Chapter 3
Glutamate Transporter Inhibitors with Photo-Controlled Activity

This chapter was published as:
Glutamate Transporter Inhibitors with Photo-Controlled Activity
# equal contribution
Abstract:

Glutamate is an important signaling molecule in the nervous system and its extracellular levels are regulated by amino acid transporters. Studies on the role of glutamate transport have benefitted from the development of small molecule inhibitors. Most inhibitors, however, cannot be remotely controlled with respect to the time and place of their action, which limits their application in biological studies. Herein, the development and evaluation of inhibitors of the prokaryotic transporter GltTk with photo-controlled activity, enabling the remote, reversible, and spatiotemporally resolved regulation of transport is reported. Based on a known inhibitor, seven inhibitors, bearing a photoswitchable azobenzene moiety, are designed and synthesized. The most promising photo-controlled inhibitor, shows in its non-irradiated form, an IC$_{50}$ of 2.5 ± 0.4 μM for transport by GltTk. Photoswitching results in a reversible drop of potency to an IC$_{50}$ of 9.1 ± 1.5 μM. This 3.6-fold difference in activity is used to demonstrate that the transporter function can be switched on and off reversibly through irradiation. As a result, this inhibitor could be a powerful tool in studying the role of glutamate transport by precisely controlling the time, and the specific tissue or groups of cells, in which the inhibitor is active.

3.1 Introduction

Glutamate transporters belong to a large family of membrane proteins that catalyze co-transport of the substrate (glutamate/aspartate/neutral amino acid) and cations$^{1,2}$. Glutamate is an important precursor in the biosynthesis of purines, glutamine, proline, arginine, alpha-ketoglutarate, and glutathione$^{3,4}$. Most importantly, in the human central nervous system (CNS), glutamate is a neurotransmitter. In order to pass a signal, the presynaptic neuron releases glutamate via exocytosis, upon which glutamate is sensed by receptors on the post-synaptic neuron$^{5}$. Subsequently, glutamate is removed by glutamate transporters, known as excitatory amino acid transporters (EAATs), to attenuate the signal$^{6}$. Accumulation of glutamate in the synapse is involved in the development of several neuro-degenerative diseases$^{7}$.

Mammalian glutamate transporters belong to the SLC1 family of membrane proteins, which is present in all the kingdoms of life, and includes the archaeal aspartate transporters GltPh and GltTk$^{1,2}$. Much of our understanding of the transport mechanism of the glutamate transporters has come from structural studies of GltPh and GltTk$^{8-16}$ that are structurally and mechanistically similar to the mammalian proteins$^{17,18}$. GltPh and GltTk however can transport only aspartate, while EAATs can use both aspartate and glutamate as a substrate$^{19}$.

Mechanistic studies on the role of glutamate transport are facilitated by the use of small molecule inhibitors$^{6,20,21}$. L-threo-β-Benzylxoyaspartate (TBOA) and (L-threo)-3-[3-[4-trifluoromethyl]benzoylaminobenzylxoxy]aspartate (TFB-TBOA) are aspartate derivatives that are most commonly used to study the role of glutamate transporters in the CNS$^{22,23}$. An impressive example was published by Xie et al.$^{24}$, where a window was installed in the skull of a mouse that was genetically modified with a fluorescent glutamate reporter protein. Upon delivering a light pulse to the eye of the mouse, increased glutamate levels
were observed shortly in the visual cortex. After injection of glutamate transporter inhibitor TBOA, the level of glutamate was higher and clearance was slower\textsuperscript{14}.

However, in experiments such as the one described above, the inhibition of glutamate transport by TBOA and TFB-TBOA is systemic and it cannot be excluded that compensation effects occur. Furthermore, due to systemic inhibition, it is difficult to study the physiology of glutamate transporters in a specific organ, tissue or group of cells of interest. To overcome this limitation, control over the activity of the inhibitor with an external stimulus would be highly desirable as it would allow to reversibly turn the inhibitor on and off at specific organs, tissues and cells at any chosen time and in a reversible manner. Such a remotely controlled inhibitor would contribute to a better understanding of the role of the glutamate transporters in health and disease, as also exemplified by a recent report by Trauner and Kavanaugh in which one of the molecules also reported here was evaluated on human EAATs\textsuperscript{25}.

*Figure 3.1:* Schematic view of photo-control over glutamate transporter activity, along the principles of photopharmacology. The yellow box represents an inactive inhibitor, which does not block the transport of the substrate (purple). By irradiation with light of wavelength $\lambda_1$, the active inhibitor can be locally formed (green cylinder), which blocks substrate transport. This process is reversible by irradiation with light of wavelength $\lambda_2$.

In recent years, bio-active molecules have been developed that can be switched on and off with light as an external stimulus (*Figure 3.1*), along the principles of photopharmacology\textsuperscript{26-28}. Photo-control over biological activity can be achieved by the introduction of a molecular photoswitch, such as azobenzene\textsuperscript{29}, into the structure of the molecule. Thermally stable trans-azobenzene (See *Figure 3.2* in blue) is a linear, (near) flat molecule; irradiation with UV light results in the isomerization of the azo bond and gives cis-azobenzene, which is less stable, non-planar, has a higher dipole moment\textsuperscript{29,30} and is more soluble in aqueous solutions than the trans isomer\textsuperscript{31}. Trans to cis isomerization can be reversed by irradiation with visible light; however, the cis-trans isomerization also happens spontaneously on a time scale of milliseconds to years, depending on the azobenzene
structure\textsuperscript{29,30}. Since \textit{trans}-azobenzene and \textit{cis}-azobenzene strongly differ in structure and polarity, they have the potential to differently influence the activity of a bio-active molecule into the structure of which they have been incorporated. This enables the reversible photoswitching between the forms of a photo-active molecule with different potency\textsuperscript{26-28}. A schematic view of possible photo-control over glutamate transporter activity using a photo-controlled inhibitor is shown in Figure 3.1. The glutamate transporter facilitates the transport of substrate, together with sodium ions\textsuperscript{12,16}. The inhibitor has two states, an inactive state (yellow) that does not bind to the transporter and an active state (green) that blocks transport. Light of specific wavelengths can be used to switch between the two states of the inhibitor and thereby a reversible photo-control over transport can be achieved, offering additional advantages of high spatiotemporal resolution possible in light delivery and the low toxicity of photons to biological systems\textsuperscript{27}. This approach has been successfully demonstrated in developing photo-controlled antibiotics\textsuperscript{32,33}, anticancer drugs\textsuperscript{34-39}, and receptor ligands\textsuperscript{40-49}, among others.

\textbf{Figure 3.2: TFB-TBOA and designed photoswitchable glutamate transporter inhibitors azo-TBOAs, with the photoswitch azobenzene marked in blue.}

Here we present the synthesis and evaluation of seven analogues of TBOA and TFB-TBOA with photo-controlled activity. The compounds were prepared using a key enzymatic step to ensure high stereocontrol in the synthesis of enantiopure precursor. Subsequently, the photochemical properties were studied and biological activity was determined using the archaeal aspartate transporter Glt\textsubscript{Tk}. \textit{p}-MeO-azo-TBOA and \textit{p}-HexO-azo-TBOA showed the best photochemical properties, in which nearly full conversion from \textit{trans} to \textit{cis} isomer can be achieved upon irradiation. The largest difference in activity between \textit{trans} and \textit{cis} isomers was observed for \textit{p}-MeO-azo-TBOA and this difference was successfully used to reversibly control the transport rate by light in\textit{situ}.

\section{3.2 Results and discussion}

\subsection{3.2.1 Design and synthesis}

Our design of photoswitchable inhibitors is based on a known EAAT inhibitor TFB-TBOA (\textbf{Figure 3.2})\textsuperscript{50} which has been widely used to study glutamate transport in the CNS\textsuperscript{20,21}. To render TFB-TBOA photoresponsive, we replaced the amide bond by a diazo moiety (\textbf{Figure 3.2}), in a photopharmacological approach known as azologization\textsuperscript{52}. An extensive SAR of TBOA has been described\textsuperscript{50} on EAAT\textsubscript{2} and EAAT\textsubscript{3} and it demonstrated that substituents at the para position are beneficial for the potency. TFB-TBOA (\textit{p}-CF\textsubscript{3}) has an affinity of 1.9
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nM and 28 nM for EAAT2 and EAAT3, respectively. Other potent inhibitors disclosed in the SAR study have either p-HexO (1.2 nM for EAAT2, 18 nM for EAAT3), p-MeO (12 nM for EAAT2 and 266 nM for EAAT3) or p-CF$_3$O (7 nM for EAAT2 and 128 nM for EAAT3) substituents at the para position. With those high potencies in mind, p-CF$_3$-azo-TBOA, p-HexO-azo-TBOA, p-MeO-azo-TBOA and p-CF$_3$O-azo-TBOA (Figure 3.2) were synthesized and evaluated in our study. The choice of MeO and HexO substituents was further expected to be beneficiary, since alklyoxy substituents in the para position of azobenzene often result in good band separation of the isomers, enabling nearly full isomerization to cis upon irradiation.$^{52}$ To evaluate the importance of the position on the ring, further azo-TBOAs with methyl substituents at the ortho, meta, and para positions were designed (Figure 3.2). Due to the difference in electronic properties and structure, all the substituents likely influence both the biological activity of cis and trans isomers and the photochemical properties such as the maximum wavelength of absorption, ratio of isomers at the photostationary states (PSS) and half-life of the cis isomer. Finally, we also sought to evaluate the p-CF$_3$ substituted compound, which is the closest to the original TFB-TBOA structure, inspired by a recent report by Trauner and Kavanaugh.$^{25}$ In their study, differences in activity between trans and cis isomers were observed on oocytes overexpressing either EAAT1, EAAT2, or EAAT3 by measuring membrane voltage. The photo-controlled glutamate transporter inhibitor was more potent in the trans configuration than in cis form.

**Figure 3.3: Synthesis of azo-TBOAs.**

The azo-TBOAs were prepared in a convergent synthesis, where the alkylating agents 4a–g and the chiral building block 17 were synthesized separately and coupled at a late stage in the synthetic route (Figure 3.3). The alkylating agents 4a–g, containing the azobenzene photoswitch, were synthesized using standard procedures, as described in the experimental procedures. The chiral building block 17 was synthesized using an enzymatic reaction, in which an optimized mutant of methylaspartate ammonia lyase (MAL)$^{53,54}$ stereoselectively aminates 2-(benzyloxy)fumaric acid 13 to (2S,3S)-2-amino-3-(benzoyloxy)succinic acid 14.$^{55}$ Subsequently, the free amine and carboxylic acid groups of compound 14 were protected and, after debenzylation, the reaction of the alcohol moiety in 17 with bromides 4a–g, followed by global deprotection, gave final compounds 1a–g (azo-TBOAs).
3.2.2 Photochemical properties

Next, the photochemical properties of the azo-TBOAs were analyzed (Figure 3.4). As determined by UV/VIS spectroscopy, all compounds absorb in the UV region, where trans-\( p \)-MeO-azo-TBOA and trans-\( p \)-HexO-azo-TBOA have an absorption maximum in DMSO of 355 nm and 353 nm, respectively (Figure 3.4B). All other trans-azo-TBOAs have an absorption maximum in the 317 – 332 nm region, slightly more blue-shifted than \( p \)-HexO-azo-TBOA and \( p \)-MeO-azo-TBOA. All azo-TBOAs could be switched for several cycles in DMSO with little fatigue observed.

A

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<th>cis/trans</th>
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Figure 3.4: Photochemical properties of azo-TBOAs. A) Photochemical properties of azo-TBOAs in DMSO. B) UV/VIS spectra of \( p \)-MeO-azo-TBOA, 20 \( \mu \)M in DMSO, thermally adapted, irradiated with \( \lambda = 365 \text{ nm} \) light for 40 s and white light for 20 s. C) UV/VIS absorbance of \( p \)-MeO-azo-TBOA at \( \lambda = 355 \text{ nm} \), 20 \( \mu \)M in DMSO, irradiated with 365 nm light and white light (WL). D)\( ^1 \)H NMR spectrum of \( p \)-MeO-azo-TBOA, 1 mg in 500 \( \mu \)l DMSO-d\(_6\), cis–trans ratio’s calculated from the \( ^1 \)H signals of Ar-\( \text{CH}_2\)-O (1) and O-\( \text{CH}_2\)-R (2). Top: thermal, cis–
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Using $^1\text{H}$ NMR spectroscopy, ratios of isomers at the photo-stationary states (PSS) of all azo-TBOAs in DMSO were determined, providing information on how much of the compound can be switched to the cis isomer upon irradiation in DMSO as a solvent. As expected, p-alkyloxy substituted azobenzenes p-MeO-azo-TBOA and p-HexO-azo-TBOA showed excellent PSS: irradiation with 365 nm light results in nearly full isomerization to the cis isomer (Figure 3.4D). In contrast, p-CF$_3$O-azo-TBOA shows only 71% cis present at PSS upon irradiation with 312 nm light. Irradiation of p-Me-azo-TBOA, m-Me-azo-TBOA and o-Me-azo-TBOA resulted in a 93%, 86%, and 90% of the cis isomer, respectively at PSS.

Surprisingly, p-CF$_3$-azo-TBOA, reported earlier$^{25}$, was in our hands unstable and small shifts in the spectra upon five cycles of irradiation were observed. When determining the ratio of isomers at PSS upon irradiation in DMSO by $^1\text{H}$ NMR spectroscopy, formation of side products was observed, which was not observed before$^{25}$. However, it must be noted that we used different wavelengths of irradiation (312 nm and 365 nm vs 350 nm$^{25}$) and the shifts in the spectrum are mainly observed for switching in DMSO and not in 50 mM KPi buffer (pH 7.4). For all other compounds, no photodegradation was observed. To confirm that the excellent switching behavior extends to biologically relevant solvents, p-MeO-azo-TBOA was dissolved in 50 mM KPi buffer (pH 7.4) and switching was studied with UV/VIS spectroscopy, showing very similar properties to those in DMSO (Figures 3.9 and 3.10). To evaluate the rate of thermal cis–trans relaxation, half-lives for all compounds were determined in DMSO at 37 °C. For all azo-TBOAs, a half-life at 37 °C in DMSO of >10 h was observed, showing that the cis isomer is relatively stable. The half-life of the cis isomer of p-MeO-azo-TBOA in 50 mM KPi buffer (pH 7.4) at 37 °C is approximately 6 h, which is shorter than in DMSO, but the isomer is still relatively stable on the timescale (4–12 min) of the experiments that were used to evaluate the biological activity of azo-TBOAs (vide infra).

3.2.3 Biological evaluation

Next, the biological activity of the synthesized azo-TBOAs was determined on the aspartate transporter Glt$_{Tk}$ from the archaeon *T. kodakarensis*, that shows 32% sequence identity with human EAATs with even higher conservation of amino acid residues in the substrate/cation binding site and therefore has been used for structural and mechanistic studies$^{12}$. Glt$_{Tk}$ catalyzes uptake of aspartate coupled to the symport of three Na$^+$ ions$^{16}$. To study the inhibition of uptake by azo-TBOAs, Glt$_{Tk}$ was purified, incorporated in liposomes and the rate of uptake of $^{14}$C-labeled aspartate into the lumen of the liposomes was assayed$^1$ in the presence and absence of the photoswitchable inhibitors.
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Figure 3.5: Biological evaluation of azo-TBOAs. A) Screening of the GltT1 inhibitory activity of azo-TBOAs at 10 μM, dark and irradiated; error bars represent the range obtained in duplicate experiments; n.s., not significant; *p < 0.05. B) IC₅₀ curves for TFB-TBOA (0.4 ± 0.1 μM), p-MeO-azo-TBOA in trans (2.5 ± 0.4 μM), cis (9.1 ± 1.5 μM) and p-HexO-azo-TBOA in trans (0.7 ± 0.1 μM) and cis (0.6 ± 0.1 μM), experiments performed in duplicate. C) Binding affinity of compounds to GltT1, determined using isothermal titration calorimetry (ITC), including the standard error.

For initial screening, [¹⁴C]aspartate was used at a concentration of 1 μM, and all azo-TBOAs were tested at 10 μM concentration, both in the dark (full trans) or irradiated (PSS) state, together with a negative control (no inhibitor) and a positive control (TFB-TBOA) (Figure 3.5A). The uninhibited uptake rate was set at 100% transporter activity. At 10 μM concentration, all para-substituted trans-azo-TBOAs showed activity in the same range as TFB-TBOA, while trans-m-Me-azo-TBOA and trans-o-Me-azo-TBOA were less potent. This shows that for a better inhibitor in the trans configuration, a substituent at the para position is preferred, in agreement with previously reported SAR for TFB-TBOA. In general, the irradiated cis-azo-TBOAs had less inhibitory effect than the corresponding trans isomers. For p-MeO-azo-TBOA, we have observed the largest difference in inhibitory activity between the cis and trans forms at 10 μM and therefore the IC₅₀ values for both cis and trans isomers were determined (Figure 3.5B), showing IC₅₀ of 2.5 ± 0.4 μM for trans and IC₅₀ = 9.1 ± 1.5 μM for cis, which represents a statistically significant 3.6-fold drop in activity upon irradiation. As compared to TFB-TBOA (IC₅₀ of 0.4 ± 0.1 μM), p-MeO-azo-TBOA lost one order of potency due to the azologization. Since p-MeO-azo-TBOA and p-HexO-azo-TBOA have nearly identical photochemical properties, also IC₅₀ was determined for p-HexO-azo-TBOA. Surprisingly, for p-HexO-azo-TBOA, we observed no differences in activity between trans and cis isomers, giving IC₅₀ values of 0.7 ± 0.1 μM and 0.6 ± 0.1 μM, respectively. This result cannot be explained by differences in photoswitching efficiency between the p-MeO- and p-HexO-substituted molecules, since in both cases the trans isomer can nearly completely be switched to the cis isomer. Interestingly, both isomers of p-HexO-azo-TBOA are nearly as active as TFB-TBOA (Figure 3.5B). To demonstrate that.
the lower activity of cis compared to trans is not because of an unexpected photodegradation effect, \( p \)-MeO-azo-TBOA was switched in several cycles in DMSO to confirm the recovery of the activity of the trans isomer (Figure 3.12).

Besides the biological activity in the uptake assay, dissociation constants (Kd) were determined using isothermal titration calorimetry (ITC)\(^{16}\) (Figure 3.5C). The affinity of the transporter substrate aspartate and the inhibitor TFB-TBOA were determined with Kd values of 0.12 ± 0.03 μM and 0.86 ± 0.19 μM, respectively. For TFB-TBOA, the affinity of 0.86 ± 0.19 μM is in the same order as the IC\(_{50}\) of 0.4 ± 0.1 μM, as determined by the uptake assay. The isomers of \( p \)-MeO-azo-TBOA have Kd of 1.89 ± 1.26 μM and 3.19 ± 0.49 μM, for the trans and cis form respectively, with no statistically significant difference between the values. Also for \( p \)-HexO-azo-TBOA no significant difference in binding was observed for the two isomers, where trans binds with an affinity of 2.56 ± 0.77 μM and cis with 4.99 ± 3.05 μM. Although the error in the ITC measurements is too large to determine whether the cis and trans isomers bind with different affinity to the transporter, the Kd values in the low micromolar range are consistent with the uptake assays. Furthermore, we expect that the observed differences in the inhibitory activity between the two different photo-isomers may not originate only from differences in binding affinity, but possibly also binding kinetics\(^{56}\).

Reversibility and temporal control are important features of bio-active molecules with photo-controlled activity, since they enable the control over time and place (tissue or group of cells) where the inhibitor is active. To test whether the 3.6-fold difference in IC\(_{50}\) values between trans and cis isomers of \( p \)-MeO-azo-TBOA is sufficient to reversibly control the transport in time, we attempted to photoswitch this inhibitor between the higher and lower potency states during the uptake assay. As shown in Figure 3.6A, the experiment was started with the cis isomer of \( p \)-MeO-azo-TBOA (weaker inhibitor) and fast uptake was observed. Upon irradiation with white light, the inhibitor was switched to the trans isomer (stronger inhibitor) and uptake was attenuated. Subsequent irradiation with UV light again resulted in switching to the weaker inhibitor and an increase in uptake rate was observed. The second irradiation with white light to the trans isomer, attenuated the uptake again. The same experiment was performed starting with the trans isomer (Figure 3.6B), showing that irradiating with UV light increases uptake rate and with white light decreases uptake rate, in a reversible manner. As controls, aspartate transport was measured in the presence of 1 vol% DMSO while continuously irradiating with UV light or with visible light (Figure 3.18). No changes in transport rate were observed, demonstrating that the proteoliposomes are not affected by light and further supporting the reversibility and temporal control of \( p \)-MeO-azo-TBOA over transport.
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Figure 3.6: Photo-control over the transport rate by switching p-MeO-azo-TBOA. A) Irradiation reversibly controls the transport, starting with cis-p-MeO-azo-TBOA, irradiated with white light (45 s) after 2.5 min, UV light (45 s) after 5.75 min and white light (45 s) after 9 min. B) Irradiation reversibly controls the transport, starting with trans-p-MeO-azo-TBOA, irradiated with UV light (45 s) after 2.5 min, white light (45 s) after 5.75 min and UV light (45 s) after 9 min. C) Transport rate of [14C]aspartate as a function of % trans-p-MeO-azo-TBOA at 50 μM. All experiments were done in duplicate.

Besides the “strong inhibitor trans” and “weak inhibitor cis” states, different cis–trans ratios between the thermal cis–trans ratio and the ratio at PSS can be obtained by dosing the duration and intensity of irradiation. To demonstrate this concept, several cis–trans ratios of p-MeO-azo-TBOA were acquired by tuning the duration of irradiation and for all mixtures their effect on the transport rate was measured (Figure 3.6C), showing a linear dependence of the transport rate on the percentage of cis isomer achieved by irradiation.

3.3 Conclusions

We present the design, synthesis and biological evaluation of inhibitors of the SLC1 transporter Glt1 with photo-controlled activity. Based on the known inhibitor TFB-TBOA, seven azo-TBOAs were synthesized using a key stereoselective enzymatic step. Of the seven azo-TBOAs, those with alkyloxy substituents at the para-position showed an excellent ratio of isomers PSS and long half-lives of the cis isomer. The largest difference in inhibitory activity was observed for p-MeO-azo-TBOA; the trans isomer is 3.6-fold more active compared to the cis isomer.
Notably, *p*-HexO-azo-TBOA shows an excellent ratio of isomers at PSS but no difference in activity between *cis* and *trans*. This means that switching from trans to *cis* or from *cis* to *trans* has no effect on the biological activity, despite the large structural change. In fact, *p*-MeO-azo-TBOA and *p*-HexO-azo-TBOA have nearly identical photochemical properties. Therefore, these compounds give insight into the relation between structure and binding to GltTk, providing important structural guidance in the rational design of new photo-controlled glutamate transporter inhibitors.

We demonstrate the reversible and temporal control over glutamate transport using photo-controlled inhibitors and light. Besides switching “on” and “off,” also intermediate transport rates between those in the presence of full *cis* and full *trans* isomers can be achieved by dosing the light, demonstrating the concept of photodosing Employing glutamate transporter inhibitors with photo-controlled activity can potentially provide a better understanding of the role of glutamate transporters in healthy tissues and disease pathology.

### 3.4 Acknowledgements

M.W.H.H and H.F. contributed equally to this work. This research was financially supported by the Netherlands Organization for Scientific Research (NWO-CW), ECHO grant 711.017.012 to W.S. and D.J.S. and KIEM grants 731.013.110 and 731.015.108 to G.J.P., the China Scholarship Council (scholarship to H.F.), and the Ministry of Education, Culture, and Science (Gravitation program 024.001.035 to B.L.F.).

### 3.5 Experimental contributions

M.W.H.H. designed the compounds, synthesized the alkylating agents, determined photochemical properties, assisted with the biological evaluation and processed data of the biological evaluation. H.F. synthesized of the chiral building block and one of the alkylating agents, reacted the protected chiral building block with the alkylating agents and purified the final compounds. R.H.H. purified the transporter and performed the biological evaluation. G.T. performed ITC measurements. V.A. purified the transporter.

### 3.6 Experimental data

#### 3.6.1 General remarks

All chemicals for synthesis were obtained from commercial sources and used as received unless stated otherwise. Solvents were reagent grade. Thin-layer chromatography (TLC) was performed using commercial Kieselgel 60, F254 silica gel plates, and components were visualized with KMnO₄ or phosphomolybdic acid reagent. Flash chromatography was performed on silica gel (Silicycle Siliaflash P60, 230-400 mesh). Drying of solutions was performed with MgSO₄ and solvents were removed with a rotary evaporator. Chemical shifts for ¹H NMR measurements were determined in CDCl₃ relative to the tetramethylsilane internal standard (TMS, δ = 0.00). Chemical shifts for ¹³C NMR
measurements were determined relative to the residual solvent peaks (CHCl$_3$, $\delta = 77.0$; DMSO-$d_6$, $\delta = 40.0$). The following abbreviations are used to indicate signal multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad signal; app, apparent. High resolution mass spectra (electrospray ionisation) spectra were obtained on a Thermo scientific LTQ Orbitrap XL. Melting points were recorded using a Buchi melting point B-545 apparatus.

### 3.6.2 Synthesis

All azo-TBOAs were synthesized by alkylation of the chiral building block with azobenzenes, which were synthesized separately. The complete synthetic route and characterization can be found in the supporting information of the published article.

#### Ethyl (E)-3-(((4-hydroxyphenyl)diazenyl)benzoate (6)

Ethyl 3-aminobenzoate (5, 3.0 mL, 27.2 g, 17 mmol) was dissolved in eq. 1 N HCl (50 mL) and NaN$_2$, (1.60 g, 23 mmol) was added. The reaction mixture was stirred in an ice-bath for 10 min. MeOH (25 mL) was added to the reaction mixture and a solution of PhOH (1.93 g, 14.6 mmol) and KOH (2.14 g, 38.1 mmol) in MeOH (20 mL) was added dropwise. The reaction mixture was stirred at room temperature for 1 h. After completion, eq. 1 N HCl (50 mL) and EtOAc (50 mL) were added to the reaction mixture and the aqueous layer was extracted with EtOAc (3 x 50 mL). The combined organic layers were concentrated in vacuo, Et$_2$O was added (100 mL) and the precipitated product was filtered off and washed with pentane. The product was obtained as an orange solid (1.58 g, 5.8 mmol, 34% yield). Mp: 141-146°C, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.44 (t, $J = 7.1$ Hz, 3H, CH$_3$), 4.38 – 4.48 (m, 2H, CH$_2$), 5.65 (s, 1H, Ar=O), 6.98 (d, $J = 6.9$ Hz, 2H, ArH), 7.58 (t, $J = 7.8$ Hz, 1H, ArH), 7.91 (d, $J = 6.9$ Hz, 2H, ArH), 8.05 (d, $J = 7.9$ Hz, 1H, ArH), 8.12 (d, $J = 7.7$ Hz, 1H, ArH), 8.52 (s, 1H, ArH). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 14.3, 61.4, 115.9, 123.9, 125.2, 126.4, 129.1, 131.1, 147.0, 152.7, 158.7, 166.4. HRMS (ESI+) calc. for. [M+H$^+$] (C$_{15}$H$_{15}$N$_2$O$_3$) 271.1077, found: 271.1074.

#### Ethyl (E)-3-((4-methoxyphenyl)diazenyl)benzoate (7b)

Compound 6 (0.70 g, 2.6 mmol) was dissolved in acetone (20 mL) and Mel (3.0 mL, 1.3 g, 9.3 mmol) and K$_2$CO$_3$ (3.7 g, 26.8 mmol) were added. The reaction mixture was stirred at 40°C overnight. After completion, Et$_2$O (50 mL) and water (50 mL) were added and the organic layer was separated, dried with MgSO$_4$ and concentrated in vacuo. The product was purified by flash chromatography (Silica gel 40 – 63 nm, 0-10% EtOAc in pentane). The product was obtained as an orange solid (0.62 g, 2.2 mmol,
4. The product was rinsed with MgSO$_4$, 162.1. HRMS were added and the organic layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were separated and the aqueous layer was extracted with EtOAc (50 mL), and sodium tartrate (5 g in 100 mL H$_2$O) were added. The reaction mixture was stirred overnight at room temperature. After completion, MeOH (5 mL), EtOAc (50 mL), sodium tartrate (5 g) were added and the resulting mixture was stirred for 1 h. The layers were separated and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with water and brine, dried with MgSO$_4$, and precipitated with pentane. The product was obtained as an orange solid (0.27 g, 1.1 mmol, 61% yield). Purified by flash chromatography (Silicagel 40 mesh). Mp: 55 - 57 °C.

(E)-3-((4-methoxyphenyl)diazenyl)phenyl)methanol (8b)

Compound 7b (0.50 g, 1.8 mmol) was dissolved in dry THF (5 mL) and the reaction mixture was cooled in an ice-bath. LiAlH$_4$ (1.8 mL of 1M solution in THF) was added and the reaction mixture was stirred overnight at room temperature. After completion, MeOH (5 mL), EtOAc (50 mL), and sodium tartrate (5 g in 100 mL H$_2$O) were added and the resulting mixture was stirred for 1 h. The layers were separated and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were separated, and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with water and brine, dried with MgSO$_4$, and precipitated with pentane. The product was obtained as an orange solid (0.27 g, 1.1 mmol, 61% yield).

(E)-1-((3-bromomethyl)phenyl)-2-(4-methoxyphenyl)diazene (4b)

Compound 8b (0.23 g, 0.94 mmol) was dissolved in DCM (10 mL) and NBS (0.25 g, 1.4 mmol) and triphenylphosphine (0.23 g, 0.94 mmol) was dissolved in DCM (10 mL) and NBS (0.25 g, 1.4 mmol) and triphenylphosphine (0.23 g, 0.94 mmol) were added. The reaction mixture was stirred overnight at room temperature. After completion, the reaction mixture was concentrated in vacuo. The product was purified by flash chromatography (Silicagel 40 - 63 nm, pentane, 0 - 50% EtOAc in pentane) and precipitated with pentane. The product was obtained as an orange solid (0.27 g, 1.1 mmol, 61% yield).

Ethyl (E)-3-((4-methoxyphenyl)diazenyl)benzoate (7c)

Compound 6 (0.70 g, 2.6 mmol) was dissolved in acetonitrile (20 mL) and 1-bromohexane (3.0 mL, 1.3 g, 93 mmol) and K$_2$CO$_3$ (3.7 g, 26.8 mmol) were added. The reaction mixture was stirred at 40 °C overnight. After completion, EtO (50 mL) and water (50 mL) were added and the organic layer was separated, dried with MgSO$_4$, and concentrated in vacuo. The product was purified by flash chromatography (Silicagel 40 - 63 nm, pentane, 0 - 10% EtOAc in pentane). The product was obtained as an orange solid (0.62 g, 2.2 mmol, 85% yield).

(E)-(3-((4-hexyloxyphenyl)diaze nyl)phenyl)methanol (8c)

Compound 7c (0.61 g, 2.1 mmol) was dissolved in dry THF (5 mL) and the reaction mixture was cooled in an ice-bath. LiAlH$_4$ (1.8 mL of 1M solution in THF) was added and the reaction mixture was stirred overnight at room temperature. After completion MeOH (5 mL), EtOAc (50 mL), sodium tartrate (5 g in 100 mL) were added to reaction mixture and stirred for 1 hour. The layers were separated and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed...
with water and brine, dried with MgSO₄ and concentrated in vacuo. The product was purified by flash chromatography (Silicagel 40 – 63 nm, pentane, 0 - 50% Et₂O in pentane) and precipitated with pentane. The product was obtained as an orange solid (0.46 g, 1.5 mmol, 88% yield): Mp: 43 - 46 °C.

1H NMR (400 MHz, CDCl₃) δ 0.88 – 0.97 (m, 3H, CH₃), 1.28 – 1.43 (m, 4H, CH₂, CH₂), 1.42 – 1.53 (m, 2H, CH₂), 1.77 – 1.88 (m, 2H, CH₂), 4.03 (t, J = 6.6 Hz, 2H, CH₂), 4.77 (s, 2H, CH₂OH), 6.99 (d, J = 9.0 Hz, 2H, ArH), 7.41 – 7.52 (m, 2H, ArH). The product was obtained as an orange solid (0.17 g, 0.8 mmol, 51% yield over two steps). Mp: 66 – 68 °C.

13C NMR (101 MHz, CDCl₃) δ 14.0, 22.6, 25.7, 29.3, 31.6, 65.0, 68.4, 114.7, 120.3, 122.3, 124.8, 128.6, 129.2, 141.9, 146.8, 153.0, 161.8. HRMS (ESI+) calc. for. [M+H]⁺: 313.1911, found: 313.1908.

(E)-1-(3-(bromomethyl)phenyl)-2-(4-hexyloxyphenyl)diazené (4c)

Compound 8c (0.36 g, 1.2 mmol) was dissolved in DCM (10 mL) and NBS (0.24 g, 1.4 mmol) and triphenylphospine (0.39 g, 1.5 mmol) were added. The reaction mixture was stirred at room temperature overnight. After completion, the reaction mixture was concentrated in vacuo. The crude product was flushed over a silica gel column (Silicagel 40 E). The product was purified by flash chromatography (Silicagel 40 E) two times. The product was obtained as an orange solid (0.32 g, 0.86 mmol, 72% yield). Mp: 43 – 46 °C.

1H NMR (400 MHz, CDCl₃) δ 0.88 (t, J = 6.7 Hz, 2H, C₂H₃), 1.77 (m, 2H, C₂H₂), 1.93 (m, 4H, CH₂), 4.03 (t, J = 6.5 Hz, 2H, CH₂), 4.56 (s, 2H, CH₂Br), 7.00 (d, J = 8.8 Hz, 2H, ArH), 7.46 (d, J = 6.0 Hz, 2H, ArH), 7.80 (s, 1H, ArH), 7.91 (m, 3H, ArH).

13C NMR (101 MHz, CDCl₃) δ 14.0, 22.6, 25.7, 29.3, 31.6, 65.0, 68.4, 114.7, 120.3, 122.3, 124.8, 128.6, 129.2, 141.9, 146.8, 153.0, 161.8. HRMS (ESI+) calc. for. [M+H]⁺ (C₁₉H₂₁N₂O) 375.1069, found: 375.1067.

Figure 3.8: Synthesis of alkylating agents 4d-g

(E)-(p-tolyldiazene)phenyl)methanol (11d)

Para-toluidine 9d (1.0 g, 9.3 mmol) was dissolved in DCM (20 mL) and water (100 mL) and Oxone (5.2 g, 29 mmol) was added. The reaction mixture was stirred at room temperature for 85 min. After completion, DCM (20 mL) was added and the aqueous layer was extracted with DCM (3 x 20 mL). The combined organic layers were washed with sat. aq. NaHCO₃, aq. 1 N HCl and brine and concentrated in vacuo. The crude product was flushed over a silica gel column (Silicagel 40 – 63 nm, pentane). Without further purification, the crude product (0.18 g, 1.4 mmol) was dissolved in acetic acid (5 mL) and (3-aminophenyl)methanol (0.3 g, 2.4 mmol) was added. The reaction mixture was stirred at 40°C overnight. After completion EtOAc (15 mL) and sat aq. NaHCO₃ (10 mL) were added and the reaction mixture was stirred overnight. EtOAc (50 mL) and water (50 mL) were added and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with sat. aq. NaHCO₃, aq. 1 N HCl and brine, dried with MgSO₄ and concentrated in vacuo. The product was purified by flash chromatography (Silicagel 40 – 63 nm, pentane, 0 – 50% Et₂O). The product was obtained as an orange solid (0.17 g, 0.8 mmol, 53% yield over two steps): Mp: 66 - 68 °C.

1H NMR (400 MHz, CDCl₃) δ 2.40 (s, 4H, CH₂, OH), 4.71 (s, 2H, CH₂OH), 7.28 (d, J = 8.1 Hz, 2H, ArH), 7.42 (m, 2H, ArH), 7.67 – 7.94 (m, 4H, ArH).

13C NMR (101 MHz, CDCl₃) δ 21.5, 64.8, 120.5, 122.4, 122.9, 129.3, 129.2, 129.4, 141.7, 142.0, 150.7, 152.8. HRMS (ESI+) calc. for. [M+H]⁺ (C₁₉H₁₄N₂O) 277.1180, found: 277.1176.
(E)-1-(3-(bromomethyl)phenyl)-2-(p-tolyl)diazene (4d)

Compound 11d (0.15 g, 0.66 mmol) was dissolved in DCM (10 mL) and NBS (0.18 g, 1.0 mmol) and triphenylphosphine (0.25 g, 0.95 mmol) were added. The reaction mixture was stirred at room temperature overnight. After completion, the reaction mixture was concentrated in vacuo. The product was purified by flash chromatography (Silicagel 40–63 nm, pentane; 0–5% EtOAc). The product was obtained as an orange solid (0.12 g, 0.42 mmol, 63% yield). Mp: 68–69 °C. H NMR (400 MHz, CDCl$_3$) $\delta$ 2.42 (s, 3H, CH$_3$), 4.55 (s, 2H, CH$_2$Br), 7.30 (d, 3J = 8.2 Hz, 2H, ArH), 7.46 (d, 3J = 5.2 Hz, 2H, ArH), 7.83 (d, 3J = 8.2 Hz, 3H, ArH), 7.91 (s, 1H, ArH). 13C NMR (101 MHz, CDCl$_3$) $\delta$ 21.6, 32.9, 122.8, 123.0, 123.2, 129.5, 129.8, 131.1, 138.8, 141.9, 150.7, 152.9. HRMS (ESI+) calc. for $\text{[M+H$^+$]}$ (C$_{14}$H$_{11}$BrN) 289.0335, found: 289.0335.

(E)-3-(4-(trifluoromethoxy)phenyl)diazene (4e)

4-(trifluoromethyl)aniline (2.00 g, 11.3 mmol) was dissolved in DCM (20 mL) and water (100 mL) and Ozone (8.0 g, 26 mmol) was added. The reaction mixture was stirred at room temperature for 1 h. After completion, DCM (20 mL) was added and the aqueous layer was extracted with DCM (3 × 20 mL). The combined organic layers were washed with sat. aq. NaHCO$_3$ and brine and concentrated in vacuo. The crude product was flushed over a silica gel column (Silicagel 40–63 nm, pentane). Without further purification, the crude product (0.73 g, 2.5 mmol) was obtained as an orange solid (0.73 g, 2.5 mmol, 22% yield). Mp: 47–48 °C. H NMR (400 MHz, CDCl$_3$) $\delta$ 7.86 (m, 5H, ArH), 7.90 (s, 1H, ArH), 7.93 (d, 3J = 7.0 Hz, 2H, ArH), 7.50 (d, 3J = 8.7 Hz, 2H, ArH). 13C NMR (101 MHz, CDCl$_3$) $\delta$ 120.7, 64.8.

(E)-1-(3-(bromomethyl)phenyl)-2-(4-(trifluoromethoxy)phenyl)diazene (4f)

Compound 11e (0.3 g, 1 mmol) was dissolved in DCM (20 mL) and NBS (1.0 g, 9.3 mmol) was dissolved in DCM (20 mL) and water (100 mL) and Oxone (5.2 mmol) were added. The reaction mixture was s stirred at room temperature overnight. After completion, DCM (20 mL) was added and the aqueous layer was extracted with DCM (3 × 20 mL). The combined organic layers were washed with sat. aq. NaHCO$_3$ and brine and concentrated in vacuo. The crude product was obtained as an orange solid (0.31 g, 0.85 mmol, 83% yield). Mp: 64–66 °C. H NMR (400 MHz, CDCl$_3$) $\delta$ 2.48 (s, 3H, CH$_3$), 4.79 (s, 2H, CH$_2$), 7.26 (d, 3J = 8.2 Hz, 2H, ArH), 7.52 (d, 3J = 7.0 Hz, 2H, ArH), 7.86 (d, 3J = 6.8 Hz, 3H, ArH), 7.92 (s, 1H, ArH). H NMR (400 MHz, CDCl$_3$) $\delta$ 2.42 (s, 3H, CH$_3$), 7.36 (d, 3J = 8.2 Hz, 2H, ArH), 7.52 (d, 3J = 7.0 Hz, 2H, ArH), 7.86 (d, 3J = 6.8 Hz, 3H, ArH). 13C NMR (101 MHz, CDCl$_3$) $\delta$ 123.3, 123.0, 123.4, 124.4, 129.6, 131.7, 139.0, 150.6, 152.3, 152.6. 19F NMR (376 MHz, CDCl$_3$) $\delta$ –57.7. HRMS (ESI+) calc. for $\text{[M+H$^+$]}$ (C$_{14}$H$_{11}$F$_3$N$_2$O) 297.0844, found: 297.0844.

(E)-3-(3-(m-tolyl)diazeny)l)phenyl)methanol (11f)

Meta-toluidine (1.0 g, 9.3 mmol) was dissolved in DCM (20 mL) and water (100 mL) and Oxone (5.2 g, 19 mmol) was added. The reaction mixture was stirred at room temperature for 90 min. After completion, DCM (20 mL) was added and the aqueous layer was extracted with DCM (3 × 20 mL). The combined organic layers were washed with sat. aq. NaHCO$_3$ and brine and concentrated in vacuo. The crude product was flushed over a silica gel column (Silicagel 40–63 nm, pentane).

Without further purification, the crude product (0.18 g, 1.4 mmol) was dissolved in acetic acid (5 mL)
and (3-aminophenyl)methanol (0.30 g, 2.4 mmol) was added. The reaction mixture was stirred at 40°C overnight. After completion EtOAc (15 mL) and sat aq. NaHCO$_3$ (10 mL) were added and the reaction mixture was stirred overnight. EtOAc (50 mL) and water (50 mL) were added and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with sat. aq. NaHCO$_3$, aq. 3 N HCl and brine, dried with MgSO$_4$ and concentrated in vacuo. The product was purified by flash chromatography (Silicagel 40 – 63 nm, pentane; 0 – 50% EtO). The product was obtained as orange oil (0.17 g, 0.8 mmol, 52% yield over two steps). $^1$H NMR (400 MHz, CDCl$_3$) δ 2.41 (s, 3H, CH$_3$), 2.77 (s, 1H, OH), 4.69 (s, 2H, CH$_2$OH), 7.25 (d, $J$ = 7.8 Hz, 2H, ArH), 7.33 – 7.46 (m, 3H, ArH), 7.69 (d, $J$ = 6.4 Hz, 2H, ArH), 7.79 (d, $J$ = 7.5 Hz, 1H, ArH), 7.83 (s, 1H, ArH). $^1$C NMR (101 MHz, CDCl$_3$) δ 21.4, 64.7, 70.5, 120.5, 120.6, 122.5, 123.0, 128.9, 129.2, 129.3, 131.3, 139.0, 142.1, 152.7, 152.8. HRMS (ESI+) calc. for. [M+H$^+$] (C$_{14}$H$_7$N$_2$O) 227.1180, found: 227.1176.

(E)-1-(3-(bromomethyl)phenyl)-2-(m-tolyl)diazene (4f)

Ortho-toluidine (9 g, 1.0 g, 9.3 mmol) was dissolved in DCM (10mL) and NBS (0.18 g, 1.0 mmol) and triphenyolphosphine (0.25 g, 0.95 mmol) were added. The reaction mixture was stirred at room temperature overnight. After completion, EtOAc (15 mL) and Na$_2$SO$_4$ (0.15 g, 0.66 mmol) were added and the mixture was stirred overnight. EtOAc (50 mL) and water (50 mL) were added and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with sat. aq. NaHCO$_3$, aq. 3 N HCl and brine, dried with MgSO$_4$ and concentrated in vacuo. The product was purified by flash chromatography (Silicagel 40 – 63 nm, pentane; 0 – 50% EtO). The product was obtained as orange oil (0.12 g, 0.42 mmol, 63% yield) $^1$H NMR (400 MHz, CDCl$_3$) δ 2.70 (s, 3H, C$_{(CH$_3$)}$, 4.59 (s, 2H, C$_{(CH$_2$OH)}$, 7.28 (d, $J$ = 8.1 Hz, 2H, ArH), 7.32 – 7.40 (m, 2H, ArH), 7.45 (t, $J$ = 8.1 Hz, 2H, ArH), 7.51 (m, 2H, ArH), 7.80 (m, 2H, ArH), 7.91 (m, 1H, ArH), 7.99 (s, 1H, ArH). $^1$C NMR (101 MHz, CDCl$_3$) δ 21.5, 63 nm, pentane; 0 – 50% EtO). The product was purified by flash chromatography (Silicagel 40 – 63 nm, pentane; 0 – 50% EtO). The product was obtained as orange oil (0.17 g, 0.8 mmol, 68% yield over two steps). $^1$H NMR (400 MHz, CDCl$_3$) δ 2.49 (s, 3H, CH$_3$), 4.59 (s, 2H, CH$_2$Br), 7.33 (d, $J$ = 7.7 Hz, 1H, ArH), 7.45 (t, $J$ = 8.0 Hz, 1H, ArH), 7.52 (m, 2H, ArH), 7.80 (m, 2H, ArH), 7.91 (m, 1H, ArH), 7.99 (s, 1H, ArH). $^1$C NMR (101 MHz, CDCl$_3$) δ 21.5, 63 nm, pentane; 0 – 50% EtO). The product was purified by flash chromatography (Silicagel 40 – 63 nm, pentane; 0 – 50% EtO). The product was obtained as orange oil (0.12 g, 0.38 mmol, 57% yield). Mp: 46 – 48°C. $^1$H NMR (400 MHz, CDCl$_3$) δ 2.73 (s, 3H, CH$_3$), 4.59 (s, 2H, CH$_2$Br), 7.28 (d, $J$ = 8.1 Hz, 2H, ArH), 7.32 – 7.40 (m, 2H, ArH).
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ArH), 7.50 (m, 2H, ArH), 7.63 (d, J = 8.3 Hz, 1H), ArH, 7.82 – 7.89 (m, 1H, ArH), 7.93 (s, 1H). 13C NMR (101 MHz, CDCl3) δ 17.5, 32.9, 115.4, 123.3, 123.4, 126.4, 129.5, 131.2, 132.3, 138.3, 138.8, 150.6, 153.2. HRMS (ESI+) calc. for. [M+H]+ (C14H14BrN2) 289.0335, found: 289.0335.

3.6.3 Analysis of the photochemical properties

Full analysis of the photochemical properties of aza-TBOAs can be found in the supporting information of the published article. Here the example for compound p-MeO-azo-TBOA is presented.

Figure 3.9: UV/VIS spectra of p-MeO-azo-TBOA 20 μM in DMSO, thermally adapted, irradiated with λ = 365 nm light for 40s and white light for 20s.

Figure 3.10: UV/VIS spectra of p-MeO-azo-TBOA 20 μM in 50μM KPi pH = 7.4, 1% DMSO, thermally adapted, irradiated with λ=365 nm light for 40s and white light for 20s.
Figure 3.11: UV/VIS absorbance of p-MeO-azo-TBOA at $\lambda = 354$ nm, ~0.1 mM in DMSO in quadruplo, measured for thermal cis-trans isomerization at 37°C.

Figure 3.12: UV/VIS absorbance of p-MeO-azo-TBOA at $\lambda = 353$ nm, 20 $\mu$M in DMSO, irradiated with 365 nm light and white light.
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Figure 3.13: $^1$H NMR spectrum of p-MeO-azo-TBOA 1 mg in 500 µl DMSO-d$_6$, cis-trans ratio's calculated from the $^1$H signals of Ar-CH$_2$-O and O-CH$_2$R protons. Top: thermal, cis-trans ratio 1:99. Bottom: irradiated with $\lambda = 365$ nm light, cis-trans ratio 96:4.

3.6.4 Uptake assay
The proteoliposomes were thawed, extruded with a 400 nm filter, spin down for 20 minutes at 80000 cpm and subsequently resuspended in 50 mM KPi pH 7.0 at a concentration of 16.7 µg protein per 120 µL buffer. 1800 µL of 50 mM NaPi pH 7.0, 1µM $^{14}$C Aspartic acid and 3 µM Valinomycin was stirred in a tube at 30 ºC and to that the transport assay was started by the addition of 10 µL proteoliposomes (1.39 µg protein) and either pure DMSO or a DMSO solution of inhibitor. From this mixture, at several time points 100 µL was taken and the transport was stopped by adding 2 mL cold 100mM LiCl and subsequently filtering over a Protran BA 85-Whatman filter. The filter was washed by 2 mL cold 100 mM LiCl and transported to a cup. (Optionally the assay was irradiated with 365 nm UV light for 45s to switch compounds p-MeO-azo-TBOA at a concentration of 50mM from trans to cis or irradiated with white light using a Thor Labs OSL1- EC Fiber Illuminator to switch compound p-MeO-azo-TBOA at a concentration of 50mM from cis to trans). To the cup, 2 mL of scintillation liquid was added and the activity of the cup was measured with a PerkinElmer Tri-Carb 2800RT liquid scintillation counter.
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**Figure 3.14:** \(^{14}C\) aspartate uptake assay, with 1% DMSO control, 10µM non-irradiated p-MeO-azo-TBOA and 10µM irradiated p-MeO-azo-TBOA.

**Figure 3.15:** \(^{14}C\) aspartate uptake assay, with an 1% DMSO control and increasing concentrations of p-MeO-azo-TBOA trans.

**Figure 3.16:** \(^{14}C\) aspartate uptake assay, with an 1% DMSO control and increasing concentrations of p-MeO-azo-TBOA cis.
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Figure 3.17: $^{14}$C aspartate uptake assay, with an 1% DMSO control and several concentrations of p-MeO-azo-TBOA in different trans-cis ratios. A 0.5 mM stock solution was irradiated and samples were removed at several timepoints. From the 0.5 mM solution, 20μL was diluted 5 times in DMSO to a total volume of 100 μL and OD$_{340}$ was recorded in a transparent 96 well plate. The 0.5 mM solution was diluted 50 times with buffer to a final concentration of 10 μM at which the uptake was measured.

Figure 3.18: $^{14}$C aspartate uptake assay, with an 1% DMSO control, continuously irradiated with UV light for 6 minutes and continuously irradiated with white light for 6 minutes.
Figure 3.19: $^{14}C$ aspartate uptake assay of p-MeO-azo-TBOA. A 0.5 mM DMSO solution of p-MeO-azo-TBOA was irradiated with UV (120 s), white light (180 s), UV light (120 s) and white light (180 s) and after every irradiation step a samples was taken and diluted 50 times in the uptake assay to a concentration of 10 µM.

Figure 3.20: $^{14}C$ aspartate uptake assay, with an 1% DMSO control and a p-MeO-azo-TBOA trans 50 µM control. Irradiation reversibly controls the transport, starting with p-MeO-azo-TBOA in trans, irradiated for 45 s with UV light after 2.5 minutes, 45 s white light after 5.75 minutes and 45 s UV light after 9 minutes.
Figure 3.21: 14C aspartate uptake assay, with an 1% DMSO control and a p-MeO-azo-TBOA cis 50 μM control. Irradiation reversibly controls the transport, starting with p-MeO-azo-TBOA in cis, irradiated for 45 s with white light after 2.5 minutes, 45 s UV light after 5.75 minutes and 45 s white light after 9 minutes.

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