Combinatorial Dapoxyl Dye Library and its Application to Site Selective Probe for Human Serum Albumin

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The combinatorial fluorescent dapoxyl dye library was prepared by both solution- and solid-phase synthesis, generating 80 unique dapoxyl derivatives. A fluorescence-based screening toward human serum albumin (HSA) found one highly sensitive HSA binder (**A41-S**) with over 55-fold intensity change. Displacement assay showed the selective binding of **A41-S** to the site I of HSA, addressing its potential to be a highly selective and sensitive HSA probe.

Introduction

Fluorescent compounds have been excellent tools for the sensitive and specific detection of diverse bioanalytes.[1] While the conventional target-oriented rational approach was successful toward diverse analytes, a combinatorial library approach on fluorescent dyes has demonstrated its unique advantages: it generated a wide range of spectral and structural diversity of fluorescent compounds that allowed for the sensitive detection of various bioanalytes such as DNA, RNA, β-amyloid protein, and organelles.[2] With success of the initial styryl fluorophore library by our group, we are expanding this combinatorial approach to different fluorophores for sensor development.[3]

Dapoxyl dyes are highly environment-sensitive fluorescent compounds.[4] Their fluorescence in water is very low, but induces a large change of fluorescence intensity, Stokes shift, and extinction coefficients depending on pH and polarity of solvents.[5] This environment-sensitive property of dapoxyl dyes has been extensively utilized for detection of different organelles including lysosome[6] and ER,[5] and of conformational and phosphorylation change of the proteins.[7] We have applied a combinatorial library approach on dapoxyl scaffold to generate the potential fluorescent sensor candidates which may induce a large fluorescence change by an analyte environment. Eighty unique structures of dapoxyl dye library were prepared by both solution- and solid-phase synthesis. A fluorescence-based high-throughput screening of dapoxyl library has identified one library member (**A41-S**) highly sensitive toward human serum albumin (HSA).

HSA is the major protein constituent of blood plasma (60% of total plasma proteins) and it is known to bind a number of endogenous compounds, thus facilitating their transport in the circulation.[8] More importantly, it binds to a diverse range of exogenous drugs, resulting in significant impact on the pharmacokinetics of drugs.[8] The affinity of a particular drug to HSA determines the concentration of free drug that diffuse from the circulation to sites of action. Therefore, drug–HSA binding is one of the important factors to consider in drug design and there is a growing effort to determine this pharmacokinetic property at the early stage of the drug discovery process.[9]

The remarkably broad range of ligand bindings, especially for neutral and negatively charged hydrophobic compounds, mainly originates from two primary binding sites (sites I and II).[10] An additional drug binding site (site III) and multiple binding sites for fatty acids also contribute to a diverse binding capability of HSA.[11] Competitive displacement of fluorescent probes has been used for the efficient determination of the binding site and affinity of drugs to HSA.[12] Although DNSA (dansylamide, selectively binding to site I) and DS (dansylsarcosine, specifically binding to site II) exhibit a large fluorescence increase upon binding to HSA and thus have been widely used for displacement assays,[10,12] higher sensitivity of fluorescent probes would increase the efficiency for determination of HSA binding drugs. Here we report a dapoxyl compound (**A41-S**) that selectively binds to site I of HSA with a high sensitivity of over ca. 55-fold fluorescence intensity change, addressing its potential to be a highly selective and sensitive HSA probe.

Results and Discussion

Synthesis of Dapoxyl Dye Library. Preparation of dapoxyl dyes was previously reported in the literature.[13–16] Mostly, two-step reactions, the synthesis of acylamino ketone and the following cyclodehydration, generate the oxazole structure, the scaffold of dapoxyl dyes (Scheme 1).[13] In particular, the second cyclodehydration step elicited more interest since its success determines functional groups accessible in the final oxazole structure. Classically, a relatively harsh coupling reagent such as sulfuric acid has been used for the cyclodehydration, known as the Robinson–Gabriel reaction.[14] Later, several milder reagents[15] including Burgess reagent[13] or Ph_3PCl_2[16] were developed for the preparation of oxazole-containing natural product.

Initially we began to construct the dapoxyl dye library by the above two-step reactions (solution-phase synthesis, Scheme 1). First, six derivatives of 2-aminoacetophenone

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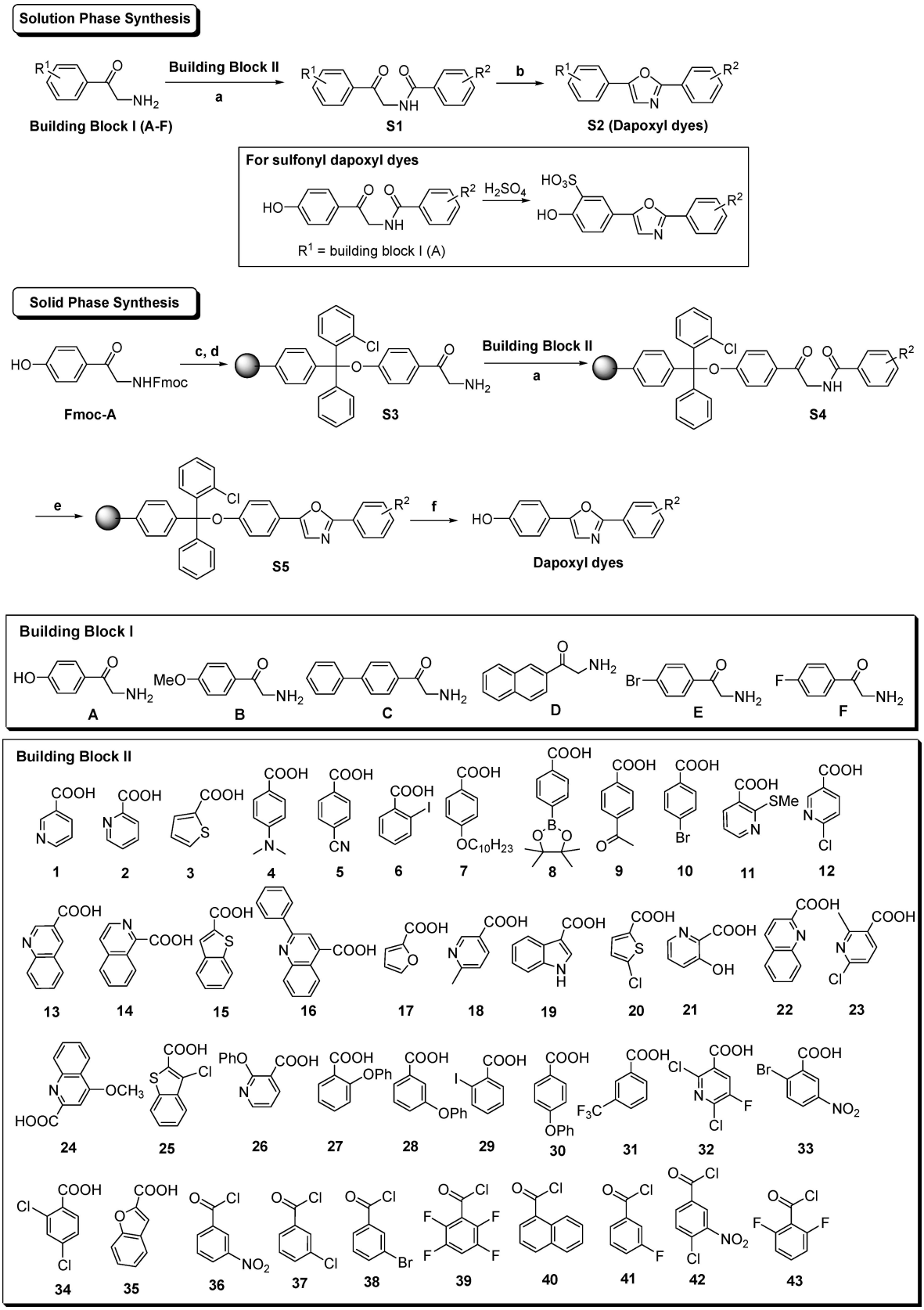
Scheme 1. Solution- and solid-phase synthesis generating dapoxyl dye library and building blocks: (a) HATU, DIEA, CH_2Cl_2 for **1–35** or pyr, CH_2Cl_2 for **36–43**; (b) Burgess reagent, microwave; (c) 2-chlorotrityl chloride resin, DIEA, THF; (d) 10% piperidine, DMF; (e) Ph_3PCl_2, TEA, CH_2Cl_2; and (f) 1% TFA, CH_2Cl_2.



(building block I) and 43 derivatives of benzoic acid/chloride (building block II) were selected for generating the acylamino ketone derivatives (**S1**). The coupling reaction of building block I and II was typically carried out under HATU/DIEA or pyridine condition, giving relatively good yields. Second, the cyclodehydration step was carried out with a neat sulfuric

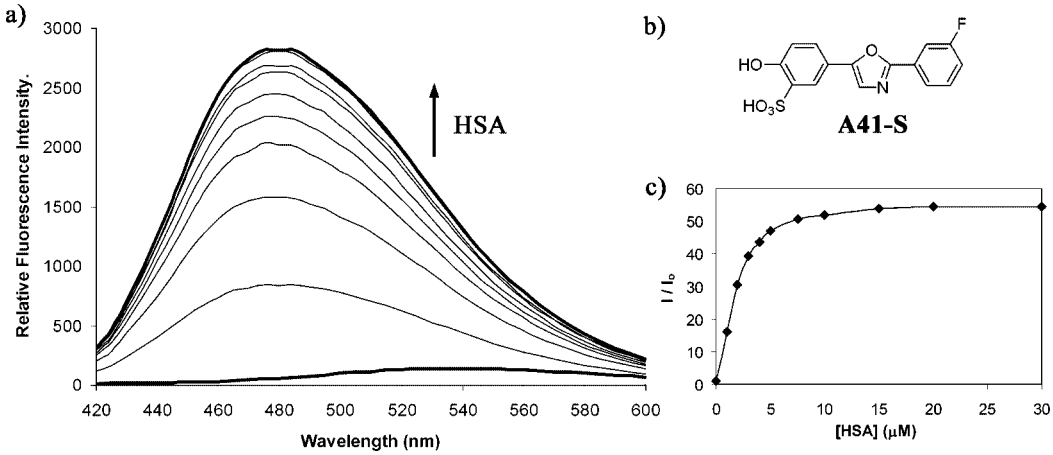


Figure 1. Fluorescence response of **A41-S** to HSA: (a) **A41-S** (5 μM) with HSA at 0, 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 30 μM; spectra were taken in 50 mM HEPES, pH 7.4, with excitation 360 nm; (b) structure of **A41-S**; (c) the corresponding intensity changes, I/I_0, are the ratio of intensity of **A41-S** with/without HSA.

acid or Burgess reagent with a microwave. In particular, the latter one was preferred since it significantly reduced the reaction time ($\sim$ 10 min) and generated the desired oxazole structures predominantly. On the other hand, sulfuric acid was useful *in situ* introducing additional functionality, sulfonic acid, on a dye. It was found that a building block I (**A**),[20] which has a *para*-phenolic OH, gave the addition of sulfonic acid on *ortho* position of OH when carrying out the cyclodehydration with sulfuric acid. Forty different dapoxyl dyes were individually prepared and purified by this solution phase synthesis route (see Supporting Information (SI), Table S1).

Next, we sought to utilize the solid-phase resin in order to expedite the library construction (solid-phase synthesis, Scheme 1). In particular, a phenolic OH of the building block I (**A**) was utilized for conjugation on the resin after Fmoc-protection of amino group. After Fmoc-deprotection (**S3**), building block IIs reacted to give 2-acylaminoacetophenone derivatives (**S4**) and they were cyclized by Burgess reagent or Ph_3PCl_2 in the presence of triethylamine to generate **S5**. While both reagents generated the desired products, Burgess reagent resulted in the cleavage of the compounds during the reaction. Therefore, Ph_3PCl_2 was utilized thereafter for the successful cyclodehydration, and the final acidic cleavage from the resin afforded dapoxyl dyes. Forty different dapoxyl dyes were further prepared in this route and purified by preparative TLC and characterized by HPLC-MS (SI, Table S1).

Binding of Dapoxyl Dyes to HSA. For primary screening, dapoxyl library compounds were screened for fluorescence intensity changes toward HSA. Notably, **A41-S** (sulfonyl A41) exhibited a highly sensitive fluorescence response toward HSA. In a physiological condition (50 mM HEPES, pH 7.4), **A41-S** (5 μM) displayed a marked dose-dependent increase upon addition of HSA by ca. 55-fold (Figure 1). Upon binding with HSA, **A41-S** showed a clear blue shift of the fluorescence emission (Em_{max} 520 to 473 nm) and a red shift of fluorescence–excitation (Ex_{max} 313 to 356 nm) (SI, Figure S2). The fluorescence of unbound **A41-S** was highly negligible compared to the bound one when excited with 360 nm, displaying a highly sensitive intensity change by HSA.

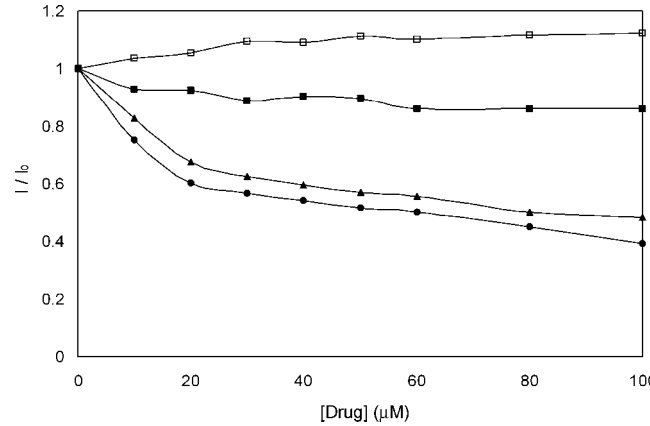


Figure 2. Displacement of **A41-S** from HSA-**A41-S** complex by the addition of site-specific drugs: **A41-S** (2 μM), HSA (20 μM), and a series of concentration of drugs were added to give the final concentration of 0, 10, 20, 30, 40, 50, 60, 80, 100 μM. □ Digitoxin (site III), ■ ibuprofen (site II), ▲ warfarin (site I), ● TIB (site I and II). I is the fluorescence intensity at drugs of each indicated concentration and I_0 is the fluorescence intensity at drugs (0 μM). I/I_0 indicates the ratio of I to I_0.

To verify binding sites of **A41-S** on HSA, the fluorescence displacement of **A41-S** from HSA was measured with site-specific binding drugs: warfarin (site I),[10] ibuprofen (site II),[10] digitoxin (sit III),[17] and triiodobenzoate (site I and II)[18] (Figure 2). **A41-S** (2 μM) bound to HSA (20 μM) was displaced by a series concentration of drugs. In a ratio of HSA to drug (1:5), warfarin displaced **A41-S** significantly by ca. 52% while ibuprofen showed a slight displacement of ca. 13%, exhibiting the selective binding of **A41-S** on site I of HSA. Digitoxin did not induce any displacement but TIB showed a clearly higher displacement by ca. 61%. The Scatchard plot from the titration of **A41-S** with HSA showed two binding sites with dissociation constants, $K_{D1} = 0.83$ and $K_{D2} = 1.98$ μM (assuming $n_1 = 1.0$ and $n_2 = 1.0$), addressing high affinity bindings with HSA (Figure 3).

Furthermore, the fluorescence response of **A41-S** to HSA was compared with two representative site I and II probes (DNSA and DS) (Figure 4). While **A41-S** and DS (site II) displayed significantly high intensity changes (over 50-fold), DNSA (site I) showed a relatively smaller intensity change

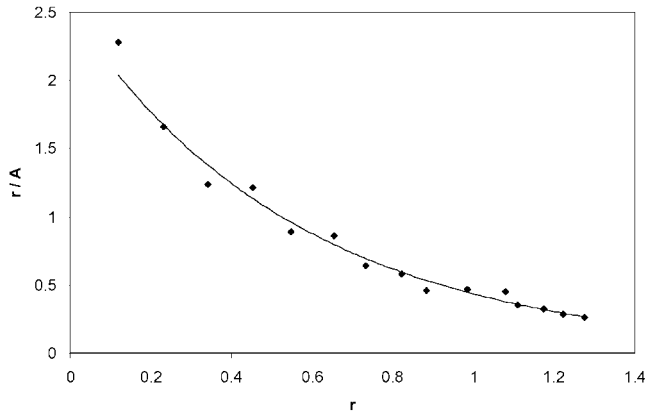


Figure 3. Scatchard plot for **A41-S** binding to HSA. HSA (8 μM) was titrated with **A41-S** (1–15 μM). The concentration of free and bound **A41-S** was calculated from fluorescence intensity and a curve was fitted to give $K_{a1} = 1.2 \times 10^6$ M^{-1}, $K_{a2} = 5.1 \times 10^5$ M^{-1}, assuming $n_1 = 1$ and $n_2 = 1$. r is the number moles of **A41-S** bound per mole of HSA and A is the concentration of free dye.

(16-fold), demonstrating that **A41-S** would be a highly sensitive probe to site I of HSA.

In conclusion, we have developed a combinatorial dapoxyl dye library using both solution- and solid-phase synthesis as a potential fluorescence sensor set. A fluorescence-based screening identified the highly sensitive and selective binding dye (**A41-S**) toward site I of HSA, demonstrating that this dapoxyl dye library has a great potential to sense macromolecules with fluorescence intensity changes. An extensive screening of this library toward various bioanalytes is in progress.

Experimental Section

General. All the chemicals and solvents were obtained from commercial suppliers and used without further purification. HSA and HSA-binding drugs were obtained from Sigma. 2-Chlorotrityl alcohol resin (1.37 mmol/g) was purchased from BeadTech Inc., Korea and chlorinated by the reported procedure.[3] All library compounds were identified by LC–MS from Agilent Technology, using a C18 column (20 × 4.0 mm), with 4 min elution using a gradient solution of CH_3CN–H_2O (containing 0.1% acetic acid), with UV detector and an electrospray ionization source. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer. All the fluorescence assays were measured with a Gemini XS fluorescent plate reader with 96-well plate. Fluorescence–excitation and emission spectra of **A41-S** were obtained with Hitachi F-2500 FL spectrophotometer.

Solution-Phase Synthesis of Dapoxyl Dyes. General Synthesis of S1. For Building Block II (1–35). To a solution of individual building block II (benzoic acid derivatives) (0.01 mmol) in dichloromethane (1 mL) were added DIEA (0.02 mmol) and HATU (3.8 mg, 0.01 mmol). After shaking for 30 min, a building block I (**A–F**) (0.01 mmol) was added to the reaction mixture. The reaction mixture was stirred at room temperature overnight and the reaction mixture was purified by column chromatography to afford compound **S1**. **For Building Block II (36–43).** To a solution of building block I (0.01 mmol) in dichloromethane (1 mL) were added pyridine (0.05 mmol) and building block II (0.01 mmol). The reaction mixture was stirred for 3 h and concentrated and purified by column chromatography to afford compound **S1**.

General Synthesis of S2 (Dapoxyl Dyes). To a solution of **S1** (0.01 mmol) in THF (0.3 mL) was added Burgess reagent (10 mg, 0.04 mmol) and the reaction mixture was heated in a microwave for 10 min. The solvent was evaporated and the reaction mixture was purified by preparative thin layer chromatography to give **S2**.

General Synthesis for Sulfonyl Dapoxyl Dyes. S1 (R^1 = Building Block I **(A)**) (0.01 mmol) was dissolved in H_2SO_4 (0.5 mL) and stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate and washed with water. The organic layer was dried over sodium sulfate. The filtrate was concentrated and purified by preparative thin layer chromatography to give the sulfonyl **S2**.

Solid-Phase Synthesis of Dapoxyl Dyes. Synthesis of S3. To a suspension of 2-chlorotrityl chloride resin (571 mg, 0.57 mmol) in THF (10 mL) were added **Fmoc-A** (637 mg, 1.71 mmol) and DIEA (304 μL). After heating at 60 °C for 6 h, the resin was filtered and washed with DMF (10 mL × 3), methanol (10 mL × 3), and dichloromethane (10 mL × 3) and then dried. The resin was suspended in 10% piperidine in DMF. After shaking for 1 h, the resin was filtered and

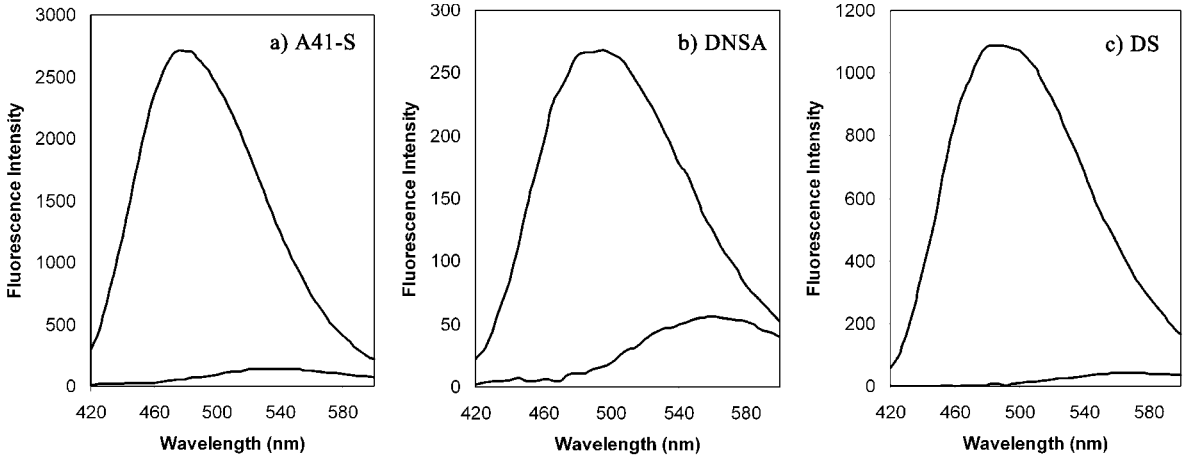


Figure 4. Comparison of fluorescence intensity changes upon HSA binding: each dye (5 μM), HSA (10 μM), 50 mM HEPES, pH 7.4, with excitation 360 nm. **A41-S**: 53-fold change at 475 nm; DNSA: 16-fold change at 490 nm; and DS: 160-fold change at 490 nm.

washed with DMF (10 mL × 3), methanol (10 mL × 3), and dichloromethane (10 mL × 3) and then dried.

General Synthesis of S4. For Building Block II (1–35). To a solution of building block II (0.1 mmol) in DMF (300 μL) were added DIEA (16 μL) and HATU (38 mg, 0.1 mmol). This reaction mixture was added to the solution of the resin **S3** (10 mg, 0.01 mmol) suspended in dichloromethane (700 μL). After shaking for 6 h at room temperature, the resin was filtered and washed with DMF (1 mL × 3), methanol (1 mL × 3), and dichloromethane (1 mL × 3) and then dried. **For Building Block II (36–43)**. To a resin **S3** (10 mg) suspended in dichloromethane (700 μL) was added pyridine (100 μL) and a building block II (0.1 mmol). After shaking for 3 h, the resin was filtered and washed with DMF (1 mL × 3), methanol (1 mL × 3), and dichloromethane (1 mL × 3) and then dried.

General Synthesis of S5 and the Cleavage from Resin. To a solution of **S4** (10 mg, 0.01 mmol) suspended in dichloromethane (0.5 mL) were added TEA (45 μL, 0.12 mmol) and Ph_3PCl_2 (40 mg, 0.1 mmol). The reaction mixture was stirred for 1 h at room temperature. The resin was filtered and washed with DMF (1 mL × 3), methanol (1 mL × 3), and dichloromethane (1 mL × 3) and then dried. The resin was treated with 1% TFA in dichloromethane (1 mL) for 20 min. The solution was drained and dried under reduced pressure.

Measurement of Binding Affinity. The binding affinity to HSA was measured and calculated by the previously reported procedures.[19] First, **A41-S** (2 μM) was titrated with a series of concentrations of HSA (1–30 μM) to measure the limiting fluorescence intensity (when **A41-S** is completely bound). The excitation was 360 nm and emission was from 420 to 600 nm. Second, HSA (8 μM) was titrated with **A41-S** (1–15 μM) and the concentration of bound dye was calculated using the equation: $Dye_b = F_o / F_l \times 2\ \mu M$ where Dye_b is the concentration of bound dye, F_o is the observed fluorescence, and F_l is the limiting fluorescence of **A41-S** (2 μM). Results were plotted and the binding parameters were obtained by iteration of the equation:

$$r = \frac{n_1 K_{a1} A}{1 + K_{a1} A} + \frac{n_2 K_{a2} A}{1 + K_{a2} A}$$

where r is the number moles of **A41-S** bound per mole of HSA, n is the number of binding site, K_a is the association constant, and A is the concentration of free dye.

Displacement of A41-S from HSA by HSA-Binding Drugs. To a solution of **A41-S** (2 μM) and HSA (20 μM), where **A41-S** is completely bound, were added a series of concentrations of drugs to reach the following concentrations: 0, 10, 20, 30, 40, 50, 60, 80, and 100 μM. Excitation was 360 nm and emission ranged from 420 to 600 nm.

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Supporting Information Available. Synthesis and characterization of **A41-S**, and structures, mass, and purity of dapoxyl library. This information is available free of charge via the internet at http://pubs.acs.org.

References and Notes

(1) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515–1566.
(2) (a) Lee, J. W.; Jung, M.; Rosania, G. R.; Chang, Y. T. *Chem. Commun.* **2003**, 1852–1853. (b) Li, Q.; Kim, Y. K.; Namm, J.; Kulkarni, A.; Rosania, G.; Ahn, Y. H.; Chang, Y. T. *Chem. Biol.* **2006**, *13*, 615–623. (c) 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. (d) Rosania, G. R.; Lee, J. W.; Ding, L.; Yoon, H. S.; Chang, Y. T. *J. Am. Chem. Soc.* **2003**, *125*, 1130–1131.
(3) (a) Ahn, Y. H.; Lee, J. S.; Chang, Y. T. *J. Am. Chem. Soc.* **2007**, *129*, 4510–4511. (b) Wang, S.; Chang, Y. T. *J. Am. Chem. Soc.* **2006**, *128*, 10380–10381.
(4) Diwu, Z.; Lu, Y. X.; Zhang, C. L.; Klaubert, D. H.; Haugland, R. P. *Photochem. Photobiol.* **1997**, *66*, 424–431.
(5) Haugland, R. P. *Handbook of Fluorescent Probes and Research Chemicals*, 9th ed.; Molecular Probes: Eugene, OR, 2002.
(6) Diwu, Z.; Chem, C. S.; Zhang, C.; Klaubert, D. H.; Haugland, R. P. *Chem. Biol.* **1999**, *6*, 411–418.
(7) Wang, Q.; Lawrence, D. S. *J. Am. Chem. Soc.* **2005**, *127*, 7684–7685.
(8) (a) Peters, T. *All About Albumin: Biochemistry, Genetics, and Medical Application*; Academic Press: San Diego, CA, 1996. (b) Kragh-Hansen, U.; Chuang, V. T. G.; Otagiri, M. *Biol. Pharm. Bull.* **2002**, *25*, 695–704.
(9) Hodgson, J. *Nat. Biotechnol.* **2001**, *19*, 722–726.
(10) (a) Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1975**, *11*, 824–832. (b) Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1976**, *12*, 1052–1061. (c) Kratochwil, N. A.; Huber, W.; Müller, F.; Kansy, M.; Gerber, P. R. *Biochem. Pharmacol.* **2002**, *64*, 1355–1374.
(11) (a) Sjoholm, I.; Ekman, B.; Kober, A.; Ljungstedt-Pahlman, I.; Seiving, B.; Sjodin, T. *Mol. Pharmacol.* **1979**, *16*, 767–777. (b) Curry, S.; Mandelkow, H.; Brick, P.; Franks, N. *Nat. Struct. Biol.* **1998**, *5*, 827–835.
(12) (a) Epps, D. E.; Raub, T. J.; Kezdy, F. J. *Anal. Biochem.* **1995**, *227*, 342–350. (b) Dalvit, C.; Flocco, M.; Stockman, B. J.; Veronesi, M. *Comb. Chem. High Throughput Screen.* **2002**, *5*, 645–650.
(13) (a) Brain, C. T.; Paul, J. M. *Synlett.* **1999**, 1642–1644. (b) Burgess, E. M.; Penton, H. R.; Taylor, E. A. *J. Org. Chem.* **1973**, *38*, 26–31.
(14) Robinson, R. *J. Chem. Soc.* **1909**, *95*, 2167.
(15) (a) Bagley, M. C.; Buck, R. T.; Hind, S. L.; Moody, C. J. *J. Chem. Soc., Perkin Trans. 1* **1998**, 591–600. (b) Wipf, P.; Yokokawa, F. *Tetrahedron Lett.* **1998**, *39*, 2223–2226. (c) Wipf, P.; Lim, S. *J. Am. Chem. Soc.* **1995**, *117*, 558–559.
(16) (a) Clapham, B.; Richard, A. J.; Wood, M. L.; Sutherland, A. J. *Tetrahedron Lett.* **1997**, *38*, 9061–9064. (b) Clapham, B.; Spanka, C.; Janda, K. D. *Org. Lett.* **2001**, *3*, 2173–2176.
(17) Irikura, M.; Takadate, A.; Goya, S.; Otagiri, M. *Chem. Pharm. Bull.* **1991**, *39*, 724–728.
(18) He, X. M.; Carter, D. C. *Nature* **1992**, *358*, 209–215.
(19) Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1973**, *9*, 649–657.
(20) Corrigan, J. R.; Langerman, M. J.; Moore, M, L. *J. Am. Chem. Soc.* **1945**, *67*, 1894–1896.

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