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Intramolecular Didehydro-Diels–Alder Reaction and Its Impact on the Structure–Function Properties of Environmentally Sensitive Fluorophores.

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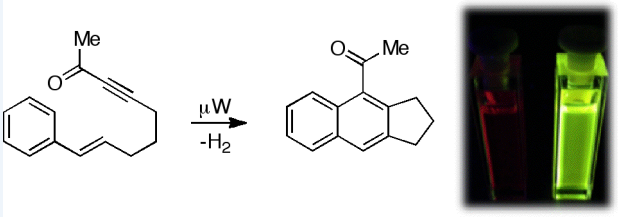
Supporting Information

CONSPECTUS: Reaction discovery plays a vital role in accessing new chemical entities and materials possessing important function.[1] In this Account, we delineate our reaction discovery program regarding the [4 + 2] cycloaddition reaction of styrene-ynes. In particular, we highlight our studies that lead to the realization of the diverging reaction mechanisms of the intramolecular didehydro-Diels–Alder (IMDDA) reaction to afford dihydronaphthalene and naphthalene products. Formation of the former involves an intermolecular hydrogen atom abstraction and isomerization, whereas the latter is formed via an unexpected elimination of H_2. Forming aromatic compounds by a unimolecular elimination of H_2 offers an environmentally benign alternative to typical oxidation protocols.



We also include in this Account ongoing work focused on expanding the scope of this reaction, mainly its application to the preparation of cyclopenta[*b*]naphthalenes. Finally, we showcase the synthetic utility of the IMDDA reaction by preparing novel environmentally sensitive fluorophores.

The choice to follow this path was largely influenced by the impact this reaction could have on our understanding of the structure–function relationships of these molecular sensors by taking advantage of a *de novo* construction and functionalization of the aromatic portion of these compounds. We were also inspired by the fact that, despite the advances that have been made in the construction of small molecule fluorophores, access to rationally designed fluorescent probes or sensors possessing varied and tuned photophysical, spectral, and chemical properties are still needed.

To this end, we report our studies to correlate fluorophore structure with photophysical property relationships for a series of solvatochromic PRODAN analogs and viscosity-sensitive cyanoacrylate analogs. The versatility of this *de novo* strategy for fluorophore synthesis was demonstrated by showing that a number of functional groups could be installed at various locations, including handles for eventual biomolecule attachment or water-solubilizing groups. Further, biothiol sensors were designed, and we expect these to be of general utility for the study of lipid dynamics in cellular membranes and for the detection of protein-binding interactions, ideal applications for these relatively hydrophobic fluorophores. Future studies will be directed toward expanding this chemistry-driven approach to the rational preparation of fluorophores with enhanced photophysical and chemical properties for application in biological systems.

INTRODUCTION

Serendipitous Intramolecular Didehydro-Diels–Alder Reaction (IMDDA)[2]

The research program described herein originates from our studies to elucidate the mechanism of a thermal [2 + 2] cycloaddition reaction of allene-ynes. An example of this reaction involves the conversion of allene-yne **1** to the bicyclo[4.2.0]octane **2** (eq 1, Scheme 1). Prior to our findings,[3] thermally disallowed [2 + 2] cycloaddition reactions of allene-ynes were rare;[4] however, B3LYP/6-31+G(d,p) calculations indicated that these reactions proceed via a stepwise diradical pathway favoring the formation of an intermediate possessing a vinyl and an allyl radical.[5] These calculations were supported by experiment, whereby incorporation of a cyclopropyl radical trap into the allene-yne precursor **3** afforded the cyclopropyl ring-opened product **4** upon heating, representing the first example of trapping proximal, carbon-centered diradicals intramolecularly under thermal conditions (eq 2). This result led us to hypothesize that "capturing judiciously designed diradical intermediates, such as **6**, generated by heating allene-yne **5**, would afford access to unsaturated polycyclic products," which in turn would be valuable for the preparation of molecularly complex targets. For example, the reaction of **6** with a tethered

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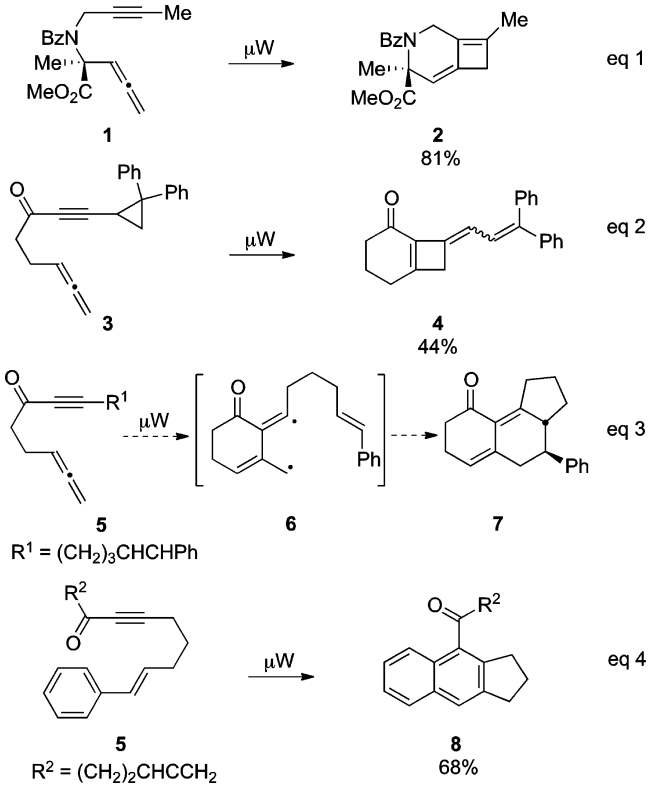
Scheme 1. Serendipitous IMDDA Reaction



styrenyl group was predicted to produce the tricyclic dienone **7** (eq 3). Curiously, microwave heating of allene-yne **5** afforded only naphthalene **8**, the product of an IMDDA reaction between the ynone and the styrenyl group; the allene remained in tact (eq 4)! While IMDDA reactions of this type are known,[6] they are limited in synthetic utility due to contamination with varying quantities of dihydronaphthalene products. On the contrary, this reaction yielded only the dehydrogenated, aromatic product **8**; an exclusivity that inspired our mechanistic investigations to better understand the IMDDA reaction. This retrosynthetically orthogonal strategy to the most commonly used protocols for the preparation of aromatic building blocks, motivated our efforts to expand the scope of this reaction. Finally, the importance of aromatic building blocks to material and biological targets cannot be overstated; in particular, we envisioned that the potential of a de novo synthesis of functionalized naphthalenes could be fully realized by the preparation of small molecule fluorophores. All phases of this study are delineated within this Account.

Expanding the Scope of the IMDDA

Ready access to uniquely substituted naphthalenes afforded by the IMDDA reaction of styrene-ynes inspired additional studies.[7] Several examples demonstrating the high level of functional group compatibility and the electronic requirements of the IMDDA reaction are highlighted in Scheme 2. Styrene-yne precursors **11a–h** were readily synthesized in two steps from aldehyde **10** and diethylbenzylphosphonate **9**. Heating **11a–h** produced cyclopenta[*b*]naphthalenes **12a–h** in high to quantitative yields. Extending the tether by one methylene unit provided a 1,2,3,4-tetrahydroanthracene in quantitative yield

Scheme 2. Functionalized Naphthalenes by Variations to the IMDDA Precursor

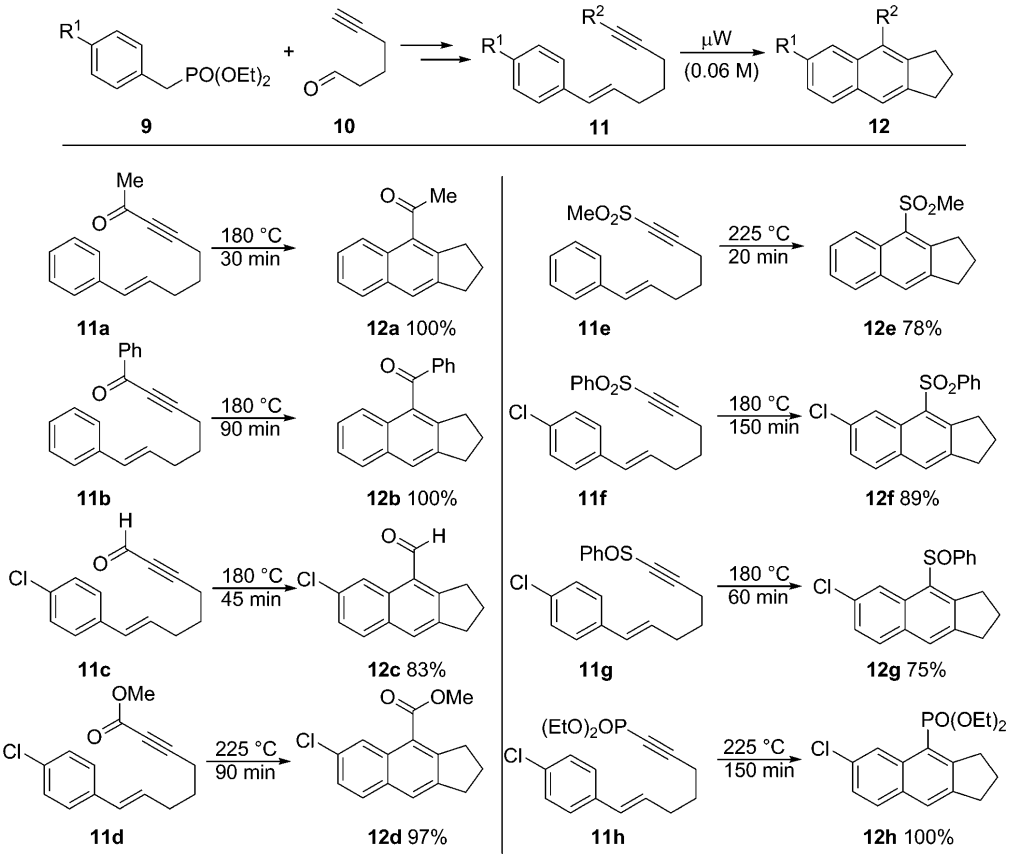


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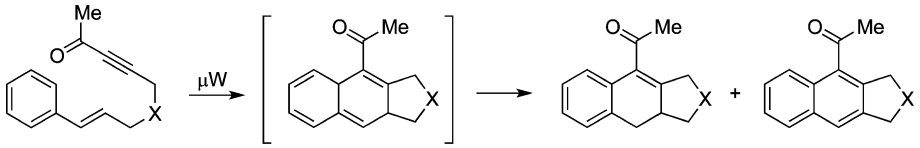
Table 1. IMDDA Reaction of Styrene-ynes with Heteroatom Tethers



11a, X = CH_2
13, X = O
15, X = NTs

12-D, X = CH_2
14-D, X = O
16-D, X = NTs

12a, X = CH_2
14-N, X = O
16-N, X = NTs

entry	styrene-yne	temp (°C)	concn (M)	time (min)	solvent	% D	% N
1	**11a**	180	0.06	30	DCE	0 (**12-D**)	100 (**12a**)
2	**13**	180	0.06	30	DCE	15 (**14-D**)	28 (**14-N**)
3	**15**	180	0.06	10	DCE	56 (**16-D**)	30 (**16-N**)
4	**15**	180	0.06	60	DCE	8 (**16-D**)	38 (**16-N**)
5	**15**	120	0.06	15	*o*-DCB	53 (**16-D**)	7 (**16-N**)
6	**15**	225	0.06	1	*o*-DCB	33 (**16-D**)	48 (**16-N**)
7	**15**	180	0.50	1	*o*-DCB	60 (**16-D**)	16 (**16-N**)

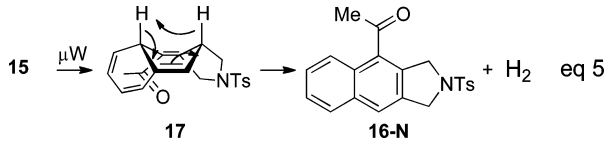
(product not depicted) and represented the first successful IMDDA reaction of a styrene-yne containing a four-atom tether. IMDDA reactions performed with substrates possessing a terminal or TMS-substituted alkyne were unsuccessful. These results support a normal demand Diels–Alder mechanism and are generally in agreement with those reported independently by Matsubara.[8]

IMDDA reactions of substrates possessing heteroatoms in the styrene-yne tether afforded very different product mixtures. For example, styrene-ynes **13** (X = O) and **15** (X = NTs) were heated using similar reaction conditions to those detailed above to produce low yields of naphthalenes **14-N** and **16-N**, in addition to the dihydronaphthalene products **14-D** and **16-D** (entries 2 and 3, Table 1). Variations to the reaction conditions for the IMDDA reaction of **15** were made to better understand the observed product selectivity.[9] First, extending the reaction time of **15** at 180 °C from 10 to 60 min afforded less dihydronaphthalene **16-D** (8% vs 56% yield); however, a proportionate increase in the quantity of naphthalene generated was not observed (entry 3 vs 4), which established that **16-N** did not form from **16-D** upon prolonged heating. Second, lowering the reaction temperature to 120 °C yielded significantly more **16-D**, while performing the reaction at 225 °C resulted in a slight favoring of the naphthalene product **16-N** (compare entries 3, 5, and 6). Third, increasing the reaction concentration to 0.50 M produced a 3.8:1 ratio of **16-D** to **16-N** compared with the 1.9:1 ratio obtained using a concentration of 0.06 M (entry 7). Finally, heating styrene-yne **15** at 180 °C in DCE produced a 1:1.9 mixture of naphthalene **16-N** to dihydronaphthalene **16-D** regardless of whether the reaction was conducted in the presence of air or degassed with argon. These results indicated the possibility that the naphthalene and dihydronaphthalene products were generated via two different mechanistic pathways and begged a more thorough understanding of the reaction.

MECHANISTIC HYPOTHESIS FOR THE IMDDA REACTION

Formation of Naphthalene Product 16-N

Further investigation of the thermal reaction of styrene-yne **15** to produce naphthalene **16-N** confirmed that the IMDDA reaction occurs *via a loss of hydrogen gas*.[9] Loss of H_2 in similar reactions proposed, but not confirmed, by Matsubara.[8] H_2 in the reaction solution was characterized and quantified by ^1H NMR spectroscopy in *o*-DCB-d_4, which showed a singlet at δ 4.73 accounting for a 5–10% yield of H_2. In addition, H_2 in the headspace of the reaction was quantified by GC, where the amount of H_2 corresponded to a 39–48% yield. Extrusion of hydrogen gas is postulated to occur via a unimolecular atom elimination from intermediate **17**, which is formed by an initial [4 + 2] cycloaddition reaction of styrene-yne **15** (eq 5). This is



17 **16-N**

an allowed process according to rules set forth by Woodward and Hoffmann when $m = 4q + 2$; specifically, m equals the number of π electrons and q is an integer. In the case of the IMDDA reaction of **15**, $m = 2$ and $q = 0$.[10] Other reactions demonstrating unimolecular elimination of H_2 have been reported, including the thermal elimination of H_2 from 1,4-cyclohexadiene to generate benzene.[11]

The formation of dihydronaphthalene **16-D** is also postulated to occur from the tetraene intermediate **17**, but via an alternate mechanistic pathway than that proposed for generation of naphthalene **16-N** (Scheme 3A). To form the isomerized product **16-D**, we presume that triplet oxygen abstracts the tris-allylic hydrogen atom of **17** to generate the radical intermediate **20**, a resonance form of **21**. It is important to point out that only a catalytic quantity of triplet oxygen is required because the newly generated radical of intermediate **21** then abstracts a hydrogen atom from another equivalent of tetraene **17** to provide dihydronaphthalene **16-D**. This intermolecular hydrogen atom transfer process was evidenced by a crossover experiment where a 1:1 mixture of **15** and pentadeuterated styrene-yne **18** was heated to afford all four possible dihydronaphthalene products **16-D** and **22–24**.[9] The proposed mechanism was further supported by experiments, which demonstrated that an increase in the reaction concentration yielded a higher quantity of **16-D** (entry 7, Table 1). Interestingly, the atmosphere of the reaction, whether argon or air, did not affect product ratios. This inconsistency can be explained by the work of Hendry, who showed that substoichiometric amounts of oxygen were all that were required for the thermal dehydrogenation of 1,4-cyclohexadiene **25** to benzene and tetraoxidane **26** (Scheme 3B).[12]

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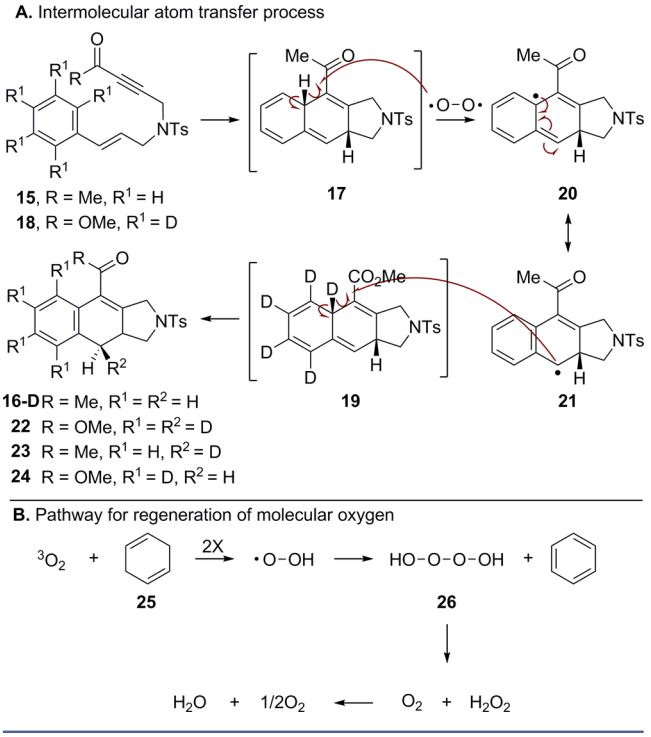
Scheme 3. Mechanism for Formation of Dihydronaphthalene



Computational Reaction Modeling

The diverging reaction pathway portrayed above for the formation of the naphthalene and dihydronaphthalene products from tetraene intermediate **17** was also supported by transition state calculations. Modeling was performed at SMD(DCE)-UM06-2X/6-31+G(d,p) level of theory with free energy estimates at 180 °C, and the tosyl group of **17** was replaced with a methanesulfonyl group to simplify the calculations.[9] Hydrogen atom abstraction by triplet oxygen from the tetraene intermediate was predicted to be more favorable than the direct expulsion of H_2 by 9.8 kcal/mol, which correlates with the data in Table 1, where lower reaction temperatures afforded predominantly dihydronaphthalene **16-D** and higher temperatures produced naphthalene **16-N** as the major product.

Prior to these experimental and computational studies, either a mechanistic rationale for the formation of the naphthalene product via the IMDDA reaction of styrene-ynes was not described, or its formation was attributed to the purported ease of dihydronaphthalene auto-oxidation. Thus, the production of naphthalene **16-N** from intermediate **17** via the direct expulsion of H_2 provided new insight into the product mixtures afforded from the IMDDA reaction of styrene-ynes. This insight has served to increase the synthetic utility of this underutilized reaction, which in turn has enabled detailed structure–function–property analysis of new environmentally sensitive fluorophores.

FLUORESCENT DYES AND THEIR IMPORTANCE IN MOLECULAR PROBES AND SENSING TECHNOLOGIES

Fluorescent compounds are valuable tools for elucidating and understanding biological systems.[13] In particular, small molecule organic fluorophores are advantageous because the small size minimizes disruption of the environment being studied. Moreover, they are being used to advance the drug discovery process in high throughput screening assays[14] and also to increase our understanding of living systems by real-time monitoring of cellular events.[15] The widespread use of these probes is enabled by the commercial availability of hundreds of fluorescent dyes.[16] Even so, each of these dyes comes with advantages and compromises that must be weighed for a particular application. While photochemical and spectroscopic properties represent some of the most important criteria for selection of a sensor, the ability to chemically modify and functionalize these dyes is also important.[17] Despite advances in the construction of small molecule fluorophores, access to rationally designed fluorescent probes or sensors with all desired photophysical, spectral, and chemical properties is still not practical. We expect that the next generation of small molecule-based fluorescent probes designed to encompass all desired elements will be developed by utilizing more modern and diverse synthetic strategies.

Design, Synthesis, and Function of Solvatochromic Fluorophores

The fluorescent spectra of many dyes are influenced by differences in the interaction between solvent and dye in the ground and excited states. One example of this behavior is solvatochromism, in which solvent-dependent spectral shifts result from changes in the dipole moment of the fluorophore upon excitation.[18] PRODAN (**27**) is one of the most well studied solvatochromic dyes because of its highly solvent-dependent spectral shifts, an example of which is the 84 nm bathochromic shift in emission maxima observed from benzene (λ_{em} 421 nm) to methanol (λ_{em} 505 nm).[19] The key structural feature that contributes to PRODAN's solvatochromic behavior is its naphthalene core, which is functionalized along the *x*-axis with dimethylamino and propionyl groups. These donor–π–acceptor (D–π–A) systems are commonly incorporated in a variety of fluorophores, but few of these dyes exhibit the same sensitivity to solvent polarity as PRODAN.

This state of the art fluorophore has inspired the preparation of various analogs, three of which, 6-acryloyl-2-dimethylaminonaphthalene (ACRYLODAN, **28**), 2-dimethylamino-6-lauroylnaphthalene (LAURDAN, **29**), and 6-dodecanoyl-2-[*N*-methyl-*N*-(carboxylmethyl)amino]naphthalene (C-LAURDAN, not shown), have seen widespread use. ACRYLODAN has been employed in direct binding assays as a kinase label to detect ligands that stabilize inactive kinase conformations,[20] while LAURDAN has been utilized to study lateral organization and heterogeneity of lipid membranes by reporting local permeation of water.[21] One drawback to using LAURDAN as a fluorescent probe is its low photostability, which limits its application to two-photon microscopy. A solution to the instability of LAURDAN was the generation of the carboxyl-modified derivative C-LAURDAN, which can be used with conventional one-photon microscopy.[22,23]

The preparation of fluorophores **28**,[24] **29**,[25] and C-LAURDAN[26] only required chemical modification of the ketone and amine functional groups; no alterations to the naphthalene core were necessary. This is typical for many naphthalene-containing fluorophores, where new derivatives are generated by slight modifications to commercially available dyes. We envisioned that a *de novo* synthesis of naphthalenes made possible by the IMDDA reaction would contribute significantly to the preparation of PRODAN analogs incorporating a wider array of functionality and substitution patterns.

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Scheme 4. IMDDA Approach to Environmentally Sensitive Fluorophores

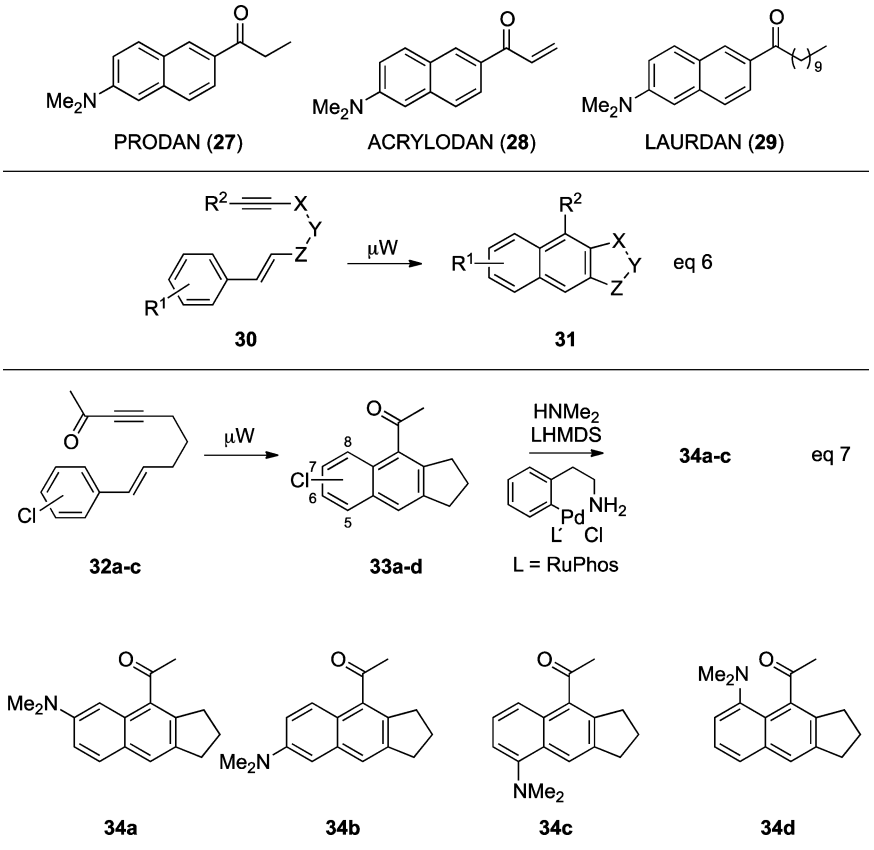
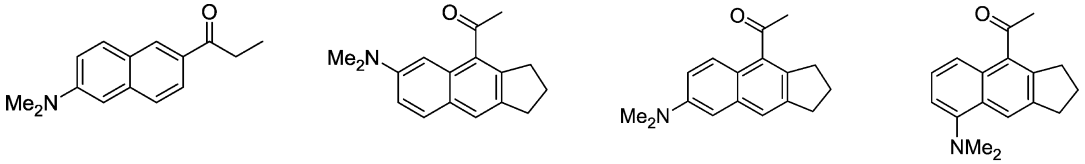


Table 2. Photophysical Properties of Fluorophores 34a–c Compared with PRODAN (27)



	27			34a			34b			34c		
solvent	λ_{abs} (nm)	λ_{em} (nm)	ϕ_f[a]	λ_{abs} (nm)	λ_{em} (nm)	ϕ_f[a]	λ_{abs} (nm)	λ_{em} (nm)	ϕ_f[a]	λ_{abs} (nm)	λ_{em} (nm)	ϕ_f[a]
toluene	346	416	0.56	376	490	0.62	368	481	0.25	322	520	0.47
CH_3CN	350	455	0.80	375	529	0.99	370	545	0.18	316	590	0.23
DMSO	357	462	0.91	377	536	0.85	374	558	0.37	334	598	0.48
EtOH	362	485	0.71	377	578	0.05	374	605	0.03	334	634	0.01

[a]Fluorescence quantum yield was measured in DCM vs PRODAN in DMSO (91%), excitation at 334 nm (10^{-5} M).

This synthetic strategy would allow access to a focused library of fluorophores that would potentially encompass the desired properties, both chemical and photophysical, to address needs of specific applications.

The IMDDA approach to functionalized naphthalenes offers structural and functional versatility through the availability of a wide array of benzene derivatives (R^1), tethering subunits (X–Y–Z), and alkynyl substituents (R^2) (eq 6, Scheme 4). In addition, thermal reaction conditions employed for the IMDDA reaction tolerate substituents that can undergo further modifications.[7] For example, precursors **32a–c**, possessing chloro substituents at the *ortho*-, *meta*-, and *para*-positions of the styrene afforded the corresponding 5-, 6-, 7-, and 8-substituted chloronaphthalenes **33a–d**, which were subjected to Buchwald–Hartwig palladium-catalyzed cross-coupling conditions to produce fluorescent dimethylaminonaphthalenes **34a–c**.[27] While the 8-chloronaphthalene **33d** was accessed via the IMDDA reaction of the *meta*-chlorosubstituted styrenyl precursor, efforts to transform **33d** to the desired dimethylamino derivative **34d** were unsuccessful.

The photophysical properties of **34a–c** were measured in solvents of increasing polarity, and their solvatochromic behavior was compared with that of PRODAN (**27**) (Table 2).[27] Several trends emerged from these measurements, the first being that bathochromic shifts in emission were observed for each fluorophore as solvent polarity increased. For example, shifts in emission when dyes **34a–c** in toluene and ethanol were compared were 88, 124, and 114 nm, respectively, which was larger than the solvatochromic shift of 69 nm noted for PRODAN. The breadth of absorption and emission maxima

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among these fluorophores was also notable, along with the large Stokes shifts observed for compound **34c**. Moreover, emission spectra of these fluorophores were all significantly red-shifted by 65 to 149 nm compared with that of PRODAN. Photobleaching was also examined for fluorophores **34a−c** in CH_2Cl_2, and all three dyes had photodegradation curves consistent with PRODAN in CH_2Cl_2. Another notable characteristic of these fluorophores was that compound **34c**, which contained a 1,5-substituted naphthalene moiety, absorbed light at much shorter wavelengths and fluoresced at much longer wavelengths than either **34a** or **34b**. Interestingly, the photophysical properties of **34b** and **34c** were nearly identical to structurally related analogs of PRODAN lacking the fused five-membered ring,[28] which indicates that appending a cyclopentane ring to the naphthalene has little effect on spectral properties of these dyes. One final comment regarding compounds **34a−c** is that the quantum yields are extremely low in EtOH, which is not the case for PRODAN. It is expected that this fluorogenic property will be useful for distinguishing between protic and aprotic environments in biological systems.

Another series of fluorophores was prepared that incorporated subtle changes to the π-acceptor and π-donor groups. Donor groups including a pyrrolidyl **35**, piperidine **37**, morpholine **38**, benzylamine **39**, aniline **40**, and *para*-methoxyaniline **41** were all installed via the Buchwald−Hartwig cross-coupling reaction, while different acceptor groups, such as aldehydes **36** and **42** and ester **43** were incorporated during synthesis of the IMDDA precursor.[27] Absorption and emission maxima along with quantum yields (not shown) for these dyes were measured in CH_2Cl_2 (Figure 1). The tertiary cyclic amino

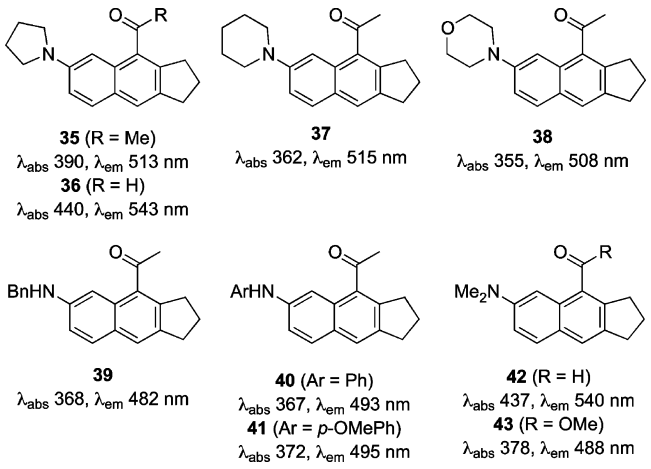

Figure 1. Photophysical effect of changing donor and acceptor moieties in DCM.

ketone series of compounds **35**, **37**, and **38** ranged in absorption maxima from 355 to 390 nm, with the pyrrolidyl-substituted dye **35** having the most red-shifted absorption maxima extending close to the visible region. While changes in absorption were observed upon alteration of the donor group, the emission maxima of these dyes remained relatively constant between 508 and 515 nm. Modifying the donor to secondary amines **39**, **40**, and **41** resulted in a significant hypsochromic shift in the emission maxima (482−495 nm), but no significant differences in absorption maxima were observed (367−372 nm). Changing the acceptor from a ketone to an aldehyde or ester caused the most significant alterations in photophysical properties, especially in the case of aldehydes **36** and **42**, which were characterized by 50−60 nm red shifts in their absorption maxima and 30 nm red shifts in their emission maxima compared with ketones **34a** and **35**. Dye **43**, with an ester as the acceptor rather than a ketone, displayed a blue-shift in the emission maximum of 22 nm. It should be noted that quantum yields for all fluorophores shown in Figure 1 were high in CH_2Cl_2 (0.82−0.99) with the exception of compound **36**.

The most common approach to increasing the absorption and emission maxima of a D−π−A fluorophore is by incorporating additional π-bonds between the donor and acceptor. ANTHRADAN, an analog of PRODAN (**27**) possessing an anthracene core rather than naphthalene, is one particular example where increased conjugation resulted in an approximate 100 nm bathochromic shift of absorption and emission spectra compared with PRODAN.[29] While extremely useful, this strategy can also be limiting due to reduced brightness of the dye associated with the presence of additional nonradiative relaxation modes and more pronounced perturbations in systems being studied resulting from the increasing size of the probe. We have demonstrated that changes to the substitution pattern of the naphthalene ring produce longer absorption and emissions wavelengths without proportionate increases in molecular size or decreases in quantum yield.

Design, Synthesis, and Function of Fluorescent Viscosity Sensors

Viscosity changes in protein-containing biofluids have been linked to a number of disease states; thus the development of robust tools to measure real-time, viscosity-sensitive behavior is needed. Fluorescent viscosity sensors form twisted intramolecular charge transfer states (TICT) upon excitation that relax to the ground state in a manner dependent upon environmental viscosity. However, the relationship between molecular rotor structure, observed photophysical properties, and environmental viscosity is not well understood. Previously, Theodorakis designed 6-aminonaphthalenyl-2-cyano-acrylate (ANCA) sensors that displayed increases in emission intensity or brightness (B), which is directly proportional to the extinction coefficient (ε) and the quantum yield ($B = \varepsilon \cdot \phi_f$), upon increases in solution viscosity and subsequently applied these sensors to the selective recognition and binding of β-amyloid plaques.[30] Cyclopenta[*b*]naphthalene cyanoacrylate (CNCA) dyes **44−46**, possessing a dimethylamino donor group in conjugation with a cyanoacrylate acceptor, were synthesized, and the effect of the placement of the donor and acceptor groups on viscosity-sensitive fluorescence emission was measured (Figure 2).[31]

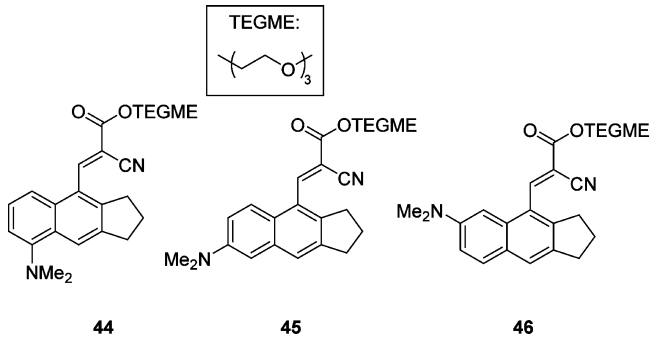

Figure 2. Cyclopenta[*b*]naphthalene cyanoacrylate (CNCA) dyes **44−46**.

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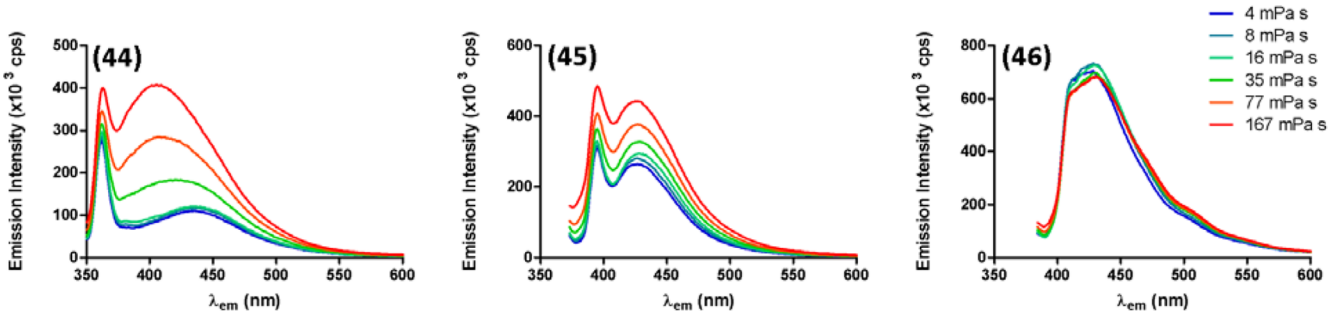

Figure 3. Emission spectra of **44**, **45**, and **46** in alcoholic mixtures of different viscosities.

The emission spectra of **44**–**46** were measured in solutions of increasing viscosity resulting from mixing varied proportions of methanol, ethylene glycol, and glycerol (Figure 3). Compounds **44** and **45** displayed two distinct emission peaks, the emission intensity of which increased with increasing solvent viscosity. Whereas a hypsochromic shift in the longer-wavelength emission band of **44** was observed at higher viscosities, no similar trend was noted for **45**. Compound **46** showed only one broad fluorescent band that exhibited no variability with solvent viscosity.[31]

A previously established characteristic of naphthalene-based molecular rotors is an inverse relationship between emission intensity and viscosity sensitivity, which is witnessed in the case of CNCA dyes **44**–**46**.[32] The most intense emission was observed for **46**, which contained a 1,7-substituted cyclopenta-[*b*]naphthalene moiety; however, this dye lacked viscosity sensitivity.[31] On the other hand, the 1,5-substituted cyclopenta-[*b*]naphthalene **44** displayed a less intense emission but a much more significant sensitivity to viscosity. CNCA dye **45** exhibited intermediate values for both parameters. The correlation between donor position and viscosity sensitivity of the CNCA dyes is attributed to the proximity of the donor and acceptor moieties. When the donor and acceptor are placed in close proximity, as in **46**, their ability to rotate decreases, which increases the energy level of TICT state causing fluorescence deexcitation without passage through the environment-sensitive TICT state. This translates to less viscosity sensitivity but increased emission.

Design, Synthesis, and Function of Attachable Solvatochromic Fluorophores

A focus of modern day biochemical research is the labeling of biomolecules with chemically reactive fluorescent probes.[17] The synthetic versatility afforded by the IMDDA reaction for the synthesis of naphthalenes, in conjunction with the Buchwald–Hartwig reaction to transform these substrates into solvato-chromic fluorescent dyes, provided an ideal platform for the bottom-up design of bioattachable fluorophores.[7,27] For these studies, a cyclopenta[*b*]naphthalenone skeleton was chosen as the ideal fluorescent scaffold because of the red-shifted absorption and emission maxima displayed by similar dyes in previous studies.[27] Furthermore, the absorption maxima of cyclopenta[*b*]naphthalenone-based dyes is located in the visible region of the electromagnetic spectrum, which provides an additional advantage for the labeling and fluorescent study of biomolecules. A 4-hydroxymethylphenyl group was selected as a linker between the chromophore and the reactive group for attachment of the biomolecule due its ease of incorporation into the chromophore, its chemical stability, and its minimal effect on photophysical properties.[33] Additionally, to utilize excitation wavelengths that were well in the visible region, the dimethylamino donor was placed at a 1,7-position on the naphthalene ring.

The synthesis of these attachable fluorophores was accomplished by first subjecting styrene-yne **47** to the IMDDA reaction conditions, followed by a Buchwald–Hartwig cross-coupling reaction to produce the corresponding TBS-protected cyclopenta[*b*]naphthalenone in 53% yield over two steps (Scheme 5).[33] Deprotection with TBAF afforded the attachable fluorophore **48** in 91% yield. In regard to optical properties, **48** showed absorption and emission maxima in CH_2Cl_2 that were much more red-shifted than other cyclopenta[*b*]naphthalene dye derivatives (Figure 1).

The synthetic versatility of the hydroxymethyl group of fluorophore **48** was demonstrated by its conversion into maleimide derivative **50**, the alkyne-containing dye **52**, and the fluorescent fatty acid analog **49**. Fluorescent adduct **50** reacted in 10 min upon exposure to *N*-(boc)-L-cysteine ethyl ester to afford conjugate **51**. Interestingly, the molecular brightness increased upon cysteine addition to 13.0×10^2 for adduct **51** from 4.0×10^2 for **48**, while other photophysical properties remained constant. A copper-catalyzed click reaction of **52** with *N*-Boc-L-*β*-azidoalanine methyl ester generated the covalently linked amino acid **53**. Fluorophores **49**–**53** all displayed similar photophysical properties to parent dye **48**.[33]

Cyclopenta[*b*]naphthalenones as Biothiol Sensors

Ligand induced modulation of kinase function is central to the development of modern therapeutics. Currently, a majority of kinase inhibitors classified as type I inhibitors target the highly conserved ATP binding site, a promiscuity that leads to a lack of kinase inhibitor selectivity.[34] There is an emerging realization that some inhibitors target the DFG-out pocket of kinases (type II and III inhibitors), which in turn stabilize an enzymatically incompetent kinase conformation. Attention to the develop-ment of chemical biology tools that distinguish between these inhibitor types has produced fluorescent labels in kinases (FliK), an assay that takes advantage of ligand induced conformational change in the glycine rich loop and is used in high throughput screening of small molecule libraries.[14] This assay involves tagging the activation loop with ACRYLODAN (**28**), a solvatochromic, thiol-reactive analog of PRODAN.[20] This fascinating application of a naphthalene-based tool to FliK was our initial inspiration for the design and preparation of a thiol-reactive fluorophore using an IMDDA approach. In addition, we expect that an ACRYLODAN analog with reduced conformational mobility may provide a reporting readout that contributes to an improved or complementary understanding of ligand binding and kinase function.

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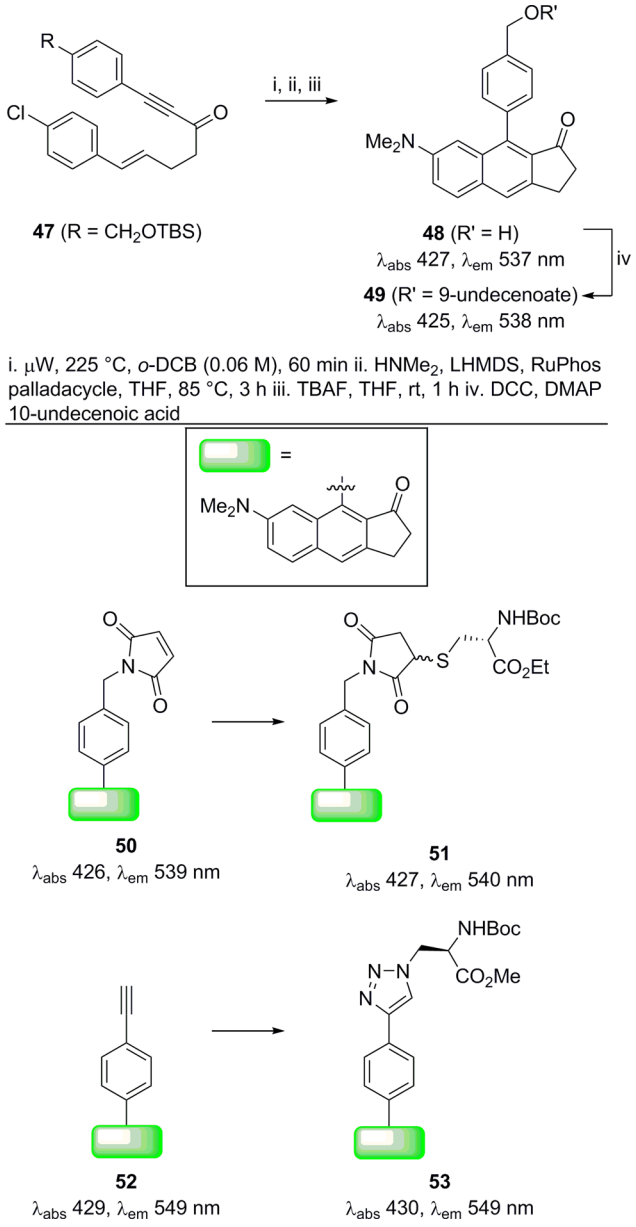
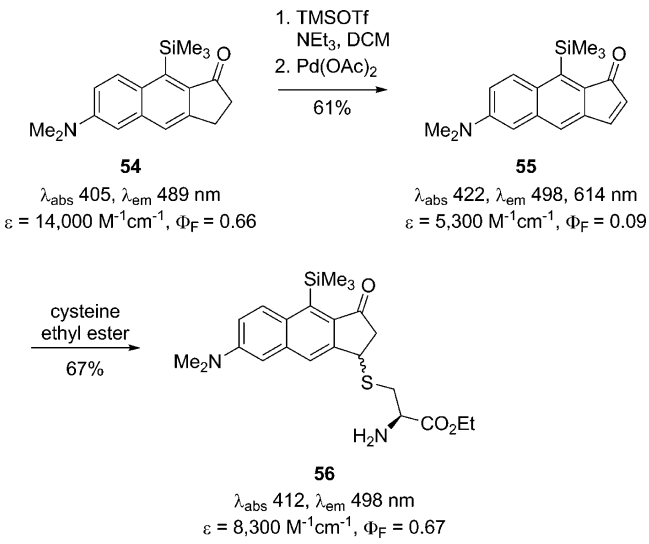
Scheme 5. Attachable Environmentally Sensitive Fluorophores



Scheme 6. Synthesis and Properties of the Fluorogenic Cyclopenta[*b*]naphthalenone 55



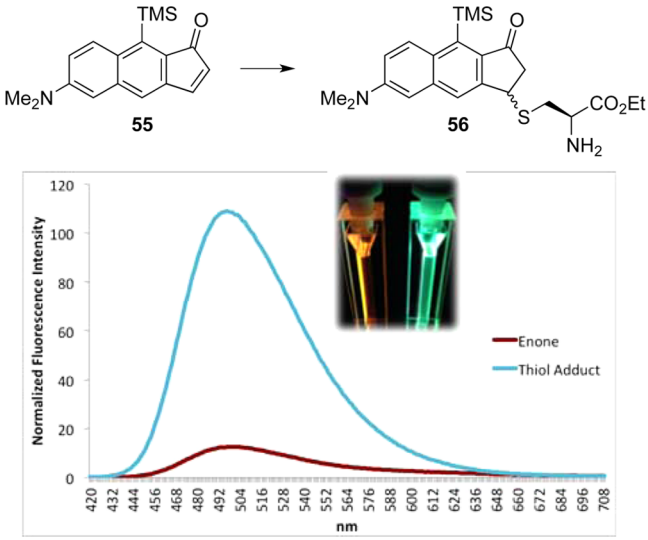


Figure 4. Overlaid emission spectra of the enone **55** (blue) and thiol adduct **56** (red) in CH_2Cl_2.

To this end, cyclopenta[*b*]naphthalanone **54** was converted to cyclopenta[*b*]naphthalenone **55** via a Saegusa oxidation (Scheme 6). Compared with dye **54**, the absorption maxima of dye **55** was only slightly affected by the additional double bond (405–422 nm). However, the molar extinction coefficient and quantum yield for **55** were substantially decreased, rendering dye **55** a potential fluorogenic probe. It should also be mentioned that like ACRYLODAN, enone **55** is a dual-emitter with the emission intensity of the respective peaks at 498 and 614 nm being highly dependent upon solvent and excitation wavelength.[35]

Next, enone **55** was reacted with cysteine ethyl ester, and after 2.5 h, the presence of enone **55** was no longer evident. The thiol adduct was purified, and fluorescence measurements were performed in CH_2Cl_2 (Figure 4). While enone **55** had an absorption maximum at 422 nm, thiol adduct **56** exhibited an absorption maximum at 412 nm, closer to the absorption maximum for ketone **54** (403 nm). An extinction coefficient for adduct **56** was calculated, and the molar absorptivity value was between that of the ketone and enone (8,300 M^{-1} cm^{-1}). The parent enone **55** displayed dual fluorescence, whereas thiol adduct **56** displayed only a single emission maximum at 498 nm, a value similar to the emission maximum for ketone **54** (487 nm). Quantum yield measurements showed that **56** was nearly as efficient as the corresponding ketone (0.66–0.67), which represented a great enhancement in fluorescence intensity compared with parent enone **55**. This enhancement is useful because the fluorescence of the dye–thiol adduct and the free dye is due mostly to the dye–thiol adduct. Thus, the spectral properties of a dye–thiol adduct can be investigated without interference from **55**. Melanie Charpenay performed the biothiol sensor work and has demonstrated that fluorophore **55** can be used to label cysteine-containing proteins. This work will be fully disclosed in the near future.

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CONCLUSION

Our foray into this area was roused by a desire to position our newly synthesized Diels–Alder adducts into existing chemical space at which point their structural likeness to that of PRODAN, a state of the art solvatochromic fluorophore, caught our attention. Venturing more deeply into the literature world of small molecule fluorophores, we emerged from this investigation with an appreciation for the profound impact that these fluorescent compounds have on the understanding of environments and biological processes at the molecular level. Furthermore, we recognized that the rapidly developing technology utilizing these fluorescent probes puts great demands on the performance of these compounds. The question we asked was, "Can we take advantage of the modular synthesis of the IMDDA precursor, the robust IMDDA reaction, and the readily modified products to design and develop solvatochromic fluorophores with enhanced and innovative function?" Preliminary data confirmed that this was possible, by the development of PRODAN analogs that are more fluorogenic in protic media, are more readily attachable to biomolecules, and have absorption and emission spectra that are more red-shifted compared with PRODAN. This approach not only supports further studies to develop smarter, more impactful, and user-friendly fluorescent sensors, but also provides an exceptional venue for expanding the scope and validating the utility of the IMDDA reaction of styrene-ynes.[36,37]

ASSOCIATED CONTENT

Supporting Information

General experimental methods, synthesis of the discussed compounds, absorbance and fluorescence spectroscopy for compounds **54**–**56**, and ^{1}H and ^{13}C NMR spectra. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.accounts.5b00126.

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Notes

The authors declare no competing financial interest.

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