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Combinatorial discovery of novel fluorescent dyes based on Dapoxyl™

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Abstract—We have developed a combinatorial method for the fast and effective synthesis of a library based on a known fluorescent dye, Dapoxyl™ using parallel solution-phase chemistry. By screening the 140 new compounds using fluorescence-based assays, we identified three new fluorescent dyes with novel properties.

Fluorescent dyes are widely used in the detection, quantitation, identification and characterization of inorganic and organic compounds, and of biological structures and processes.[1] Recently, the use of fluorescent dyes to label biologically important molecules such as DNA, proteins, drugs etc. has grown markedly, particularly as a result of advances in biotechnology.[2] Little, however, is known regarding how subtle structural changes in a dye give rise to profound effects in its fluorescence properties, and in many cases these effects are less than trivial to predict. Until now,[3] most research directed towards the discovery of new fluorescent dyes has been limited to the synthesis of individual compounds and studies of their fluorescence properties, one compound at a time.[4] This, as a result, has greatly hindered the rapid development of novel fluorescent dyes. Dapoxyl™ dyes (2-pyridyl-5-aryl-oxazoles) and analogues are commercially available and are used widely as pH indicators and molecular probes.[4] They have attracted our attention not only because of their environment-dependent sensitivity, but also their unique properties which allows for their selective accumulation in acidic organs such as lysosomes.[5]

Over the last 15 years, combinatorial chemistry has grown to become one of the most powerful tools used in the areas of drug discovery, solid-state materials, homogenous and heterogeneous catalysts, polymeric materials and so on.[6] Combinatorial approaches, based on both solid-phase and solution-phase chemistry may be readily adaptable to automation, representing a cost-effective tool in the field of preparative chemistry, which increases throughput and reagent diversity.[7] In this paper, we describe the development of a fast, parallel synthesis of fluorescent dyes based on the core structure of Dapoxyl™. By combining the ability of combinatorial chemistry to manipulate substituted groups on the dye efficiently, together with available instrumentation for high-throughput, fluorescence-based screening, we hoped to identify new dyes that possess novel fluorescence properties such as a large Stokes shift, high quantum yields and favorable pH and solvent profiles.

The synthesis of the dye library is shown in Fig. 1. First, individual analogues of nicotinic acid **A** (27 in total) were reacted with individual analogues of 2-aminoacetophenone **B** (nine in total; Fig. 1) in the presence of 1,3-diisopropylcarbodiimide (DIC), using parallel solution-phase chemistry, to generate amides **C**, which, without further purification, were first evaporated to dryness, then concentrated sulfuric acid was added.[8] The mixture was stirred overnight to afford **D**. Purification of **D** was achieved by addition of ice water and 25% ammonia solution to the crude product, and the resulting aqueous solution was extracted with ethyl acetate. The organic layer was washed with sat. NaCl and evaporated to give the final product **D** in 40–80% purity for subsequent fluorescence screenings. In all, with this solution-phased parallel approach, a total of 140 new compounds of type **D** were successfully synthesized (as judged by TLC of the products and their fluorescence spectra), of which more than 20% were

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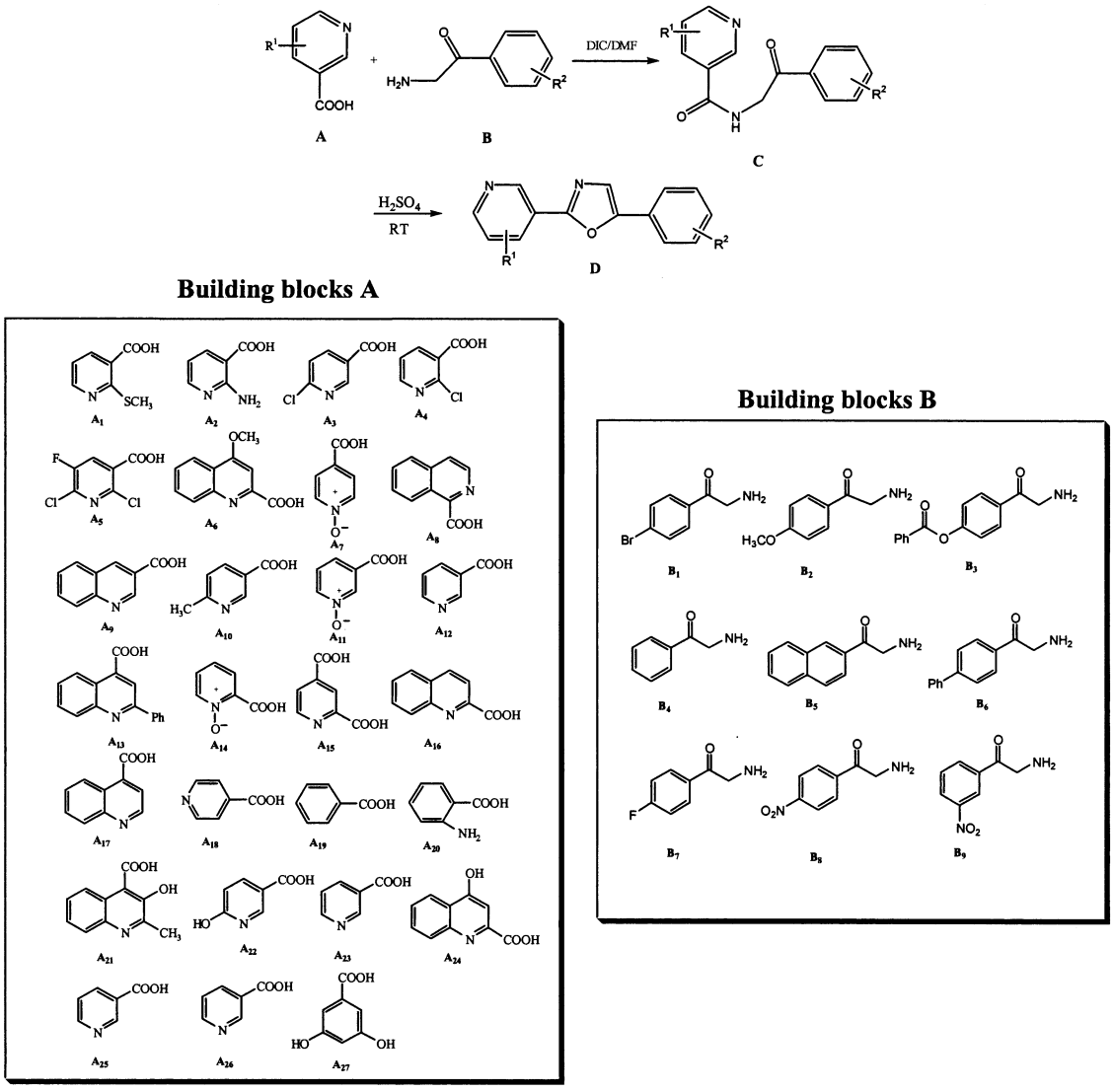

Figure 1. Synthesis of the Dapoxyl™ library with building blocks **A** and **B**.

randomly chosen and further characterized with LC-MS to confirm unambiguously their purity and identity (Table 1).

Synthesis of some of the compounds (listed in Table 1) was either not attempted or attempted unsuccessfully, probably because most of them contain competing functional groups (-COOH or -NH_2) that interfere with the regiospecific coupling between **A** and **B**. Other groups, such as -OH in $\mathbf{A_{24}}$, were more tolerable and thus in some cases required no protection in order to carry out the condensation and the subsequent cyclization step to generate the desired products (e.g. $\mathbf{A_{24}B_4}$). Since our approach was to develop a simple and efficient synthetic route for rapid access to a large number of new compounds for subsequent fluorescence screening, no attempt was made to introduce appropriate protecting groups in some of the building blocks or their corresponding intermediates.

All 140 compounds were screened for desired fluorescence properties. The use of a fluorescence-based microplate reader made it simple to carry out the screening in a 96-well format in a rapid yet automated fashion. Both excitation and emission spectra of the compounds in different solvents under different pH conditions (e.g. DMF, chloroform, methanol:water (1:1), methanol and pH 4, 7, 10 buffer solutions[9]) were recorded. The determined excitation maxima ($\lambda_{ex}=270$–380 nm) and the emission maxima ($\lambda_{em}=380$–480 nm) covered a wide range of values. To our surprise, replacement of an aromatic ring in the dye structure with fused rings (i.e. $\mathbf{A_{8,9,13}B_{1-9}}$) did not lead to drastic red/blue shifts of excitation/emission wavelengths, which have often been observed in other dyes, further indicating the unpredictable effect of different substituents on the dye structure on its fluorescence properties. Out of all compounds screened, we identified three compounds ($\mathbf{A_2B_1}$, $\mathbf{A_2B_7}$, $\mathbf{A_{18}B_7}$; Fig. 2(a)) which have unique fluorescence properties. They all have large Stokes shifts and show strong fluorescence in aqueous buffers under different pH conditions, and in the presence of organic solvents where most other compounds have little or no fluorescence. In order to confirm this

Table 1. Analogues of Dapoxyl™ synthesized

	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}	A_{12}	A_{13}	A_{14}
B_1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
B_2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
B_3	✓	x	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
B_4	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
B_5	✓	x	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
B_6	✓	x	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
B_7	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
B_8	x	✓	x	x	x	x	x	x	x	x	x	x	x	x
B_9	x	✓	x	x	x	x	x	x	x	x	x	x	x	x

	A_{15}	A_{16}	A_{17}	A_{18}	A_{19}	A_{20}	A_{21}	A_{22}	A_{23}	A_{24}	A_{25}	A_{26}	A_{27}
B_1	✓	✓	✓	✓	x	x	✓	x	x	x	x	x	x
B_2	✓	✓	✓	✓	x	x	✓	x	x	x	x	x	x
B_3	✓	✓	✓	✓	x	x	x	x	x	x	x	x	x
B_4	✓	✓	✓	✓	x	✓	x	✓	✓	✓	✓	✓	✓
B_5	✓	✓	✓	✓	x	x	x	x	x	x	x	x	x
B_6	✓	✓	✓	✓	x	x	x	x	x	x	x	x	x
B_7	✓	✓	✓	✓	x	x	✓	x	x	x	x	x	x
B_8	x	x	x	x	✓	x	x	x	x	x	x	x	x
B_9	x	x	x	x	✓	x	x	x	x	x	x	x	x

✓: Synthesized successfully (as judged by TLC and fluorescence); x: not synthesized or attempted unsuccessfully.

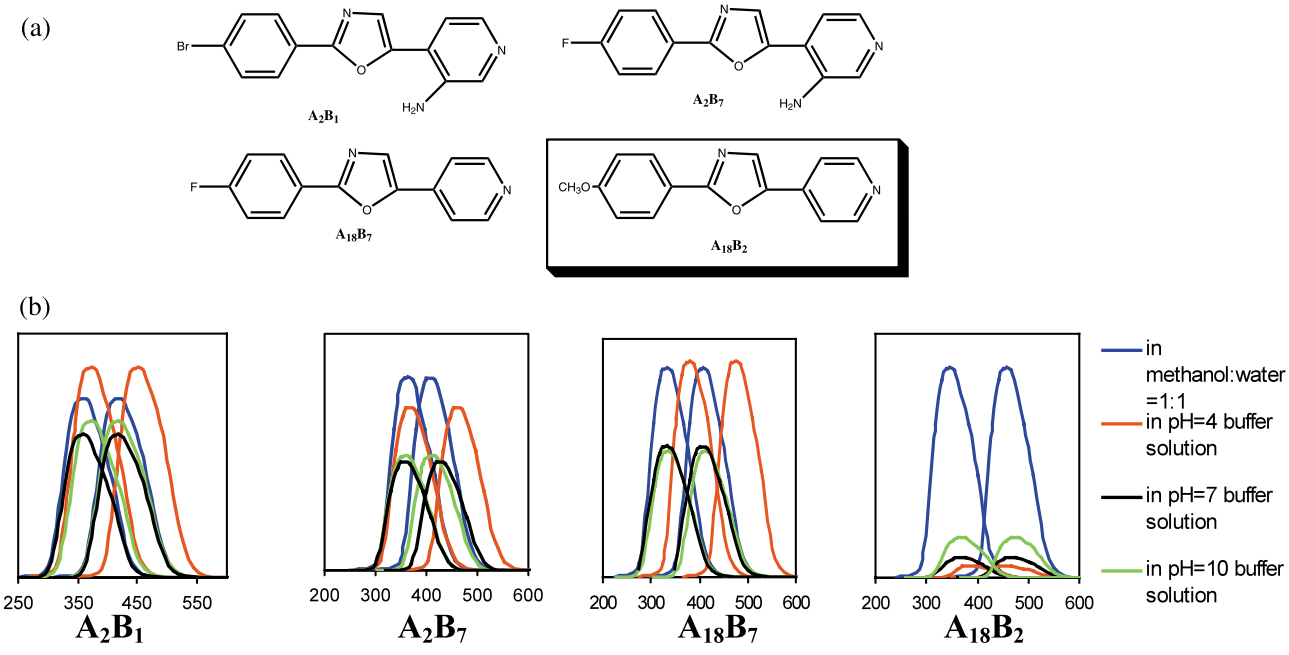

Figure 2. (a) Structures of selected dyes; (b) spectra of the dyes. Each graph represents spectra of a dye under different conditions (shown in different colors), where both excitation (left spectrum) and emission (right spectrum) under the same conditions, are represented with the same color.

unambiguously, we subsequently synthesized these three compounds individually, purified them to homogeneity (by HPLC) and further characterized their fluorescence properties with a conventional fluorimeter. For comparison, compound $\mathbf{A_{18}B_2}$, which does not show the same pH- and solvent-independent fluorescence profiles from our screenings, was also synthesized.

As shown in Fig. 2(b) and Table 2, $\mathbf{A_2B_1}$, $\mathbf{A_2B_7}$ and $\mathbf{A_{18}B_7}$ all show strong fluorescence under different pH conditions in aqueous solutions and in the presence of organic solvents such as 50% methanol, in good agreement with results obtained from the high-throughput, 96-well-format screening, further validating both the synthetic and the 96-well screening strategies we have adopted. It is worth noting that all three compounds contain halogen groups on their aromatic rings. It had previously been demonstrated that halogen substitution affects the pH profile of some known fluorescent dyes.[1] The reference compound, $\mathbf{A_{18}B_2}$ on the other hand, shows strong pH-

Table 2. Fluorescence properties of selected dyes in different solutions[9]

	Methanol:water (1:1)[a]			pH 4		pH 7		pH 10	
	ε_0 ($\times 10^{-3}$)	$\lambda_{ex}/_{em}$ (nm)	φ_f	$\lambda_{ex}/_{em}$ (nm)	φ_f	$\lambda_{ex}/_{em}$ (nm)		$\lambda_{ex}/_{em}$ (nm)	φ_f
$\mathbf{A_2B_1}$	20.3	350/410	0.63	364/444	0.66	350/410	0.50	354/410	0.55
$\mathbf{A_2B_7}$	28.4	352/400	0.88	360/453	0.74	350/400	0.49	350/420	0.52
$\mathbf{A_{18}B_7}$	23.9	325/400	0.74	373/477	0.60	325/400	0.59	328/406	0.39
$\mathbf{A_{18}B_2}$[b]	**23.9**	**339/450**	**0.74**	**380/450**	**0.04**	**360/460**	**0.07**	**340/466**	**0.14**

[a] The quantum yields were determined using 2-pyridyl-5-phenyloxazole in methanol: water (1:1) as the reference standard ($\varphi_f = 0.88$).
[b] An example of a dye screened which does not have the desired fluorescence profile.

as well as solvent-dependent fluorescence profiles, correlating well with those generated from microplate screenings. Quantum yields, as well as ε_0 of the compounds were also determined and showed similar values to that of the original Dapoxyl™.

In conclusion, we have developed a new method for fast and effective synthesis of a fluorescent dye library based on the known dye Dapoxyl™, using parallel solution-phase chemistry. By combining this method with high-throughput fluorescence screening, we have identified three new dyes that possess novel fluorescence properties. We are currently assessing other dyes in this library for novel fluorescence properties. This approach may be general and applicable to the screening of other fluorescent dye libraries.

Acknowledgements

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8. Representative example of dye synthesis. Synthesis of 2-pyridyl-5-(4-fluorophenyl)oxazole ($\mathbf{A_{18}B_7}$): Isonicotinic acid $\mathbf{A_{18}}$ (0.02 mmol) and 2-amino-4′-fluoroacetophenone $\mathbf{B_7}$ (0.02 mmol) were dissolved in 0.5 mL DMF, and 50 μL DIC was added to the solution. After 1 h, the reaction was filtered and evaporated under vacuum. Concentrated sulfuric acid (0.5 mL) was added and the reaction was stirred overnight before quenching with 5 mL of ice water and 2 mL of 25% ammonia. The product was extracted into 30 mL of ethyl acetate, followed by washing with a sat. NaCl solution, and dried with anhyd. $MgSO_4$. Evaporation in vacuo gave the crude product with purity typically ranging from 40 to 80%. Further purification and characterization were performed on an LCQ-MAT LC-MS (Finnigan, USA). HPLC was performed using Phenomenex RP-18 (analytic: 5 μm, 4.6×250 mm; semi-preparative: 5 μm, 10×250 mm) columns with an acetonitrile–water gradient (with 0.1% TFA). Fluorescence was recorded using a SpectraMAX™ Gemini XS fluorescence plate reader (Molecular Devices, USA). Mp 152–153°C, ^{1}H NMR (300 MHz, $CDCl_3$, 25°C): $\delta = 8.78$ (broad s, 2H), 7.96 (broad s, 2H), 7.73 (m, 2H), 7.47 (s, 1H), 7.18 (m, 2H). ESI-MS: m/z (%): 241.3 (100) [M^++1].
9. Compounds were dissolved in an organic solvent (MeOH or DMSO). A drop of the solution was added to 2 mL of freshly prepared aqueous buffer solutions (Thomas Scientific, USA). The amount of organic solvent was estimated to be <0.5% of the total volume.