Diversity-Oriented Synthesis

Expanding the Functional Group Compatibility of Small-Molecule Microarrays: Discovery of Novel Calmodulin Ligands**

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Small molecules derived from diversity-oriented synthesis (DOS) that perturb the functions of proteins are facilitating explorations in biology. Small-molecule microarrays[1a,b,2] have proven robust and scalable[3] for the discovery of small-molecule–protein interactions that lead to small-molecule modulators of protein function.[1,4] Chlorinated slides have been used to print primary-alcohol-containing DOS-derived compounds.[2b] However, inspection of the literature suggests that synthetic routes yielding libraries of carboxylic acids or phenols outnumber those yielding primary alcohols.[5] In our own laboratory, DOS pathways leading to skeletally diverse products that use phenolic benzaldehydes as the first set of diversity elements have been developed,[6] and the phenolic products do not attach to chlorinated slides. We now report a new method for the covalent capture on a glass slide of phenols as well as compounds containing functional groups of at least comparable acidity.

A suitable surface must capture compounds of interest from 1-nL microcontact-printed spots in the absence of other reagents or additives and must be stable to handling in the atmosphere.

To address these requirements we focused on the development of diazobenzylidene-functionalized glass slides. Diazobenzylidenes are known to react selectively with heteroatoms that bear an acidic proton. Initial transfer of a proton from the heteroatom to the methine carbon atom of the diazobenzylidene is followed by nucleophilic displacement of N_2 by the heteroatom.

The glass slides derivatized with a diazobenzylidene moiety were prepared by coupling the toluenesulfonylhydrazone derived from 4-carboxybenzaldehyde (**1**) to γ-aminopropylsilane slides through formation of an amide bond (PyBOP, *i*Pr_2NEt, DMF) to yield a surface **B** as shown in Figure 1. Subsequent base-induced elimination yields the putative diazobenzylidene-derivatized glass slides (**C**).[7] Functionalization of these slides as small-molecule microarrays (**D**) was then facile with compounds containing acidic protons, such as phenols, carboxylic acids, and sulfonamides.

To test this approach, a robotic microarrayer was used to spot solutions of tetramethylrhodamine (**3**) in DMF, as well as synthetic FKBP12 ligands derivatized so as to present a phenol (**2a**), carboxylic acid (**2b**), primary alcohol (**2c**),

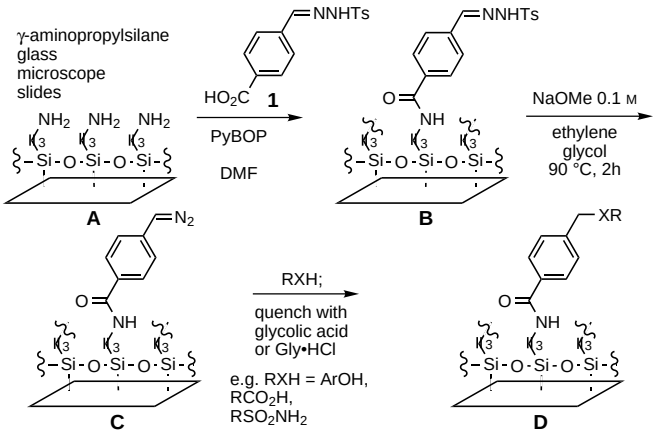

Figure 1. Preparation of diazobenzylidene-derivatized glass slides and covalent attachement of functional groups that bear an acidic proton. BOP = 1-benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate, Ts = toluene-4-sulfonyl.

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secondary alcohol (**2d**), and methyl ether (**2e**). The slides were then quenched with glycolic acid, washed extensively with DMF, THF, methanol, and phosphate-buffered saline, and probed with Cy5-FKBP12 (1 μg mL^{-1}),[8] a protein known to bind to derivatives **2a–e** (approximate dissociation constants for FKPB12 of 10 nM).[9] As shown in Figure 2, the visualization of the Cy5-FKBP12–small-molecule complexes are consistent with the immobilization of tetramethylrhodamine, phenol **2a**, and carboxylic acid **2b**, but not of **2c, 2d,** or **2e**.[10]

To test the reactivity of these diazobenzylidene slides further, 100 μM solutions[11] of biotin derivatives **4a–i** in DMF were printed in triplicate, with tetramethylrhodamine printed for reference. Probing this slide with 100 nM Cy5-streptavidin provided the differentially illuminated slide depicted in Figure 3. On the basis of these data, compounds **4c–i** were surmised to be attached to the glass slide, while **4a** and **4b** were not. Therefore, we conclude that functional groups that bear a proton with a p$K_a < 11$ (pK_a in DMSO < 19) are covalently attached to these slides, while those that bear a proton with a p$K_a > 16$ (pK_a in DMSO > 28) are not.

To demonstrate the ability of this covalent slide-capture method to identify new binding interactions between a protein and DOS-derived small molecules, 6336 phenol-containing fused bicycles and tetracycles,[5d] prepared in an encoded,[12] one-bead-one-stock solution[13] format, were printed and probed with Cy5-calmodulin (Figure 4).[14]

In order to prioritize the microarray positives prior to resynthesis, we have found it useful to retest qualitatively for the ability of the corresponding compound in solution to bind to the immobilized protein using surface plasmon resonance (SPR) spectroscopy (BIAcore). The initial secondary SPR screening was performed using compound (2 μL of a 1 mM DMF stock) directly from the original stock solutions used for microarray production. Of the 16 compounds on the microarray deemed positives, 13 showed qualitative binding to immobilized calmodulin based on the initial BIAcore analysis.[15] From this set, compounds **5, 6**, and **7** were resynthesized, purified, and their K_D values for calmodulin determined by SPR using steady-state affinity analysis. Compound **5** had a K_D value of 0.121 ± 0.03 μM, **6** a K_D value of 1.06 ± 0.09 μM, and **7** a K_D vlue of 20.7 ± 1 μM (Scheme 1).

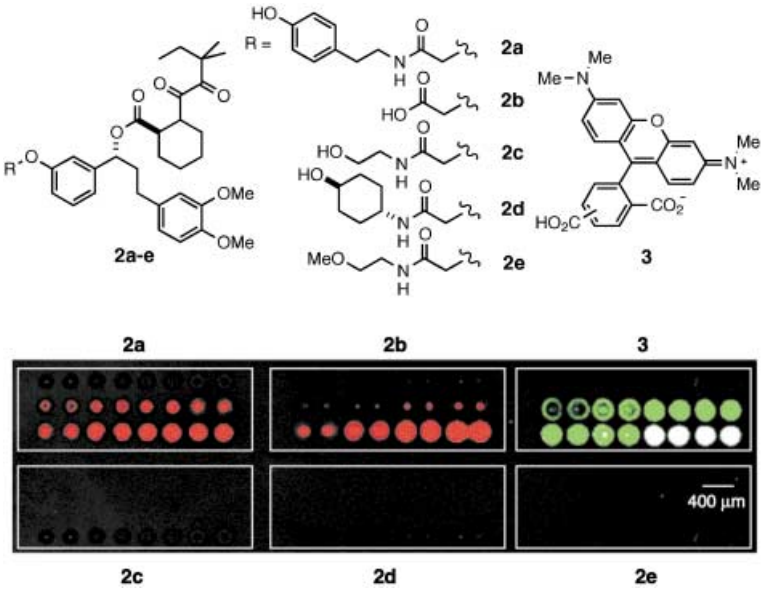


Figure 2. FKBP12 ligands (**2a–e**) and tetramethylrhodamine (**3**) printed on diazobenzylidene-derivatized slides. The DMF solutions were spotted in duplicate in serial twofold dilutions from 2 mM (lower right corner) to 1 μM (upper left corner).

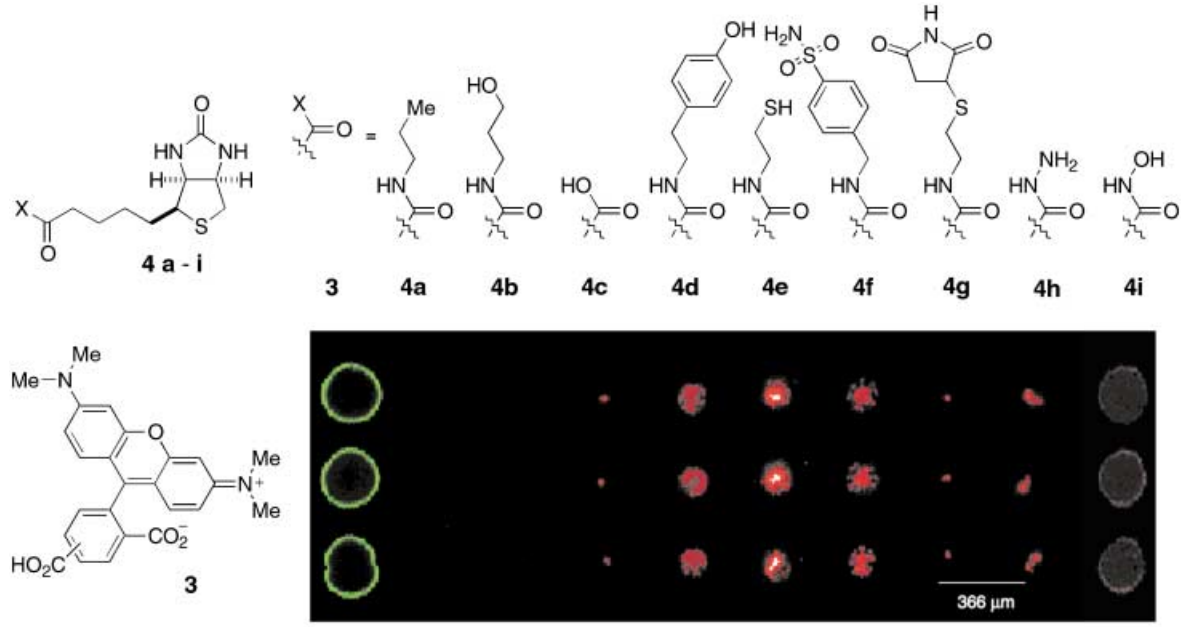


Figure 3. Tetramethylrhodamine (**3**) and biotin derivatives (**4a–i**) printed in triplicate at 100 μM and probed with 100 ng mL^{-1} Cy5-streptavidin. Estimated pK_a values (H_2O/DMSO): **4b** (16/28), **4c** (5/12), **4d** (10/18), **4e** (10/17), **4f** (10/16), **4g** (10/15), **4h** (11/19), **4i** (9/14).

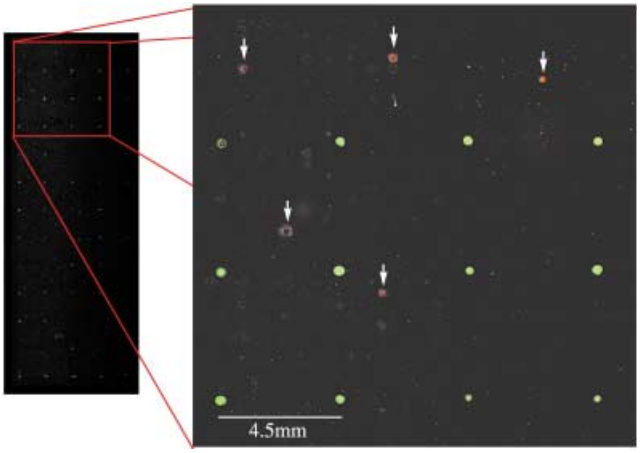
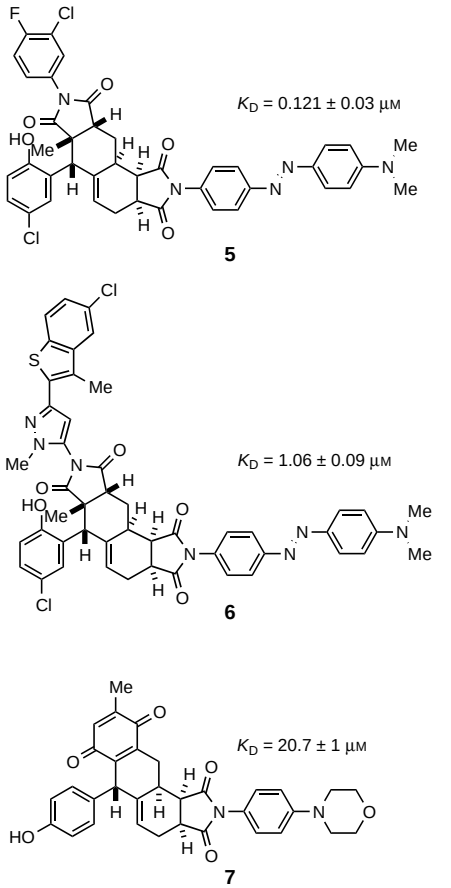


Figure 4. Diels–Alder polycycle array (6336 small molecules in total, approximately 1296 small molecules in the expanded view) probed with 3 $\mu g\,mL^{-1}$ Cy5-conjugated calmodulin. The positives for Cy5-calmodulin are false-colored red, and are indicated by white arrows. Tetramethylrhodamine makers, printed in the lower right-hand corner of each 12×12 subarray, are false-colored green.



Scheme 1. K_D values of calmodulin binders determined by surface plasmon resonance spectroscopy (BIAcore).

In conclusion, nonbiased[16] phenol-containing fused bicycles and tetracycles were immobilized as microarrays on diazobenzylidene slides. When they were probed with a protein, the observed positives corresponded well to binding interactions observed by SPR, where the protein is immobilized and the compound is free in solution.

Many sets of skeletally diverse small molecules derived from solid-phase, diversity-oriented syntheses should now be available in the manner described for high-density protein-binding assays that require minute quantities of synthetic compound. Also, the activated slides are simple and inexpensive to generate in large quantities and can be stored.[17] In contrast to the method previously reported for the covalent capture of primary alcohols,[2b] this new method includes an 11-atom spacer that may allow compounds to bind to deep pockets in proteins. Additionally, carboxylic acids and phenols are commonly found in collections of compounds currently prepared for screening.[5]

Diazobenzylidene slides serve a complementary function to chlorinated slides: the former captures phenols and carboxylic acids, but not primary alcohols, whereas the latter captures primary alcohols, but not phenols or carboxylic acids. The development of this new surface means that a significant number of compounds found in screening collections are now compatible with this high-throughput small-molecule–protein binding assay.

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[7] Diazobenzylidene slides can be left at room temperature in the dark for at least three weeks with no noticeable deterioration in performance. They are typically stored at −20 °C.

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[10] **2a** and **3** were detectable when printed at as low as 15 μM, whereas **2b** was detectable when printed at as low as 31 μM.

[11] Compounds printed from aqueous solutions were also captured.

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[14] Cy5 detection was chosen to avoid conflict with compound autofluorescence in the Cy3 channel.

[15] Negative controls showed no effect. See the Supporting Information for details on BIAcore analysis.

[16] That is, not designed to interact with a given protein or class of proteins using structural motifs known to favor such binding.

[17] Printed slides are typically stored at −20 °C, with no noticeable deterioration over at least two months.

