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Colorimetric Probe for the Detection of Thiols: The Dihydroazulene/Vinylheptafulvene System

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A new application of the dihydroazulene/vinylheptafulvene (DHA/VHF) system as a chemodosimeter for thiols is reported herein. A color change visible to the naked eye can be detected in acetonitrile/water (4:1) in the presence of cysteine and alkanethiols but not with other amino acids. The higher reactivity of DHA towards cysteine compared with VHF has been demonstrated. The easy synthesis of the main core and its versatility in terms of structural and physicochemical changes are essential elements for developing a new family of thiol-specific probes with higher selectivity and sensitivity than the reported prototype.

Introduction

The key role that cysteine, homocysteine, and glutathione play in biological systems has stimulated the development of numerous optical probes for thiols in recent years.[1,2] Abnormal levels of cysteine are associated with many syndromes, from neurotoxicity to edema, slow growth in children, liver damage, skin lesions, and hair depigmentation.[3] In addition, altered levels of cysteine have been discovered in the late stages of HIV infection and other diseases associated with the loss of skeletal muscle mass.[4] Recently, the high potential of cysteine-reactive small molecules in drug discovery as inhibitors of cysteine protease enzymes has been pointed out.[5]

Most of the optical probes reported to date for thiols are chemodosimeters, which are molecules that react with the thiol functionality through a specific and irreversible reaction leading to the formation of a covalent bond between the probe and the thiol group, and producing an observable signal.[6] Of the various detection techniques, the most convenient and cheapest method is the detection of such a signal by the naked eye through a color change.

Michael addition is one of the possible reactions that is often exploited by such optical probes, taking advantage of the strong nucleophilicity of the sulfur atom in the thiol moiety.[1] In addition, the reactivity of thiols towards the cyano group of a malononitrile moiety has recently been reported by Zhao and co-workers for the detection of thiols.[2b]

In this context we became interested in investigating the behavior of the dihydroazulene/vinylheptafulvene system (DHA/VHF, **1**/**2**; Scheme 1) towards thiols, attracted by its peculiar structural features. DHA **1** contains two cyano groups at C-1, whereas VHF **2** has the two cyano groups conjugated to an unsaturated moiety. DHA **1** and VHF **2** represent a photo/thermoswitch system. Thus, DHA **1** is a yellow photochromic compound that undergoes a light-induced 10-electron retro-electrocyclization to the red VHF **2** (Scheme 1 and Figure 1, first vial on the left).[7] The back-reaction to the parent DHA can be realized by a thermally induced ring-closure and, more recently, by treatment with mild Lewis acids that enhance the rate of the thermal conversion of VHF into DHA.[8] The significant structural difference between DHA and VHF, as reflected in their different colors and hence electronic properties, has made the system interesting as a light-controlled molecular switch for molecular electronics.[9] The system is particularly attractive as it is simple to synthesize on a large scale,[10] and it exhibits both tunable optical properties (and hence colors) and switching abilities if properly functionalized.[11] To the best of our knowledge, it has never been explored for its effective recognition capabilities. We therefore became interested in elucidating how the two isomers would behave in the presence of a thiol. Here we show that the system can indeed be employed as a chemodosimeter in which a thiol like cysteine can conveniently be detected by color changes visible to the naked eye. Moreover, it should be mentioned that as thiols are often used as anchoring groups in molecular electronics for adhering molecular wires and switches to gold electrodes,[12] it is important to establish the reactivity of DHA and VHF towards free thiols in order to explore the system within this field as part of our ongoing efforts.[9]

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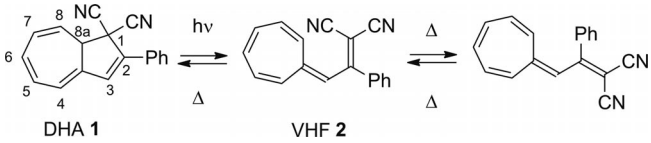


Scheme 1. DHA/VHF system.

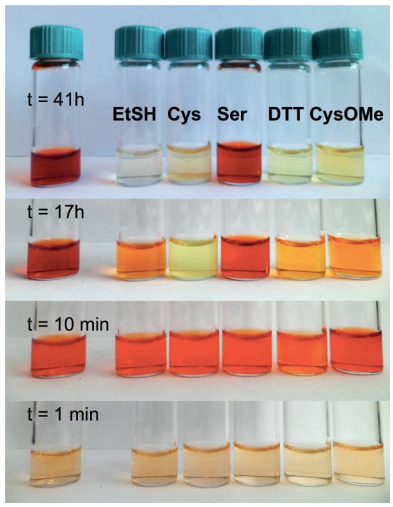
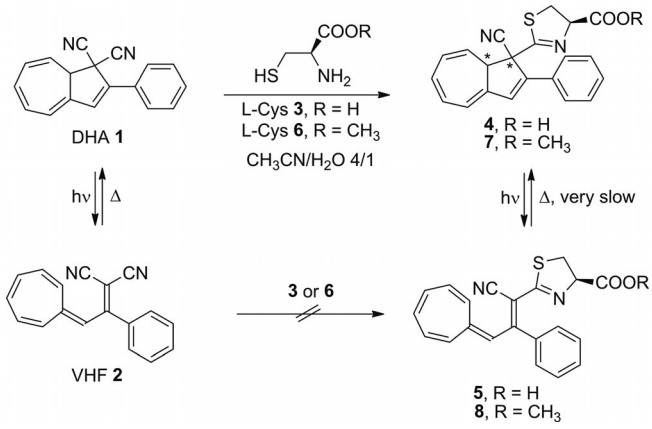


Figure 1. Color changes of a 1.0 mM solution of DHA/VHF (left) in CH_3CN/H_2O (4:1) upon addition of 10 equiv of EtSH, Cys, Ser, DTT and Cys-OMe (at t = 1, 10 min, 17 h, 41 h).

Results and Discussion

Upon dissolving solid cysteine **3** (Cys) in a freshly prepared 1 mM solution of DHA/VHF **1/2**[13] in acetonitrile/water (4:1), a color change from orange to a slightly yellow solution was observed with the naked eye over time (see Figure 1). In contrast, no obvious color change was detected upon addition of other amino acids such as serine or alanine. This simple experiment suggests that the DHA/VHF system reacts with the thiol group and can be used as a dosimeter to detect Cys and other important biological thiols.

To investigate the source of the color change, mass spectrometric analysis (MALDI-TOF) of the solution was performed. Two peaks at m/z = 361 and 315 were observed (see the Supporting Information). The signal at m/z = 361 agrees with the 4,5-dihydro-1,3-thiazolyl structure **4** $[M + H]^+$ suggested in Scheme 2 and can be explained by the reaction of the thiol group of Cys **3** with one of the cyano groups of DHA **1** followed by a condensation/cyclization that also involves the amino group of Cys **3**. The mass spectrum also shows a signal at m/z = 315, which may correspond to the loss of CO_2H from **4**; this decarboxylation likely occurs during the MALDI ionization. The formation of a 4,5-dihydro-1,3-thiazole ring by reaction of a cyano group with a 1,2-aminothiol moiety is known in the literature[2b] for being the last step in the synthesis of luciferin, a common substrate for firefly luciferase[14] and it has recently been used as a biocompatible condensation reaction in living cells.[15]



Scheme 2. Proposed compounds formed in the presence of Cys **3** or the Cys methyl ester **6**.

To shed light on which isomer of the DHA/VHF system is reactive towards thiols, we studied the change in the reaction mixture over time by UV/Vis spectroscopy. UV/Vis spectra were recorded at room temp. in acetonitrile/water (4:1) after adding an excess of Cys **3** (50 equiv.) to a 5.5×10^{-2} mM solution containing only DHA **1** and to a 5.5×10^{-2} mM solution containing only VHF **2**. The absorption spectra of DHA **1** in the presence of Cys **3**, with the cuvette kept in the dark, show clear broadening and a decrease in the characteristic DHA absorption at 356 nm; a small redshift of the absorption is observed after 24 h, but then a small blueshift is observed in the following 24 h (Figure 2, solid lines). The scattering observed in the UV/Vis curve recorded after 48 h (Figure 2, blue line) corresponds to the precipitation of a white solid, most likely due to both cysteine aggregation and further reactions of **4**, as confirmed below in NMR studies. Two days after the addition of Cys **3** to DHA **1**, the slightly yellow solution was irradiated at 357 nm for 20 min; this resulted only in a stronger yellow color, no red color typical of the parent VHF **2** was observed.

Thus, the original DHA **1** had undergone complete reaction with Cys **3** during the 2 d in the dark. The UV/Vis absorption spectrum recorded after irradiation (Figure 2, dotted line) revealed, however, the presence of a new species with an absorption maximum at 402 nm, which is blueshifted relative to the absorption band of the parent VHF **2** (λ_{max} = 478 nm, Figure 3). Moreover, a characteristic absorption at 293 nm is observed[2b] and the spectrum has been ascribed to the proposed structure of compound **5** in which the original cyano group of VHF **2** has been replaced by a less electron-withdrawing group, provoking a blueshift of more than 70 nm. In contrast, this new group does not really affect the absorption maximum of **4**, which has an absorption maximum just slightly shifted by 2–3 nm. Next

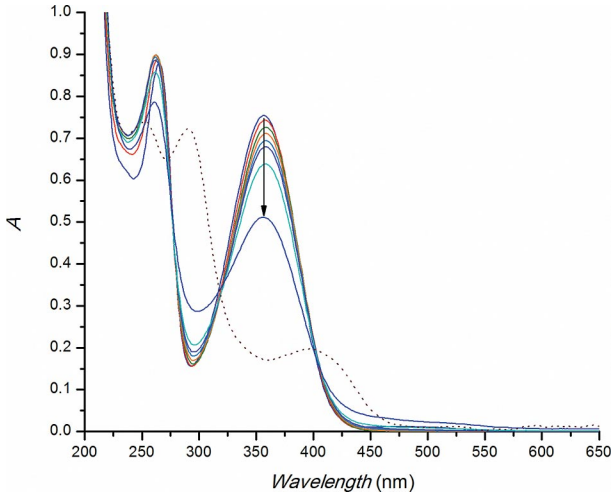


Figure 2. Absorption spectra of the reaction in the dark between DHA (**1**; 5.5×10^{-2} mM) and Cys (**3**; 50 equiv.) in CH_3CN/H_2O (4:1) at 25 °C for 2 d (solid lines, recorded, from top to bottom, at t = 0, 4, 8, 12, 16, 20, 24, 48 h). The dotted line shows the absorption spectra of the final solution (48 h) after selective irradiation at 357 nm for 20 min.

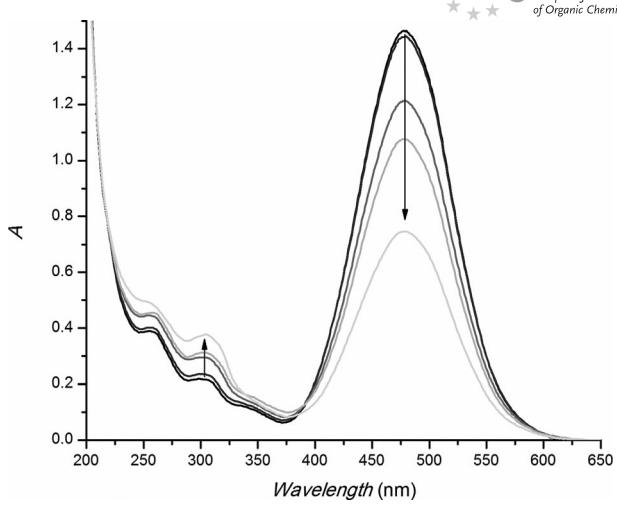


Figure 3. Absorption spectra of the reaction between VHF **2** (5.5×10^{-2} mM) and Cys (**3**; 50 equiv.) in CH_3CN/H_2O (4:1) upon irradiation at 366 nm at room temp. for 2 d (recorded, from top to bottom, at t = 0, 4, 21, 26, 54 h).

we studied the absorption properties of a solution of VHF **2** and Cys **3** (50 equiv.) constantly irradiated by a UV lamp at 366 nm for 48 h. As is evident from Figure 3, the intensity of the VHF absorption decreases somewhat, possibly due to some decomposition, but possibly also due to Michael attack on the dicyanoethylene unit {see below for experiments on 2-[2-(cyclohepta-2,4,6-trienyl)-1-phenylethylidene]malononitrile, a VHF precursor (see S10 in the Supporting Information)}, but the spectrum presented in Figure 2 (dotted line), tentatively assigned to VHF **5**, is not observed. In other words, it seems that only a cyano group in DHA **1** is able to react with Cys **3** to form a 4,5-dihydro-1,3-thiazole unit. Indeed, the cyano groups on the malononitrile moiety **1** are better electrophiles than those on **2**.[16]

To validate the hypothesis described above for the reaction between DHA **1** and Cys **3** in the dark, NMR experiments were performed under these conditions. The reactions between DHA **1** and Cys **3** and between DHA **1** and Cys methyl ester **6** (both in an NMR tube; [DHA] = 23 mM in CD_3CN/D_2O (4:1); [DHA]/[Cys] ≈ 1:1.2) were monitored for 2 d, the latter allowing easier spectral interpretation. The ^{1}H NMR spectra are rather complicated, but it is possible to detect the presence of two main products in the diagnostic region (δ = 5.2–5.3 ppm) of CH-α of the amino acid (Figure 4). In fact, both DHA **1** and Cys **3/6** are chiral molecules, and – although DHA **1** was used as a racemic mixture – Cys was chosen as a single enantiomer of the natural L series. DHA derivatives **4** and **7** described in Scheme 2 have three stereocenters, namely C-1 and C-8a on the "DHA" core and CH-α of the amino acid. Although the third stereocenter in L-Cys is well defined, the others are not and can theoretically give rise to four different diastereomers.

An accurate description of the ^{1}H NMR spectra depicted in Figure 4 is certainly merited. The ^{1}H NMR spectra of Cys methyl ester **6** (spectrum 1) and DHA **1** (spectrum 2) are shown for direct comparison. Spectra 3, 4 and 5 were recorded for the reaction between DHA **1** and Cys **6** in the dark after 5 , 24, and 48 h, respectively. In spectrum 5, recorded after 48 h, selected signals typical of DHA **1** are almost undetectable, and signals from the modified DHA **7**, present as two main diastereomers, are dominant. In particular, the singlet arising from 3-H at δ = 7.1 ppm of **1** (spectrum 2, Figure 4) is slightly shifted and transformed into two singlets almost overlapping at δ = 7.05 ppm (spectrum 5), the doublet arising from the protons of the phenyl ring at δ =7.8 ppm in **1** (spectrum 2) is split into two doublets at δ = 7.7 and 7.6 ppm (spectrum 5), and the multiplet arising from 8a-H at δ = 3.8 ppm in **1** is shifted to δ = 3.3 ppm and again split into two multiplets in spectrum 5. Most of the signals of the diastereomers **7** are overlapping; nevertheless, it is possible to select some more signals that are diagnostic of the presence of two main products. In fact, the two doublets of doublets at $\delta \approx$ 5.2–5.3 ppm can be ascribed to CH-α of the amino acid in the two different diastereomers, and two methoxy groups can also be detected in the region of $\delta \approx$ 3.8 ppm (see the Supporting Information). When using an excess of Cys, as in the UV/Vis studies, a white precipitate, insoluble in most common organic solvents, formed, which completely removed the DHA derivative **4** or **7** from solution in a few days.

Cys methyl ester **6** (1 equiv.) was also used in the reaction with DHA/VHF on a preparative scale aiming at isolating the products **7** and **8** (Scheme 2). The presence of several diastereomers of **7** with similar or overlapping R_f values inevitably affected a proper purification, as reflected in the ^{1}H NMR spectrum reported in the Supporting Information. The mass spectrum recorded with the micrOTOF-Q II spectrometer interfaced to an HPLC instrument exhibits a peak at m/z = 375 (see the Supporting Information),

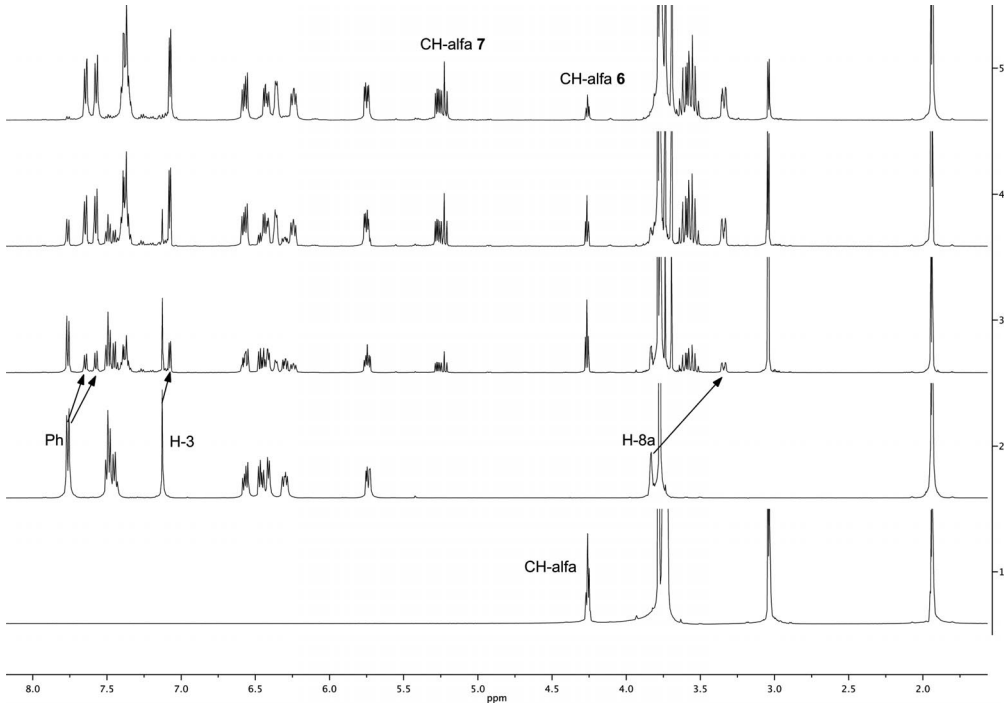


Figure 4. ^{1}H NMR spectra in CD_3CN/D_2O (4:1) recorded for the reaction of DHA **1** (23 mM) with Cys methyl ester **6** (1.2 equiv.). From bottom to top: only **6**, only **1**, **1** + **6** at t = 5, 24, 48 h.

in agreement with the calculated value for $[M + H]^+$ of the proposed structure **7**.[17] Moreover, when the NMR tube was exposed to light, conversion of the different diastereomers of **7** into a main single product, tentatively assigned to **8**, was detected together with decomposition products (see the Supporting Information). Indeed, the characteristic signal of 8a-H of a "DHA" form at δ = 3.45 ppm disappears, the two CH-α signals of the amino acid at δ = 5.24 and 5.32 ppm merge into one signal at δ = 4.6 ppm, and the AB part of the ABX system CH-α/CH_2-S at δ = 3.6 ppm appears much simpler than before. Moreover, the two singlets of the methoxy groups merge into one.

The ^{13}C NMR spectrum supports the proposed structure of compound **7**, present as two main diastereomers. All the signals are double (1:1 ratio in accordance to the ^{1}H NMR spectrum) and by comparison with the ^{13}C NMR spectrum of the parent DHA **1**, disappearance of one cyano group (resonance in the characteristic region at δ = 115 ppm) and appearance of the signals of four new carbon atoms at $\delta \approx$ 170–175 ppm, in accordance with the formation of an S–C=N group and the presence of an ester, is clearly detected (see the Supporting Information). 2D NMR experiments were performed, and the HMBC spectrum revealed correlation peaks between those new carbon atoms and both the CH-α and the CH_2-S protons of the amino acid, which supports the formation of the 4,5-dihydro-1,3-thiazole ring (see the Supporting Information).

Because NMR studies on VHF **2** in the presence of Cys **3** were always affected by the presence of traces of DHA **1**, we investigated the reactivity of 2-[2-(cyclohepta-2,4,6-trienyl)-1-phenylethylidene]malononitrile, an intermediate in the synthesis of DHA **1**[10] that still presents two cyano groups conjugated to a double bond (see the Supporting Information). In this case we can detect the Michael addition of Cys **3** to the double bond through the disappearance of selected and characteristic signals both in the ^{1}H and ^{13}C NMR spectra (see the Supporting Information). This observation also suggests the preferred Michael addition of Cys **3** to VHF **2** in the total absence of DHA **1**, as did the UV studies (Figure 3).

From the above experiments, we can conclude that in the dark the first transformation involves one cyano group on DHA **1**, which reacts with the 1-amino-2-mercapto moiety of Cys to give a 4,5-dihydro-1,3-thiazole ring, proved to be responsible for the color change. Thereafter, DHA derivative **4** can still react with Cys producing an insoluble white solid. As for VHF **2**, we cannot demonstrate neither reactivity nor inertness to Cys, but we showed that 2-[2-(cyclohepta-2,4,6-trienyl)-1-phenylethylidene]malononitrile undergoes Michael addition with Cys. Efforts to perform the reaction on a preparative scale were not successful in terms of the purification of the single diastereomers, but proved a quantitative transformation of DHA **1** into DHA **7** and demonstrated the faster reactivity of DHA **1** compared with VHF **2**, even in the presence of light. Hence we can state that the color change of the DHA/VHF system in the presence of Cys reported in Figure 1 is provoked by the reaction of DHA **1** with Cys and by the formation of a 4,5-dihydro-1,3-thiazole ring.

Fluorescence measurements to monitor the transformation of DHA **1** into DHA **4** were performed at 77 K (Figure 5) to retard the photoswitch of DHA **1** into VHF **2** and

of **4** into **5**. Indeed, compound **4**, like its parent DHA **1**,[18] does not show any significant emission at room temperature due to its photoconversion into **5**. DHA **1** shows a broad emission band at 77 K in the 400–700 nm range (λ_{max} = 475 nm, Figure 5 and the Supporting Information). After reaction with Cys **3**, DHA **1** is converted into compound **4** and, upon excitation at 356 nm, the emission maximum shifts to a higher wavelength (λ_{max} = 499 nm, Figure 5 and the Supporting Information). As for the absorption spectrum, the first excitation peak of **4** (268 nm) does not significantly differ from that of DHA **1**, whereas the second broader peak shifts towards a higher wavelength (ca. 367 nm). Irradiation of **4** with UV light provokes the transformation into **5** and results in a new emission peak at a higher wavelength (λ_{max} = 532 nm, see the Supporting Information). Interestingly, although VHF **2** is non-emitting,[18] compound **5** shows a weak emission at room temperature (λ_{max} = 520 nm) that is strongly enhanced at 77 K. The similarity between the excitation and the absorption spectra of the converted compound **5** supports the hypothesis that the fluorescence originates from the S_1–S_0 transition that is not observed in VHF **2**.[18]

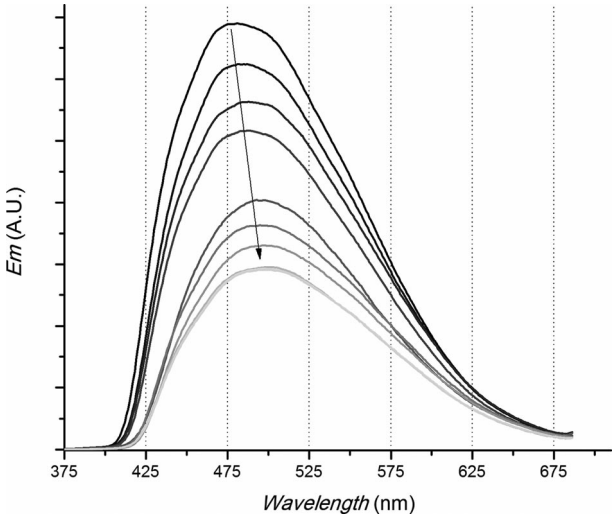


Figure 5. Fluorescence spectral changes over time of DHA **1** (5.5×10^{-2} mM) upon addition of Cys **3** (50 equiv.). The spectra were recorded at 77 K with λ_{ex} = 356 nm (from top to bottom: only **1**, then **1** + **3** at t = 0, 4, 8, 20, 24, 28, 40, 44, 48 h).

Experiments performed with dithiothreitol (DTT),[19] an important antioxidant agent, and ethanethiol, chosen as an example of a simple alkanethiol, support the general, but lower, reactivity of DHA **1** towards thiols as well as the ability to detect the reaction with the naked eye (Figure 1). Nevertheless, because both these thiols lack the amino moiety, the reaction mechanism is different, although not yet investigated in detail. Preliminary experiments again suggested that nucleophilic attack on the cyano group is preferred to a Michael addition on the malononitrile system.

Conclusions

We have reported herein a new important application of the DHA/VHF system **1/2** as a naked-eye probe for thiols. It has been shown that DHA **1** reacts with cysteine to produce a visible color change. In fact, even when present in only traces in a deep-orange solution of VHF **2**, DHA **1** still reacts with thiols, shifting the equilibrium of the DHA/VHF system towards its side in the presence of light. This reactivity provokes an evident and visible color change in solution and a redshift in the emission of DHA **1** after reaction with Cys **3** that allows our system to be classified as a new chemodosimeter for the detection of thiols. The simple synthesis of the scaffold, its versatility in terms of structural changes and consequent chemical and optical properties, as demonstrated by the numerous derivatives reported in the last few years,[20] lead us to believe that the system has much promise. Herein, its value as a thiol-specific probe has been demonstrated for the parent system **1/2**, which opens up access to a new family of probes for thiols, and we expect to improve sensitivity, selectivity, and the properties of the sensing system.

Experimental Section

General: All solvents and reagents were purchased commercially and used without further purification. NMR spectra used for the characterization of products were recorded with Bruker 300 and 500 MHz spectrometers. The NMR spectra are referenced to the solvent. MALDI-TOF MS were recorded with a Bruker Daltonics mass spectrometer by using 2,5-dihydroxybenzene as the matrix. Spectra were calibrated by using a peptide standard solution. HRMS were recorded with a Q-Tof mass spectrometer with PEG as the reference mass. HPLC–MS analysis was performed by using a Dionex UltiMate 3000 RSCLC System U-HPLC machine coupled with a Bruker micrOTOF-Q II mass spectrometer. Solution absorption spectra were recorded with a Cary 50-Bio Varian UV/Vis spectrophotometer with pure solvent as the baseline. Fluorescence spectra were recorded by using a FluoroLog-3 spectrophotometer at 77 K and an excitation and emission slit width of 3 nm. The emission and excitation spectra were corrected for instrumental function. UV/Vis and fluorescence spectra were recorded by starting with a 5.5×10^{-2} mM solution of DHA **1** in CH_3CN/H_2O (4:1) after addition of 50 equiv. of Cys **3**. DHA **1** was prepared according to a literature procedure.[10]

Synthesis of Compound 7: L-Cys-Me **6** (75 mg, 0.44 mmol) was added to a solution of DHA **1** (108 mg, 0.42 mmol) in CH_3CN/H_2O (4:1, 20 mL). The mixture was stirred at room temp. for 2 d and then concentrated to dryness, redissolved in CH_2Cl_2 (25 mL), washed with water (2 × 20 mL), and dried with $MgSO_4$. Evaporation of the solvent gave an orange oil that was purified by flash column chromatography in the dark (SiO_2, from 70% CH_2Cl_2 in heptane to 100% CH_2Cl_2) to give compound **7** (120 mg, 0.32 mmol, 76%) as a 1:1 mixture of two inseparable diastereomers. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.76–7.68 (m, 2 H, Ph), 7.67–7.61 (m, 2 H, Ph), 7.51–7.32 (m, 6 H, Ph), 6.94 (s, 1 H, 3-H), 6.93 (s, 1 H, 3-H), 6.65–6.53 (m, 2 H, 4-H or 5-H), 6.46 (dd, J = 6.0, 1.1 Hz, 1 H, 6-H), 6.44 (dd, J = 6.0, 1.1 Hz, 1 H, 6-H), 6.37–6.32 (m, 2 H, 5-H or 4-H), 6.29–6.23 (m, 2 H, 7-H), 5.96 (d, J = 4.0 Hz, 1 H, 8-H), 5.94 (d, J = 4.0 Hz, 1 H, 8-H), 5.32 (dd, J = 9.7, 5.6 Hz, 1 H, CH-α), 5.24 (dd, J = 9.4, 8.6 Hz, 1 H, CH-α), 3.84 (s, 3 H,

CH_3), 3.77 (s, 3 H, CH_3), 3.68–3.52 (m, 4 H, 2 CH_2S), 3.45 (dt, J = 3.9, 2.0 Hz, 1 H, 8a-H), 3.42 (dt, J = 3.9, 2.0 Hz, 1 H, 8a-H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 174.7, 173.9, 170.5, 170.4, 145.0, 140.24, 140.18, 132.2, 132.2, 132.0, 131.1, 131.0, 130.4, 130.3, 129.5, 129.4, 128.94, 128.98, 126.80, 126.77, 126.6, 126.4, 121.33, 121.2, 119.7, 119.5, 117.0, 78.1, 77.6, 59.2, 59.0, 52.99, 52.96, 51.44, 51.38, 37.1, 36.6 ppm. MS (EI) of the HPLC peak eluted at t = 8.71 min: 375.15 $[M + H]^+$, 397.14 $[M + Na]^+$, 771.26 $[2 M + Na]^+$.

Supporting Information (see footnote on the first page of this article): 1D and 2D NMR spectra of **7**, HPLC/MALDI-TOF MS of **7**, fluorescence spectra of **1**, **4**, and **5**, MALDI-TOF MS spectrum of vial of **1** + **3** after 24 h, ^1H and ^{13}C NMR spectra of the reaction of 2-[2-(cyclohepta-2,4,6-trienyl)-1-phenylethylidene]malononitrile with excess of Cys **3**.

Acknowledgments

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[13] When DHA **1** is dissolved in the solvent mixture in the presence of daylight, conversion into VHF **2** starts immediately and can be detected by a change in the color of solution from yellow to orange. VHF thermally reverts back to DHA and the composition of the equilibrium mixture depends on both the light intensity and the temperature. The exclusive presence of VHF **2** occurs only after selective irradiation at 350 nm by using a 150 W xenon arc lamp equipped with a monochromator for 1 h.

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[17] Two more peaks are present in the HPLC chromatogram, one corresponding to the mass of the carboxylic acid **4**, formed in the HPLC column because of formic acid in the eluent mixture, and one corresponding to the ethyl ester of **7**, most likely formed by transesterification (see the Supporting Information).

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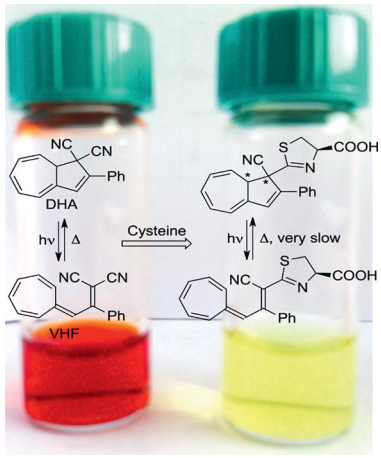
Can the dihydroazulene/vinylheptafulvene (DHA/VHF) photo/thermoswitch system be used as a chemodosimeter for thiols? A new application for this versatile system has been discovered.



Keywords: Sensors / Thiols / Molecular recognition / Chromophores

