Planarizable push–pull fluorescent probes, also referred to as flipper probes, have been introduced as conceptually innovative mechanophores that report on forces in their local environment in lipid bilayer membranes. The best flipper probes respond to a change from liquid disordered to solid ordered membranes with a red shift in excitation of 50–90 nm. A simultaneous increase in fluorescence lifetime and negligible background fluorescence from the aqueous phase qualifies these fluorescent probes for meaningful imaging in live cells. Here, we report that the replacement of methyl with isobutyl substituents along the scaffold of a dithienothiophene dimer strongly reduces fluorescence intensity but increases solvatochromism slightly. These trends imply that the large substituents in “leucine flippers” hinder the planarization in the first excited state to result in twisted intramolecular charge transfer (TICT). As a result of this overtwisting, the leucine flippers form interesting fluorescent micelles in water but fail to respond to changes in membrane order. These dramatic changes in function provide one of the [...]


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Planarizable Push–Pull Probes: Overtwisted Flipper Mechanophores
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Introduction

The concept of planarizable push–pull probes has been inspired by the change of color of lobsters during cooking. The idea is to combine the planarization and the polarization of twisted fluorophores in the ground state (Figure 1a). Their planarization by mechanical or other forces exerted by the surrounding environment should then result in large shifts in excitation, whereas emission from the planar first excited state should be mechano-insensitive. This mechanism is distinct from and somewhat complementary to solvatochromism, twisted intramolecular charge transfer (TICT), photoinduced electron transfer (PET), and excited-state intramolecular proton transfer (ESIPT) of other membrane probes and related systems, which mostly report in emission.

The currently best flipper probe 1, the result of quite extensive optimization, is composed of two dithienothiophene (DTT) monomers (Figure 1b). These “fluorescent flippers” are bright enough to keep emitting when twisted out of conjugation and have a large enough surface for extensive mechanical interactions with the environment. The push–pull system is established with accepting “sulfone” and donating “sulfide” bridges in the DTT and further supported by cyano acceptors and intriguing chalcogen-bonding thienyl ether donors. The headgroup further contains an essential triazole to prevent elimination and a carboxylate to produce an overall amphiphilic probe. This is essential to deliver and orient flipper probes in membranes.

Deplanarization of the DTT dimer 1 is achieved by chalcogen-bond repulsion between methyl groups and endocyclic sulfur atoms. The deplanarization of conjugated oligomers like 1 in the ground state is reported by a blue shift of the absorption or excitation maxima. The subsequent planarization of the twisted mechanophore in confined space results in the corresponding red shift in excitation. Consistent with mechanical planarization in the ground state, increasingly red-shifted excitation was found for flipper probe 1 in lipid bilayer membranes of increasing order. Such mechanosensitivity was of interest to image so far invisible forces in biological systems. However, in single crystals, face-to-face π-stacked mechanophores 1 are

Figure 1. a) The concept of planarizable push–pull probes together with b) the structure of the currently best flipper probe 1 and the “leucine flipper” 2 introduced here.
already fully planarized.\textsuperscript{[7]} This observation suggested that increasing twisting could perhaps further increase the mechano-sensitivity of flipper probe 1. Here, we report that this is unlikely. Already the replacement of methyl by isobutyl groups, an overall smaller change, is shown to afford with "leucine flipper" 2 a fluorophore that has lost all functional relevance. This quite spectacular response, from best to worst, provides a wonderful illustration for the high sensitivity of fluorescent membrane probes, mechanophores, and beyond, to small changes of their structure.

Results and Discussion

The synthesis of leucine flipper 2 required strategic reconsideration because the convenient traditional approach from methylthiophene was not applicable.\textsuperscript{[6,8]} Tetrabromothiophene 3 was selected as the starting point instead (Scheme 1).\textsuperscript{[12]} A cascade condensation with aldehyde 4 and ethyl 2-thioacetate (5) afforded the new DTT core 6 with the two desired isobutyl substituents in good yield.\textsuperscript{[12,13]} Ester hydrolysis followed by de-carboxylation liberated the ortho positions in DTT 7 for further elaboration along the routes developed for the original probe 1.\textsuperscript{[6,6]} The first step was a formylation. The resulting aldehyde 8 was reduced, and protection of the resulting thienyl alcohol with a silyl group led to 9. To access the accepting flipper, the same aldehyde 8 was converted into nitrile 10. Bromination with NBS readily gave DTT 11, which was oxidized with mCPBA to DTT 12 with a sulfone instead of a sulfide bridge. The electron-rich DTT 12 and the electron-poor DTT 12 were then connected by Stille coupling. The resulting twisted dimer 13 was obtained in good yield. Removal of the silyl protecting group and reaction of thienyl alcohol 14 with bromide 15 gave alkyne 16, ready for cycloaddition with azide 17 to yield leucine flipper 2. Detailed procedures and full characterization of all new compounds can be found in the Supporting Information.\textsuperscript{[14]}

This synthetic approach provides an alternative route to flipper probes in general. It will be of use also to produce probes such as flipper 1 on a larger scale because it is more cost effective.

In toluene, the absorption maximum of the hydrophobic precursor 13 of leucine flipper 2, was at $\lambda_{abs} = 403$ nm (Figure 2a). This was $\Delta \lambda_{abs} = -10$ nm blue-shifted compared to 18, the previously reported "methyl" homolog of 13. Moreover, the maximum of 13 extended more toward the blue, whereas the maximum of 18 showed a distinct shoulder toward the red. The differences supported that in solution, the ground state of leucine flipper 2 is more deplanarized than that of flipper 1. Consistent with previous observations,\textsuperscript{[6]} the absorption spectra showed little dependence on solvent polarity, and excitation and absorption spectra were nearly identical.

The emission maximum of the hydrophobic, not amphiphilic pre-leucine flipper 13 in toluene was at $\lambda_{em} = 562$ nm (Figure 2b, solid, blue). The slight red shift $\Delta \lambda_{em} = +6$ nm compared to the original 18 supports that the blue shift in absorption of 13 originates from ground-state deplanarization and not from unrelated effects.\textsuperscript{[13]}

A red shift in the emission of 13 compared to the less twisted original 18 was observed also in more polar solvents such as...
as EtOAc (Figure 2b, solid cyan vs. dotted green spectra, Figure S1). The spectral shift in emission on going from hexane to DMSO was calculated to be 3778 cm⁻¹ for leucine flipper 13 and 3147 cm⁻¹ for original 18. Quantitative Lippert analysis of their Stokes shifts as a function of solvent polarity gave the apparent difference in dipole moments of the ground and the excited states (Figure S2). The obtained Δμₑ = 14.8 D of leucine flipper 13 clearly exceeded the Δμₑ = 13.6 D of original 18 (Figure 3).

Using Rhodamine 6G as an established standard, we found that the fluorescence quantum yield ϕ = 4.4% obtained for leucine flipper 13 in EtOAc was clearly below the ϕ = 35% of the original 18 (Figure 3). Both the decreasing quantum yield and the increasing positive solvatochromism with the increasing twist of the push–pull fluorophore implied the emergence of twisted excited states. With the original flipper probe 1, planarization of the excited state upon intramolecular charge transfer (ICT) has been confirmed experimentally by time-resolved fluorescence emission spectroscopy. The planar first excited state of push–pull chromophores is characterized by bright fluorescence, high quantum yields, and long fluorescence lifetimes. However, several twistable push–pull chromophores have been shown not to relax into planar ICT but into twisted excited states with fully decoupled aromatic rings that are perpendicular to each other. These TICT states are characterized by strong solvatochromism due to full charge separation and poor fluorescence due to non-radiative decay. The here identified trends with flipper probes thus suggest that with increasing “pretwisting” in the ground state, exited state relaxation into planar ICT states becomes increasingly unfavorable and TICT states start to dominate (Figure 3). This interpretation that “pretwisting” in the ground state enables TICT is consistent with previously reported results with simpler model systems.

One of the hallmarks of the mechanosensitive membrane probe 1 is complete fluorescence quenching in water (Figure 4a, solid line). This is essential for measurements in cells and model membranes without disturbance of background fluorescence. In sharp contrast, leucine flipper 2 showed intense fluorescence in water (Figure 4b, solid line). In water, both amphiphiles are expected to self-assemble into micelles. In micellar 1, fluorescence quenching presumably originates from face-to-face π stacking of the mechanophores. The preserved fluorescence of micellar 2 suggested that the bulky isobutyl groups along the scaffold prevent π stacking.

Fluorescence quenching upon self-assembly as seen with mechanophore 1 is a general phenomenon. Fluorescent micelles, other nanoparticles, and also solids have attracted recent attention for this reason. For the fluorescent micelles formed by the twisted push–pull amphiphile 2 in water, a fluorescent quantum yield of ϕ = 4.2% was measured. Using Rhodamine 6G as an established standard, we found an unchanged ϕ = 4.4% for 13 in EtOAc.

A standard experiment to determine aggregation-induced emission (AIE) was used to obtain deeper insight into the formation of fluorescent micelles. As expected, the fluorescence intensity of 2 increased nonlinearly with the increasing percentage of water in MeOH/water mixtures (Figure 5a, filled circles). The formation of fluorescent micelles around 60% water in MeOH coincided with a blue shift of the excitation maximum by Δλₑx = −8 nm (Figure 5a, empty circles). Although the precise origin of this small shift will remain unknown, it certainly demonstrated that the micelle formation does not cause planarization of the overtwisted leucine flipper 2. The concentration dependence in buffer was linear, suggesting that the critical micelle concentration (cmc) is below 250 nm (Figure 5b). Fluorescent micelles of 2 could be observed directly as small (at resolution limit), fairly homogeneous spheres in fluorescent micrographs (Figure 5c).

Partitioning into membranes was measured with large unilamellar vesicles (LUVs) composed of dipalmitoylphosphatidylcholine (DPPC) at 55 °C, that is, well above the temperature of the transition into the liquid-disordered (Ld) phase at 41 °C. Not emitting in water, the partitioning of the original probe 1 into
DPPC membranes caused the known dramatic fluorescence recovery (Figure 4a).\textsuperscript{[3]} Once again, the behavior of leucine flipper 2 could not be more different: The strong red shift in excitation $\Delta \lambda_{ex} > 0$ plus intensity $I_{o}\lambda_{ex} > 1$ in water did not change much in the presence of L$_d$ DPPC membranes except for general contributions from scattering (Figure 4b).

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With fluorescence being unresponsive also to partitioning (Figure 4b), the possibility that leucine flipper 2 did not enter the L$_d$ DPPC membranes and remained in micellar form in water instead could not be excluded. However, the preparation of DPPC LUVs in the presence of leucine flipper 2 gave the same result. Similar, but clearly less pronounced loss in mechanosensitivity upon overtwisting was observed previously during twistome screening in the quaterthiophene series.\textsuperscript{[3]}

**Conclusion**

In summary, we report that the replacement of methyl groups by isobutyl groups along the DTT dimer scaffold completely destroys the activity of fluorescent flipper probes. The best flipper probes: 1) show a red shift in excitation and an increase in intensity and lifetime upon transition from L$_o$ into solid-ordered (S$_d$) DPPC membranes are the characteristics of operational flipper probes (Figure 4c).\textsuperscript{[4,6]} Flipper 1, the current best, features $\Delta \lambda_{ex} = +50$–90 nm and $I_{o}\lambda_{ex}/I_{o} > 2.7$, the latter being also reflected in an increase in fluorescence lifetime $\tau_{o}/\tau_{o} = 2.1$, $\tau_{o} = 5.8$ ns (Figure 4c).\textsuperscript{[5]} The new leucine flipper 2 simply did not respond to the phase transition from L$_o$ into S$_d$ DPPC membranes (Figure 4d). The contrast between the two could not be stronger: the properties of 2 could not be worse, all mechanosensitivity of 1 was completely lost. With fluorescence being unresponsive also to partitioning (Figure 4b), the possibility that leucine flipper 2 did not enter the L$_d$ DPPC membranes and remained in micellar form in water instead could not be excluded. However, the preparation of DPPC LUVs in the presence of leucine flipper 2 gave the same result. Similar, but clearly less pronounced loss in mechanosensitivity upon overtwisting was observed previously during twistome screening in the quaterthiophene series.\textsuperscript{[3]}

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**Conflict of interest**

The authors declare no conflict of interest.

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