z*: calcd for C$_{16}$H$_{23}$N$_3$NaO$_6$: 376.1479 [*M*+Na]$^+$; found: 376.1484.

2-Mono-*tert*-butoxycarbonyl (Boc)-guanidinocarbonyl-5-[(*R*)-1-carbamoyl-2-methylpropylcarbamoyl]-3-methylpyrrole-4-propionic acid methyl ester (5): A solution of compound **4** (680 mg, 1.93 mmol), HCTU (865 mg, 2.10 mmol) and Boc-guanidine (334 mg, 2.10 mmol) in CH$_2$Cl$_2$/DMF 5:1 (24 mL) with NMM (1 mL) was stirred for 1 h at room temperature. Water (20 mL) was added, the phases were separated and the aqueous phase was extracted with CH$_2$Cl$_2$ (3 × 20 mL). The combined organic phases were dried with Na$_2$SO$_4$, the solid was filtered, and the solvent was removed under reduced pressure. The resulting solid was purified by flash column chromatography (silica gel, CH$_2$Cl$_2$/MeOH 20:1) to give **5** (536 mg, 1.08 mmol, 56 %) as a colorless solid. M.p. 149 °C (decomp.); ^{1}H NMR (400 MHz, CDCl$_3$): δ = 1.04 (dd, $^3J_{H-H}$ = 6.76, $^4J_{H-H}$ = 2.76 Hz, 6H; CHCH(C*H*$_3$)$_2$), 1.49 (s, 9H; C(C*H*$_3$)$_3$), 2.35 (s, 3H; pyrrole-C*H*$_3$), 2.26–2.38 (m, 1H; CHC*H*(CH$_3$)$_2$), 2.67 (t, $^3J_{H-H}$ = 7.02 Hz, 2H; pyrrole-CH$_2$C*H*$_2$), 3.01 (m, 2H; pyrrole-C*H*$_2$), 3.64 (s, 3H; OC*H*$_3$), 4.44 (t, 1H, $^3J_{H-H}$ = 8.02 Hz; C*H*CH(CH$_3$)$_2$), 6.26 (s, 1H), 6.54 (s, 1H), 8.39 (d, $^3J_{H-H}$ = 8.20 Hz, 1H; amide-N*H*), 8.71 (s, 1H), 10.3 ppm (s, 1H; pyrrole-N*H*); ^{13}C NMR (100 MHz, CDCl$_3$): δ = 10.7 (pyrrole-*C*H$_3$), 18.9 (CHCH(*C*H$_3$)$_2$), 20.3 (pyrrole-*C*H$_2$), 28.1 (C(*C*H$_3$)$_3$), 30.1 (CH*C*H(CH$_3$)$_2$), 34.1 (pyrrole-CH$_2$*C*H$_2$), 52.1 (OC*H*$_3$), 59.5 (*C*HCH(CH$_3$)$_2$), 119.5, 128.4 (pyrrole-*C*), 162.1, 174.9 (carbonyl-*C*). IR (solid): $\tilde{\nu}$ = 3391 (w), 1725 (w), 1672 (w), 1625 (s), 1541 (m), 1434 (m), 1309 (m), 1238 (s), 1147 (s), 835 (s), 625 (m), 611 cm^{-1} (w); HRMS (pos. ESI): *m*/*z*: calcd for C$_{22}$H$_{34}$N$_6$NaO$_7$: 517.2381 [*M*+Na]$^+$; found: 517.2378.

2-Mono-Boc-guanidinocarbonyl-5-[(*R*)-1-carbamoyl-2-methylpropylcarbamoyl]-3-methylpyrrol-4-propionic acid (6): A mixture of compound **5** (250 mg, 0.505 mmol) and lithium hydroxide (36.5 mg, 1.52 mmol) in THF/H$_2$O 5:1 (24 mL) was stirred for 5 h at room temperature. The solvent was evaporated under reduced pressure. HCl (1 M) was added to the residue until pH 4 was reached. The precipitate was filtered off and lyophilized to give **6** (150 mg, 0.312 mmol, 62 %) as a colorless solid. M.p. 216 °C (decomp.); ^{1}H NMR (400 MHz, CDCl$_3$): δ = 0.91 (d, $^3J_{H-H}$ = 6.72 Hz, 6H; CHCH(C*H*$_3$)$_2$), 1.49 (s, 9H; C(C*H*$_3$)$_3$), 2.03–2.11 (m, 1H; CHC*H*(CH$_3$)$_2$), 2.31 (s, 3H; pyrrole-C*H*$_3$), 2.38 (t, $^3J_{H-H}$ = 7.82 Hz, 2H;

pyrrole-CH_2CH_2), 2.86–2.92 (m, 2H; pyrrole-CH_2), 4.30 (t, 1H, $^3J_{H-H}$= 7.64 Hz; CHCH(CH_3)$_2$), 7.03 (s, 1H), 7.48 (s, 1H), 8.18 (d, $^3J_{H-H}$= 8.60 Hz, 1H; amide-NH), 8.81 (s, 1H), 9.58 (s, 1H), 11.66 ppm (s, 1H; pyrrole-NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ=10.2 (pyrrole-CH_3), 18.3 (CHCH(CH_3)$_2$), 19.9 (pyrrole-CH_2), 27.7 (C(CH_3)$_3$), 30.2 (CHCH(CH_3)$_2$), 34.4 (pyrrole-CH_2CH_2), 57.7 (CHCH(CH_3)$_2$), 67.0 (C(CH_3)$_3$), 127.8 (pyrrole-C), 160.2, 173.1, 174.3 ppm (carbonyl-C); IR (solid): $\tilde{\nu}$=3318 (w), 2965 (w), 1670 (m), 1626 (s), 1537 (s), 1409 (m), 1285 (s), 1147 (s), 1091 (w), 845 (w), 754 (m), 619 cm^{-1} (m); HRMS (pos. ESI): m/z: calcd for $C_{21}H_{32}N_6NaO_7$: 503.2225 [M+Na]$^+$; found: 503.2230.

2-Mono-Boc-guanidinocarbonyl-3-methyl-4-ethyl-pyrrol-5-[(*R*)-3-methyl-2-carbamoyl]butylamide (8): A solution of **6** (110 mg, 0.229 mmol), **7** (190 mg, 0.288 mmol), and HCTU (119 mg, 0.288 mmol) in CH_2Cl_2/DMF 2:1 (15 mL) with NMM (0.5 mL) was stirred at room temperature for 3 d. Water (15 mL) was added and the phases were separated. The aqueous phase was extracted with CH_2Cl_2 (3×15 mL), the combined organic phases were dried with $MgSO_4$, and the solid was filtered off. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate 1:1+ 1% HOAc) to give **8** (153 mg, 0.136 mmol, 59%) as a colorless solid. M.p. 92 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.88 (t, $^3J_{H-H}$=6.82 Hz, 9H; CH_2CH_3), 1.04 (d, $^3J_{H-H}$=6.72 Hz, 6H; CHCH(CH_3)$_2$), 1.18–1.37 (m, 49H; CH_2 δ-λ), 1.39–1.47 (m, 6H; $OCH_2CH_2CH_2$), 1.49 (s, 8H; C(CH_3)$_3$), 1.67–1.80 (m, 6H; OCH_2CH_2), 2.25–2.33 (m, 1H; CHCH(CH_3)$_2$), 2.35 (s, 3H; pyrrole-CH_3), 2.45–2.66 (m, 2H; pyrrole-CH_2CH_2), 2.95–3.13 (m, 2H; pyrrole-CH_2), 3.87–3.91 (m, 6H; OCH_2), 4.19–4.30 (m, 2H; benzyl-CH_2), 4.46 (t, 1H, $^3J_{H-H}$=7.58 Hz; CHCH(CH_3)$_2$), 6.38 (s, 2H; phenyl-CH), 6.71 (s, 1H), 8.60 ppm (br s, 1H; amide-NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ=10.9 (pyrrole-CH_3), 14.2 (CH_2CH_3), 19.5 (CHCH(CH_3)$_2$), 22.8 (pyrrole-CH_2), 22.8 (λ-CH_2), 26.3 (γ-CH_2), 28.1 (C(CH_3)$_3$), 29.5, 29.5, 29.6, 29.8, 29.8, 29.8, 29.9, 29.9, 29.9, 30.5 (δ-ι CH_2), 32.1 (pyrrole-CH_2CH_2), 44.2 (benzyl- CH_2), 59.6 (CHCH(CH_3)$_2$), 69.3, 73.6 (α-CH_2), 105.9 (phenyl-C_o), 133.1 (pyrrole-C), 137.7 (phenyl-$Ci_{,p}$), 153.4 (phenyl-C_m), 161.8, 172.9, 175.0 ppm (carbonyl-C); IR (solid): νÜ=>=3330 (w), 2917 (s), 2850 (s), 1673 (m), 1628 (s), 1536 (m), 1465 (w), 1437 (m), 1369 (w), 1320 (m), 1287 (m), 1217 (m), 1150 (s), 1115 (s), 1092 (w), 1018 (w), 852 (w), 816 (w), 754 (m), 720 (m), 655 (w), 624 cm^{-1} (m); HRMS (pos. ESI): m/z: calcd for $C_{64}H_{112}N_7O_9$: 1122.8516 [M+H]$^+$; found: 1122.8516.

5-[(*R*)-1-Carbamoyl-2-methylpropylcarbamoyl]-3-methyl-4-{2-[3,4,5-tris-(dodecyl-oxy)phenyl]methylcarbamoyl}ethyl-pyrrole-2-carbonylguanidinium chloride (9): A solution of **8** (40.0 mg, 35.7 μmol in CH_2Cl_2/TFA 4:1 (20 mL) was stirred at room temperature for 4 h. The solvent was evaporated under reduced pressure. The resulting solid was lyophilized four times with 5% HCl to give **9** (37.7 mg, 35.6 μmol, 100%) as a colorless solid. M.p.: 237 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.87 (t, $^3J_{H-H}$= 6.82 Hz, 9H; CH_2CH_3), 1.05 (s, 6H; CHCH(CH_3)$_2$),1.15–1.37 (s, 48H; CH_2 δ-λ), 1.38–1.48 (s, 6H; $OCH_2CH_2CH_2$), 1.67–1.80 (m, 6H; OCH_2CH_2), 2.24 (s, 3H; pyrrole-CH_3), 2.50–2.64 (s, 2H; pyrrole-CH_2CH_2), 2.85–3.10 (m, 2H; pyrrole-CH_2), 3.82–3.95 (m, 6H; OCH_2), 4.07–4.17 (m, 1H; CHCH(CH_3)$_2$), 4.18–4.35 (m, 2H; benzyl-CH_2), 6.42 (s, 2H; phenyl-CH), 6.76 (s, 1H), 8.33 (s, 2H), 8.88 (s, 1H), 11.3 (s, 1H), 11.5 ppm (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=10.3 (pyrrole-CH_3), 14.3 (CH_2CH_3), 19.4 (pyrrole-CH_2), 19.7 (CHCH(CH_3)$_2$), 22.8 (λ-CH_2), 26.3 (γ-CH_2), 29.5, 29.8, 30.3, 30.5, 30.6 (δ-ι CH_2), 32.1 (pyrrole-CH_2CH_2), 44.9 (benzyl-CH_2), 60.8 (CHCH(CH_3)$_2$), 69.1, 73.9 (α-CH_2), 112.2 (phenyl-C), 128.5, 142.0 (pyrrole-C), 160.4, 172.6, 176.8 ppm (carbonyl-C); IR (solid): $\tilde{\nu}$=3306 (w), 2922 (s), 2852 (s), 1639 (m), 1488 (w), 1464 (m), 1431 (m), 1377 (w), 1285 (s), 1202 (s), 1118 (s), 835 (w), 799 (w), 755 (w), 720 cm^{-1} (m); HRMS (pos. ESI): m/z: calcd for $C_{59}H_{104}N_7O_7$: 1022.7992 [M+H]$^+$; found: 1022.7992.

Extraction experiments: The extraction experiments were carried out in 4 mL glass vials. The organic phase consisted of 1 mL of a solution of receptor **9** in chloroform or pure chloroform, respectively, for the control experiment. The aqueous solutions consisted of 10 mM solutions of the acetylated amino acids valine, tryptophan, and tyrosine in 100 mM BIS-TRIS buffer. The solutions were adjusted to pH 6 by the addition of 0.1 mM HCl and 0.1 mM NaOH. The different phases were put together in the glass vials and shaken manually for 10 min. Afterwards, they were left to stand for another 10 min or until the foam had disappeared. Samples of 50 μL were taken out of the aqueous phase and either directly injected into the HPLC with an UV detector or in the case of tryptophan and tyrosine diluted tenfold and then injected. Spectra were recorded at 220 nm. For the injection, the 20 μL sample loop was used to measure exactly equal quantities. The experiments were carried out twice and the average value was determined. Solutions of defined substrate concentration were used for calibration of the UV-detector.

U-tube transport experiments: The form of the U-tubes was as seen in Figure 3. The stirring bar was 7 mm long and 2 mm in diameter. The organic phase consisted of 3.5 mL of a 1 mM solution of receptor **9** in chloroform or pure chloroform, respectively, for the control experiments. The stirring bar was rotated at 1250 rpm. In the single substrate experiments, the source phase consisted of 3 mL of a 50 mM solution of the respective substrate in 100 mM BIS-TRIS buffer, which was adjusted to pH 6 by the addition of 0.1 mM HCl and 0.1 mM NaOH. The receiving phase consisted of 3 mL of 100 mM BIS-TRIS buffer that was adjusted to pH 8. In the active-transport experiments, the source phase consisted of 3 mL of a 50 mM solution of Ac-Phe-OH in 100 mM BIS-TRIS buffer at pH 6 and the receiving phase consisted of 3 mL of a 50 mM solution of Ac-Phe-OH in 100 mM BIS-TRIS buffer at pH 8. In the competitive experiments, the source phase consisted of 3 mL of a solution 2 mM in concentration of each of the employed substrates Ac-Ala-OH, Ac-Val-OH, Ac-Phe-OH, Ac-Tyr-OH, and Ac-Trp-OH. The receiving phase consisted of 3 mL of 100 mM BIS-TRIS buffer that was adjusted to pH 8. Prior to the experiments, the organic phase was equilibrated with the source phase by manually shaking the two solutions for 5 min in a 10 mL glass vial. The two solutions were then left to stand to separate and afterwards they were filled into the U-tube. The U-tube's upper ends were sealed and the experiment was started by stirring. To measure the concentration of substrate, samples of 50 μL were taken of the aqueous phases and injected into the HPLC with a UV detector or in the case of tryptophan and tyrosine diluted tenfold and then injected. Spectra were recorded at 220 nm. For the injection, the 20 μL sample loop was used to measure exactly equal quantities. The experiments were carried out twice and the average value was determined. Solutions of defined substrate concentration were used for calibration of the UV detector.

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