Split−Pool Synthesis of 1,3-Dioxanes Leading to Arrayed Stock Solutions of Single Compounds Sufficient for Multiple Phenotypic and Protein-Binding Assays

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Abstract: Diversity-oriented organic synthesis offers the promise of advancing chemical genetics, where small molecules are used to explore biology. While the split−pool synthetic method is theoretically the most effective approach for the production of large collections of small molecules, it has not been widely adopted due to numerous technical and analytical hurdles. We have developed a split−pool synthesis leading to an array of stock solutions of single 1,3-dioxanes. The quantities of compounds are sufficient for hundreds of phenotypic and protein-binding assays. The average concentration of these stock solutions derived from a single synthesis bead was determined to be 5.4 mM in 5 μL of DMSO. A mass spectrometric strategy to identify the structure of molecules from a split−pool synthesis was shown to be highly accurate. Individual members of the 1,3-dioxane library have activity in a variety of phenotypic and protein-binding assays. The procedure developed in this study allows many assays to be performed with compounds derived from individual synthesis beads. The synthetic compounds identified in these assays should serve as useful probes of cellular and organismal processes.

Introduction

Small molecules provide a means to modulate rapidly the circuitry of biological networks.[1−3] Such compounds can be discovered using either phenotypic[4] or protein-binding assays.[5] Phenotypic assays can be used to identify small molecules that modulate a specific cellular or organismal pathway without prior knowledge of the protein components of the pathway. Protein-binding assays, often used in drug discovery efforts, can also be used to identify reagents for exploring protein function in subsequent biological assays. By determining the pathways and processes altered by the small molecule, the functions of its target can be elucidated. Both strategies are capable of providing insight into complex processes.

The use of small molecules to dissect biological function is being accelerated by high-throughput screening of large collections of small molecules.[4] Advances in robotics and in the miniaturization of phenotypic and protein-binding assays have facilitated rapid screening of large compound collections. However, the production of small molecule libraries has not matched the advances in screening technology. The development of solid-phase organic synthesis has increased productivity in organic synthesis through simplification of purification protocols, permitting reactions to be automated and run in parallel.[6,7] This approach has gained widespread acceptance because milligram quantities of each small molecule can be generated for screening in multiple biological assays; however, issues of cost and labor typically limit library sizes to hundreds of compounds. Millions of distinct compounds can be synthesized through a variation of solid-phase synthesis that treats each solid-phase particle (commonly, derivatized polystyrene beads) as a separate reaction vessel. By splitting and pooling[8−10] the collection of synthesis beads over a reaction sequence, all possible combinations of a large matrix of reagents and building blocks can be accessed, generating an enormous amplification in the number of different compounds produced for a small number of reactions.[11] Despite the introduction of the split−pool synthetic method nearly a decade ago, it has not gained widespread use due to challenges in compound identification, the minute quantities of released compounds, and the resulting tendency to screen molecules as mixtures.[11] By addressing these issues, we have developed a split−pool synthetic approach to generate arrayed stock solutions of single 1,3-dioxane compounds sufficient for multiple phenotypic and protein-binding assays. Synthesis of a small demonstration library of 1890 molecules (a precursor step to a larger 50 000 compound

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(1) Mitchison, T. J. *Chem. Biol.* **1994**, *1*, 3−6.
(2) Schreiber, S. L. *Bioorg. Med. Chem.* **1998**, *6*, 1127−1152.
(3) See also http://www-schreiber.chem.harvard.edu.
(4) Mayer, T. U.; Kapoor, T. M.; Haggarty, S. J.; King, R. W.; Schreiber, S. L.; Mitchison, T. J. *Science* **1999**, *286*, 971−974.
(5) MacBeath, G.; Koehler, A. N.; Schreiber, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 7967−7968.
(6) Bunin, B. A.; Ellman, J. A. *J. Am. Chem. Soc.* **1992**, *114*, 10997−10998.
(7) DeWitt, S. H.; Kiely, J. S.; Stankovic, C. J.; Schroeder, M. C.; Reynolds Cody, D. M.; Pavia, M. R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6909−6913.
(8) Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487−493.
(9) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82−84.
(10) Houghten, R. A.; Clemencia, P.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84−86.
(11) Tan, D. S.; Foley, M. A.; Stockwell, B. R.; Shair, M. D.; Schreiber, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 9073−9087.

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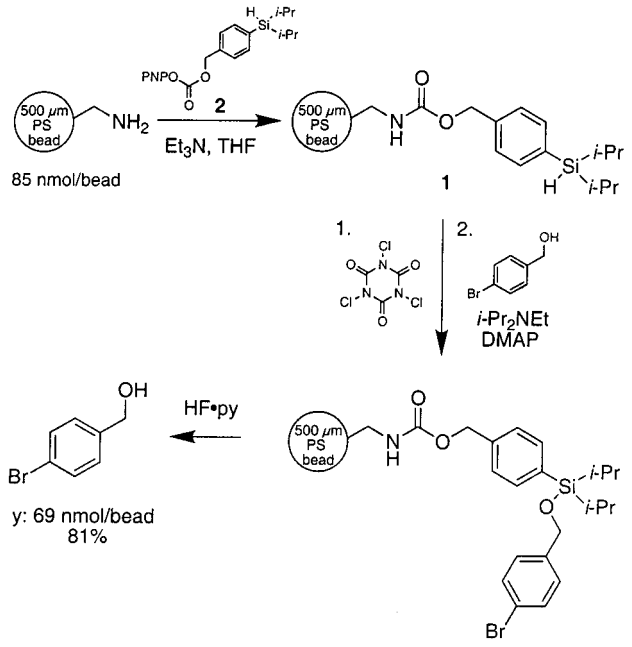
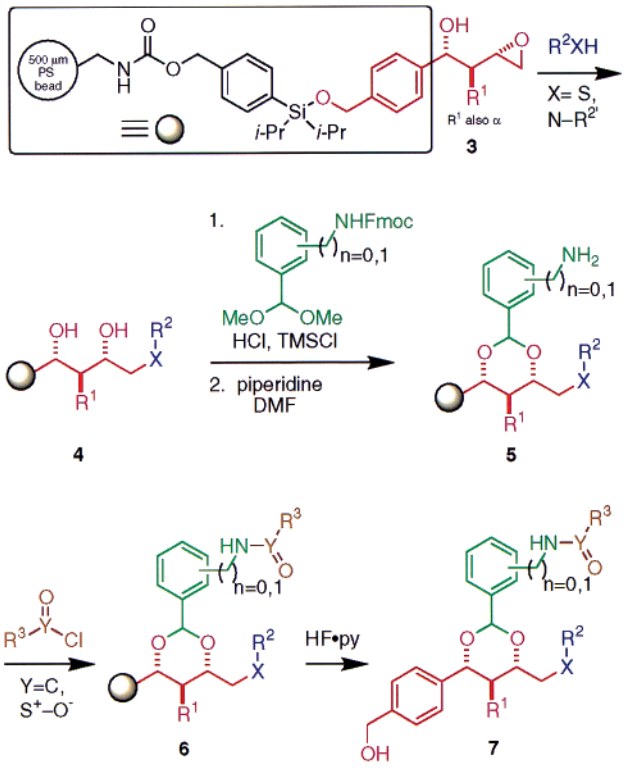
Scheme 1



Scheme 2



library), structure determination of active compounds, and biological activities in multiple assays are discussed.

Results and Discussion

High-Capacity Solid Support and a Silicon Linker Enable the Synthesis of Small Molecules in Quantities Sufficient for Multiple Phenotypic and Protein-Binding Assays. A fundamental challenge to the production of stock solutions from a split−pool library suitable for multiple biological assays is the release of sufficient compound from the synthesis resin. To generate the concentrations of small molecule necessary for phenotypic assays in cell culture and in multicellular organisms, 5−10 mM stock solutions in 5−10 μL of DMSO or DMF were deemed desirable.[12] When using miniaturized assays (assay volumes 2−40 μL), this permits hundreds of assays to be performed at screening concentrations of up to 100 μM after dilution into an assay plate. To obtain sufficient quantities of arrayed small molecule stock solutions for multiple assays, 500 μm aminomethyl polystyrene beads (Rapp Polymere- Tubingen, Germany) with a loading capacity of 85 nmol/bead were used. These beads have ∼5-fold larger diameter than the commonly used synthesis resin, with ∼100-fold greater quantity of attached small molecule. Synthesis resin with a capacity of 50−100 nmol/bead is sufficient to generate 10 mM stock solutions in 5 μL of DMSO.

An acid- and base-stable diisopropylphenylsilyl ether linker (**1**) was developed for the 1,3-dioxane synthesis to permit mild fluoride-mediated cleavage of the small molecules (Scheme 1). Despite the availability of many acid- and base-stable linkers,[13] few of these exhibit stability to both of these sets of reaction conditions as required by our 1,3-dioxane library synthesis (Scheme 2). Additionally, chemically robust linkers typically require harsh cleavage conditions that are not compatible with a wide variety of chemical functionality present in the 1,3-dioxane library. To avoid postsynthetic purification strategies, the reagent used for cleavage of the small molecule at the end of the synthesis should be removed easily, preferably by evaporation, further limiting the possible linker chemistries to be used. We focused on alkylsilyl ether chemistry which is widely used in organic synthesis because silyl ethers are often stable to both acid and base, but are cleaved under mild conditions with fluoride. A common source of fluoride, HF•pyridine (HF•py), can be quenched with TMSOMe,[14] yielding volatile byproducts thereby obviating the need for purification after compound cleavage. The diisopropylphenylsilyl linker **1** was developed with these considerations in mind.[15]

The diisopropylphenylsilane linker, activated as a *p*-nitrophenyl carbonate (**2**), was attached to aminomethyl polystyrene synthesis resin through a carbamate linkage. Oxidation of silane **1** with trichloroisocyanuric acid generated a silyl chloride that was reacted with alcohol building blocks. To illustrate the intrinsic yield of attachment and release for silyl linker **1**, 4-bromobenzyl alcohol was attached and then cleaved with HF•py in 81% yield, releasing 69 nmol/bead on average. This amount is sufficient to prepare ∼10 mM stock solutions by addition of 5−10 μL of DMSO. Due to the development of miniaturized phenotypic and protein-binding assays using robotic liquid transfer of 4−40 nL droplets with blunt-end pins[16] or 1 nL droplets with quill pins,[5] respectively, these stock solutions can be assayed hundreds of times at 50−100 μM assay concentrations.

Split−Pool Library Synthesis. The discovery of small molecule partners for uncharacterized proteins can provide powerful tools to explore biology. While many guidelines exist

(12) Abbreviations not cited in text: DMAP = 4-(dimethylamino)-pyridine, DMF = *N*,*N*-dimethylformamide, DMSO = dimethyl sulfoxide, Fmoc = 9-fluorenylmethoxycarbonyl, PNP = *p*-nitrophenyl, PS = polystyrene, py = pyridine, THF = tetrahydrofuran, TMSCl = chlorotrimethylsilane, TMSOMe = methoxytrimethylsilane.

(13) Guillier, F.; Orain, D.; Bradley, M. *Chem. Rev.* **2000**, *100*, 2091−2157.

(14) Hu, Y.; Porco, J. A. *Tetrahedron Lett.* **1998**, *39*, 2711−2714.

(15) An all-carbon version of this linker has been developed. Tallarico, J. A.; Depew, K. D.; Westwood, N. J.; Pelish, H. E.; Lindsley, C. W.; Shair, M. D.; Schreiber, S. L.; Foley, M. A., submitted.

(16) King, R. W. Unpublished results.

for the molecular structure of drugs,[17] the only generally accepted criterion for structures of protein-binding small molecules is that they should contain elements to restrict their degrees of conformational freedom. Bias toward "pharmacophore" or natural product structures can provide inspiration for a synthesis,[6,18] but there is no inherent requirement to adhere to these structural motifs for the synthesis of small molecule partners to uncharacterized proteins. Some considerations for such diversity-oriented organic syntheses have been provided.[19]

The 1,3-dioxane structure (Scheme 2) was selected for split−pool synthesis because it is a rigid core that can be synthesized stereoselectively with high purity in the presence of diverse ancillary functional groups. Building blocks for the library were selected through a series of quality control experiments involving liquid chromatography−mass spectrometry (LC-MS) analysis of the building blocks in model reactions on 500 μm polystyrene beads.[20] The building blocks that underwent test reactions with >90% purity were selected for the synthesis. Although our intention is to synthesize a library of 50 000 1,3-dioxane molecules, we first demonstrated our strategy for producing split−pool libraries as arrayed stock solutions by synthesizing a 1890 (theoretical) member library synthesized from a subset of the tested building blocks.

Three γ,δ-epoxy alcohols (Figure 1a) were attached to the polystyrene solid support through the diisopropylphenylsilyl ether linkage in 90% of the theoretical yield (theoretical yield based on loading/cleavage sequence of 4-bromobenzyl alcohol). The epoxy alcohol derivatized resin (**3**) was pooled and then split into 30 vessels with a diverse set of secondary amine and thiol building blocks (Figure 1b) to generate 90 different 1,3-diols (**4**) in quantitative yield, a portion of which was set aside for screening in biological assays. The solid supported 1,3-diols were pooled and split into two portions that were reacted with Fmoc-aminodimethyl acetal building blocks (Figure 1c) in 0.05 M HCl in dioxane and TMSCl to furnish 180 Fmoc-amino-1,3-dioxanes in 85−95% yield. The use of TMSCl as a dehydrating agent was important for consistently driving the reaction to completion. The diisopropylphenylsilyl ether linkage was stable to 0.05 M HCl in anhydrous dioxane for 4 h; however, yield and purity were substantially reduced with higher HCl concentrations or with longer reaction time. Dimethylacetal building blocks were used for 1,3-dioxane formation because the corresponding aldehydes reacted slowly when forming the *cis,cis*-5-methyl-1,3-dioxanes. This is presumably due to the development of four gauche interactions with the axial C5 methyl group as no difficulties were observed in forming the *trans,trans*-5-phenyl-1,3-dioxanes which have only two gauche interactions with the C5 phenyl group. Dimethylacetal building blocks led to the unwanted formation of mixed acetals with hydroxyl functionality present in the nucleophile building blocks. Because it was considered desirable to maintain free hydroxyl functionality due to considerations of molecular diversity, these acyclic acetals were removed by treatment of the resin with 0.2 M pyridinium *p*-toluenesulfonate in 9:1 THF−MeOH. The resin was then pooled and treated with piperidine to effect Fmoc removal and washed with TMSCl to protect any free hydroxyls, and the solid supported amines (**5**) were split and reacted with 10 electrophiles (Figure 1d) to generate 1800 amides, ureas, thioureas, and sulfonamides (**6**). Two equivalents of aminomethyl polystyrene were used for the synthesis, thus 3780 compounds (3600 1,3-dioxanes and 180 1,3-diols) were synthesized in 2 days with only 48 reactions.

Concentrations of Arrayed Stock Solutions. Traditionally, split−pool libraries are screened as mixtures, requiring deconvolution strategies to identify the active compounds.[21,22] Mixtures are prone to high false positive and false negative frequency,[11] and they demand considerable labor when identifying the active components from multiple assays. To segregate each synthetic compound for the preparation of arrayed stock solutions, the collection of beads with attached 1,3-dioxanes was distributed into eleven 384 well polypropylene plates using a bead arraying tool.[23] The 1,3-dioxanes (**7**) were released from the beads by treatment with HF·py for 1.5 h followed by TMSOMe to quench the excess HF as volatile byproducts. Solvent evaporation and addition of 5 μL of DMSO generated stock solutions of individual compounds. The concentration of a representative stock solution from the library was spectrophotometrically determined to be 6.7 mM by comparison to a standard curve calculated from a purified bulk sample of the relevant compound (**8** in Figure 2).

To study bead-to-bead variability of the amount of released compound, nine synthesis beads with attached 1,3-dioxane **8** were synthesized, arrayed, cleaved under the above conditions, and analyzed with spectrophotometry. After the beads were soaked in 5 μL of DMSO for 14 days, the stock solution concentrations were between 1.9 and 10.1 mM (median concentration, 6.2 mM; mean concentration, 5.4 mM). This variance in the concentration is likely the result of small variations in bead diameter which, due to the cubic relationship of diameter to volume, leads to a spread in the amount of compound attached within the bead and, thus, in the concentration of the stock solutions.

Bulk cleavage of the beads used above liberated 59 nmol/bead of compound **8**, which was determined by dividing the amount of isolated compound after cleavage, bead washing, and chromatography by the number of beads cleaved. Using this value, the theoretical mean stock solution concentration in 5 μL of DMSO is 11.9 mM. The discrepancy between the theoretical mean concentration of 11.9 mM and the observed mean concentration of 5.4 mM can be attributed to several factors. The lower than expected concentration is due, in part, to the absorption of atmospheric water by the DMSO stock solutions over time leading to an increase in solution volume with a corresponding decrease in concentration. Over 14 days, the stock solutions were observed to undergo a volume increase of ~40%. In the future, wet DMSO or solvents that absorb less water will be tested. Correction for the volume increase would give a mean concentration of 7.6 mM, still less than the theoretical mean concentration. The remaining difference in observed versus theoretical concentration is likely due to inefficient extraction of the cleaved compound out of the bead. We have observed that DMSO does not swell polystyrene beads, and extraction of compound remaining in the bead after cleavage may be slow, especially for large diameter beads. The use of solvents with better swelling and extracting properties has been found to diminish this concentration discrepancy in a model system.[24]

Despite concentrations below the theoretical value, the dilution of these stock solutions into cell based assays permit,

(17) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **1997**, *23*, 3−25.
(18) Lindsley, C. W.; Chan, L. K.; Goess, B. C.; Joseph, R.; Shair, M. D. *J. Am. Chem. Soc*. **2000**, *122*, 422−423.
(19) Schreiber, S. L. *Science* **2000**, *287*, 1964−1969.
(20) See Supporting Information.
(21) Erb, E.; Janda, K. D.; Brenner, S. *Proc. Natl. Acad. Sci., U.S.A.* **1994**, *91*, 11422−11426.
(22) Freier, S. M.; Konings, D. A. M.; Wyatt, J. R.; Ecker, D. J. *J. Med. Chem.* **1995**, *38*, 344−352.
(23) Walling, L.; King, R. W. Unpublished results.
(24) Clemons, P. A.; Schreiber, S. L. Unpublished results.

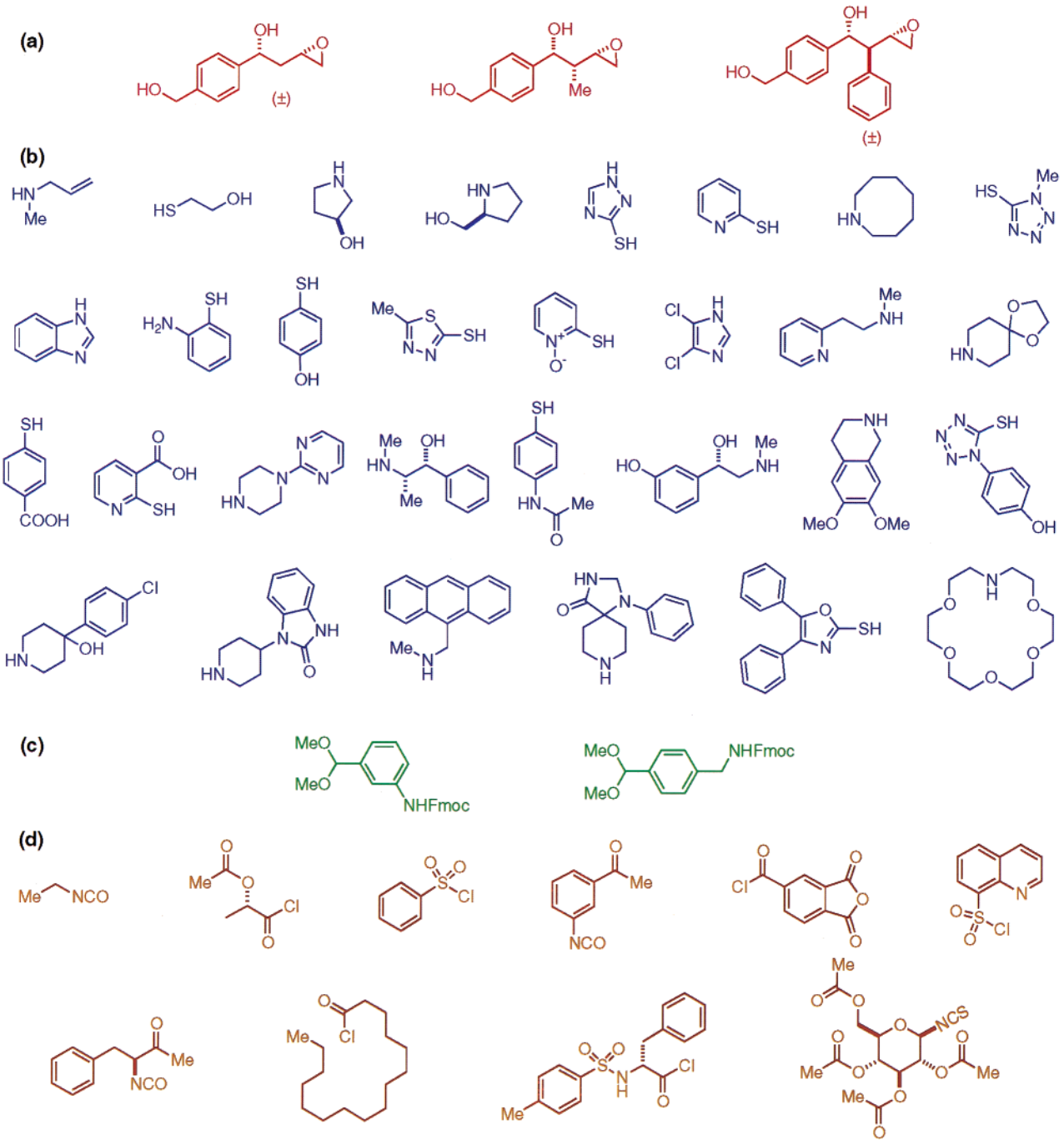


Figure 1. Building blocks used for a 1890 member 1,3-dioxane library: (a) γ,δ-epoxy alcohol building blocks, (b) amine and thiol building blocks, (c) Fmoc-amino dimethylacetal building blocks, and (d) electrophile building blocks.

on average, 50 μM screening concentrations at 1% final DMSO concentration. We have found that this is sufficient to discover biologically active molecules in a wide variety of protein-binding and phenotypic assays using the one compound−one bead approach.

Postsynthetic Purity Analysis of the Arrayed Stock Solutions. The postsynthetic purity of the library was analyzed by liquid chromatography on 10 randomly selected stock solutions from each of the 10 final acylation reactions (100 beads). Of the selected stock solutions, 47% were >90% pure after 4 synthetic steps and 76% were >70% pure. Incomplete acylation, over-acylation, and oxidation were responsible for 23% of the solutions being <90% pure. These inefficiencies with the final acylation step have been addressed by slight alterations in the synthetic method. Specifically, the acylation reaction times have been extended to ensure the completion of this reaction in all cases, and the triethylsilyl protecting group is used instead of trimethylsilyl protection to prevent acylation of ancillary hydroxyl groups in the final step of the synthesis.

It appears that, excluding the final acylation reaction, the molecules were synthesized reliably with high purity (most undesired products could be attributed to the acylation step). Although all of the building blocks used for the acylation reactions were shown to give the desired product with >90% purity in test systems, it is not surprising that reaction performance for the full range of building block combinations yields some compounds with lower than expected purity. This difficulty in predicting reaction success is magnified in a split−

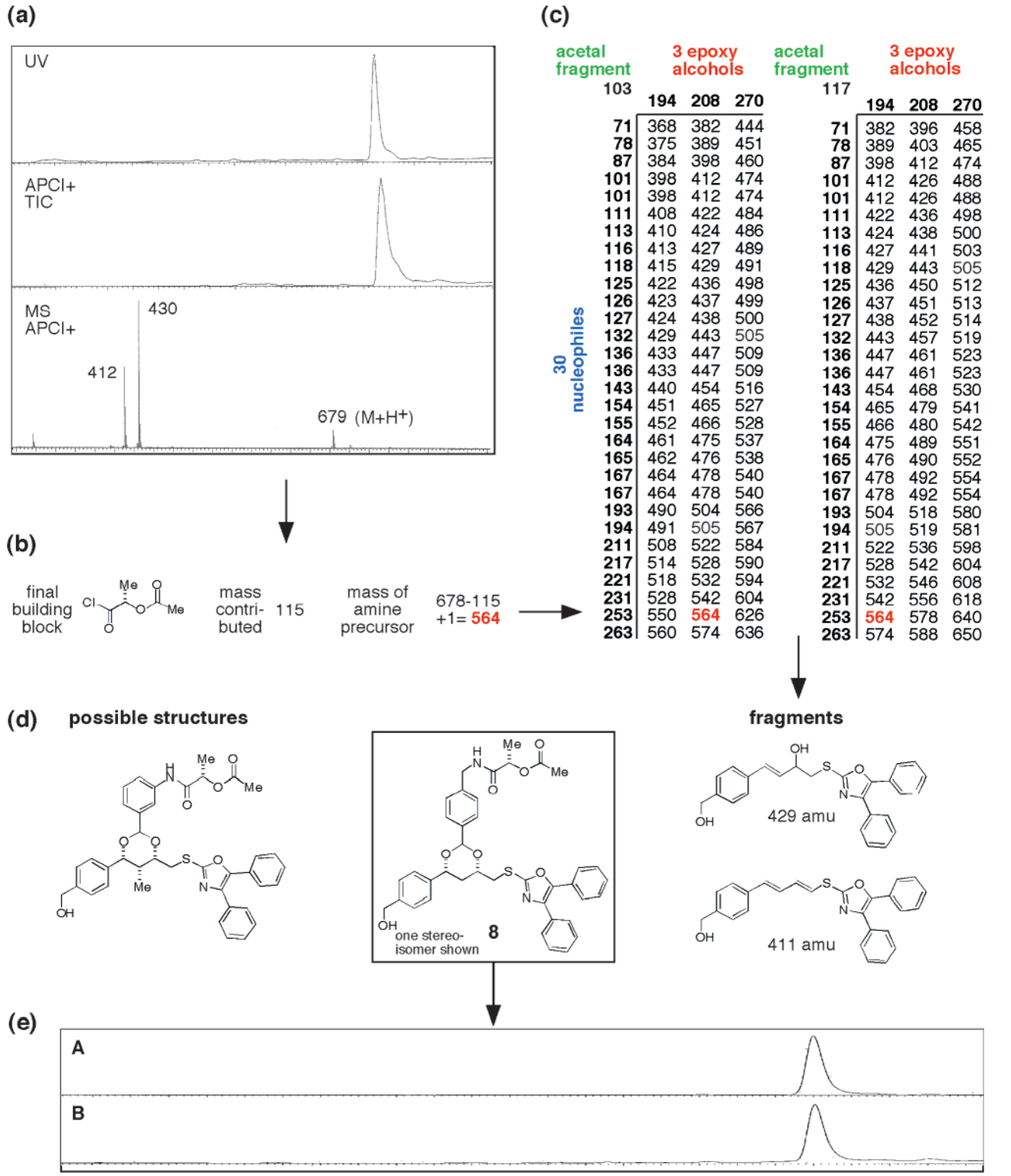


Figure 2. Structure determination procedure for the 1,3-dioxane library. (a) From LC-MS: UV absorbance trace, total ion count (TIC) trace (APCI+), and mass spectrum under the major peak. The molecular ion (M + 1) is 679 amu. (b) Determination of precursor amine mass. (c) All possible combinations of epoxy alcohol, nucleophile, and acetal building block masses. The mass being referenced, 564 amu (shown in red), results from two possible combinations of building blocks. (d) The mass 564 amu corresponds to a combination of building blocks represented in the two possible structures. Fragments with masses of 429 and 411 amu are consistent only with structure **8**. (e) Sample from the synthesis of the proposed structure **8** (trace A) shows the same retention time as a mixture of the synthesized compound and a sample from the original stock solution (trace B).

pool synthesis during the final diversity steps. Because it is untenable to perform reaction optimization on every reaction in a library synthesis, this postsynthesis quality control analysis will be essential to producing the larger 50 000 compound library with the highest possible purity. These considerations lead us to predict that future libraries based on slight optimizations of this chemistry will show >90% purity for ~70% of the compounds.

Use of Mass Spectrometry for Molecular Structure Determination. The structures of compounds in the stock solutions

were determined by using LC-MS[25,26] with atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). Direct infusion APCI and ESI-MS analysis was not successful due to competing signals from byproducts derived from extensive manipulations of organic solvents in plastic vessels that are required by the use of HF·py. Liquid chromatography was used to separate the small molecule of interest from these largely polymeric impurities. One difficulty in using mass as a unique identifier for compounds in split−pool libraries is the limited number of integer masses in the range relevant to small molecules (250−1000 amu). Our strategy to decrease the mass redundancy was to segregate the beads from the final 10 synthetic reactions into separate stock plates, thereby reducing the problem of distinguishing between 1800 possible masses to distinguishing between 180 masses.[27]

A representative example of the structure determination procedure is illustrated in Figure 2. After LC-MS analysis of a portion (5%) of the stock solution, the molecular ion was identified (Figure 2a). Because the beads were not pooled after the last synthetic step, the mass of the final building block was known. Subtraction of this mass from the molecular ion gave the mass of the 1,3-dioxane intermediate, **5** (Figure 2b). Comparison to a table of the 180 possible 1,3-dioxane masses (Figure 2c) generated from all combinations of the epoxy alcohol, nucleophile, and acetal fragments allowed, in this case, two possible molecular structures with the same mass to be assigned (Figure 2d). In most cases, the structures of molecules with redundant integer masses were distinguished by a reliable fragmentation of the 1,3-dioxane ring involving elimination of the aldehyde fragment. The appearance of peaks at 429 and 411 amu corresponds, respectively, to the products from fragmentation of the 1,3-dioxane ring and from subsequent elimination of water. These fragments can be used to unambiguously distinguish between the two 1,3-dioxane constitutional isomers. The proposed 1,3-dioxane, **8**, was synthesized and liquid chromatography showed that a mixture of the synthesized compound and the compound from the stock solution gave only one peak that had an identical retention time with the synthesized compound **8** (Figure 2e).

Features such as isotope patterns and LC retention times were also used in some cases to resolve redundancies. Mass considerations did not influence the choice of desired building blocks, and some mass redundancies existed even after fragment analysis. In these cases two or more molecules would be synthesized and tested separately. The reliability of this structure determination approach was assessed by identification of a molecule from each of the final 10 acylation reactions and resynthesis of the proposed structure. Comparison of the retention times by LC-MS showed that this procedure predicted the correct structure 10 out of 10 times.

Identification of Biologically Active 1,3-Dioxane Molecules and Related Structures. Arrayed stock solutions of single compounds from a split−pool library permit compounds to be tested individually in multiple assays. Using less than 10 vol % of the 1,3-dioxane stock solutions, we have performed five phenotypic assays and over 50 protein-binding experiments in duplicate. We report the results of these screens here, but in most cases, the macromolecular targets of the 1,3-dioxanes have not yet been pursued. However, these experiments confirm that molecules from the library are entering cells, interacting directly with protein targets, and displaying sufficient cellular stability to manifest an observable change, validating our approach to screen from split−pool libraries in multiple assays.

Biologically active 1,3-dioxanes have been identified in phenotypic assays in cell culture, zebrafish, and *Xenopus laevis* oocyte extract (Figure 3). Three structurally related phthallic anhydride derivatives (one example, compound **9**, is shown) showed inhibitory activity in a miniaturized (4 μL assay volume) *Xenopus laevis* oocyte extract assay that indicates modulation of the cyclin B degradation pathway.[28] A similar cyclin B degradation assay in HeLa cells (30 μL assay volume) revealed sulfonamide **10** to have inhibitory activity.[29] Neither set of compounds was active in both assay systems.

High throughput microscopy of HeLa cells in approximately 2500 single-compound assays with compounds from the 1,3-dioxane library showed cell detachment (induced by **11**) and an altered actin staining phenotype (induced by **12**).[30] The cellular basis of these phenotypes has not been investigated. Encouragingly, no phenotypes consistent with mitotic arrest were observed.

A cytoblot assay[31] in HeLa cells based on the accumulation of phosphorylated nucleolin protein has proven useful for identification of compounds that disrupt mitotic cell cycle progression, including ones that act by novel mechanisms.[4,32] No molecules in the 1,3-dioxane library led to the accumulation of phosphorylated nucleolin protein. Consistent with observations in the cell staining assays (see above), this indicates that the compounds in the library do not target the mitotic machinery. This is in contrast to a previous study with a collection of compounds having, in general, less structural complexity.[32] A significant percentage of the simple compounds target tubulin and either stabilize or destabilize its polymerized form, the microtubule.

To determine the effect of these compounds on whole organism development, a phenotypic assay was performed with use of 16-cell zebrafish (*Danio rerio*) embryos[33] with 1300 compounds from the 1,3-dioxane library. Embryos treated with a 1,3-diol precursor (**13**) to the dioxanes at 60 μM developed folds in the anterior trunk region of the notochord at 18 h post-fertilization (Figure 3b; embryo shown at 24 h post-fertilization for clarity). The folded notochord phenotype has also been observed through genetic mutant screens[34] for the *gul*^{m208} and *lev*^{m531} mutations. The 1,3-diol (**13**) may target these gene products or other proteins involved in the same biological pathway. Dissection of the pathways involved in notochord development may be complemented by small molecules that modulate these pathways, in particular by using the logic of chemical epistasis.[35]

In addition to phenotypic assays, over 50 protein-binding assays have been performed with the 1,3-dioxane library using small molecule microarrays.[5,36] The microarrays were con-

(25) Brummel, C. L.; Lee, I. N. W.; Zhou, Y.; Benkovic, S. J.; Winograd, N. *Science* **1994**, *264*, 399−402.

(26) Hughes, I. *J. Med. Chem.* **1998**, *41*, 3804−3811.

(27) Our use of LC-MS for structure determination is most amenable to moderately sized split−pool libraries (100−5000 compounds). For larger libraries we have recently demonstrated (Stavenger, R. A.; Schreiber, S. L. Unpublished results) the combined use of LC-MS structure determination and molecular encoding (adapted from: Still, W. C.; et al. *Proc. Natl. Acad. Sci., U.S.A.* **1993**, *90*, 10922−10926).

(28) Peters, N.; King, R. W. Unpublished results.

(29) Hoyt, J.; King, R. W. Unpublished results.

(30) Yarrow, J.; Field, C. Unpublished results.

(31) Stockwell, B. R.; Haggarty, S. J.; Schreiber, S. L. *Chem. Biol.* **1999**, *6*, 71−83.

(32) Haggarty, S. J.; Mayer, T. U.; Miyamoto, D. T.; Fathi, R.; King, R. W.; Mitchison, T. J.; Schreiber, S. L. *Chem. Biol.* **2000**, *7*, 275−286.

(33) Peterson, R. T.; Link, B. A.; Dowling, J. E.; Schreiber, S. L. *Proc. Natl. Acad. Sci., U.S.A.* **2000**, *97*, 12965−12969.

(34) Stemple, D. L.; Driever, W. *Development* **1996**, *123*, 117−128

(35) Shamji, A. F.; Kuruvilla, F. G.; Schreiber S. L. *Current Biol.* **2000**, *10*, 1574−1581.

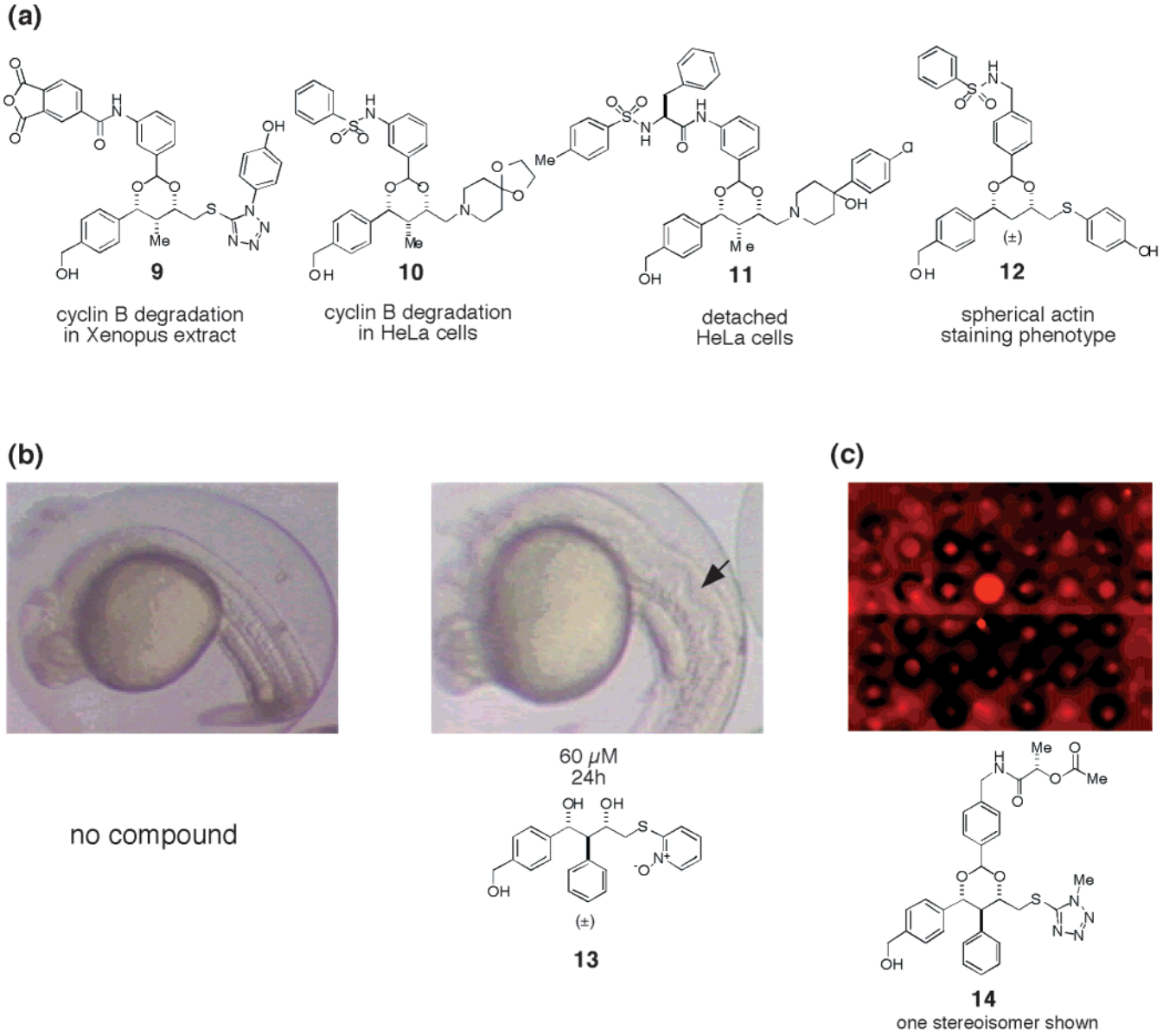


Figure 3. Molecules identified to show activity in phenotypic and protein-binding assays: (a) molecules showing activity in a variety of phenotypic assays in *Xenopus laevis* extract and in HeLa cells; (b) 1,3-diol **13** causes a wavy notocord phenotype (arrow) in zebrafish embryos 24 h post-fertilization; and (c) FKBP12 ligand identified using a small molecule microarray (a magnified portion of the array is shown).

structed by covalent attachment of 1 nL of each stock solution to a glass slide using a contact printing robot. Labeled proteins were used to probe the microarray for small molecule partners. Small molecules that bind to human FKBP12 (**14**), histone deacetylase-1, calmodulin, and a variety of fusion proteins derived from the yeast proteome and glutathione-*S*-transferase have been identified.[37] We are currently studying the functional effect and genomewide specificity in yeast of small molecule partners discovered by this technique for the yeast transcriptional repressor, Ure2p.

Conclusions

We have developed a split−pool synthesis of 1,3-dioxanes leading to the production of arrayed stock solutions compatible with multiple phenotypic and protein-binding assays. The use of high-capacity synthesis beads and chemically robust silicon linkers is crucial to performing multiple assays with individual compounds derived from a single synthesis bead. We also demonstrate an effective use of mass spectrometry to identify biologically active small molecules. The use of fragmentation to distinguish molecules with identical integer masses is of note and is widely applicable to many classes of molecule, making it an important consideration in the planning of a split−pool library. The synthesis of this small split−pool library has permitted analysis of compounds derived from single synthesis beads. These studies should enable optimization of a synthesis of 50 000 1,3-dioxanes that is under development.

We are currently using phenotypic and protein-binding assays, the latter using small molecule microarrays, to identify biologically active 1,3-dioxanes. Less than 10 vol % of the 1,3-dioxane library stock solutions has been used to perform five phenotypic assays and over 50 protein-binding experiments thus far. These preliminary results illustrate the capacity of the process described herein to link diversity-oriented organic synthesis and the exploration of biology in an effective way. Further significant improvements and developments in the one bead-one stock solution approach will be reported soon.

(36) Hergenrother, P. J.; Depew, K. M.; Schreiber, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 7849−7850.
(37) Koehler, A. N.; Schreiber, S. L. Unpublished results.

Experimental Section

See Supporting Information.

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Supporting Information Available: Experimental procedures for library synthesis, the method for building block selection, a procedure for compound cleavage from the bead and structure determination using LC-MS, and spectral characterization of biologically active 1,3-dioxane molecules (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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