Article

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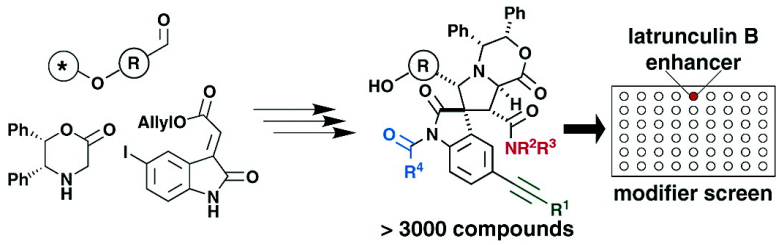

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A Library of Spirooxindoles Based on a Stereoselective Three-Component Coupling Reaction

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Abstract: A collection of structurally complex and chemically diverse small molecules is a useful tool to explore cell circuitry. In this article, we report the split-pool synthesis of more than 3000 spirooxindoles on high capacity macrobeads. The key reaction to assemble the spirooxindole core stereoselectively is a Lewis acid variant of the Williams' three-component coupling. After formation, the skeleton was elaborated using Sonogashira couplings, amide forming reactions, and N-acylations of γ-lactams. The final library was analyzed by sampling individual macrobeads and by using binomial confidence limits. It was determined that at least 82% of the library compounds should have better than 80% purity. To demonstrate the utility of our discovery process, a high-throughput chemical genetic modifier screen was performed using stock solutions of the resultant products. A number of positives were identified as enhancers of the cellular actions of latrunculin B, an actin polymerization inhibitor. Through resynthesis, we confirmed one of the positives and demonstrated that, in yeast cells, it has an EC_{50} in the sub-micromolar range.

Introduction

A goal of chemical genetics is to find small molecules that modulate the individual functions of gene products with high potency and high specificity.[1] Nature has provided effective examples via natural products, which in turn have stimulated the development of target-oriented synthesis (TOS). However, synthetic chemists have shown that Nature has no monopoly on such molecules. An unproven but intuitive hypothesis is that synthetic compounds that embody features characteristic of natural products may prove equally suited as modulators: rigidity, from either covalent bonding or hydrogen bonding to reduce the conformational flexibility of the molecule; stereochemistry, to fit into the chiral active sites of proteins; and multiple hydrophilic and hydrophobic groups, to provide the enthalphic driving force for protein binding.[2]

Our laboratory has been interested in exploring whether diversity-oriented synthesis (DOS), especially using a "one-support, one-stock solution" format,[3] might be useful in testing the above hypothesis. This approach has already led to the discovery of many small molecules having novel biological properties.[4] By combining this approach with analysis tools, e.g., from graph theory[5] and an Internet-accessible analysis environment (ChemBank),[6] we hope not only to have chemistry inform biology but also to have biology inform chemistry. Chemists can gain insight into underlying property−activity relationships from the systematic collection and analysis of matrices of data involving different small molecules, assay measurements, and cell states.[7]

DOS aims to synthesize compounds whose diversity results from variations in skeletons and stereochemistry. In addition, products having functionalities that enable follow-up chemistry to be performed effectively and systematically, for example, using appending processes, are highly valued. The latter point follows from the expectation that DOS compounds will be screened in assays and that bioactive compounds identified in this manner will be modified in ways that facilitate their use as cellular probes.[8] The selection of reactions to be incorporated in DOS pathways is thus critical to the value of the resultant library as a tool to investigate biology. Complexity-generating reactions are appealing, because molecules embodying the aforementioned features of natural products can be assembled

(1) (a) Schreiber, S. L. *Science* **2000**, *287*, 1964−1969. (b) Schreiber, S. L. *Chem. Eng. News* **2003**, *81* (9), 51−61.
(2) For discussions of libraries inspired by natural products, see: (a) Hall, D. G.; Manku, S.; Wang, F. *J. Comb. Chem.* **2001**, *3*, 125−150. (b) Nicolaou, K. C.; Pfefferkorn, J. A. *Biopolymers* **2001**, *60*, 171−193. (c) Breinbauer, R.; Vetter, I. R.; Waldmann, H. *Angew. Chem., Int. Ed.* **2002**, *41*, 2878−2890. (d) Boldi, A. M. *Curr. Opin. Chem. Biol.* **2004**, *8*, 281−286.
(3) (a) Blackwell, H. B.; Pérez, L.; Stavenger, R. A.; Tallarico, J. A.; Cope-Eatough, E.; Foley, M. A.; Schreiber, S. L. *Chem. Biol.* **2001**, *8*, 1167−1182. (b) Clemons, P. A.; Koehler, A. N.; Wagner, B. K.; Sprigings, T. G.; Spring, D. R.; King, R. W.; Schreiber, S. L.; Foley, M. A. *Chem. Biol.* **2001**, *8*, 1183−1195.
(4) (a) Pelish, H. E.; Westwood, N. J.; Feng, Y.; Kirchhausen, T.; Shair, M. D. *J. Am. Chem. Soc.* **2001**, *123*, 6740−6741. (b) Spring, D. R.; Krishnan, S.; Blackwell, H. E.; Schreiber, S. L. *J. Am. Chem. Soc.* **2002**, *124*, 1354−1363. (c) Kuruvilla, F. G.; Shamji, A. F.; Sternson, S. M.; Hergenrother, P. J.; Schreiber, S. L. *Nature* **2002**, *416*, 653−657. (d) Wong, J. C.; Hong, R.; Schreiber, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 5586−5587. (e) Koehler, A. N.; Shamji, A. F.; Schreiber, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 8420−8421.
(5) Haggarty, S. J.; Clemons, P. A.; Schreiber, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 10543−10545.
(6) (a) ChemBank. http://chembank.med.harvard.edu (accessed August 2004). (b) Strausberg, R. L.; Schreiber, S. L. *Science* **2003**, *300*, 294−295.
(7) Kim, Y.-K.; Arai, M. A.; Arai, T.; Lamenzo, J. O.; Patterson, N.; Clemons, P. A.; Schreiber, S. L. *J. Am. Chem. Soc.*, in press.
(8) Burke, M. D.; Schreiber, S. L. *Angew. Chem., Int. Ed.* **2004**, *43*, 46−58.

10.1021/ja045089d CCC: $27.50

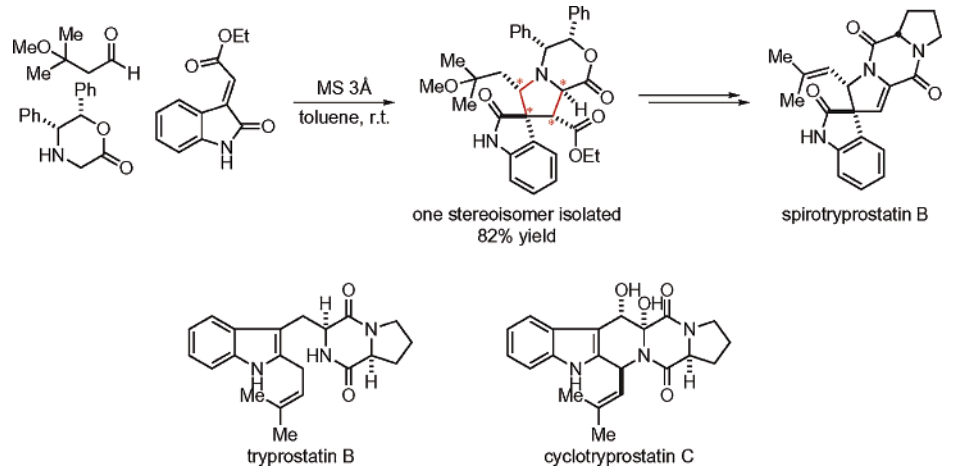


Figure 1. Outline of the Williams' synthesis of (−)-spirotryprostatin B and structures of two additional diketopiperazines from *Aspergillus fumigatus*.

from simple building blocks. Multicomponent coupling reactions are illustrative;[9] however, stereoselective versions are not yet routine.[10]

We were therefore inspired by the report of Williams and co-workers in which a stereoselective three-component reaction (3-CR) was used as the key step in the TOS of the natural product (−)-spirotryprostatin B.[11] A spirocyclic oxindole-pyrrolidine core was constructed with the simultaneous creation of three bonds and four stereogenic centers in one single chemical step. The spiro fusion between the 3-position of the pyrrolidine ring and the 3′-position of the oxindole ring distinguishes spirotryprostatins[12] from structurally related tryprostatins[13] and cyclotryprostatins (Figure 1).[14] These tryptophan-derived natural products were all isolated from the fermentation broth of *Aspergillus fumigatus* by Osada.[15]

In the original conception of this synthetic pathway, we were attracted to several features of the Williams 3-CR. We recognized the theoretical possibility of varying the stereochemistry of cycloadducts in a partially systematic manner by exploiting the stereospecific nature of [2 + 3]-cycloaddition reactions (cf., E vs Z dipolarophiles) and the influence of auxiliaries or catalysts, although we anticipated that control over endo−exo selectivity might be formidable. We envisioned skeletal diversity arising by the use of alternative dipolarophiles and by the removal of the auxiliary, yielding an amino acid that we perceived as a substrate for subsequent skeleton-determining reactions. Here, we report several advances that address the initial challenges necessary to realize these theoretical advantages. The results, involving a pilot split-pool synthesis[16] of more than 3000 single-skeleton spirooxindole products, should enable more extensive stereochemical and skeletal diversification in future studies. Numerous technical and tactical advances were developed that are prerequisites for future studies, including a Lewis acid mediated variant of the Williams 3-CR that proceeds on macrobeads,[17] and biological assays showing that synthetic compounds having structural features in common with compounds in Figure 1 are indeed useful as probes. These advances enabled the synthesis of compounds that derive structural diversification through appending processes.

Results and Discussion

In this initial use of the title reaction, spirooxindoles were elaborated using building blocks having diverse properties and orthogonal chemical reactivities. The spirooxindole skeleton is assembled in the first step, through a highly diastereoselective 3-CR using macrobead-supported aldehydes, either enantiomer of the Williams' chiral auxiliary **1**,[18] and isatin-derived dipolarophiles bearing the allyl ester (**2** and **3**).

Optimization of the 3-CR. We were unable to achieve the 3-CR on macrobeads using the conditions shown to be suc-

(9) For reviews, see: (a) Weber, L.; Illgen, K.; Almstetter, M. *Synlett* **1999**, 366−374. (b) Bienaymé, H.; Hulme, C.; Oddon, G.; Schmitt, P. *Chem.—Eur. J.* **2000**, *6*, 3321−3329. (c) Dömling, A.; Ugi, I. *Angew. Chem., Int. Ed.* **2000**, *39*, 3168−3210. (d) von Wangelin, A. J.; Neumann, H.; Gördes, D.; Klaus, S.; Strübing, D.; Beller, M. *Chem.—Eur. J.* **2003**, *9*, 4286−4294.

(10) Some examples include: (a) Mannich reaction: List, B.; Pojarliev, P.; Biller, W. T.; Martin, H. J. *J. Am. Chem. Soc.* **2002**, *124*, 827−833. Notz, W.; Tanaka, F.; Watanabe, S.; Chowdari, N. S.; Turner, J. M.; Thayumanavan, R.; Barbas, C. F., III. *J. Org. Chem.* **2003**, *68*, 9624−9634. (b) Tandem aza[4 + 2]-cycloaddition/allylboration: Touré, B. B.; Hoveyda, H. R.; Tailor, J.; Ulaczyk-Lesanko, A.; Hall, D. G. *Chem.—Eur. J.* **2003**, *9*, 466−474. (c) Addition of organoboronic acids to in situ generated imines: Petasis, N. A.; Zavialov, I. A. *J. Am. Chem. Soc.* **1997**, *119*, 445−446. (d) 1,3-Dipolar cycloaddition: Fokas, D.; Ryan, W. J.; Casebier, D. S.; Coffen, D. L. *Tetrahedron Lett.* **1998**, *39*, 2235−2238. (e) Passerini reaction: Frey, R.; Galbraith, S. G.; Guelfi, S.; Lamberth, C.; Zeller, M. *Synlett* **2003**, 1536−1538. (f) Propargylamine synthesis: Gommermann, N.; Koradin, C.; Polborn, K.; Knochel, P. *Angew. Chem., Int. Ed.* **2003**, *42*, 5763−5766. (g) aza-Baylis−Hillman reaction: Balan, D.; Adolfsson, H. *Tetrahedron Lett.* **2003**, *44*, 2521−2524.

(11) (a) Sebahar, P. R.; Williams, R. M. *J. Am. Chem. Soc.* **2000**, *122*, 5666−5667. (b) Sebahar, P. R.; Osada, H.; Usui, T.; Williams, R. M. *Tetrahedron* **2002**, *58*, 6311−6322.

(12) (a) Cui, C.-B.; Kakeya, H.; Osada, H. *J. Antibiot.* **1996**, *49*, 832−835. (b) Cui, C.-B.; Kakeya, H.; Osada, H. *Tetrahedron* **1996**, *52*, 12651−12666.

(13) (a) Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Ubukata, M.; Takahashi, I.; Isono, K.; Osada, H. *J. Antibiot.* **1995**, *48*, 1382−1384. (b) Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Osada, H. *J. Antibiot.* **1996**, *49*, 527−533. (c) Cui, C.-B.; Kakeya, H.; Osada, H. *J. Antibiot.* **1996**, *49*, 534−540.

(14) Cui, C.-B.; Kakeya, H.; Osada, H. *Tetrahedron* **1997**, *53*, 59−72.

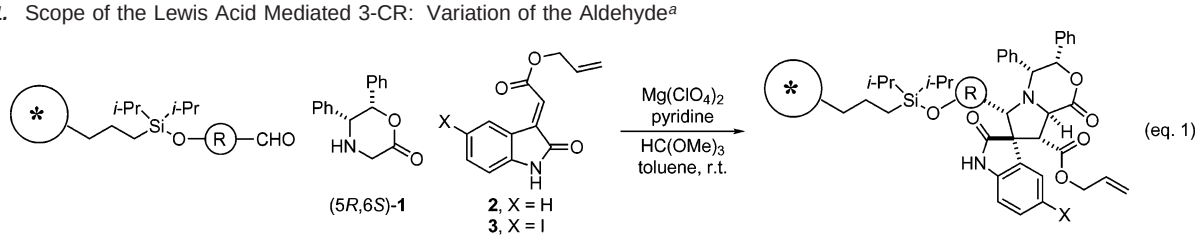
(15) The intriguing scaffold and antimitotic activities of spirotryprostatins have attracted the attention of synthetic chemists. For total syntheses of spirotryprostatin B, see: (a) von Nussbaum, F.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2000**, *39*, 2175−2178. (b) Reference 11a. (c) Wang, H.; Ganesan, A. *J. Org. Chem.* **2000**, *65*, 4685−4693. (d) Overman, L. E.; Rosen, M. D. *Angew. Chem., Int. Ed.* **2000**, *39*, 4596−4599. (e) Bagul, T. D.; Lakshmaiah, G.; Kawabata, T.; Fuji, K. *Org. Lett.* **2002**, *4*, 249−251. (f) Meyers, C.; Carreira, E. M. *Angew. Chem., Int. Ed.* **2003**, *42*, 694−696.

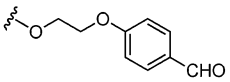
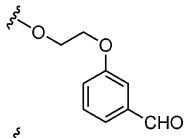
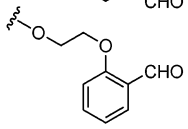
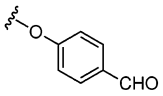
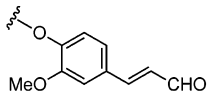
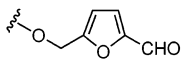
(16) (a) Furka, A.; Sebestyén, F.; Asgedom, M.; Dibó, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487−493. (b) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82−84. (c) Houghten, R. A.; Clemencia, P.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84−86. (d) Bunin, B. A.; Ellman, J. A. *J. Am. Chem. Soc.* **1992**, *114*, 10997−10998.

(17) Tallarico, J. A.; Depew, K. M.; Pelish, H. E.; Westwood, N. J.; Lindsley, C. W.; Shair, M. D.; Schreiber, S. L.; Foley, M. A. *J. Comb. Chem.* **2001**, *3*, 312−318.

(18) Both enantiomers of Boc-protected Williams' chiral auxiliary **1** are commercially available.

Table 1. Scope of the Lewis Acid Mediated 3-CR: Variation of the Aldehyde[a]



entry	O—R—CHO	dipolarophile	conv. (%)	d.r.	product
1		**2**	89	88:12	**4a**
2		**3**	89	> 95:5	**5a**
3		**2**	>95	>95:5	**4b**
4		**3**	>95	> 95:5	**5b**
5		**2**	>95	>95:5	**4c**
6		**3**	>95	> 95:5	**5c**
7		**2**	>95	91:9	**4d**
8		**3**	94	92:8	**5d**
9		**2**	>95	77:23	**4e**
10		**3**	>95	72:28	**5e**
11		**2**	>95	82:18	**4f**
12		**3**	>95	85:15	**5f**

[a] Conversion determined relative to residual aldehyde. d.r. determined by ^{1}H NMR.

cessful in solution (MS 3 Å, toluene). We therefore examined various mild Lewis acids to promote the reaction. Most of these were found to be effective accelerants for the reaction, furnishing the 3-CR product in high purity and excellent diastereoselectivity.[19] To minimize the cleavage of the loaded aldehyde from the silicon linker by adventitious acid, a base was added to the reaction as a buffer. Among the amine bases examined, pyridine was found both to prevent cleavage and to promote the reaction.[20] Finally, molecular sieves were replaced with methyl orthoformate as the dehydrating agent for operational simplicity.

With the new reaction conditions (eq 1), aromatic aldehydes of various substitution patterns (Table 1, entries 1−8) are excellent substrates, affording good levels of diastereoselectivity (≥88:12) and conversion (≥89%).[21] α,β-Unsaturated aldehydes (entries 9 and 10) and heteroaromatic aldehydes (entries 11 and 12) offer lower levels of diastereoselectivity, so we decided to exclude them from the library synthesis.[22]

We planned to increase the stereochemical diversity of the products further by using either diastereomer (*E*/*Z*) of the dipolarophile in the 3-CR. The major obstacle, however, is the challenging synthesis of the *Z*-dipolarophiles. The *E*-dipolarophiles have been reported to isomerize into the *Z*-isomers with $AlCl_3$,[23] but, in our hands, the reaction is capricious and does not work for the majority of the substrates that we have examined. As a result, *Z*-dipolarophiles were not included in the subsequent library synthesis. We note that this shortcoming represents an important challenge for subsequent research. The stereospecificity of the key cycloaddition reaction offers the possibility of at least partial stereochemical diversification of the resulting products. Exploiting this possibility and imparting further stereochemical diversification through, for example, alteration of the relative face selectivity in the reaction, constitute challenging but important goals of future research.

Library Synthesis. I. Reaction Survey and Building Block Selection. Split-pool synthesis is the most effective method to generate a desired library in the fewest chemical operations, but it involves solid-phase synthesis and hence introduces a different set of criteria for evaluation. While solid-phase synthesis reactions can be driven to completion by excess reagents, the desired products are immobilized and cannot be separated from unreacted starting material or purified from

(19) Lewis acids examined that are capable of promoting the reaction include: LiOTf, $Mg(OTf)_2$, $Mg(ClO_4)_2$, $Sc(OTf)_3$, $Y(OTf)_3$, $La(OTf)_3$, $Yb(OTf)_3$, $Cu(OTf)_2$, AgOTf, $Zn(OTf)_2$, $Sn(OTf)_2$, $In(OTf)_3$, and $Bi(OTf)_3$. Strong Lewis acids cleave the silicon linker on the macrobead and, thus, were not examined.

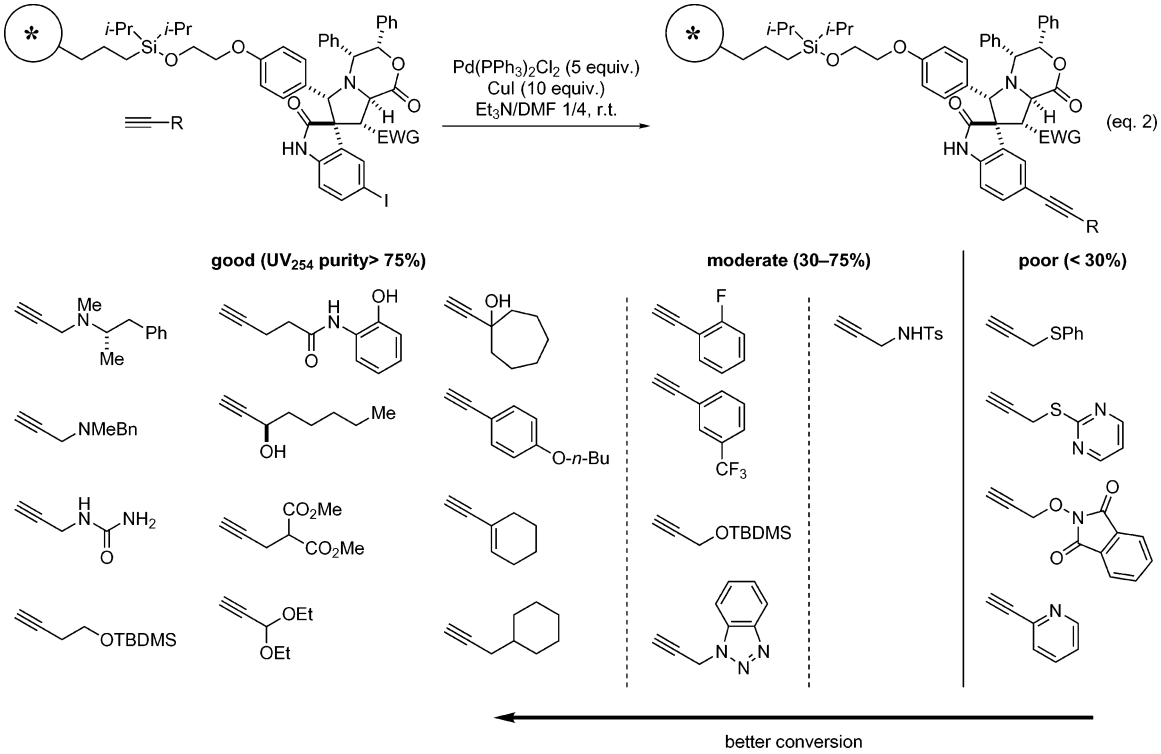
(20) Other amine bases that were examined include 2,6-lutidine, 2,6-di-*tert*-butyl-4-methylpyridine, diisopropylethylamine, and 2,2′-bipyridine.

(21) The stereochemistry of the product was assigned using NOE analyses, and it is consistent with Williams' results reported in ref 11b.

(22) Our protocol also works with other dipolarophiles bearing electron-withdrawing substituents such as ketones, nitriles, and amides.

(23) Faita, G.; Mella, M.; Righetti, P.; Tacconi, G. *Tetrahedron* **1994**, *50*, 10955−10962.

Scheme 1. Test Results of Alkyne Building Blocks for Sonogashira Coupling



immobilized byproducts. Hence, the reactions used should convert the immobilized substrates into products with high purity.

Reactions that are general, are insensitive to minor alterations of reaction conditions, and have broad functional-group compatibility are preferred in DOS. Reactions are also evaluated based on their ability to modify the initial chemical properties of the starting material, e.g., the number of rotatable bonds (cyclization reactions), or the value of cLogP (replacing a hydrophobic group with a hydrophilic one).[24] Unfortunately, the two goals are often conflicting, so the challenge of pathway development is to select reactions that strike a balance.[25]

The spirooxindole scaffold is densely packed with functionalities. We therefore sought orthogonal methods to derivatize the products without use of protecting groups. Three different approaches were explored and demonstrated to be practical: (a) palladium-catalyzed carbon−carbon bond formation with concomitant allyl ester cleavage; (b) amide formation between the resultant carboxylic acid with amines; and (c) N-acylation of γ-lactams with electrophiles.

Several palladium-catalyzed coupling reactions were studied, and while Suzuki and Stille reactions proceed to some extent, Sonogashira coupling with aromatic iodide-bearing spirooxindoles was found to be the most reliable (eq 2). We surveyed the scope of the alkynes and classified their reactivities as outlined in Scheme 1. The classification is only approximate, as the reactivity of some alkynes is sensitive to minor structural changes in the spirooxindole.

The cleavage of the ester group proved to be difficult, presumably due to steric hindrance.[26] While a standard palladium-based protocol with thiosalicylic acid did not succeed with spirooxindoles bearing the allyl ester, we discovered that, during Sonogashira coupling, the allyl ester was cleaved concomitant with the carbon−carbon bond formation. If the alkyne is omitted from the reaction conditions altogether, the aromatic iodide is left intact while the allyl ester is cleaved to the acid.

Relatively high levels of palladium and copper were used in the reaction to ensure reliable coupling.[27] Although the products are immobilized on macrobeads and the excess reagents can in theory be washed away, substantial amounts of metal can still be retained. To obtain reliable cellular screening results, it is desirable to keep the levels of adventitious metal to a minimum.

The thiourea moiety has been reported to be effective for scavenging several late-transition elements, presumably due to its soft nitrogen and sulfur atoms.[28] Glyoxal bis(thiosemicarbazone), which contains two thiourea subunits, was found to be a good scavenger for our purpose. Color serves as a useful indicator for the completion of metal removal, as the light yellow solution turns deep red on exposure to copper and deep yellow to palladium (Figure 2).[29] Hence, after reaction, the macrobeads were washed iteratively with a DMF solution of the scavenger until the solution retained its native light yellow color.

(24) cLogP is defined as the calculated log P value of a compound, which is the logarithm of its partition coefficient between *n*-octanol and water [log($c_{octanol}/c_{water}$)]. It is a measure of the hydrophilicity of the compound.

(25) For discussions on the factors governing the selection of reactions in a split-pool library, see: Marx, M. A.; Grillot, A.-L.; Louer, C. T.; Beaver, K. A.; Bartlett, P. A. *J. Am. Chem. Soc.* **1997**, *119*, 6153−6167.

(26) We have installed other esters (e.g., methyl, ethyl, *tert*-butyl, benzyl, *p*-methoxybenzyl) in the 3-CR product and explored their corresponding deprotection methods, but none show the desired reactivity.

(27) We could have conceivably used the more potent palladium catalysts that are based on bulky phosphines/carbenes to lower the catalyst loading. (For a review, see: Littke, A. F.; Fu, G. C. *Angew. Chem., Int. Ed.* **2002**, *41*, 4176−4211.) However, in the library synthesis, electrophoric tags that contain multiple aryl chlorides are used for encoding, and it is quite likely that these tags would be activated as well.

(28) A case study was presented by Silicycle, a company that markets silica-bound metal scavengers. http://www.silicycle.com/html/english/products/product_line.php?cat_id=15 (accessed August 2004).

(29) Amberlite resins have been coated with glyoxal bis(thiosemicarbazone) to extract metal ions such as Hg(II), Pd(II), Cu(II), Cd(II), and Pb(II) from aqueous solutions. See: Hoshi, S.; Fujisawa, H.; Nakamura, K.; Nakata, S.; Uto, M.; Akatsuka, K. *Talanta* **1994**, *41*, 503−507.

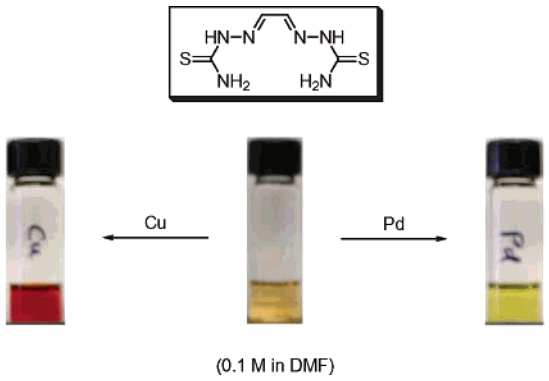


Figure 2. Glyoxal bis(thiosemicarbazone) as colorimetric indicator for copper and palladium.

To establish the efficiency of glyoxal bis(thiosemicarbazone) as a metal scavenger quantitatively, we conducted a Sonogashira coupling to yield a product with several ligating heteroatoms that are likely to extract some degree of metal from the reaction mixture (Scheme 2). After the reaction, the macrobeads were then separated into two portions, one of which was washed with the scavenger solution. Compared with the control experiment in which the beads were washed with the same sequence of solvents without the scavenger, the copper content of the cleaved product was lowered by 98% to 26 ppm and the palladium content by 87% to 330 ppm.[30]

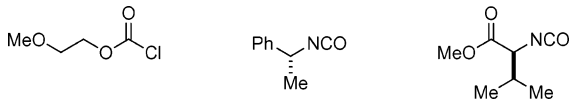


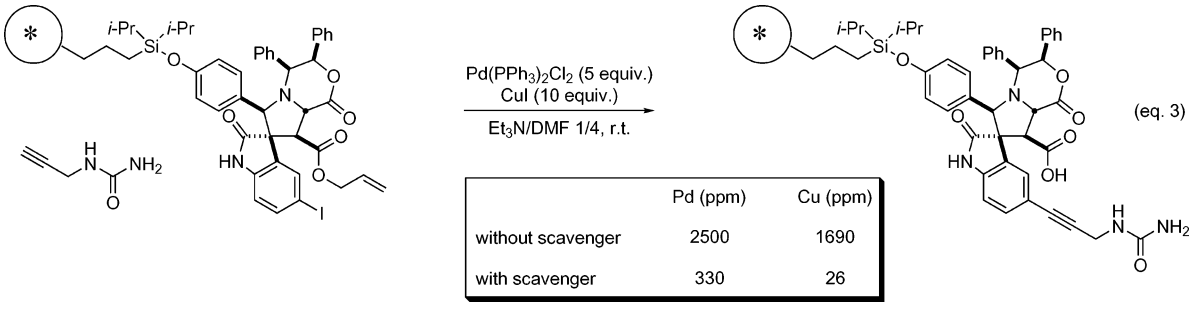
Figure 3. Acylating reagents for which reaction conditions were optimized.

Next, various methods to effect amide coupling were explored. Standard coupling reagents PyBOP[31]/(*i*-Pr)$_2$NEt are consistently effective over a broad range of amines. Most of the amines that present difficulties contain α-heteroatoms or are sterically demanding (Scheme 3).

Acid chlorides, chloroformates, and isocyanates were screened for the electrophilic capping of the γ-lactam nitrogen.[32] We identified three electrophiles from a panel of isocyanates and chloroformates during a preliminary screen of reactivity (Figure 3). The optimal reaction conditions for N-acylation depend on the electrophile used. By experimenting with changes in solvent, amine base, or addition of DMAP[33] as catalyst, we found conditions that provide good conversions for individual acylating reagents. Difficulty in identifying more general conditions suggests that N-acylation can be sensitive toward changes in substrate structure, an expected result given the increasing diversity of library members.

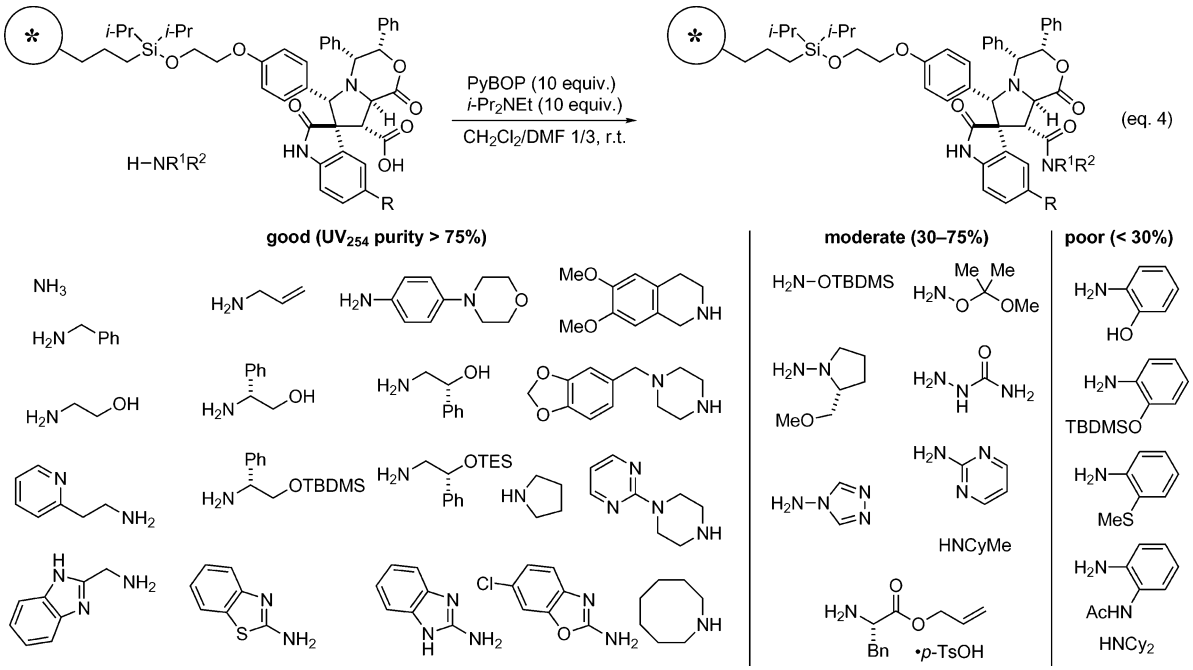
Library Synthesis. II. Preparation. Once the above three reaction pathways were validated with a range of building blocks, a library synthesis was planned according to Scheme 4.

Scheme 2. Effectiveness of Scavenger in Reducing Metal Contents from Sonogashira Coupling

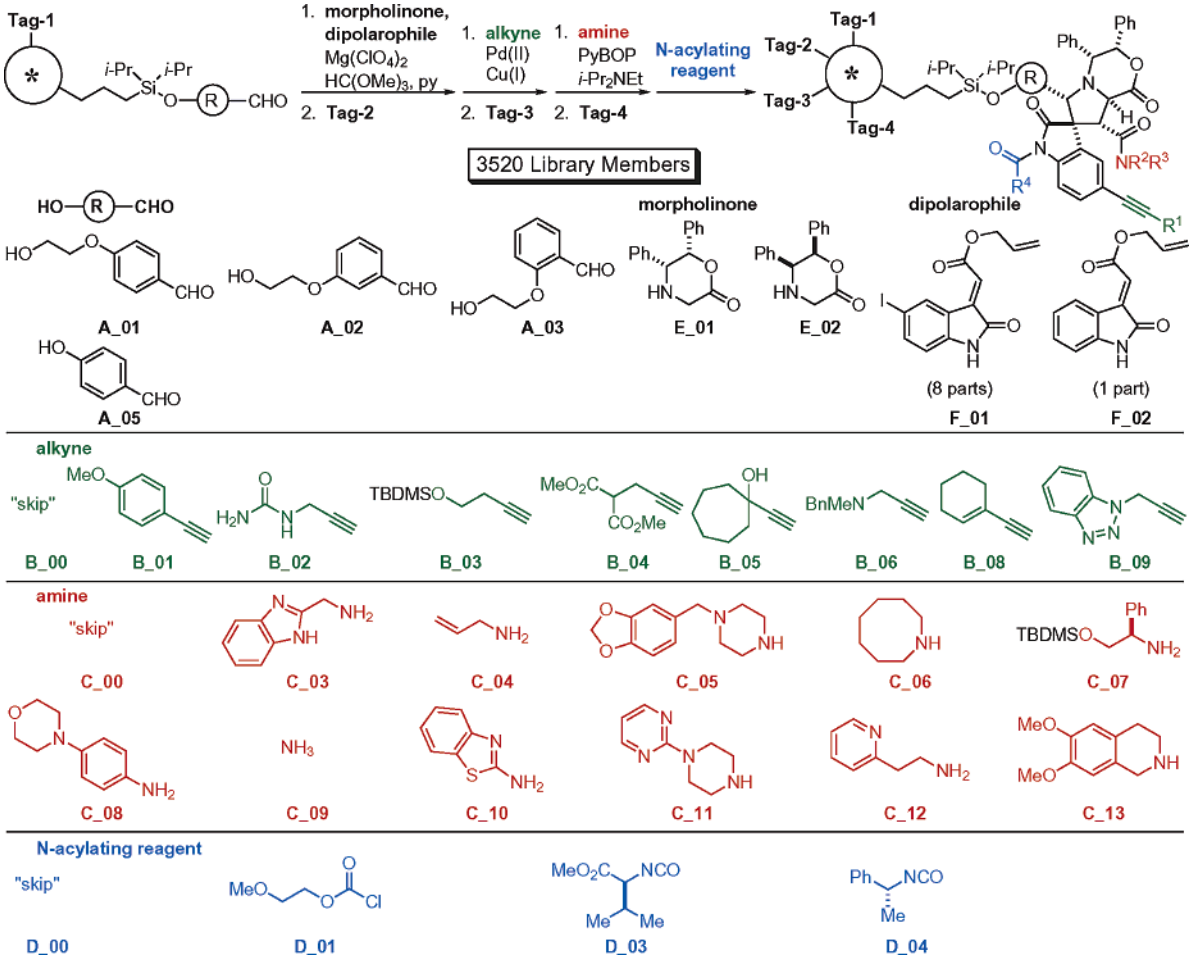


	Pd (ppm)	Cu (ppm)
without scavenger	2500	1690
with scavenger	330	26

Scheme 3. Test Results of Amine Building Blocks for Amide Formation



Scheme 4. Library Scheme and Final Building Block Selection[a]



[a] The TBDMS groups in black are removed upon cleaving the products from macrobeads.

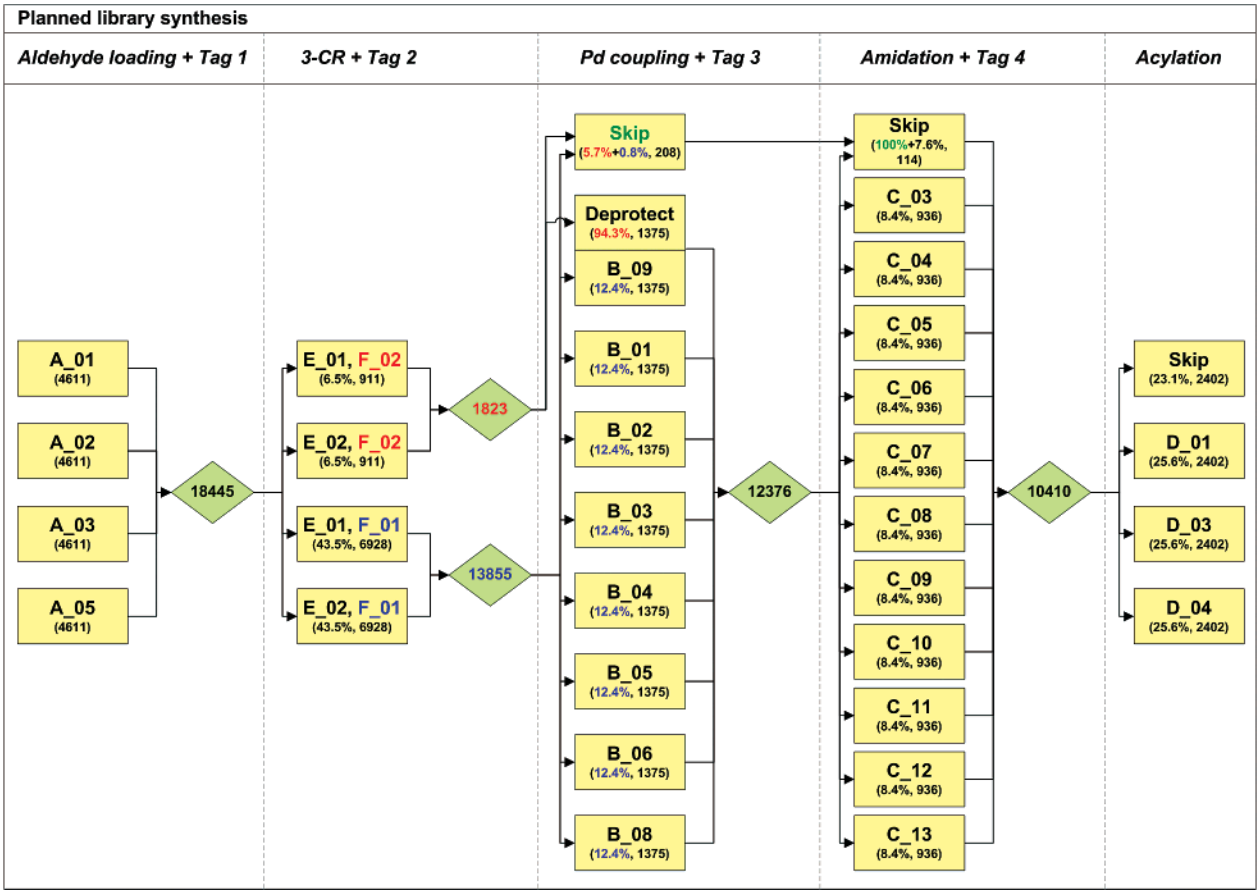
Starting from macrobead-supported aldehydes,[34] a Lewis acid mediated, stereoselective coupling was performed with 4 aldehydes, 2 morpholinones, and 2 dipolarophiles to yield 16 spirooxindole cores (4 × 2 × 2). The sequence of reaction for the eight cores containing the aryl iodide was as follows: (a) Sonogashira coupling with concomitant ester cleavage (skip + 8 alkynes); (b) amidation (skip + 11 amines); and (c) N-acylation (skip + 3 N-acylating reagents). This sequence generated 8 × (1 + 8 × 12) × 4 = 3104 members.[35] The sequence of reaction for the other eight cores without the aryl iodide was as follows: (a) ester cleavage (skip + deprotection); (b) amidation (skip + 11 amines); and (c) N-acylation (skip + 3 N-acylating reagents). This sequence generated another 8 × (1 + 1 × 12) × 4 = 416 members, so the complete library contains 3520 theoretical members.

Building blocks were selected based on intuition to maximize diversity (e.g., functional groups, heterocycles, degree of substitution) and on calculations to diversify the cLogP values of the library members. The analysis was performed in a linear fashion, but ultimately we hope to use software tools in development that allow the selection of building blocks based on their ability to diversify the desired chemical properties of all library members. To verify that the building blocks selected were viable in the synthesis pathway, thirteen library members, with each building block represented at least once, were randomly selected for synthesis on the macrobead support and full characterization.

Split-pool synthesis allows us to maximize operational efficiency. To ensure that each library member is equally represented, the macrobeads are split and pooled in different proportions. Hence, we devised a flowchart to streamline the process (Scheme 5). Each yellow rectangle represents a reaction vessel with the identity of the building block. In parentheses are shown the percentage of the pool that should go into the vessel and the number of intact beads expected after the reaction.[36] Each green diamond denotes a pool, and the number shown is the projected total number of beads. The flowchart not only helps the logistics of moving macrobeads through the library realization process but also can be useful to describe some late changes to the process itself.

(30) Analyzed by ICP-MS (West Coast Analytical Labs, CA).
(31) (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.
(32) In one model study, a spirooxindole was treated with 3-methoxybenzoyl chloride to give the acylated product in high yield. Although the product can be cleaved from macrobeads and characterized, it appears prone to hydrolysis upon prolonged storage as a 10 mM solution in DMSO. Hence, we excluded acid chlorides from further studies.
(33) *N*,*N*-(Dimethylamino)pyridine.
(34) The aldehydes were loaded on 500−600 μm polystyrene beads at a level of 150−200 nmol per bead.
(35) After the "skip" step in Sonogashira coupling, the allyl ester remained intact. Thus, amidation is not possible, and only one library member was generated.
(36) Based on the nature of the reaction, a percent loss was assigned to estimate bead loss or breakage during the reaction.

Scheme 5. Flowchart Guiding the Split-Pool Synthesis of the Library



The compatibility of the pathway with the chemical encoding procedure used to record the chemical history of the beads was next determined.[37] One combination of the three building blocks was selected, and the synthesis was performed with encoding at each step. Successful decoding directly from the bead after compound cleavage demonstrated that the operating chemical encoding procedure could be used with this library synthesis.[38]

Library Synthesis. III. Realization. With preparation work completed, we were ready to realize the library. The four aromatic aldehydes **A1**, **A2**, **A3**, and **A5** were loaded onto the macrobeads according to the reported procedure.[3b] Since no purification or isolation of intermediates is possible during a solid-phase split-pool synthesis, we tested each chemical reaction for high conversion and purity before the beads were transferred to the next step. Three beads were sampled from each reaction vessel and the compounds cleaved from them. The crude products thus obtained were then analyzed by LC/MS and ^{1}H NMR.[39] Each library member contains a pyrrolidine moiety that ensures the detection of the molecular ion (M+1) with electrospray ionization (ESI) detection in LC/MS. In addition, the high loading of the macrobeads (100−200 nmol/bead) allowed us to acquire a diagnostic NMR spectrum of material from a single macrobead within 15 min.

The following example from the Sonogashira coupling of spirooxindoles to 1-propargyl-1*H*-benzotriazole illustrates our analysis (Figure 4). Using the above analytical protocol, we determined that the transformation was incomplete in the first run, so the beads were resubmitted to the same reaction conditions until satisfactory conversion was accomplished. In addition to the progress of the reaction, we monitored the beads for effective tagging as well. After every encoding step, three beads were again retrieved and the compounds cleaved from them. Both the bead and the crude product were analyzed for proper tagging, and it was determined that compound decoding is more reliable than bead decoding for identifying library members.[38a]

During library realization, we discovered that there were encoding problems associated with building blocks **C3** and **C12**. We decided to keep the beads from these two reactions away from the pool and to perform the final N-acylation on them in a parallel fashion. We can reflect this change in the flowchart by disconnecting the corresponding reaction vessels from the pool step and connecting each of them to two parallel processes.[40] The change to the numbers in the flowchart is minimal, as only the numbers of beads entering the last pool and exiting the final N-acylation require modification. As a result of this change, the number of library members was reduced to 3232.

Library Synthesis. IV. Postsynthesis Quality Control. We used a sampling approach to estimate the quality of the library.

(37) For accounts of the development of chemical-encoding strategy, see: (a) Ohlmeyer, M. H. J.; Swanson, R. N.; Dillard, L. W.; Reader, J. C.; Asouline, G.; Kobayashi, R.; Wigler, M.; Still, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10922−10926. (b) Nestler, H. P.; Bartlett, P. A.; Still, W. C. *J. Org. Chem.* **1994**, *59*, 4723−4724.

(38) For details regarding the operation of chemical encoding, see: (a) Blackwell, H. E.; Pérez, L.; Schreiber, S. L. *Angew. Chem., Int. Ed.* **2001**, *40*, 3421−3425. (b) Reference 3a.

(39) The frequency of ^{1}H NMR analysis depends on the reliability of the reactions involved and the nature of the products.

(40) The flowchart depicting the realized library is provided in the Supporting Information.

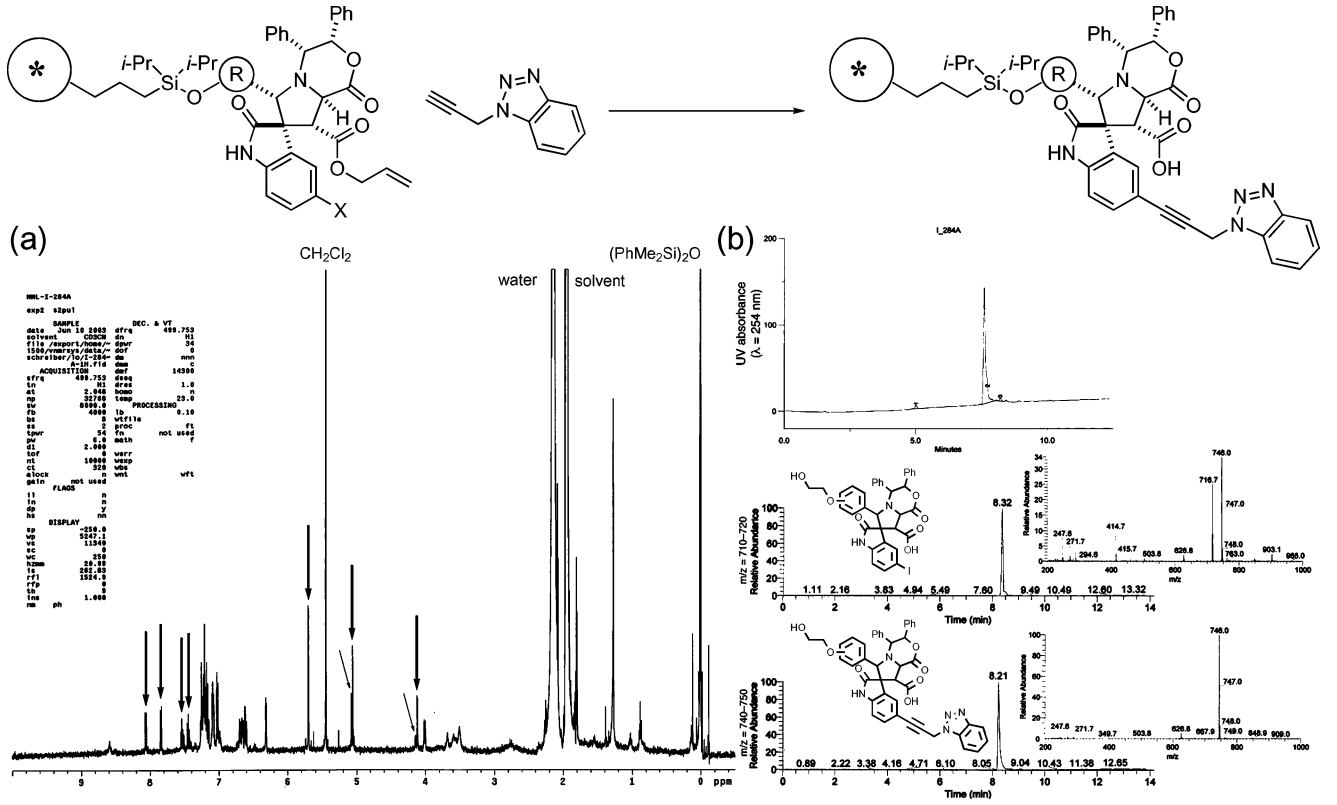


Figure 4. (a) ^{1}H NMR of the crude product from one macrobead. The bold arrows indicate signals corresponding to the product. The regular arrows indicate signals corresponding to the deprotected intermediate. $(PhMe_2Si)_2O$ was used as an internal standard for determining the loading level. (b) LC/MS of the corresponding sample. One peak was shown in the UV trace, but the peak actually consists of the product (MW = 745) and the deprotected intermediate (MW = 715). There was a 0.6 min shift between the UV trace and the MS trace.

Dolle has reported a similar approach to the same problem.[41] The sample was recorded "pure" if the UV trace at $\lambda = 254$ nm suggests a purity better than 80%, and the predominant compound has a molecular mass that matches one of the possible combinations. Within a sample of size n, the number of pure samples should follow a hypergeometric distribution, which can be approximated by a binomial distribution.[42] The confidence intervals for the population purity p can be readily determined from the number of pure samples and the sample size.[43] Since we are interested only in the lower limit, the one-sided lower limit at the 95% confidence interval is determined by setting $\alpha = 0.10$.

Figure 5 shows the purity analysis of the samples. Since the pools that are in parallel (**C3**/**D0**, **C3**/**D1**, **C12**/**D0**, **C12**/**D1**) are expected to contribute only about one-tenth of the number of compounds from the main pools, we normalized them so that each of these pools contributes only one datum point. Hence the total number of samples analyzed was 44, and the adjusted number of pure compounds was between 40 and 41.[44] The lower limit of purity for the whole library thus calculated is approximately 82% (one-sided 95% confidence interval), satisfying our target figure of 80%.[45]

As the final step prior to moving library members to a collection maintained at the screening center sponsored by the Initiative for Chemical Genetics (ICG), the macrobeads were arrayed into 384-well plates. Compounds were cleaved with HF•pyridine and eluted from the solid phase in an automated fashion, a process referred to as formatting.[3b] Altogether 20 plates of compounds were formatted. A final quality control was performed with the formatted compounds, which revealed that the purity of the library was lowered. This observation was expected, since the formatting process involves a number of automated liquid-handling steps and solution transfers. Using a less stringent criterion, 45 out of 59 wells had purity better than 70% and were recorded as pure. The lower limit of purity thus determined was 65%. By removing the plate that contains the building block combination **C3**/**D1**, we improved the lower limit to 70%. The remaining 19 plates of library compounds are now stored as part of the ICG DOS collection, available for screening by the scientific community.[46] The library was also printed on glass slides, enabling its use in small-molecule microarray assays.[47]

(41) Dolle, R. E.; Guo, J.; O'Brien, L.; Jin, Y.; Piznik, M.; Bowman, K. J.; Li, W.; Egan, W. J.; Cavallaro, C. L.; Roughton, A. L.; Zhao, Q.; Reader, J. C.; Orlowski, M.; Jacob-Samuel, B.; DiIanni Carroll, C. *J. Comb. Chem.* **2000**, 716−731.

(42) The multiplicative finite population correction factor for the confidence limit is given as $\sqrt{((N-n)/(N-1))}$, where N is the number of beads in the whole library and n is the number of beads in the sample. The factor is greater than 0.99 and hence approximated by 1.

(43) *Intercooled Stata 8.0 for Macintosh*; Stata Corporation: College Station, TX, 2003.

(44) For pools **C3**/**D0**, **C3**/**D1**, **C12**/**D0**, and **C12**/**D1**, the number of pure compounds coming out from each of them can have a value of either 0 or 1. Based on the analysis on the macrobeads from these pools, we assign a value of 1 for **C3**/**D0** ($p_1 = 0.875$), a value of 0 for **C3**/**D1** ($p_2 = 0$), and a value of 1 for **C12**/**D0** ($p_3 = 1.0$). Since pool **C12**/**D1** gives a p_4 value of 0.6, we cannot assign a definite value of either 0 or 1. We therefore report the total number of pure compounds as a range and calculate the lower limit in each case.

(45) The average of the two lower limits from 40 pure compounds (80.4%) and 41 pure compounds (83.3%).

(46) For a listing of libraries available for screening at the ICG, see http://iccb.med.harvard.edu/chemistry/compound_libraries/libraries_available.htm (accessed August 2004).

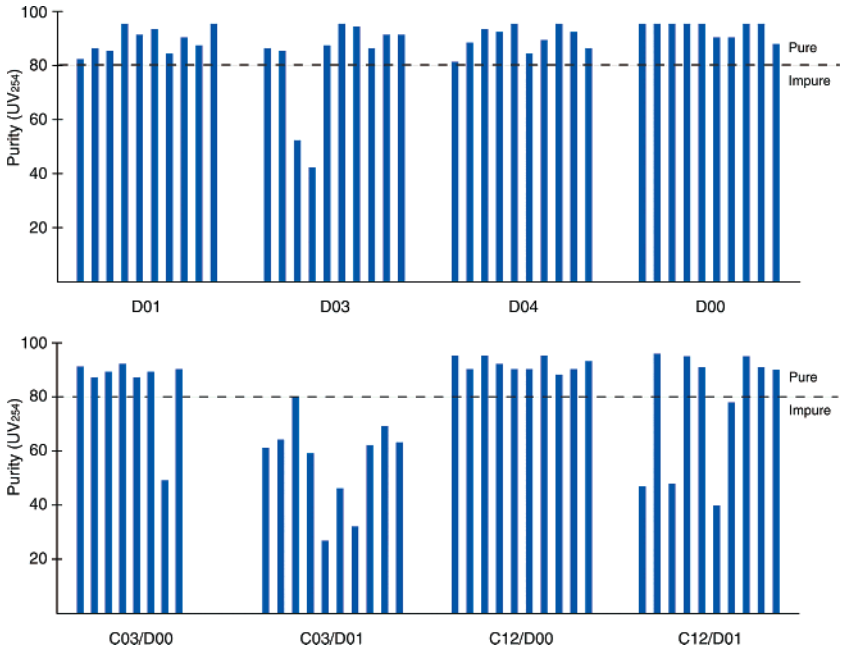


Figure 5. Purity analysis of the library before formatting.

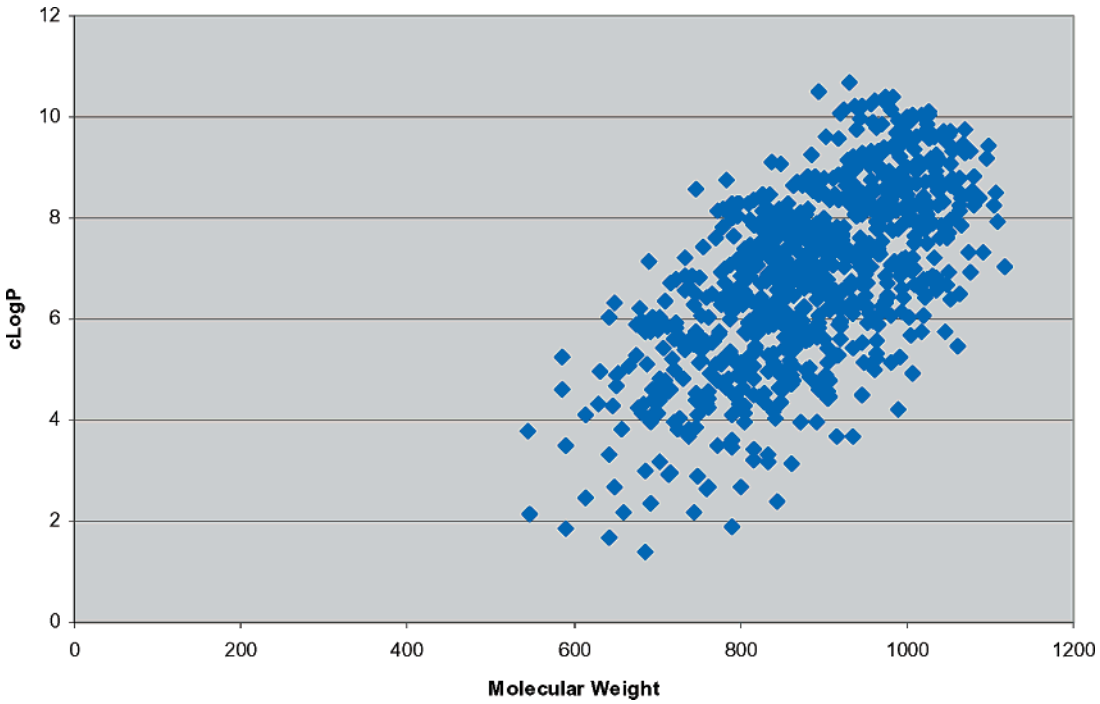
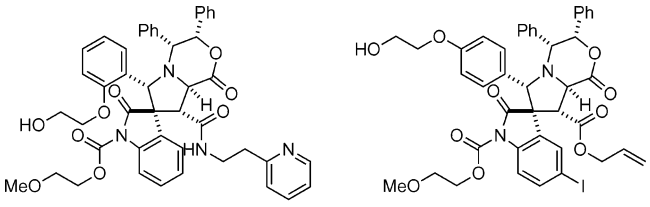


Figure 6. Plot of cLogP versus molecular weight of the final library members.

Preliminary Results from Cell-Based Screening. For a small-molecule library to be broadly useful in chemical genetics, members must be able to cross cell membranes and to bind specifically to protein targets. An examination of two key chemical properties of our realized library reveals that the members tend to be more lipophilic than those deemed "druglike" and have molecular weights more reminiscent of natural products than druglike compounds (Figure 6).[48] To demonstrate the value of these compounds as effective probes, we used a chemical genetic modifier screen to search for bioactive compounds.[49] This type of screen identifies compounds that enhance or suppress cellular phenotypes, for example, those induced by a small molecule with a known mechanism of action.

An assay was developed to identify enhancers of the growth arrest induced by latrunculin B,[50] a natural product that

(47) McPherson, O. M.; Koehler, A. K. Broad Institute, Cambridge, MA. Unpublished work.
(48) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **1997**, *23*, 3−25.
(49) For a general review on screening, see: (a) Stockwell, B. R. *Nat. Rev. Genet.* **2000**, *1*, 116−125. For examples of chemical genetic modifier screens, see: (b) Koeller, K. M.; Haggarty, S. J.; Perkins, B. D.; Leykin, I.; Wong, J. C.; Kao, J. M.-C.; Schreiber, S. L. *Chem. Biol.* **2003**, *10*, 397−410. (c) Butcher, R. A.; Schreiber, S. L. *Chem. Biol.* **2003**, *10*, 521−531. (d) Haggarty, S. J.; Koeller, K. M.; Kau, T. R.; Silver, P. A.; Roberge, M.; Schreiber, S. L. *Chem. Biol.* **2003**, *10*, 1267−1279. (e) Huang, J.; Zhu H.; Haggarty, S. J.; Spring, D. R.; Snyder, M.; Schreiber, S. L. Submitted for publication.



9
MW = 796
clog P = 5.51

7i
MW = 858
clog P = 7.48

Figure 7. Spirooxindoles screened in follow-up assays for latrunculin B enhancement.

sequesters monomeric actin and prevents the formation of actin microfilaments.[51] Latrunculin B has been a valuable tool in elucidating the roles of the actin cytoskeleton in both yeast and mammalian cells.[52] Small molecules that perturb this system should provide further insight into its regulation.

The initial screen was performed in 384-well plates using wild-type yeast growing in a nutrient-rich medium. Latrunculin B was added to a final concentration of 7 μM, followed by pin transfer of library stock solutions to a final concentration of approximately 33 μM. Ten formatted stock plates were used for the initial screen, resulting in 3520 individual assays. The assay plates were allowed to stand at room temperature, and yeast growth was quantitated over a span of 24 to 96 h. If the small molecule enhances the effect of latrunculin B, the growth of the yeast in the assay well will be retarded. By comparing growth in the assay wells with that in the control wells lacking library members, 36 compounds were scored as enhancers. Thirty-three were successfully decoded, revealing that these positives represented 19 unique chemical structures.[53]

A crude structure−activity relationship can be deduced from these initial positives. Two building blocks, aldehyde **A3** (28/33) and the (5*R*,6*S*)-morpholinone **E1** (28/33), dominate the positives. All positives were derived from the dipolarophile **F2**, which is not substituted at the 5-position of the indole ring, even though these members should constitute only 12% of the library. These results hint that the positives might be acting through a common target or mechanism. Based on these observations and the relative strengths of the positives, compound **9** was chosen for resynthesis and follow-up assays (Figure 7). For these assays, the amount of latrunculin B was reduced to 1.0 μM, which is 12.5% of the experimentally determined EC_{50} value.[54] At this concentration, latrunculin B alone has no observable effect on yeast growth. When **9** was added to this same medium, the inhibitory effect of latrunculin B is significantly enhanced. The EC_{50} value[55] for **9** was determined to be 550 ± 50 nM.

Two additional experiments were performed as controls. First, when yeast cells are treated with **9** alone, up to the solubility limit of 30 μM, yeast growth is unaffected. This experiment shows that **9** does not show the same phenotype as latrunculin B and that it is synthetic lethal with latrunculin B. Second, we chose a member of the library **7i**, which has a median molecular weight and cLogP, to serve as a negative control. Yeast cells treated with **7i** alone or with a combination of **7i** and latrunculin B show no inhibition of growth relative to untreated yeast, confirming that the activity of **9** is not a general property of the spirooxindole core. Further studies of **9** and the elucidation of its mechanism are underway. For the moment, the experiments confirm that small molecules emanating from the synthetic pathway described herein are capable of yielding novel probes of cell circuitry, in this case of the actin regulatory network.

Conclusion

We have developed a stereoselective, Lewis acid mediated variant of the Williams 3-CR that is suitable for macrobead-supported aldehydes. The diversity of these structurally complex and stereochemically diverse products can be further increased by efficient Sonogashira coupling, amide formation, and N-acylation of γ-lactams. A library of 3232 theoretical spirooxindoles was synthesized, and through sampling of individual macrobeads, it was determined that at least 82% of the library compounds have greater than 80% purity. To demonstrate our overall discovery process, a cell-based screen was performed with library members to identify enhancers of latrunculin B, an actin polymerization inhibitor. Through resynthesis, one of the positives was confirmed and found to have an EC_{50} in the sub-micromolar range. One of several important goals that emerged from these studies is the use of computed properties of virtual products to guide the selection of subunits and appendages, so that the actual products are distributed across regions of chemical space of greatest promise for interrogation.

Acknowledgment. We thank Robert M. Williams for a generous gift of Boc-protected **1** and for sharing unpublished results with us. We thank Paul Clemons for generating computed properties of the library. We thank Leticia Castro, Jennifer Raggio, Kerry Pierce, and James Roger for their expert assistance in library decoding, formatting, and quality control. We thank Justin Klekota and Newton Y.-L. Wai for discussions concerning sampling statistics. We acknowledge NIH/NCI Grant 2P01 CA78048-06A1 for support of this research and thank the NCI for sponsorship of the ICG. S.N. and C.S.N. were supported by JSPS and NSF fellowships, respectively. S.L.S. is an Investigator with the Howard Hughes Medical Institute at Harvard University.

Supporting Information Available: Experimental procedure for library synthesis, characterization data of 3-CR products and demonstration compounds, assay protocols, structures of assay positives. This material is available free of charge via the Internet at http://pubs.acs.org. The SDF file containing the product structure and 6 computed chemical properties for each member of the realized library can be downloaded at http://people.med.harvard.edu/~pclemons/DOS/props/props.htm.

JA045089D

(50) Detailed procedures for the high-throughput assay and corresponding follow-up assays are available in the Supporting Information.
(51) Spector, I.; Shochet, N. R.; Kashman, Y.; Groweiss, A. *Science* **1983**, *219*, 493−495.
(52) Peterson, J. R.; Mitchison, T. J. *Chem. Biol.* **2002**, *9*, 1275−1285.
(53) See Supporting Information for the structures of these positives.
(54) Effective concentration that produces 50% of the maximum possible response for the small molecule.
(55) Defined as the concentration required to restore latrunculin B to show the same phenotype at its EC_{50} value 8 μM.