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Peptide Conjugated Dihydroazulene/Vinylheptafulvene Photoswitches in Aqueous Environment

Brian P. Corbet[+, a, b] J. Malte Schlüter[+, a] Elena R. Cotroneo,[a] Stefano Crespi,[c] and Nadja A. Simeth*[a, b]

Nadja Simeth was nominated to be part of this collection by EurJOC Board Member Burkhard König.

Light-responsive molecules have seen a major advance in modulating biological functions in recent years. Especially photoswitches are highly attractive building blocks due to the reversible nature of their light-mediated reactivity. They are frequently used to affect both the properties of small bioactive compounds and biomacromolecules if incorporated suitably. Despite their success in a plethora of applications, only a limited set of photochromic core structures is routinely employed and a large number of photochromic couples are under-investigated in biological context. Broadening the toolbox of photoswitches available to modulate biological activity would open new avenues and unlock the full potential of photoswitchable molecules for biological studies. In this work, we explore the photochemical and thermal properties of the dihydroazulene/vinylheptafulvene photochromic couple as peptide conjugates in aqueous environment.

Introduction

Light is the ideal stimulus to modulate biological systems, due to its unparalleled spatiotemporal resolution, high tunability, and bio-orthogonality.[1–5] Thus, the introduction of photoswitches – molecules that can be reversibly isomerized with light – as a tool for controlling biomolecular processes has seen much advancement in recent years.[3,6,7] Specifically, the light-induced change in electronic properties and geometry of the photoresponsive core structure can be harvested to regulate the properties of a bioactive molecule of choice. For instance, photoswitches were applied as light-controllable antibiotics,[8,9] DNA-binders,[10,11] microtubule modulators,[12,13] and as enzyme inhibitors.[14–16] Moreover, the large structural change in geometry of the photoswitchable unit can be translated onto a larger structure, such as a biomacromolecule, when incorporated in a strategically suitable position.[1] Thus, it can be used to efficiently alter the properties of, for instance, nucleic acids,[17] peptides,[18] and proteins[14] by use of light as external stimulus. Photoswitches such as (heteroaryl) azobenzenes,[19,20] diarylethenes,[21] and spiropyrans[22] have been studied previously in biological contexts. Also, fulgides and fulgimides, (stiff)stilbenes, and hemi(thio)indigos have been studied in aqueous media and as bio-modulators.[6]

The usefulness of a specific photoswitch relies on many factors, such as photochemical efficiency, absorption wavelength, thermal lifetime, and the (bio-)reactivity of all involved intermediates and the ideal properties differ according to the intended application. These parameters depend on the employed photoswitch, environmental effects such as solvent properties, as well as on the position and nature of the substituents attached to the photochromic core to incorporate them into the biological target system. A crucial aspect of the successful application of the – mostly lipophilic – photochromic core structures is their limited solubility in aqueous media. The addition of solubilizing groups to known photochromic cores has been one of the successful strategies to overcome the solubility issues. Functional groups such as sulfonates, amines, or polyethylene glycol chains increase the hydrophilicity of molecules.[6] Frequently employed photoswitches such as azobenzenes have already seen a significant amount of investigation and a number of strategies for water solubilization.[6,23–25] However, other photochromic core moieties have not received a similar amount of attention and are consequently not yet available to modulate biological functionalities.[6] Therefore, the expansion of the toolbox of photoswitches conjugated to biomolecules and studied in aqueous media would lead to major advances in the field.

[a] Dr. B. P. Corbet,[+] J. M. Schlüter,[+] E. R. Cotroneo, Prof. Dr. N. A. Simeth
Institute for Organic and Biomolecular Chemistry,
Department of Chemistry, University of Göttingen,
Tammannstr. 2, 37077 Göttingen, Germany
E-mail: nadja.simeth@uni-goettingen.de
https://www.uni-goettingen.de/en/start/649877.html
[b] Dr. B. P. Corbet,[+] Prof. Dr. N. A. Simeth
Cluster of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells" (MBExC),
University of Göttingen,
37075 Göttingen, Germany
[c] Dr. S. Crespi
Ångström Laboratory, Department of Chemistry,
University of Uppsala,
1, 751 20 Uppsala, Sweden
[+] These authors contributed equally to this work.

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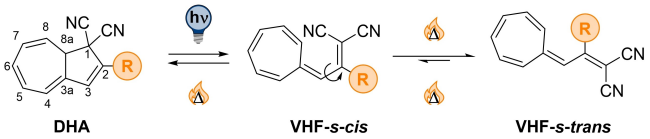
The dihydroazulene/vinylheptafulvene (DHA/VHF) photochromic pair (Scheme 1) is a fascinating photoswitch with a core consisting of a seven- and five-membered ring in its closed, DHA form.[26] Upon light irradiation, the molecule isomerises in a 10π-electrocyclic ring-opening reaction to the open VHF-*s-cis* form (Scheme 1), which transforms rapidly in a thermal reaction to the more stable VHF-*s-trans* conformation.[27–29] The DHA→VHF photoisomerization is reported to be highly efficient exhibiting high quantum yields (QYs, Φ). The back reaction towards the DHA state happens thermally and is associated with a thermal lifetime τ. The isomerization process is accompanied by a large change in geometrical and electronic properties and is thus highly attractive for the modulation of bioactive structures, and especially biomacromolecules with light.

DHA/VHF photoswitches have already been studied, individually or in combination with other photoswitches, in the context of electrochemiluminescence,[30] aggregate-induced emission,[31] and energy storage,[32] among others. Also, the effects of substituents at the seven or five membered rings, as well as effects of the nitrile functionalities and the R group (Scheme 1, R) on the photoswitching and thermal reactions, has seen a significant amount of attention.[27,28,33–35] As far as we know, however, the photochromic couple was only used once to modulate a bioactive molecule[36] and its properties were hardly explored in biologically relevant media.[36,37]

Here, we present a small library of DHA/VHF photochromic compounds bearing different substituents on the R group.

Specifically, we focused on the conjugation of these switches to amino acids (AAs) using both liquid and solid phase supported peptide synthesis. The obtained molecules were studied in detail regarding their photochemical and thermal behaviour in polar (protic) media and buffer systems. Lastly, we demonstrate the incorporation of the photoswitch into the ALFA peptide, consisting of 17 AAs, retaining the switching properties of the molecule.

Nadja A. Simeth pursued her doctorate studies with Burkhard König at the University of Regensburg as a fellow of the Studienstiftung des Deutschen Volkes and graduated in 2018 summa cum laude. She afterwards joined the group of Ben L. Feringa at the University of Groningen as a postdoc supported by a Feodor-Lynen Fellowship of the Humboldt Foundation. In autumn 2021, she was appointed as assistant professor at the University of Göttingen. She is interested in the design of smart drugs, biochemical probes and labels, as well as photoresponsive supramolecular architectures and biohybrid systems.



Scheme 1. DHA/VHF (photo)switching. Upon irradiation with light, the ring-closed DHA isomer undergoes a 10π-electrocyclic ring-opening to form the VHF-s-*cis* and s-trans forms, which interconvert on a subpicosecond time scale.[29]

Results and Discussion

Synthesis

The synthesis of the DHA photoswitches, based on modified literature procedures,[35,38,39] makes use of a modular synthesis, where various substituted acetophenones (Scheme 2, **1**) can be easily transformed in their corresponding malononitrile condensed products (**2**, 75%–quant. yield). Subsequently, these were submitted to a condensation reaction with tropone followed by a ring closing reaction to obtain **DHA1**-**DHA9**, besides **DHA-7** and **DHA-8**. The yield ranged between 6 and 22% and is comparable to the ones reported in the literature.[35,38,39] Upon analysing the products, we found that the low yield was partially due to the formation of a rearrangement product in a side reaction, which can be rationalized computationally (*vide infra*).

Next, we treated the acetylated derivatives **DHA-5** and **DHA-6** with hydrochloric acid to liberate the heteroatoms as synthetic handles for further functionalization as we were interested to couple the photoswitchable cores to AAs and peptides. While **DHA-6** could be converted into **DHA-7** quantitatively adapting a reported protocol,[39] **DHA-5** degraded under the same conditions. With the NH_2-derivative **DHA-7** and the COOH-derivative **DHA-9** in hand, we tested them as building blocks in liquid phase peptide coupling reactions. Though, the aniline **DHA-7** showed some reactivity with Fmoc-protected AAs under standard conditions,[40] the product mixtures were too diverse to isolate the desired products.

Consequently, we focused on **DHA-9** and converted the molecule in liquid phase employing 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT) as coupling agent with morpholine and differently C-terminal protected AAs to give **DHA-MO**, **DHA-Gly-OEt**, **DHA-Ala-O*t*Bu**, **DHA-Lys(Boc)-O*t*Bu** (5–20% yield, Scheme 2 and SI). Both the alanine and the lysine derivative were then C-terminal and side chain deprotected using TFA to give the corresponding free AA analogues **DHA-Ala** and **DHA-Lys**.

Using solid phase peptide synthesis (SPPS), **DHA-9** was also reacted with a resin preloaded with Glu, to directly give and **DHA-Glu** after cleavage from the solid phase (Scheme 2).

Computational analysis of side product formation

During the synthesis of the DHA/VHFs *via* the tropone route, a significant amount of a side product was observed multiple times. Occasionally, this compound was the main product of

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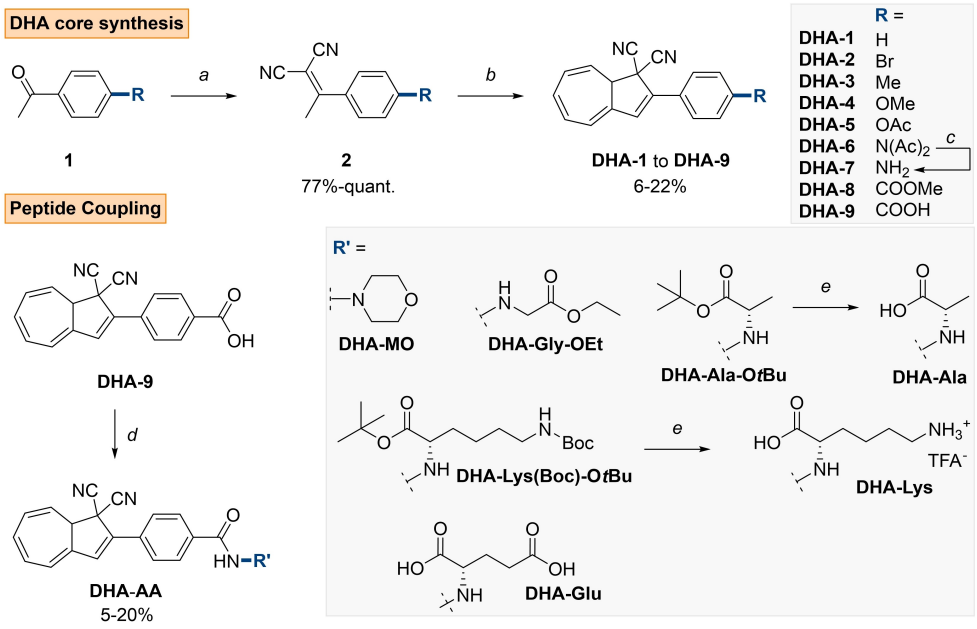

Scheme 2. Synthesis of DHA photoswitches. Conditions: a) malononitrile, NH_4OAc, toluene, Dean-Stark, *or* malononitrile, HMDS, AcOH, 70 °C, b) tropone, Ac_2O, reflux, *or* 1. tropylium tetrafluoroborate, CH_2Cl_2, NEt_3, −78 °C, 2. Ph_3CBF_4, DCE, 80 °C, 3. toluene, NEt_3, Δ; c) HCl, H_2O, EtOH, reflux; d) Solid- and liquid phase peptide coupling (for details see SI).

the reaction. The observed compound is an isomer of the desired product with a styryl motif (**3** in Figure 1A). We hypothesized that the side product could have originated from a reaction with benzaldehyde, which in turn is formed by ring contraction of tropone. We decided to explore the feasibility of this ring contraction by computationally exploring the reactivity of tropone with acetic anhydride (**A** in Figure 1B), the solvent used for the synthesis of our target molecule. Consequently, we modelled the reaction at the DSD-BLYP-D3BJ/def2-QZVPP//r^{2}SCAN-3c level, introducing implicit solvent corrections using the CPCM model for acetic anhydride (*vide infra*). The oxygen in tropone is nucleophilic enough to react with acetic anhydride due to the polar nature of the C=O bond, thanks to the mesomeric structure with a positive charge on the ring, granting aromaticity to the 7-membered ring.[41] The reaction proceeds with a barrier of 27.9 kcal/mol ($\mathbf{TS_{AB}}$) which leads to the formation of product **B**, where the acetate from the acetic anhydride is also incorporated in the final product. This reaction is endergonic (8.1 kcal/mol), and leads to the formation of the ring-closed norcardiene **C**,[42] via a small energetic barrier ($\mathbf{TS_{BC}}$). **C** can rearrange with concomitant three-membered ring opening and migration of the acetate from the ring to the benzylic carbon of product **D**, via $\mathbf{TS_{CD}}$. At high temperature, acetic anhydride decomposes to form acetic acid and ketene.[43] Protonation of **B** leads to an alternative pathway (leading to the protonated **C**′ *via* $\mathbf{TS_{BC'}}$) with overall lower barriers for the rearrangement ($\mathbf{TS_{CD'}}$), that ultimately forms **D**′, the benzyl cationic product and acetic acid. Both **D** and **D**′ are thermodynamically favoured products (overall ΔGs −16.0 and −27.6 kcal/mol) and will eventually lead to the formation of benzaldehyde in the reaction environment.

Photophysical characterization and (photo)chemical properties

With a series of DHA photoswitches in hand, we studied their photophysical and photochemical properties as well as the thermal stability of the metastable VHF form in various solvents. In particular, we focused on polar and polar protic solvents relevant for a potential biological application of functionalized DHAs to understand their properties in such media. MeCN and MeOH represent classical solvents for UV-Vis and NMR spectroscopy and allow comparability between the different methods and reported values. DMSO was selected as it is often used to prepare stock solutions of biologically active samples for *in vitro* and *in cellulo* experiments and PBS buffer was chosen as it is a standard buffer system for biological studies. To also bring the more lipophilic compounds into this medium, up to 20% DMSO was used as a co-solvent.

The absorption maximum (λ_{max}) of the DHA isomers are generally around 360–380 nm. Comparing the absorption maxima, we observed a slight bathochromic shift in the more polar solvents DMSO and PBS compared to MeCN for most derivatives (Table 1). The molar attenuation coefficient ε, however, was not affected in a similar way. It is interesting to notice how the electron-donating character of the substituent on the benzene ring influences the absorption wavelength, especially in DMSO (**DHA-7** has a λ_{max} at 408 nm). The first electronic transition possesses a π-π* character which is distributed along the entirety of the π scaffold (see SI). Excitation increases the quinoidal character of the benzene ring, hence the different electronic nature of the substituents will stabilize the LUMO and decrease the S0 to S1 transition energy. This hypothesis is supported by the results from computational

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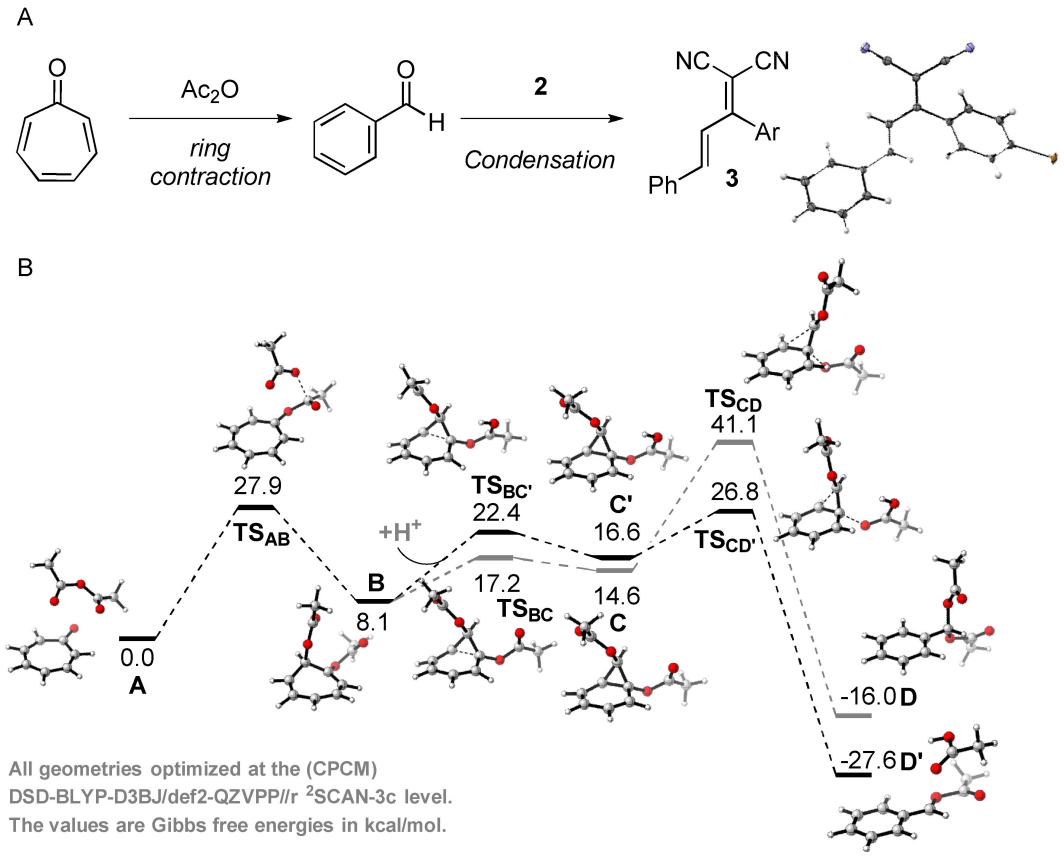

Figure 1. A. Reaction leading to the side product (**3**) formation. The X-Ray structure of **3** with Ar = *p*-C_6H_4Br is presented (ellipsoids of 50% probability and explicit protons included). B. DFT-calculated pathways for the formation of benzaldehyde from tropone.

analysis of the absorption spectrum of a number of DHAs (see SI). The amino acid conjugates, based on **DHA-9** do not show a shift in the λ_{max} of the photoswitch, as is to be expected. Moreover, the ε decreases slightly in most amino acid conjugates and strongly in the case of **DHA-Lys**.

Upon irradiation with 365 nm or 405 nm, respectively, the ring-closed DHA could be converted close to quantitatively into the corresponding VHF isomers for all derivatives (Table 1). The process is accompanied by a decrease of the electronically lowest transition band of the DHA around 370 nm and an increase of a new band in the visible region of the UV-Vis absorption spectrum around 485 nm (Figure 2 and SI). Analogous to the closed DHA isomer, the more polar solvents induce a red-shift in the transition band (Table 1). **DHA-9** and the amino acid conjugates show a λ_{max} for the open VHF form around 500 nm in buffer.

In contrast to the photophysical properties, the photochemical properties and the thermal stability of the VHF form showed a strong solvent dependency. The ring-opening QY ($\Phi^{C\rightarrow O}$, Table 1), generally lies between 0.4 and 0.6 with commonly comparable results between MeCN and DMSO. For a number of photoswitches the QY could not be accurately determined in PBS buffer. However, the amino acid conjugates provided much more reliable results, likely due to their increased hydrophilicity. The **DHA-AA** conjugates show an especially high QY in MeOH. Interestingly, the relatively apolar,

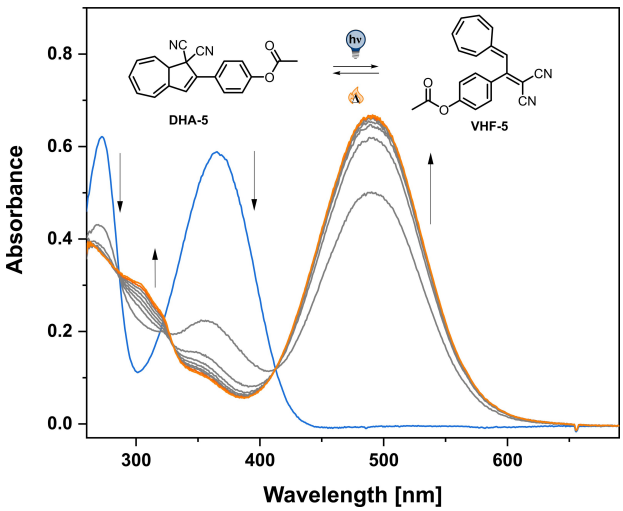

Figure 2. Photochemical Ring-opening reaction of **DHA-5** in DMSO (33 μM) upon irradiation with a 365 nm LED.

protected **DHA-Ala-O*t*Bu** showed a major solvent dependency on the QY with 0.88 in DMSO and 0.20 in buffer.

The VHF isomer undergoes a thermal ring-closing reaction back to the DHA isomer and the thermal half-life of the VHF form was in the range from 2–5 h in MeCN and drastically

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Table 1. Overview of (photo)physical properties of the DHA photoswitches in different solvents.

Compound	R-Substituent	Solvent	λ_{max} (DHA) [nm][a]	$\varepsilon(\lambda_{max})$[a]	λ_{max} (VHF) [nm][a]	$\Phi^{C\rightarrow O}$[a]	PSD[b]	$t_{1/2}$ [h][a]
DHA-1	H	MeCN	357	15630	474	0.58	>95 %*O*	4.13
		DMSO	365	17440	487	0.53	–	1.02
		PBS[c]	362	–	502	n.d.	–	0.20
DHA-2	Br	MeCN	362	12620	477	0.51	–	2.67
		DMSO	369	18440	496	0.43	–	0.87
		PBS[c]	368	–	494	0.37 [e]	–	0.63
DHA-3	Me	MeCN	358	19930	472	0.45	>95 %*O*	4.25
		DMSO	367	19980	485	0.43	–	1.55
		PBS[c]	381	–	496	0.60 [e]	–	0.27
DHA-4	OMe	MeCN	366	19010	471	0.29	–	4.25
		DMSO	375	17780	485	0.35	–	1.43
		PBS	380	–	495	0.73 [e,f]	–	0.84
DHA-5	OAc	MeCN	369	16720	476	0.57	>95 %*O*	3.27
		DMSO	365	14500	489	0.52	–	0.93
		PBS	365	–	496	n.d.	–	0.94
DHA-6	$N(Ac)_2$	MeCN	362	16020	478	0.62	>95 %*O*	2.67
		DMSO	369	15830	485	0.59	–	0.85
		PBS	361	–	503	0.94[e]	–	0.12
DHA-7	NH_2	MeCN	390	19930	475	0.14[f]	>95 %*O*	5.43
		DMSO	408	19980	473	0.59[f]	–	2.38
		PBS	381	–	482	n.d.	–	0.27
DHA-8	COOMe	MeCN	368	18660	478	0.68	>95 %*O*	2.27
		DMSO	378	17710	500	0.36	–	0.62
		PBS	371	–	505	n.d.	–	0.20
DHA-9	COOH	DMSO	376	17666	498	0.45	–	0.82
		PBS	365	16999	501	0.42	–	0.14
		MeOH	365	14500	477	0.93	>95 %*O*	1.78
DHA-MO	Morpholine	MeOH	360	14451	477	n.d.[d]	>95 %*O*	1.48
		PBS	364	11506	503	0.59	–	0.12
DHA-Gly-OEt	GlycineOEt	MeOH	363	14433	478	0.68	>95 %*O*	2.46
		PBS	366	–	506	0.47	–	0.10
DHA-Ala-O*t*Bu	Alanine-O*t*Bu	MeOH	363	10715	478	n.d.[d]	92 %*O*	1.61
		DMSO	373	11610	496	0.88	–	–
		PBS	376	–	506	0.20	–	n.d.
DHA-Lys(Boc)-O*t*Bu	Lysine(Boc)-O*t*Bu	MeOH	362	10541	479	n.d.[d]	>95 %*O*	2.47
		PBS	369	–	498	n.d.[d]	–	1.33
DHA-Glu	Glutamic acid	MeOH	364	–	479	0.67	>95 %*O*	–
		PBS	365	16658	504	0.51	–	0.21
DHA-Ala	Alanine	MeOH	364	15274	478	0.67	>95 %*O*	–
		PBS	365	23593	502	n.d.[d]	–	0.17
DHA-Lys	Lysine·TFA	MeOH	364	8109	479	n.d.[d]	>95 %*O*	–
		PBS	365	9308	504	0.72	–	0.12

[a] Determined by UV-Vis Spectroscopy at 20 °C. [b] Photostationary Distribution (PSD), determined by ^{1}H-NMR spectroscopy, given in % of the open form in the respective deuterated solvent. [c] 10% DMSO in PBS. [d] Isomerization proceeded too fast for the quantum yield determination using our setup. [e] 20% DMSO in PBS. [f] irradiated with 405 nm LED.

decreased to 0.5–1.5 h in DMSO, whereas in PBS buffer the thermal half-life further decreased up to 7 min. The transition state for the thermal ring-closing reaction from the VHF-s-cis to the DHA isomer has a more polar nature than the open and closed form. The increased solvent polarity further stabilizes the transition state, decreases the barrier and speeds up the thermal reaction.[27,44] For the DHA-AAs, a similar trend was found with fast thermal back-reactions in an aqueous environment.

Conjugation to ALFA via solid phase peptide synthesis

Due to the promising characteristics of the different **DHA-AA** derivatives regarding their solubility and photochemical properties in PBS buffer, we decided to explore the possibility of SPPS to couple **DHA-9** to a medium-sized peptide. As model structure, we chose the 17-AA-based ALFA peptide (Figure 3A) as it is highly helical, biologically compatible, hydrophilic peptide with no net charge.[45,46] The biohybrid molecule was obtained directly after cleavage from the resin and purified *via* HPLC. Upon irradiation with 365 nm light, the isolated **DHA-ALFA** could be photoisomerized into its VHF analogue (Figure 3B). Similar to the **DHA-AA** dimers, the light-responsive peptide shows a fast rate of photoisomerization. This makes it the bio-hybrid construct a valuable proof-of-principle system and highlights the propensity of DHA photoswitches to reversibly address and modulate activity and functionality in biologically relevant systems.

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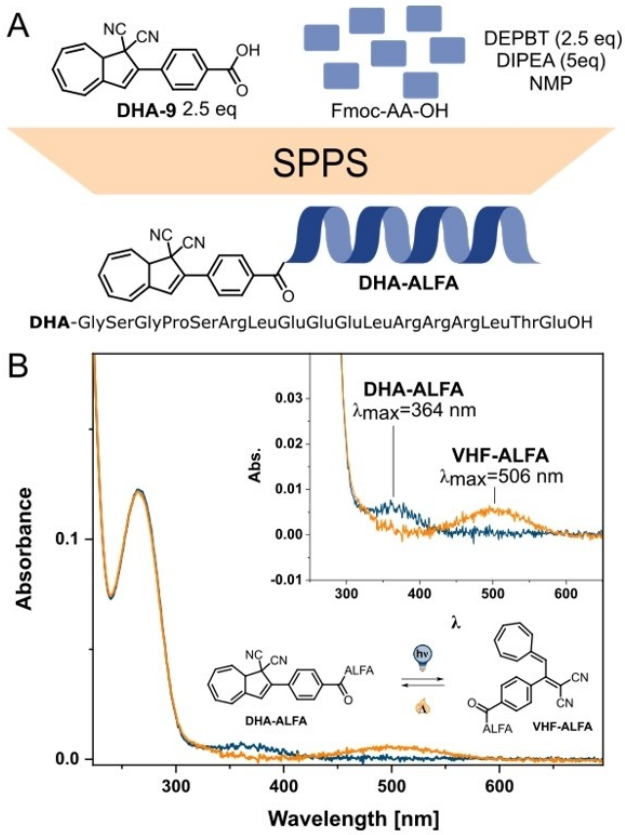

Figure 3. A. SPPS of **DHA-ALFA** and B. A UV Vis spectrum of **DHA-ALFA** in PBS buffer (blue) and after irradiation with 365 nm LED (orange).

Conclusion

In summary, we synthesized a series of 17 dihydroazulene photoswitches bearing different substituents on the six-membered ring. Specifically, we focused on functional groups that can be employed for further derivatization with different amino acids and short peptides. While the *para*-NH_2 moiety resulted in inefficient coupling yields, the *para*-COOH group allowed standard peptide coupling protocols, both in liquid phase and by SPPS.

We evaluated the photochemical and thermal characteristics of our library using a combination of UV-Vis and NMR spectroscopy in different solvent to assess their behaviour in biologically relevant media such as solvent-water mixtures and PBS buffer. We found that the lowest electronic transition was slightly affected by both the type of R substituent and the nature of the solvent. Moreover, $\Phi^{c\rightarrow t}$ and the thermal half-life $t_{1/2}$ showed a strong dependency on the environment used. While in many cases, the QY was increased in polar and polar protic media, the stability of the metastable VHF form decreased from the hour to the minute range in PBS buffer. Consequently, the DHA/VHF photochromic couple has the potential be employed as a fast-responsive (photo)switch in aqueous environment and biological media using both light and thermal stimuli.

Finally, we used our protocol to couple the carboxylic acid derivative **DHA-9** to the ALFA peptide using microwave assisted SPPS. The compound was successfully obtained using standard protocols and isolated by reversed phase HPLC. The DHA-ALFA conjugate showed excellent (photo)switching properties in PBS buffer, underlining the potential of DHA photoswitches for the light-controlled modulation of biological systems. We believe that the findings of this study will open new avenues in the synthesis and design of photoswitchable building blocks for the light-mediated regulation of complex biosystems.

Experimental Section

Synthesis and characterisation

All synthetic details and compound characterisation data can be found in the Supporting Information.

Computational analysis

Density functional theory (DFT) optimization of minima and transition states, frequency and single-point calculations were carried out with the ORCA 5.0.3 quantum chemical package.[47,48] All calculations were performed with acetic anhydride as implicit solvent within the Conductor-like continuum polarizable model (CPCM),[49] using $\varepsilon = 21$ and refractive index = 1.386. All optimizations and thermochemical corrections were performed at the r^{2}SCAN-3c level.[50] All the transition states were confirmed by performing an IRC analysis and, in this way, connected to the respective reagents and products. The electronic energy was corrected using DSD-BLYP[51] with relaxed MP2 density, using the D3 dispersion correction with Becke-Johnson damping[52,53] scheme (D3BJ), the def2-QZVPP basis set[54] and the auxiliary def2/J and def2-QZVPP/C bases.[55,56] TD-DFT analysis of **DHA-1**, **DHA-4**, **DHA-6** and **DHA-7** was performed at the DSD-BLYP-D3/def2-TZVPP level on the r^{2}SCAN-3c optimized geometries. More details can be found in the supporting information. The cartesian coordinates are deposited on figshare (DOI: 10.6084/m9.figshare.21231152).

Crystallographic data

Deposition Number 2215266 (for **3**) contains the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service www.ccdc.cam.ac.uk/structures.

Author Contributions

BPC and JMS synthesized and characterized the core structures. BPC and ERC performed solid and liquid phase peptide synthesis and isolated the compounds. BPC and SC performed DFT calculations. BPC and NAS wrote the manuscript. All authors discussed the results and agreed to the final version of the manuscript.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Dihydroazulene · Vinylheptafulvene · Photochemistry · Photopharmacology · Photoswitches

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