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A Pore-Forming Tripeptide as an Extraordinarily Active Anion Channel

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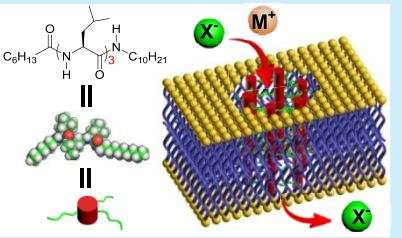
Supporting Information

ABSTRACT: Compared to the most active anion-transporting channel that requires a channel:lipid molar ratio of 1:330 (0.3 mol % relative to lipid) to achieve 50% activity, a structurally simple pore-forming tripeptide **6L$_3$10** was found to exhibit an extraordinarily strong ability to self-assemble into stable possibly barrel-shaped exceptionally active channels, with record-low EC_{50} values of 4.0, 3.0, 1.6, 2.6, and 2.6 nM (e.g., 0.005−0.013 mol % relative to lipid) for Cl^-, Br^-, I^-, NO_3^-, and ClO_4^-, respectively.



In Nature, ≥3% of genes in the known genomes encode amino acids that make up α-helix bundles.[1a] These α-helix bundles[1] play an important role in promoting intermolecular protein−protein interactions or inducing intramolecular protein folding, forming oligomeric protein complexes such as pore-forming toxins[2a] and protein channels[2b,c] in the cell membrane. They have also provided inspirational sources for *de novo* protein design research[1b−e,3] and creation of a range of supramolecular materials.[4]

A structural look into any α-helix bundle reveals a multitude of balanced noncovalent forces (e.g., van der Waals interactions, $\pi-\pi$ stacking, salt bridges, and hydrogen bonds) among the side chains arrayed around the exterior of the helical cores as the driving forces for bundle formation.[1f,g] We recently hypothesized that this association mode involving side chain−side chain interactions might be used to drive the formation of barrel-shaped channels from rodlike molecules, containing well-aligned hydrophobic exterior side chains.[5a] We tested this hypothesis using two types of molecules, i.e., amidated monopeptides[5a] and trimesic amides,[5b] that both can self-assemble into one-dimensional stacks via intermolecular H-bonds, inducing exterior alkyl side chains into well-ordered trigonal or hexagonal arrangements for further association. We found that both types of channels prefer anions over cations but differ greatly from each other in ion transport activity and selectivity. More specifically, whereas the most active monopeptide [**6L10** (Figure 1a)] displays very high activity (EC_{50} = 0.31 mol % relative to lipids) but low selectivity (NO_3^-/Cl^- = 2.7) for NO_3^-,[5a] the most selective trimesic amide exhibits high selectivity (ClO_4^-/Cl^- > 100) but low activity (EC_{50} = 9.6 mol %).[5b]

It might be worth emphasizing that these monopeptide channels represent the very first types of anion transporters that mimic the distinctive ability of some naturally occurring PFPs to assemble into pore-containing ensembles for ion transport, yet differing from both natural PFPs[6] and their synthetic derivatives,[7] which are all charged molecular species, these monopeptide channels are completely neutral. In addition, both natural and synthetic PFPs typically consist of 12−50 amino acids,[6a,b] and only on some extremely rare occasions in which these PFPs are modified with a long hydrophobic lipid moiety[6c] can they be shortened to contain two to four amino acids.[6d,7a] Otherwise, charged PFPs generally require at least 12 amino acids to function as ion transporters.

In this work, we expand the scope of this largely unexplored strategy that relies on the side chain−side chain interactions as a novel means for forming anion channels. Because the hydrophobic side chains aligning the channel's interior interact more favorably with hydrated anions than cations, these channels should preferentially transport anions over cations. On the basis of the highly active monopeptide-based anion channel **6L10**, we report here a simple pore-forming tripeptide **6L$_3$10** with a surprisingly stronger ability to self-assemble, via intermolecular H-bonds and van der Waals forces, into stable pore-forming ensembles in a lipid membrane for anion transport in an extremely efficient manner. Specifically, **6L$_3$10**, containing three leucine residues, exhibits anion-transporting activities 14−28 times those of dipeptide **6L$_2$10**, which is 1.6−4.4 times more active than monopeptide **6L10**. As such, dramatic increases in activity were seen between di- and tripeptides. In the case of iodide (or chloride) transport, **6L$_3$10** is 17 (or 23) and 125 (or 66) times as active as **6L$_2$10**

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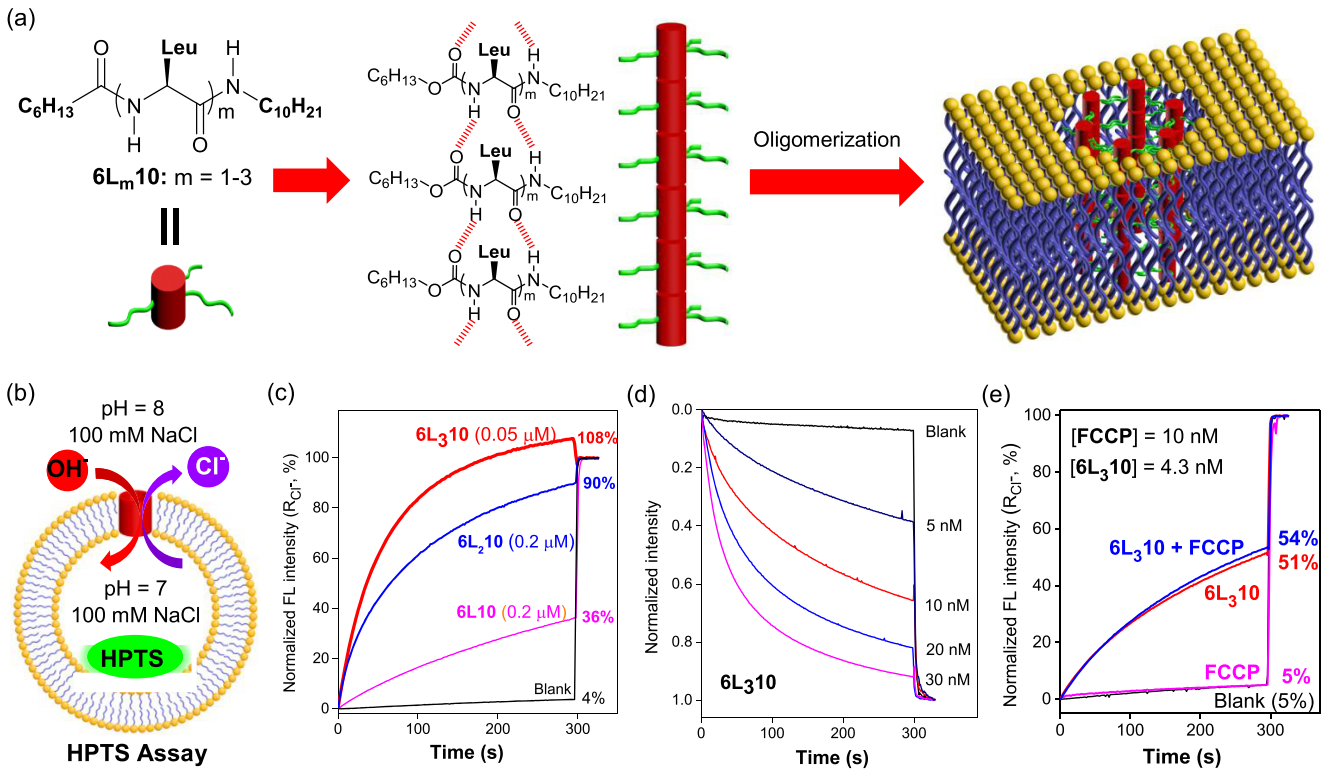

Figure 1. (a) Structures of short peptides (**6L$_m$10**, where m = 1−3) that can form α-helix-like structures with side chains arrayed around the exterior for mediating further assembly into tubular bundles for anion transport. (b) EYPC-based pH-sensitive HPTS assay for the anion transport study. (c). Normalized anion transport activities (R_{Cl^-}) of **6L$_m$10** (m = 1−3) at 0.2 or 0.05 μM; $R_{Cl^-} = (I_{Cl^-} - I_0)/(I_{Triton} - I_0)$. (d) Cl^--sensitive SPQ assay to confirm the anions as the species transported by **6L$_3$10**. (e) FCCP-based HPTS assay, pointing to co-transport of H^+ and Cl^- by **6L$_3$10**.

and **6L10**, respectively. Unfortunately, anion transport cannot be studied on a poorly soluble tetrapeptide (**6L$_4$10**).

6L10 was identified earlier as the most active anion transporter among 112 monopeptides, with anion transport selectivity of $Cl^- < I^- < Br^- < ClO_4^- < NO_3^-$ and an EC_{50} value of 0.31 mol % relative to lipids for NO_3^-.[5a] We thus wonder how an extension of the peptidic backbone by including more of the same amino acid residues would affect the anion transport activities and selectivities. In this regard, we synthesized two leucine-based peptide molecules, **6L$_2$10** and **6L$_3$10** (Figure 1a). The ion transport activity was evaluated using a pH-sensitive HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid) assay (Figure 1b). In this assay, ion transport induces proton efflux or hydroxide influx, either of which causes an increase in the fluorescence intensity of the HPTS dye inside 120 nm large unilamellar vesicles (LUVs). The extent of such an increase is well-correlated to the ion transport activity.

Pleasantly surprisingly, **6L$_2$10** and **6L$_3$10** transport ions at a rate much faster than that of **6L10**, with **6L$_3$10** being the most active (Figure 1c). The well-established Hill analyses were carried out to derive EC_{50} values at which 50% ion transport activity is reached (Figure S1). **6L$_2$10** and **6L$_3$10** exhibit low EC_{50} values of 97 and 4.0 nM, respectively. The later value is 26 times smaller than the EC_{50} value of 0.11 μM determined under the identical conditions for a unimolecular chloride channel having a high molecular weight of 3507 Da.[8] Further considering that superactive PFPs are defined as having an EC_{50} value of 1:1000 in terms of channel:lipid molar ratio,[7c] this low EC_{50} value of 4.0 nM, which corresponds to a channel:lipid molar ratio of 1:8000 or 0.013 mol %, firmly establishes the extraordinarily high ion-transporting activities of **6L$_3$10**.

CF dye [5(6)-carboxyfluorescein], having a dimension of <1.0 nm (Figure S2) and undergoing strong self-quenching at 50 mM, was trapped inside LUVs. Using this assay, we found that membrane-lytic melittin results in efflux of 36% and 94% CF dye from LUVs at 25 and 125 nM, respectively, but incorporation of **6L$_3$10** at a concentration as high as 2.5 μM produces an only negligible CF efflux of 3% (Figure S3). These comparative studies clearly support well-maintained membrane integrity in the presence of **6L$_3$10** at high concentrations and a pore diameter of <1 nm.

To elucidate the ion transport mechanism and species, we performed five sets of LUV-based assays (Figure 1d,e and Figure S4). First, HPTS assays with extravesicular MCl salts varying from LiCl to CsCl were carried out (Figure S4a). Highly similar overall transport activities among the five ions suggest the metal ions are either not or nearly equally transported by **6L$_3$10**. The latter possibility was evidently ruled out using a HPTS assay with intra- and extravesicular regions containing 0 and 200 mM Na_2SO_4, respectively (Figure S4b). That is, while gA elicits a high cation transport activity at an extremely low concentration of 1 nM, **6L$_3$10** clearly transports neither Na^+ nor H^+ at a high concentration of 4 μM. Using a chloride-sensitive SPQ dye, the Cl^--transporting ability of **6L$_3$10** is confirmed by concentration-dependent rapidly increased quenching of the SPQ dye with an increase in **6L$_3$10** concentration from 5 to 30 nM (Figure 1d).

From the three lines of experimental evidence described above, we can conclude that the Na^+-based transport

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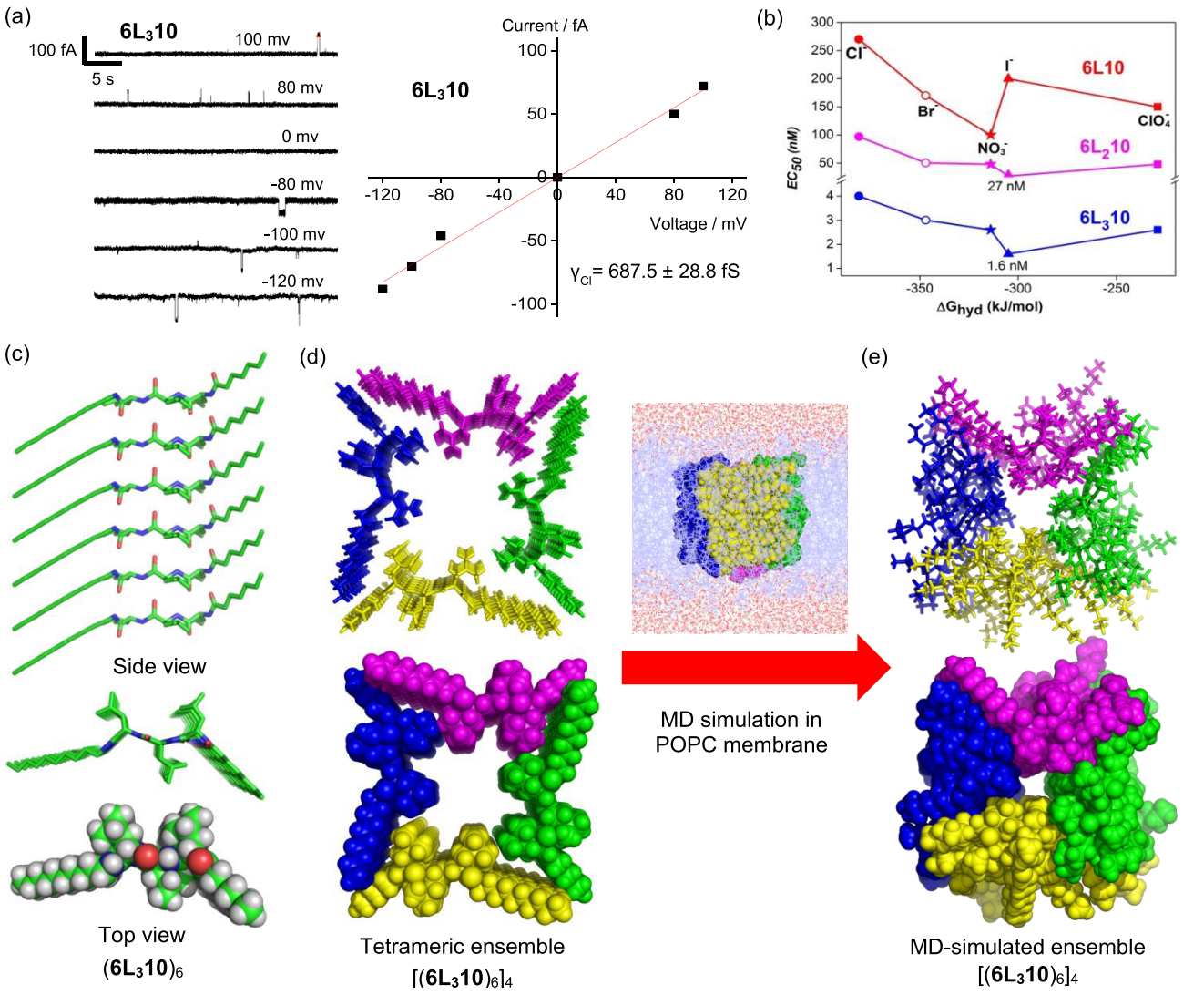

Figure 2. (a) Single-channel current traces recorded for **6L$_3$10** at various voltages in symmetric baths (*cis* chamber = *trans* chamber = 1 M KCl) and determined Cl^- conductance (γ_{Cl^-}). (b) **6L$_3$10**-mediated anion selectivities vs hydration energies with Na^+ ions as counterions. (c) One-dimensional structure of (**6L$_3$10**)$_6$ determined using the COMPASS force field.[10] (d) Initial structure of a tetrameric ensemble [(**6L$_3$10**)$_6$]$_4$ used for molecular dynamics (MD) simulations in POPC membranes. (e) MD-simulated pore-forming tetrameric ensemble [(**6L$_3$10**)$_6$]$_4$. POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) is a model lipid for EYPC.

mechanisms (e.g., M^+/H^+ antiport or M^+/OH^- symport) are not applicable to **6L$_3$10**-mediated ion transport and that any mechanism to account for the observed ion transport behavior must have the Cl^- anion as the transport species.

FCCP, a potent proton carrier, was employed to assess the relative transport rates between Cl^- and H^+ or OH^- (Figure 1e). If the **6L$_3$10**-mediated rate of transport of H^+ is much slower than that of Cl^-, the presence of FCCP will increase the rate of transport of H^+, consequently leading to a significant increase in the fluorescence intensity. This apparently did not occur experimentally as one observes a net increase of only 3% between transport activities mediated by **6L$_3$10** alone and **6L$_3$10** in the presence of FCCP (51−54%), suggesting co-transport of H^+ and Cl^- by **6L$_3$10**.[9] This, together with all four sets of fluorescent lipid bilayer experiments described above, supports H^+/Cl^- symport as the major transport mechanism and species for **6L$_3$10**-mediated chloride transport.

To examine whether **6L$_3$10** functions as a carrier or a channel for transporting chloride anions across the membrane, we carried out single-channel conductance experiments using a planar lipid bilayer workstation. The chloride-transporting behavior of **6L$_3$10** was evaluated in symmetric baths [*cis* chamber = *trans* chamber = 1 M KCl (Figure 2a)]. The observed single-channel current traces at various voltages unequivocally confirm that **6L$_3$10** functions as a channel, rather than a carrier, for chloride transport. Using a linear current−voltage (I−V) curve, the Cl^- conductance value (γ_{Cl^-}) for **6L$_3$10** was calculated to be 687.5 ± 28.8 fS (Figure 2a).

Using the LUV scheme shown in Figure 1b where both intra- and extravesicular regions contain varied NaX salts (X = Cl^-, Br^-, I^-, NO_3^-, and ClO^-) at 100 mM, we have determined the anion selectivities for **6L$_2$10** and **6L$_3$10** (Figure 2b). One common trend shared by NO_3^--selective (**6L10**) and I^--selective (**6L$_2$10** and **6L$_3$10**) channels is that they all transport the other four anions faster than chloride anions. Coincidentally, **6L$_2$10** and **6L$_3$10** share the same anion selectivity pattern; anion transport activity increases in the following order: $Cl^- < Br^- < NO_3^- = ClO_4^- < I^-$. Further

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comparisons among the EC_{50} values reveal that **6L$_2$10** is more active in anion transport than **6L10** by 1.1−6.4-fold (average of 3.3), and going from di- to tripeptides dramatically increases the activities by an additional 17−23-fold (average of 18). Notably, **6L$_3$10** displays the largest enhancement of 124-fold in iodide transport (EC_{50} = 1.6 nM) versus that of **6L10** (EC_{50} = 200 nM). This low value of 1.6 nM translates into a channel:lipid molar ratio of 1:20000 (or 0.005 mol % relative to lipid), which, to the best of our knowledge, is the lowest molar ratio among the existing synthetic anion channels.[5,8,10]

Because a single molecule of **6L$_3$10** is too small to span the lipid hydrophobic region, we proposed that six molecules of **6L$_3$10**, with an interchain distance of ∼4.8 Å, are needed to form a one-dimensional (1D) H-bonded structure [(**6L$_3$10**)$_6$ (Figure 2c)] to fully span the hydrophobic thickness of ∼28 Å of the POPC membrane. This is followed by assembly of multiple such 1D structures via side chain−side chain interactions into pore-forming ensembles of various sizes. Using the 1D structure of (**6L$_3$10**)$_6$ computationally optimized by the COMPASS force field (Figure 2a),[11] we constructed trimeric, tetrameric, pentameric, and hexameric ensembles, comprising three to six 1D structures, respectively. Each ensemble further contains two subensembles that differ only by how the constituent 1D structures interact with each other. These eight ensembles were then embedded in a bilayer having dimensions of 70 Å (width) × 70 Å (length) × 74 Å (height) that comprises 128 POPC molecules and 2 × 2397 water molecules. This simulation system was subjected to a standard molecular dynamics (MD) simulation. Among these eight MD-simulated structures, only one tetrameric ensemble, [(**6L$_3$10**)$_6$]$_4$, ends up with a sizable pore of ∼4 Å (Figure 2e). A quick look into all four 1D structures in the tetrameric ensemble reveals the persistent formation of all intermolecular H-bonds that are supposed to form among 24 molecules of **6L$_3$10**, but we wish to emphasize here that the actual pore size and association modes among these 1D structures might differ from the MD result. Further considering that the inter-columnar interactions among 1D columnar stacks, each consisting of six tripeptide molecules of **6L$_3$10**, should be much stronger than those between any 1D stack and the surrounding flexible lipid molecules, we believe it is highly unlikely that lipid molecules form part of the channel's wall. Thus, these MD results suggest the appropriateness of applying the barrel-stave model to account for anion conduction by structurally simple short peptides such as **6L$_3$10**, which carries no sophisticated functional groups for anion binding and transporting via a single-channel mechanism.

In summary, inspired by the way in which α-helices form α-helix bundles, we have successfully designed and discovered a novel class of structurally simple pore-forming peptides, with the backbone consisting of only a few neutral amino acid residues. The most active tripeptide **6L$_3$10** achieves 50% ion transport activity in iodide and chloride transports at record-low concentrations of 1.6 and 4.0 nM (e.g., channel:lipid molar ratios of 0.005 and 0.013 mol %), respectively. If we take into consideration the fact that six molecules of **6L$_3$10** are needed to form a functional 1D channel structure, these low EC_{50} values can be additionally decreased by 5-fold in terms of the effective channel concentration. In this scenario, we are truly not aware of any other existing synthetic cation/anion channels that can reach such high overall ion transport activities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b01723.

Synthetic procedures and a full set of characterization data, including ^{1}H NMR, ^{13}C NMR, and MS data as well as a complete set from an ion transport study (PDF)

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Notes

The authors declare no competing financial interest.

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