ChemPubSoc Europe

DOI: 10.1002/chem.201405767

CHEMISTRY A European Journal Full Paper

Photopharmacology

Photoreversible Prodrugs and Protags: Switching the Release of Maleimides by Using Light under Physiological Conditions

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Abstract: A water-soluble furyl-substituted diarylethene derivative has been prepared that can undergo reversible Diels–Alder reactions with maleimides to yield photoswitchable Diels–Alder adducts. Employing bioorthogonal visible light, the release of therapeutically effective concentrations of maleimide-based reactive inhibitors or labels from these "prodrugs" or "protags" could be photoreversibly triggered in buffered, aqueous solution at body temperature. It is shown how the release properties can be fine-tuned and a thorough investigation of the release dynamics is presented. Our system should allow for spatiotemporal control over the inhibition and labeling of specific protein targets and is ready to be surveyed in living organisms.

Introduction

Today's drug administration often faces challenges associated with poor selectivity, resulting in high toxicity and drug resistance, both of which have their origin in the limited spatiotemporal control offered by conventional drugs. This means that there is generally no possibility to control where and when the drug is active, inside or outside the organism. However, light as a non-invasive stimulus with its superior spatial, temporal, as well as energetic resolution in combination with its high orthogonality to biological processes, makes for an excellent gate to control drug activity with higher precision.[1–4] Outstanding examples have been reported in the literature that exploit light-gated molecular switches to turn drug-activity "on" and "off"[5–18] and recently the term "photopharmacology" has been coined, which describes this entire emerging field.[4]

The implementation of photoswitchability into pharmacologically active chemical entities is only limited by (bio)chemists' imagination and several clever designs have been reported that incorporate particular azobenzene moieties into drugs.[2,4] We have selected here a more general approach, exploring the versatile use of maleimide electrophiles as reactive inhibitors and labeling agents.

DNA topoisomerase II (TOP2) is an enzyme that catalyzes the interconversion of different DNA topoisomers (e.g., supercoiled or catenated DNA) by religation of the DNA strands thus promoting chromosome disentanglement.[19,20] Therefore, TOP2 makes for an ideal target in anticancer therapy as its inhibition can evoke cell death.[21,22] Most TOP2 inhibitors that are employed today are classified as TOP2 poisons that stabilize the so-called "cleaved complex" of TOP2 and DNA thus inhibiting enzyme turnover.[21–23] However, as TOP2 poisons can provoke unwanted secondary malignancies, it is sometimes necessary to inhibit the formation of stable cleaved complexes between TOP2 and DNA alongside pharmacotherapy.[24] Recently, the effect of different maleimide derivatives on TOP2 action was assessed and strong inhibiting properties with effective concentrations showing activity in the micromolar range could be verified.[25,26] More importantly, these maleimide derivatives have been shown to antagonize the toxicity caused by TOP2 poisons, such as etoposide.[26] Since succinimide was not found to have an effect on the TOP2 catalytic cycle, it was reasoned that the inhibition is caused by thiol-ene ligation of exposed cysteine residues. The formed covalent modification is assumed to decrease the overall available concentration of catalytically active TOP2 and thus to antagonize TOP2-related side effects preventing uncontrolled DNA cleavage.[26]

As Michael acceptors exhibit a high and rather nonselective alkylation potential, it would be advantageous to introduce a new level of control over the ability of maleimide to undergo thiol ligation reactions. The Diels–Alder reaction with furan seems well-suited for this task as it masks the reactive C=C double bond of maleimide and can readily be rendered reversible at physiological temperatures.[27,28] Furthermore, diarylethenes (DAEs) with their thermal stability and high fatigue resistance, in combination with large optical changes between their ring-open (**o**) and -closed (**c**) forms, constitute ideal gates to photocontrol function on the molecular level.[29–31] Consequently, we recently reported on achieving photocontrol over the reversible Diels–Alder reaction between a furyl-substituted diarylethene (DAE) and maleimide.[32] Here, we use this prototypical system as a phototriggerable release system as conceptually depicted in Figure 1.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201405767.

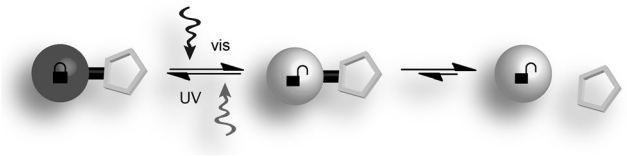
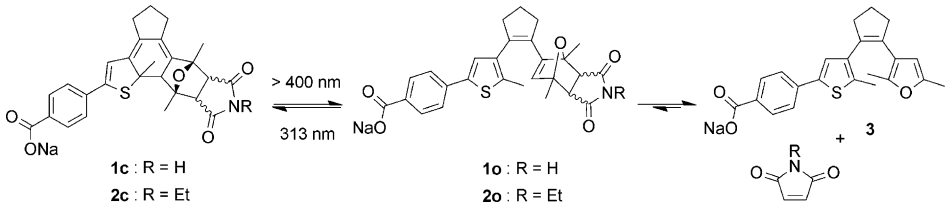


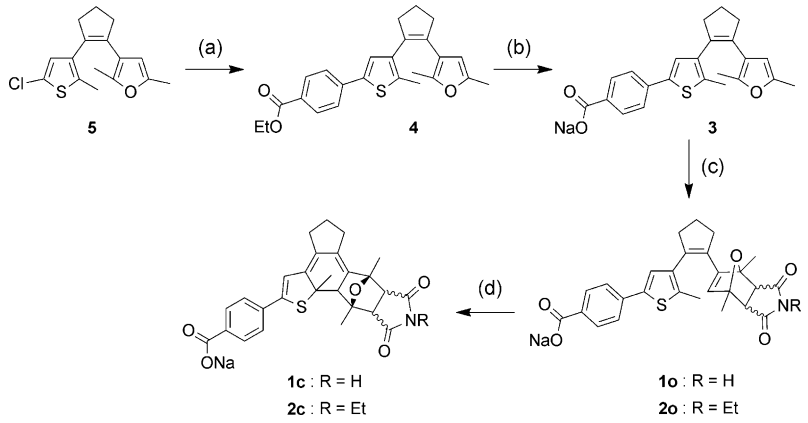
Figure 1. Conceptual depiction of the photoreversible activation of a release system relying on a subsequent thermal equilibrium.

The locked Diels–Alder adduct is thereby transformed into its active state by irradiation with visible light. A subsequent thermal equilibrium then governs the released amount of maleimide over a given period of time. The thermal equilibrium enables long-term release of the active drug, that is, the generation of a "depot effect". This is desirable as conventional drug administration usually suffers from an initially high but comparatively short bioavailability of the drug as the reactive sites are generally metabolized quickly. Another important difference compared to conventional irreversible photocaged systems is that the release can be stopped at any time by locking the Diels–Alder adduct with UV light.

Specifically, we report here on the successful implementation of water-soluble ring-closed Diels–Alder adducts **1c** and **2c** of the furyl-substituted DAE **3** and maleimide or *N*-ethylmaleimide for their release in buffered aqueous solution at body temperature triggered by visible light (Scheme 1). To the best of our knowledge, the ring-closed Diels–Alder adducts **1c** and **2c** are the first examples of water-soluble photoreversible protecting groups for maleimides. Here, we show how the release properties can be fine-tuned and we provide a thorough investigation of the release dynamics.



Scheme 1. Photoreversible activation of the release of maleimide or *N*-ethylmaleimide from their respective ring-closed Diels–Alder adducts by irradiation with visible light ($\lambda_{irr} > 400$ nm).



Scheme 2. Synthesis of target DAEs **1** and **2**: a) i) *n*-BuLi, (THF), RT, 30 min, ii) $B(OBu)_3$, (THF), RT, 1 h, iii) ethyl 4-bromobenzoate, $[Pd(PPh_3)_4]$, Na_2CO_3, (THF/H_2O), 65 °C, 1 d, 67%; b) NaOH, (EtOH), 65 °C, 3 h, 75%; c) maleimide, (EtOH), RT, 1 d, quant. or *N*-ethylmaleimide, (EtOH), 75 °C, 6 h, quant.; d) 10^{-4} M, (PBS), $\lambda = 313$ nm, 25 °C, 10 min, quant.

Results and Discussion

Synthesis

The pathway towards Diels–Alder adducts **1c** and **2c** started with the synthesis of ethyl benzoate **4** from the established chloro precursor **5** by Pd-catalyzed cross-coupling after in situ halogen–metal exchange and borylation (Scheme 2).[32,33] Subsequent saponification of ester **4** yielded the sodium carboxylate **3**, which was treated with either maleimide or *N*-ethylmaleimide to yield ring-open Diels–Alder adducts **1o** and **2o**. Expediently, the fraction of *endo* and *exo* stereoisomers produced in the Diels–Alder reaction of **3** with the two different maleimides can be tuned conveniently by varying the reaction temperature. Whereas furan **3** and maleimide were treated at room temperature for 1 d to yield a mixture consisting of 40% of the *endo* and 60% of the *exo* isomer for adduct **1o**, stirring **3** and *N*-ethylmaleimide at 70 °C for 6 h almost exclusively (97%) led to the *exo* stereoisomer of **2o**. Though the *endo*/*exo* ratio can be fine-tuned regardless of the maleimide derivative employed, these two combinations were chosen to gain insight into their different release kinetics, because the retro-Diels–Alder reaction of the *endo* stereoisomer is expected to take place at an increased rate compared to the *exo* isomer. Ring-closed forms **1c** and **2c** were generated in situ and on demand just before the release experiment by using 313 nm wavelength light to irradiate a 10^{-4} M solution of the ring-open forms **1o** or **2o** until they reached the photostationary state (PSS).

Photochemical properties

The molar absorptivities and absorption maxima of adducts **1** and **2**, and their composition at the PSS are summarized in Table 1. Importantly, all irradiation experiments were performed in phosphate-buffered saline (PBS), successfully verify-

Table 1. Molar absorptivities, absorption maxima, and composition of the PSS of adducts **1** and **2** in phosphate-buffered saline.

Adduct	ε [L mol^{-1} cm^{-1}] ring-open	ring-closed	λ_{max} [nm] ring-open	ring-closed	$PSS_{313\,nm}$[a] [%]
1	16700	11500	316	412	97
2	18500	12600	316	412	98

[a] Amount of ring-closed isomer in the PSS upon irradiation with UV light as determined by UPLC.

ing that switches **1** and **2** are applicable under in vitro and potentially in vivo conditions. Both derivatives almost quantitatively convert to their respective ring-closed forms **1c** and **2c** at the PSS after irradiation with UV light. This can be observed in Figure 2 and is indicated by the appearance of the characteristic bands at 412 nm. Consequently, employing light of a wavelength higher than 400 nm induces the cycloreversion reaction from **1c** to **1o** and from **2c** to **2o**, respectively, thereby regenerating the ring-open isomers (insets in Figure 2).

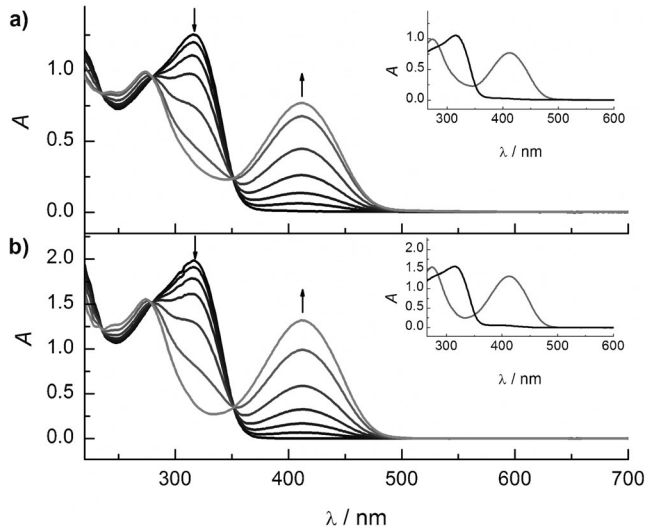


Figure 2. UV/Vis absorption spectra for a) Compound **1** ($c \approx 7 \times 10^{-5}$ M) and b) Compound **2** ($c \approx 10^{-4}$ M) in phosphate-buffered saline at 25 °C upon irradiation with UV light ($\lambda_{irr} = 313$ nm, 9 min). Insets show respective subsequent irradiations with visible light ($\lambda_{irr} > 400$ nm, 20 min).

Although the almost quantitative character of these ring-opening processes was confirmed by UPLC measurements, the absorption bands at 316 nm are not completely regenerated in the ring-open forms. We attribute this phenomenon to the presence of (kinetically trapped) aggregates of the initial ring-open form that are associated with altered spectral signatures and cannot fully be restored after a complete switching cycle.

Photoinduced release of maleimide derivatives

The conceptual validity of triggering the release of maleimide derivatives from their respective adducts **1o** and **2o** was proven by generation of their presumably pharmacologically inactive ring-closed forms **1c** and **2c** through in situ irradiation of a 10^{-4} M solution of the corresponding ring-open forms until the PSS was reached (gray lines in Figure 3).

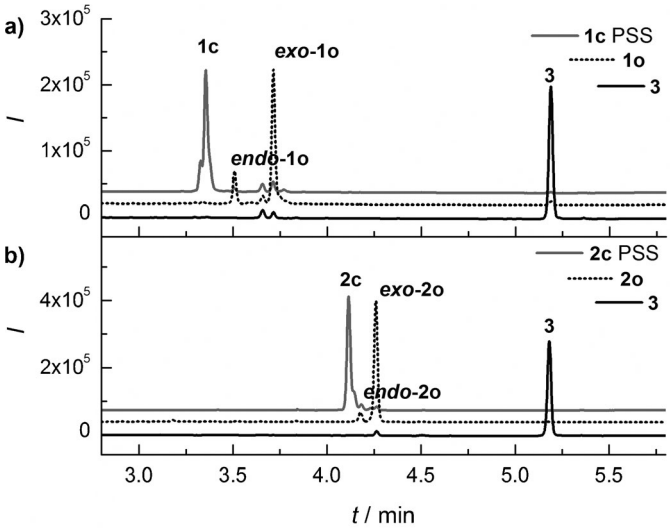


Figure 3. UPLC-MS DAD traces at 280 nm of the ring-closed Diels–Alder adducts in PBS at the PSS, the ring-open Diels–Alder adducts after irradiation with visible light ($\lambda_{irr} > 400$ nm, 20 min), and the successful release of a) maleimide after 3 h at 80 °C for **1c** and **1o** and b) *N*-ethylmaleimide after 5 h at 80 °C for **2c** and **2o**.

The ring-closed derivatives were then irradiated with visible light ($\lambda_{irr} > 400$ nm) to regenerate the corresponding active ring-open forms **1o** and **2o** (dotted lines in Figure 3) and subsequently heated at 80 °C to release maleimide or *N*-ethylmaleimide over time (black lines in Figure 3). Hence, the release of maleimide derivatives can be carried out quantitatively in phosphate-buffered saline at elevated temperatures.

However, 80 °C is nowhere near physiological temperatures and thus the release of maleimide from **1o** was investigated in detail at lower temperatures ranging from 40 °C to 80 °C (see the Supporting Information, Figure S1). As expected, the retro-Diels–Alder reaction becomes increasingly slower with decreasing temperatures. Nevertheless, a continuous release of maleimide takes place even at 40 °C to an extent of about 50% after 92 h.

To gain further insight into the release kinetics, the individual evolution of the *endo* and *exo* adducts of **1o** (60% *exo*, 40% *endo*) and **2o** (97% *exo*, 3% *endo*) in 10^{-4} M phosphate-buffered saline solution was followed over time by integrating the diode array detection (DAD) signal of the corresponding ultra-performance liquid chromatography (UPLC) traces at $\lambda =$ 280 nm in which the Diels–Alder adducts as well as DAE **3** exhibit approximately the same molar absorptivities (Figure 4).

A number of expected features can be extracted from this set of Figures: 1) The release of maleimide is only slightly slower at 37 °C than it is at 40 °C, and 2) the release of maleimide from the *endo* stereoisomer is clearly faster than from the corresponding *exo* stereoisomer. As the retro Diels–Alder reaction is a unimolecular process, it was furthermore reasoned

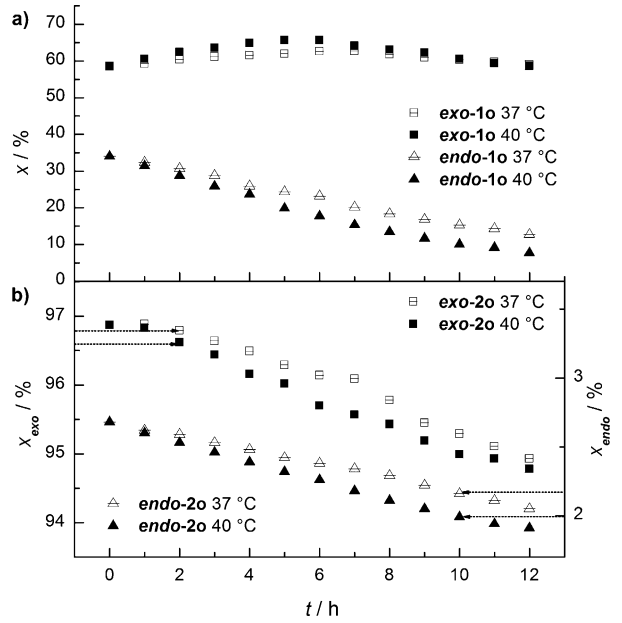


Figure 4. Retro-Diels–Alder reaction at 37 °C and 40 °C in PBS ($c = 1 \times 10^{-4}$ M) visualized by the evolution of the molar fraction *x* of the *endo* and *exo* adduct over time as determined by UPLC measurements of a) **1o** to **3** and maleimide and b) **2o** to **3** and *N*-ethylmaleimide. Ratios recorded at $t = 0$ h slightly deviate from the ratios determined directly after synthesis as the retro-Diels–Alder reaction had already started.

that at low concentrations the bimolecular forward Diels–Alder reaction can be neglected and the release should therefore follow first-order kinetics. However, from Figure 4 it can be shown that the evolution of the individual concentrations does not follow the exponential decay expected for a first-order reaction. Instead, for the retro-Diels–Alder reaction of **1o** to **3**, a slight intermediate increase in the amount of *exo* stereoisomer present was observed.

As the direct interconversion of the stereoisomers is not possible without performing both the forward and backward Diels–Alder reaction,[27] it is reasonable to assume that an additional reaction step is involved. One way to rationalize the intermediate formation of *exo*-**1o** is to assume aggregation in aqueous solution, in which released maleimide is trapped inside an aggregate and its effective concentration in the vicinity of unreacted **3** is increased such that the forward Diels–Alder reaction becomes relevant and not negligible. To find out whether the observed phenomenon is indeed related to aggregation, the release of maleimide from **1o** (60% *exo*, 40% *endo*) in a deaggregating solvent mixture (H_2O/THF/MeCN = 2:1:1) was followed at 40 °C by UPLC measurements (the Supporting Information, Figure S2). As opposed to the experiments carried out in PBS, the amount of *exo*-**1o** is not increasing in the deaggregating solvent mixture but conversely follows the initially expected exponential rate law, strongly suggesting the existence of aggregates in phosphate-buffered saline solution.

Inconveniently, the determination of the actual rate constants for the retro-Diels–Alder reaction in PBS thus yields no noteworthy insight into the release dynamics as the retro-Diels–Alder reaction apparently is not the rate-determining step. Thus, it becomes necessary to compare the released amounts of the respective maleimide derivatives directly. From the data presented in Figure 4, the overall respective concentrations of released maleimide and *N*-ethylmaleimide can be calculated at any point of the reaction and the resulting graphs are shown in Figure 5.

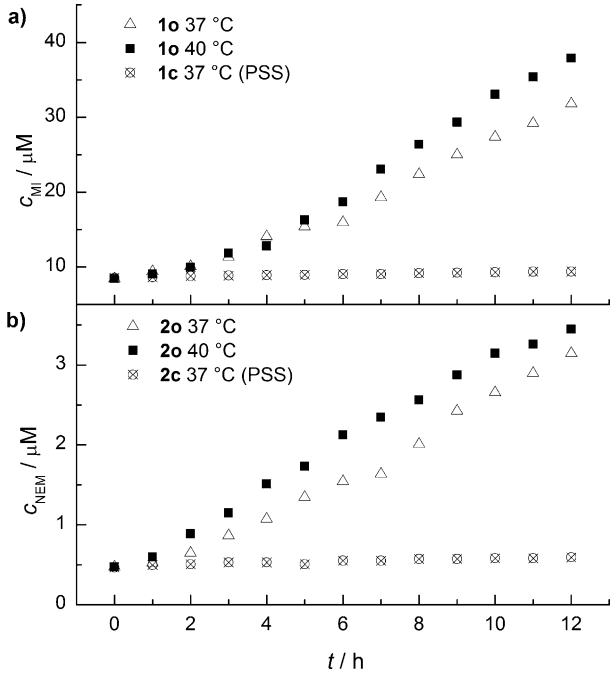


Figure 5. Retro-Diels–Alder reactions at 37 and 40 °C, respectively, in PBS ($c = 1 \times 10^{-4}$ M) visualized by the evolution of the concentration *c* of a) maleimide released from **1o** and b) *N*-ethylmaleimide released from **2o** over time compared to the release from the respective closed form adducts **1c** and **2c**.

Because the release from the *endo* adduct is expected to be considerably faster than that from the *exo* adduct, the release of maleimide from **1o** (consisting of 40% *endo* isomer) was found to be 46 μM d^{-1} at 37 °C (59 μM d^{-1} at 40 °C), not surprisingly much higher than the release of *N*-ethylmaleimide from **2o** (consisting of 3% *endo* isomer) of 5 μM d^{-1} at 37 °C (6 μM d^{-1} at 40 °C). These daily concentrations are estimated from the release that was monitored over 12 h and most importantly lie in the range suitable for therapeutic in vitro and in vivo applications.[26] Furthermore, these results show the possibility to conveniently fine-tune the amount of the released maleimide derivative over time by synthesizing Diels–Alder adducts with varying *endo*/*exo* stereoisomer compositions: Whereas a higher *endo* fraction ensures a larger initial release of maleimide, a higher *exo* fraction leads to a slower release creating a more pronounced "depot effect".

In addition, the data in Figure 5 show that the release from the deactivated ring-closed form is negligible and can be disregarded. Another advantageous feature potentially useful for in vivo application is the increased release rate at 40 °C compared

to 37 °C. As tumor tissue tends to exhibit higher metabolic activity than unaffected tissue, the release of maleimide derivatives could potentially be preferentially stimulated and hence localized in these areas.

To verify that the released species in fact corresponds to the reactive maleimide and not to some potentially pharmacologically inactive hydrolysis products, the release of the maleimides from **1o** and **2o** was monitored by ^{1}H NMR spectroscopy (see example shown for **1o** in Figure 6).

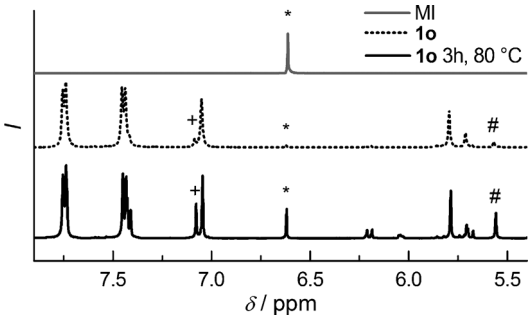


Figure 6. Details of the ^{1}H NMR spectra of **1o** ($c \approx 1 \times 10^{-2}$ M, D_2O/CD_3CN = 1:1) before and after heating for 3 h at 80 °C compared to the maleimide reference. (+) = Thienyl-H of **3**, (*) = CH of MI, (#) = furyl-H of **3**. For **1o**, a minor retro-Diels–Alder reaction already started before the NMR spectrum could be recorded.

Most importantly, the signal of the reactive C=C double bond of maleimide can be clearly retrieved after heating when compared to the reference sample, proving the release of the active maleimide species. However, only about 50% of the expected released amount of maleimide can be detected when the corresponding signals are integrated and compared to the likewise released diene **3**. This can be attributed to partial hydrolysis of the reactive maleimide moiety to diverse byproducts and is a common phenomenon at elevated temperatures and high concentrations (compare area around $\delta = 6$ ppm in Figure 6).[34] Yet, we found that heating pure maleimide at physiological temperatures in aqueous solution over multiple days does not induce degradation (see Figure S3 in the Supporting Information) and thus we reason that the calculated daily concentrations from Figure 5 are valid nonetheless.

In addition to the irreversible release of the maleimides, the reversibility of the switching process in DAEs in combination with the slow thermal release allows for the "on" and "off" switching of the maleimide liberation in situ (Figure 7). Besides the activation of the retro-Diels–Alder reaction by ring-opening **1c** to **1o** with visible light, the release of maleimide could subsequently be inhibited to a large extent by ring-closing unreacted **1o** back to **1c** at the PSS by UV light in situ rendering this system potentially useful in the context of photopharmacology in which the prodrug could be switched "off" after the desired therapeutic effect has been observed. The system then remains inactive for the chosen time and can subsequently be reactivated on demand allowing for the release of maleimide again.

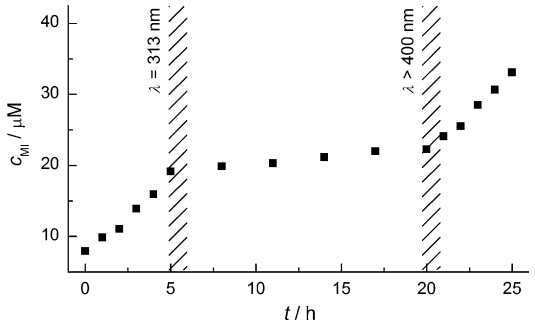


Figure 7. Switching the release of maleimide in situ "off" from **1o** to **1c** (λ_{irr} = 313 nm for 9 min) and "on" again from **1c** to **1o** (λ_{irr} > 400 nm for 20 min) at 40 °C in PBS ($c = 1 \times 10^{-4}$ M) as visualized by the evolution of the concentration of maleimide c_{MI} over time.

Conclusion

We have successfully demonstrated the use of a photoswitch to control the release of a reactive chemical entity (drug or tag) with light under physiological conditions. For this purpose, a water-soluble furyl-substituted DAE (**3**), which can undergo reversible Diels–Alder reactions with maleimide and *N*-ethylmaleimide to the corresponding adducts (**1o** and **2o**), was designed and synthesized. These Diels–Alder adducts exhibit outstanding photochromic properties and could almost be quantitatively transformed to their ring-closed forms (**1c** and **2c**). These "locked" adducts were shown to efficiently impede the retro-Diels–Alder reaction back to the starting materials yet readily regenerate their active, ring-open forms (**1o** and **2o**) upon irradiation with highly bioorthogonal visible light. The unlocked ring-open adducts (**1o** and **2o**) were shown to release their respective maleimide under physiological conditions (phosphate-buffered saline solution, body temperature) with time, achieving calculated daily concentrations in the μM range that clearly lie in the reported therapeutic window of these drugs. Not only could the extent of the "depot effect", that is, delayed release, conveniently be tuned through the adjustment of the *endo*/*exo* stereoisomer ratio in the syntheses of the Diels–Alder adducts, it could also be shown that after a certain release time residual Diels–Alder adduct could be reversibly deactivated by irradiation with UV light in situ through ring-closing remaining **1o** or **2o** to **1c** or **2c**. This combination of reversibly toggling a release system between its inactive "locked" to its active "unlocked" state, from which a specific chemical reagent can be released through a thermal equilibration, is best described as photoswitchable "prodrug" or "protag". Eventually, the presented system could prove to be a potent photoswitchable prodrug for maleimide-based reactive inhibitors, for example, showing a light-dependent pharmacological effect on TOP2 action, as well as becoming a powerful tool in chemical biology in which photoswitchable protags could allow for control over the labeling of specific proteins at specific locations and precise times in living cells.

Acknowledgements

Generous support by the German Research Foundation (DFG through SFB 658) and the European Research Council through ERC-2012-STG_308117 (Light4Function) is gratefully acknowledged. BASF AG, Bayer Industry Services, and Sasol Germany are thanked for generous donations of chemicals.

Keywords: drug delivery · inhibitors · pericyclic reactions · photochemistry · prodrugs

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Received: October 21, 2014
Published online on February 4, 2015

