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Discovery of Fluorescent Cyanopyridine and Deazalumazine Dyes Using Small Molecule Macroarrays

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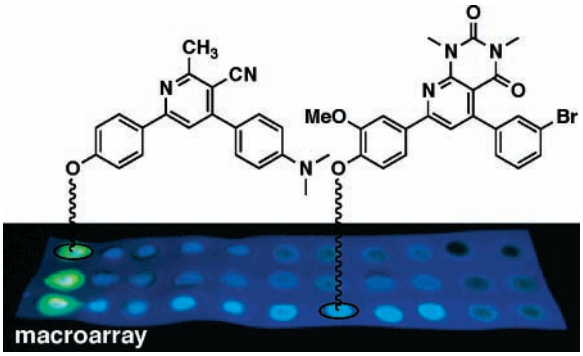
ABSTRACT



Small molecule macroarrays of cyanopyridines and deazalumazines were generated in high purities via spatially addressed synthesis on planar cellulose supports. Examination of the spectral properties of the heterocycles both on and off of the planar support revealed a set of promising new fluorescent dyes that exhibit high quantum yields, low pH dependence, and high sensitivity to solvent polarity.

Fluorescent probes have become essential tools for the investigation of biological systems.[1] Techniques such as fluorescence polarization and fluorescence resonance energy transfer have been used to expand significantly our understanding of biomolecular interactions in vitro and in vivo.[2] Likewise, the emergence of environmentally sensitive fluorophores has enabled real-time imaging of complex cellular processes.[3] As the applications for fluorescent probes continue to increase, so does the need for dyes with diverse spectral and physical properties. To date, however, the design of fluorescent dyes has been largely an empirical process. Combinatorial methods have the potential to dramatically expedite the synthesis of new dye classes, and their application in dye discovery has emerged as an active area of research.[4] These combinatorial syntheses are streamlined further when coupled to rapid screening techniques to identify desirable dye properties. Recently, we developed a combinatorial synthesis platform based on microwave-assisted SPOT-synthesis[5] that generates "small molecule macroarrays".[6,7] We reasoned that the spectral properties of

(1) (a) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906−918. (b) Tsien, R. Y. In *Fluorescent and Photochemical Probes of Dynamic Biochemical Signals Inside Living Cells*; Czarnik, A. W., Ed.; American Chemical Society: Washington, DC, 1993; pp 130−146.

(2) (a) Yan, Y.; Marriott, G. *Curr. Opin. Chem. Biol.* **2003**, *7*, 635−640. (b) Hahn, K.; Toutchkine, A. *Curr. Opin. Cell Biol.* **2002**, *14*, 167−172.

(3) Toutchkine, A.; Kraynov, V.; Hahn, K. *J. Am. Chem. Soc.* **2003**, *125*, 4132−4145 and references therein.

(4) For recent examples, see: (a) Li, Q.; Lee, J.-S.; Ha, C.; Park, C. B.; Yang, G.; Gan, W. B.; Chang, Y.-T. *Angew. Chem., Int. Ed.* **2004**, *43*, 6331−6335. (b) Rosania, G. R.; Lee, J. W.; Ding, L.; Yoon, H.-S.; Chang, Y.-T. *J. Am. Chem. Soc.* **2003**, *125*, 1130−1131. (c) Schiedel, M. S.; Briehn, C. A.; Bauerle, P. *Angew. Chem., Int. Ed.* **2001**, *40*, 4677−4680.

(5) Frank, R. *Tetrahedron* **1992**, *48*, 9217−9232.

(6) Bowman, M. D.; Jeske, R. C.; Blackwell, H. E. *Org. Lett.* **2004**, *6*, 2019−2022.

(7) Lin, Q.; O'Neill, J. C.; Blackwell, H. E. *Org. Lett.* **2005**, *7*, 4455−4458.

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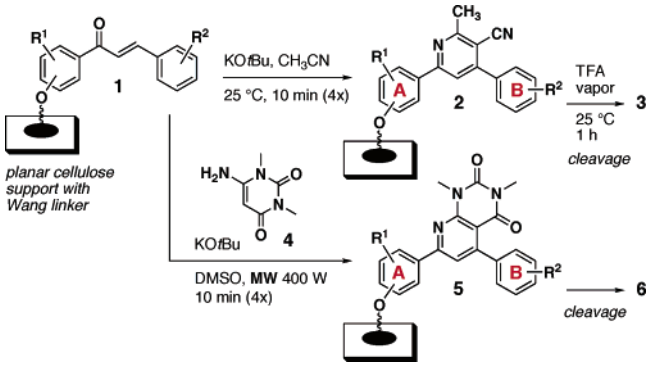
fluorescent dyes could be evaluated post-synthesis on these macroarrays by straightforward visualization techniques. Here, we report the realization of this approach and the identification of a promising set of new cyanopyridine- and deazalumazine-based fluorophores.

We previously reported the synthesis of a library of 1,3-diphenylpropenones, or chalcones (**1**), using our small molecule macroarray platform.[6] In this work, we used planar cellulose (i.e., standard chromatography paper) derivatized with an acid-cleavable, Wang-type linker as our solid-support system.[8] Spatially addressed SPOT-synthesis of chalcones on this support was straightforward and afforded high purity products after cleavage (spot size 0.3 cm^2; loading ca. 100 nmol compound/spot). This chalcone macroarray provided the foundation for the present study, as we found that the chalcones could be transformed readily into a variety of nitrogen-containing heterocycles on this support platform using one-step condensation reactions.[6,9] Numerous members of these heterocyclic classes are fluorescent yet remain largely uncharacterized as dye molecules.[10] We therefore sought to apply our array platform for the systematic synthesis and screening of these compounds.

We selected two structure classes for our initial investigations, cyanopyridines (**2**) and deazalumazines (**5**). Both heterocyclic macroarrays were generated from a 36-member chalcone macroarray **1** that was synthesized as previously reported (Scheme 1).[6,11] The chalcone array (**1**) was designed

Scheme 1. Synthesis of Heterocyclic Macroarrays **2** and **5** from Chalcone Macroarray **1**[a]



[a] For the structures of R^1 and R^2 in **2** and **5**, see Table 1.

to contain a broad range of functionality (at R^1 and R^2) to assist in the derivation of structure−activity relationships (SAR) from both on- and off-support fluorescence screens of the heterocyclic arrays.

Chalcone macroarray **1** was converted easily into cyanopyridine macroarray **2** via treatment with 3-aminocrotononitrile generated in situ from acetonitrile and potassium *tert*-butoxide (Scheme 1). Spotting a presonicated mixture[12] of these reagents (6 μL aliquots) onto the individual spots of chalcone array **1** and allowing the array to sit for 10 min at room temperature (process repeated four times) gave cyanopyridines **3** in good to excellent purity (70−90%; as determined post-cleavage, Table 1).[11] This route was compatible with the range of functionality displayed in the A (R^1) and B (R^2) rings of the initial chalcone array (**1**).

Table 1. Structures, Purity Data, and Spectral Properties of Selected Cyanopyridines **3** and Deazalumazines **6**[a]

entry	R^1	R^2	purity (%)[b]	λ_{ex} (nm)	λ_{em} (nm)	ϕ_f[c]
3a	4-OH	H	81	341	433	0.07
3b	4-OH	2-F	81	342	430	0.06
3c	4-OH	4-F	83	341	430	0.07
3d	4-OH	4-Br	82	342	440	0.04
3e	**4-OH**	**4-OMe**	**70**	**339**	**423**	**0.12**
3f	**4-OH**	**4-NMe_2**	**74**	**366**	**524**	**0.09**
3g	3-OMe, 4-OH	H	74	351	469	0.03
3h	3-OMe, 4-OH	3-OMe	89	351	473	0.02
3i	3-OMe, 4-OH	4-F	71	351	466	0.04
3j	3-OMe, 4-OH	4-Br	70	352	475	0.02
3k	3-OMe, 4-OH	4-OMe	78	351	461	0.06
3l	3-OMe, 4-OH	4-NMe_2	87	348	533	0.09
3m	3-OH	4-OMe	84	326	420	0.01
3n[d]	**4-OMe**	**2-F**	**>95**	**336**	**419**	**0.71**
3o[d]	**4-OMe**	**4-OMe**	**>95**	**335**	**409**	**0.77**
3p[d]	3-OMe	4-OMe	>95	306	417	0.38
3q[d]	**3, 4-OMe**	**4-OMe**	**>95**	**344**	**448**	**0.74**
6a	4-OH	H	77	362	446	0.15
6b	4-OH	2-F	79	363	443	0.18
6c	**4-OH**	**4-F**	**78**	**364**	**445**	**0.19**
6d	4-OH	4-Br	78	364	450	0.09
6e	4-OH	4-OMe	82	365	438	0.03
6g	3-OMe, 4-OH	H	82	368	480	0.06
6h	3-OMe, 4-OH	3-OMe	78	370	482	0.06
6i	3-OMe, 4-OH	4-F	76	370	483	0.07
6j	3-OMe, 4-OH	4-Br	76	372	495	0.05
6k	3-OMe, 4-OH	4-OMe	77	372	474	0.06
6r	3-OH	4-F	87	367	441	0.05
6s	3-OH	4-Cl	84	356	440	0.01
6t[d]	**4-OMe**	**4-F**	**>95**	**360**	**424**	**0.19**

[a] Data recorded from crude samples after cleavage from the macroarray. [b] Determined by integration of HPLC traces with UV detection at 254 nm. [c] Relative quantum yields measured in ethanol. External standard: coumarin 120 (ϕ_f = 0.88, λ_{ex} = 354 nm, λ_{em} = 435 nm in ethanol). Error = ±15%. [d] Synthesized in solution. See text and ref 11.

The deazalumazine macroarray (**5**) was constructed in like fashion to the cyanopyridine array (**2**) by application of a solution of aminouracil (**4**) and potassium *tert*-butoxide onto individual spots of chalcone array **1** (Scheme 1). However, in this case, allowing the spotted membrane to stand at room temperature for 10 min (and repeating the procedure four times) gave only 10−15% conversion to deazalumazine products (**5**). As we have found that microwave (MW)

(8) Whatman 1Chr chromatography paper is our standard planar support for macroarray synthesis (thickness = 0.34 mm; cost ~ 0.5¢/cm^2).

(9) Powers, D. G.; Casebier, D. S.; Fokas, D.; Ryan, W. J.; Troth, J. R.; Coffen, D. L. *Tetrahedron* **1998**, *54*, 4085−4096.

(10) Matsui, M.; Oji, A.; Hiramatsu, K.; Shibata, K.; Muramatsu, H. *J. Chem. Soc., Perkin Trans. 2* **1992**, 201−206.

(11) Full experimental details, compound characterization methods, and analytical data for all compounds can be found in Supporting Information.

(12) Marzinzik, A. L.; Felder, E. R. *J. Org. Chem.* **1998**, *63*, 723−727.

heating can dramatically accelerate reactions on planar cellulose supports,[6,7,13] we examined these conditions for deazalumazine macroarray (**5**) generation. By spotting the array with reagents and subjecting the array to MW heating (400 W, 10 min) four times in succession,[14] complete conversions to deazalumazines **6** were obtained. Product purities for **6** ranged from 70% to 90% (as determined post-cleavage; Table 1) with only one exception: chalcones (**1**) with *p*-dimethylamino substituents in the B ring (R^2 = 4-NMe_2) were found to be unreactive. These precursors could not be converted to deazalumazines under a wide range of MW-assisted conditions; we speculate that the increased electron density of these chalcones (**1**) is inhibiting nucleophilic attack on the α,β-unsaturated system.

Prior to compound cleavage, we evaluated the fluorescent properties of the cyanopyridines (**2**) and deazalumazines (**5**) in the macroarray format. We found that simple visual inspection of the arrays under irradiation from a handheld UV lamp (at 254 nm)[15] provided a straightforward primary screen to determine if the compounds were fluorescent (Figure 1). In some cases, this visual assay also facilitated

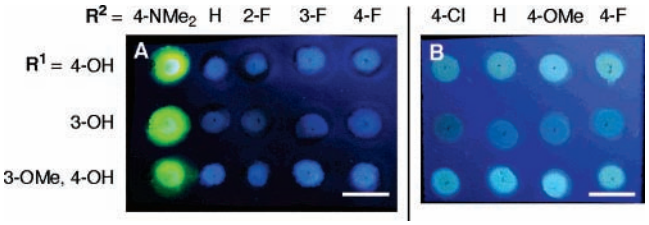


Figure 1. Subsections of (A) cyanopyridine macroarray **2** and (B) deazalumazine macroarray **5** irradiated at 254 nm using a handheld UV lamp.[15] Graphics were obtained with a digital camera and edited in AdobePhotoshop.[11] Scale: white line = 1 cm.

qualitative SAR to be derived across the entire macroarray.

Analysis of the irradiated cyanopyridine array **2** revealed two important SAR trends (Figure 1A). First, the presence of a *m*-hydroxyl group on the A ring (R^1) of cyanopyridines **2** (middle row) caused a modest reduction in fluorescence intensity relative to the para position (top row). Second, and more strikingly, a *p*-dimethylamino substituent on the B ring (R^2) caused a shift from blue to bright yellow-green fluorescence for the cyanopyridines **2** (far left column). For the deazalumazine macroarray **5**, we observed a similar diminution in fluorescence intensity when the A ring (R^1) possessed a *m*-hydroxyl substituent (Figure 1B). Contribution of the *p*-dimethylamino groups could not be assessed, however, due to the inaccessibility of these deazalumazines via our synthetic method (see above).

To facilitate more quantitative analysis of these fluorophores, the array members were each punched-out of the support, cleaved using trifluoroacetic acid (TFA) vapor (Scheme 1), and evaluated in solution.[11] We found that the quantity of cyanopyridine **3** or deazalumazine **6** obtained from a single spot (~100 nmol) was sufficient to determine excitation and emission spectra, as well as relative quantum yields (Table 1). Further, the good to high compound purities enabled the examination of these materials post-cleavage without further purification steps.

Overall, our quantitative SAR study largely correlated with that determined qualitatively under the UV lamp. The deazalumazines (**6**) demonstrated markedly higher quantum yields than the cyanopyridines (**3**) (Table 1). As we had observed visually, the presence of a hydroxyl or other electron donating group in the para position of the A ring greatly enhanced fluorescence in both dye classes (**3a**−**l** and **6a**−**k**), whereas the deazalumazines and cyanopyridines that contained only a *m*-hydroxyl group (**3m** and **6s**) had very low quantum yields (<0.01). Incorporation of additional electron-donating groups on the A ring of either compound class produced higher wavelength emissions but concomitant reductions in quantum yields.

Most alterations to the B ring on either **3** or **6** had little effect on their emission spectra (Table 1). However, addition of a *p*-dimethylamino substituent to the cyanopyridines (**3**) had a profound effect on their spectral characteristics. Fluorescence spectra of **3f** and **3l** displayed significant red shifts (91 and 64 nm, respectively) compared to the other cyanopyridines (**3**) studied. Again, these data correlated with our initial visual screen of macroarray **2** (Figure 1A). Finally, both the cyanopyridines (**3**) and deazalumazines (**6**) exhibited uniformly large Stokes shifts (90−120 nm).[16] Large Stokes shifts are highly desirable in fluorescent dyes because they minimize reabsorption of emitted light by the dye itself.[17] This property suggests the potential utility of cyanopyridines and deazalumazines as dye molecules.

For further analysis of these two dye classes, three representative compounds (**3e**, **3f**, and **6c**) were synthesized on a larger scale in solution.[11] As expected, their spectral characteristics closely matched those generated using the macroarray format. We next investigated the pH dependence of these spectral properties. Buffered ethanolic solutions of **3e**, **3f**, and **6c** were studied over a wide pH range (1.6−10), and the excitation and emission wavelengths were found to be constant over this range. While the quantum yields of **3e** and **6c** were nearly constant from low to neutral pH, the quantum yield of **3f** varied, albeit only slightly, increasing from 0.06 at pH 1.6 to 0.09 at pH 7.6. At pH > 7.6, the quantum yields of all three fluorophores began to diminish.

We reasoned that the reduction in quantum yield at higher pH was due to deprotonation of the phenols in **3e**, **3f**, and

(13) MW heating is seeing increasing use in solid-phase combinatorial synthesis: (a) Blackwell, H. E. *Org. Biomol. Chem.* **2003**, *1*, 1251−1255. (b) Kappe, C. O.; Dallinger, D. *Nat. Rev. Drug Discov.* **2006**, *5*, 51−63.

(14) All MW-assisted reactions were performed in a Milestone MicroSYNTH Labstation multimodal MW reactor using power (wattage) control. Temperature control was not possible due to the low solvent volumes used (i.e., microliter scale). See Supporting Information for full details.

(15) C. Entela Mineralight Lamp; model UVGL-58.

(16) Fluorescein, one of the most commonly used fluorophores, has a Stokes shift of 19 nm. For a recent report of fluorescein derivatives, see: Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 4888−4894.

(17) (a) Jameson, D. M.; Croney, J. C.; Moens, P. D. *Methods Enzymol.* **2003**, *360*, 1−43. (b) Lacowicz, J. R. *Principles of Fluorescence Spectroscopy*; Kluwer Academic/Plenum Publishers: New York, 1999.

6c. Therefore, we investigated the effects of replacing the hydroxyl group on the A ring in these compounds with a methoxy group. Methoxy analogs **3n**, **3o**, **3q**, and **6t** were synthesized and evaluated (Table 1). Gratifyingly, the quantum yields of these dyes were found to be totally independent of pH. More notable, however, was that the quantum yields for the cyanopyridines **3n**, **3o**, and **3q** had *increased by 5- to 10-fold* relative to their parent hydroxyl cyanopyridines. These quantum yields are significant as they are now comparable to widely utilized dyes based on coumarin[18] and fluorescein.[16]

To further explore the utility of cyanopyridines (**3**) and deazalumazines (**6**) as dyes, we examined their sensitivity to solvent polarity. The spectral characteristics of several compounds were evaluated in a range of solvents, and compounds **3e**, **3f**, and **6c** were found to display unique properties (Table 2).[19] Emission spectra for all three compounds underwent a significant bathochromic shift in fluorescence wavelength as solvent polarity increased (e.g., spectra for dimethylamino cyanopyridine **3f** shown in Figure 2). The quantum yield of **6c** also increased with increasing solvent polarity. However, the quantum yield trend for the cyanopyridines **3e** and **3f** was reversed and significantly more pronounced. For example, the quantum yields of **3e** and **3f** increased by at least 4-fold in THF over those in ethanol.

Table 2. Influence of Solvent on Dye Spectral Properties

entry	solvent	λ_{ex} (nm)	λ_{em} (nm)	ϕ_f[a]
3e	THF	339	405	0.66
3e	$CHCl_3$	333	404	0.58
3e	DMSO	344	440	0.31
3e	EtOH	341	424	0.15
3f	THF	373	486	0.53
3f	$CHCl_3$	372	471	0.53
3f	DMSO	379	538	0.17
3f	EtOH	379	526	0.10
6c	THF	361	417	0.12
6c	$CHCl_3$	361	417	0.13
6c	DMSO	363	454	0.23
6c	EtOH	363	442	0.23

[a] Relative quantum yields. External standard: coumarin 120 ($\phi_f = 0.88$, $\lambda_{ex} = 354$ nm, $\lambda_{em} = 435$ nm in ethanol). Error = ±10%.

The dramatic spectral changes for cyanopyridine **3f** in different solvents could actually be observed visually (Figure 2), even in solvents with similar polarities (e.g., $CHCl_3$ and THF). Dialkylamino groups have previously been found to engender environmental sensitivity in numerous probe molecules.[20] The sensitivity of cyanopyridine **3f** to the polarity of its environment, coupled with its pH-independent behavior, make it an attractive candidate for further development as a new bioprobe.[21]

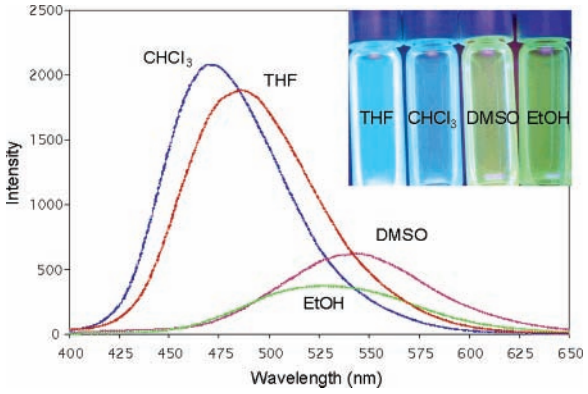


Figure 2. Emission spectra of cyanopyridine **3f** irradiated at 340 nm in various solvents. Top inset: solutions of **3f** (1 μM) in various solvents irradiated at 366 nm using a handheld UV lamp.

In summary, we have constructed macroarrays of heterocyclic fluorophores via efficient, spatially addressed synthesis on planar supports. The quantity of product generated on the array (~100 nmol) was found to be sufficient for full spectral characterization. By screening both on and off of the support, we rapidly identified a set of lead compounds with desirable fluorescent properties. Further solution-phase analysis and simple structural modifications of these initial leads afforded a set of dyes with promising solvatofluorochromic properties (**3f**) and exceptionally high quantum yields (**3n**, **3o**, and **3q**). These results underscore the value of small molecule macroarrays for fluorescent dye discovery and development. Ongoing work in our laboratory is directed at the further derivatization of the cyanopyridine dye scaffolds to improve their aqueous solubility and facilitate their attachment to biomolecules.

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Supporting Information Available: Full experimental details for macroarray construction, solution-phase synthesis of model compounds, and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) Katerinopoulos, H. E. *Curr. Pharm. Des.* **2004**, *10*, 3835−3852.
(19) See Supporting Information for full panel of compounds tested.
(20) Weber, G.; Farris, F. J. *Biochemistry* **1979**, *18*, 3075−3078.
(21) For a recent example of a fluorescent polarity probe used to study proteins, see: Cohen, B. E.; Pralle, A.; Yao, X.; Swaminath, G.; Gandhi, C. S.; Jan, Y. N.; Kobilka, B. K.; Isacoff, E. Y.; Jan, L. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 965−970.