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Research Paper

A one-bead, one-stock solution approach to chemical genetics: part 1

Helen E. Blackwell [a,b,1], Lucy Pérez [a,b,1], Robert A. Stavenger [a,b,2], John A. Tallarico [c], Elaine Cope Eatough [c,3], Michael A. Foley [c,3,4], Stuart L. Schreiber [a,b,c,*]

[a] *Howard Hughes Medical Institute at Harvard University, 12 Oxford St., Cambridge, MA 02138, USA*
[b] *Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford St., Cambridge, MA 02138, USA*
[c] *Institute of Chemistry and Cell Biology (ICCB), Harvard Medical School, 250 Longwood Ave., Boston, MA 02115, USA*

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Abstract

Background: In chemical genetics, small molecules instead of genetic mutations are used to modulate the functions of proteins rapidly and conditionally, thereby allowing many biological processes to be explored. This approach requires the identification of compounds that regulate pathways and bind to proteins with high specificity. Structurally complex and diverse small molecules can be prepared using diversity-oriented synthesis, and the split-pool strategy allows their spatial segregation on individual polymer beads, but typically in quantities that limit their usefulness.

Results: We report full details of the first phase of our platform development, including the synthesis of a high-capacity solid-phase bead/linker system, the development of a reliable library encoding strategy, and the design of compound decoding methods both from macrobeads and stock solutions. This phase was validated by the analysis of an enantioselective, diversity-oriented synthesis resulting in an encoded 4320-member library of structurally complex dihydropyrancarboxamides.

Conclusions: An efficient and accessible approach to split-pool, diversity-oriented synthesis using high-capacity macrobeads as individual microreactors has been developed. Each macrobead contains sufficient compound to generate a stock solution amenable to many biological assays, and reliable library encoding allows for rapid compound structure elucidation post-synthesis. This 'one-bead, one-stock solution' strategy is a central element of a technology platform aimed at advancing chemical genetics.

Keywords: Chemical genetics; Diversity-oriented synthesis; Library encoding/decoding; Polystyrene macrobead; Split-pool synthesis

1. Introduction

Genetics has been a primary contributor to our understanding of biology. Both forward and reverse genetics rely upon mutant alleles to gain insights into pathways

Abbreviations: APCI, atmospheric pressure chemical ionization; BB, building blocks; BSA, *N*,*O*-bis(trimethylsilyl)acetimide; CAN, ceric ammonium nitrate; CH_2Cl_2, dichloromethane; CH_3CN, acetonitrile; DMF, dimethylformamide; GC, gas chromatography; GC/ECD, gas chromatograph equipped with an electron capture detector; HF/py, hydrogen fluoride/pyridine; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LC/MS, tandem liquid chromatography/mass spectroscopy; MeOH, methanol; MS, mass spectroscopy; NMR, nuclear magnetic resonance; PS, polystyrene; THF, tetrahydrofuran; TMSOMe, methoxytrimethylsilane; UV, ultraviolet

[1] These authors contributed equally to this work.
[2] Present address: GlaxoSmithKline, 1250 S. Collegeville Rd., Collegeville, PA 19426, USA.
[3] Present address: Infinity Pharmaceuticals, 650 Albany St., Boston, MA 02115, USA.
[4] Also corresponding author.

* Corresponding author.
E-mail addresses: mfoley@infinitypharm.com (M.A. Foley), sls@slsiris.harvard.edu (S.L. Schreiber).

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or processes of interest. Small molecules have also been used to gain insights into biology in ways that are analogous to either forward or reverse genetics. Many of these advances (for example, Carlsson's use of chlorpromazine to explore the dopamine receptor [1] and Borisy's use of colchicine to discover tubulin [2]) have been brought to light on a case-by-case basis. In recent years, progress has been made in developing a systematic way to explore biology with small molecules, the chemical genetic approach [3,4]. Key elements to systematic and accessible chemical genetics are the processes by which small molecules are synthesized, screened, and further developed.

Biological processes can be explored with collections of small molecules using either phenotypic [5] or protein-binding assays [6,7]. Concomitant with the development of such assays, structurally complex and diverse compounds having the potential to modulate many processes need to be obtained. Diversity-oriented synthesis [8], solid-phase purification [9], and the split-pool strategy [10] have allowed for the efficient synthesis of such compounds during the library realization phase of research [11]. A key issue not yet satisfactorily resolved, however, is the formatting of these compounds. Typical synthesis beads yield insufficient quantities of compounds to allow many experiments to be performed with the amounts derived from a single bead. This has led to assaying pools of compounds, which is non-optimal for many reasons. A notable exception is the platform developed by IRORI relying on a variation of split-pool synthesis named directed sorting and using various sized 'Kans' [12]. These have the capacity to entrain sufficient numbers of beads so as to allow multiple milligram quantities of compounds to be derived from individual Kans. A NanoKan system has recently been reported in the context of split-pool syntheses of benzofuran and related compounds bearing structural similarities to natural products [13]. However, from the perspective of our research and training goals in chemical genetics, the IRORI system suffers from two shortcomings, both relating to cost. First, given our ability to perform highly miniaturized and effective phenotypic [5] and protein-binding assays [6,7], we calculate that an optimal sample size of compound derived from an individual bead (of which a Kan is a functional equivalent) is closer to 0.1 mg. Since the majority of compounds synthesized in a split-pool synthesis will not score as a positive in an assay, over-synthesis is costly and wasteful. Second, we aimed to develop a simple and inexpensive system, one, for example, that could be re-established by a trainee at a second site within the constraints of a typical academic budget. If chemical genetics is to have the broad applicability of biochemistry and genetics as a vehicle to explore biology, it must be portable, accessible, and cost-contained. This is an issue that also faces genomics as it evolves into a broadly applicable means to explore biology.

The system that we have developed is outlined in Fig. 1. Small molecules are synthesized on polystyrene (PS) macrobeads, which serve as individual reaction vessels during split-pool library syntheses, delivering arrayed ~5 mM stock solutions upon compound cleavage and resuspension in high-density assay plates [14]. Here we describe the first phase of the development of a two-part 'one-bead, one-stock solution' technology platform, including a scaled synthesis of a high-capacity solid-phase bead/linker system and the development of a reliable library encoding/decoding strategy. This phase was validated by the analysis of an enantioselective, diversity-oriented synthesis resulting

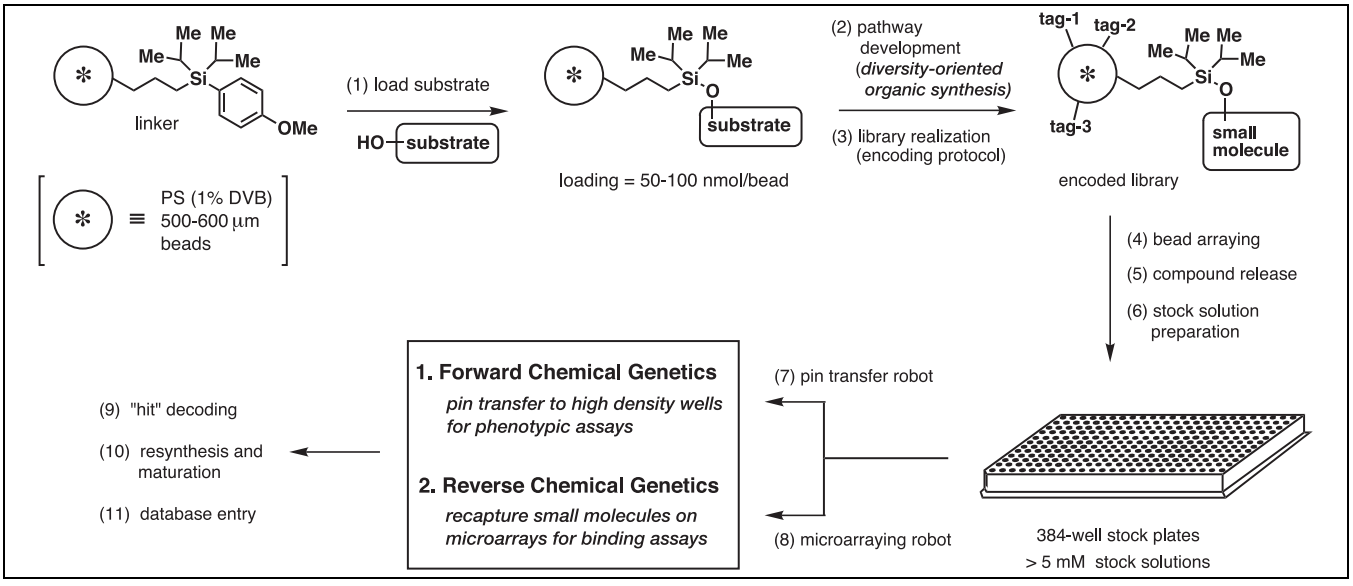

Fig. 1. Outline of a 'one-bead, one-stock solution' technology platform directed toward chemical genetics. DVB = divinylbenzene.

in an encoded 4320-member library of structurally complex dihydropyrancarboxamides (**12**) whose synthesis has been reported [15]. A full account of the second phase incorporating: (1) bead arraying, (2) automated compound cleavage, elution, and resuspension as segregated stock solutions, and (3) assay format design and annotation, follows this paper in this journal issue [16].

Our current technology platform evolved from an earlier, only partially successful plan [17] that attached compounds to 90 μm TentaGel beads [18], a poly(ethyleneglycol)–PS copolymer, via a photolabile linker developed by Geysen and co-workers [19]. This bead/linker combination allowed compounds to be released from the polymeric supports in the presence of aqueous media and living cells [20] by exposure to long wavelength (365 nm) ultraviolet (UV) light. Each split-pool synthesis step was encoded with electrophoric tags using the method introduced by Still and co-workers (see below) [21,22]. The limitations of producing encoded small molecules on TentaGel beads became apparent at the compound screening stage [17]. First, we observed that the manual arraying of TentaGel beads in a one bead per well format was not reliable, which resulted in a variable distribution of beads in the assay plate wells (i.e. from 0 to 10 beads per well). After compound cleavage, numerous wells appeared to contain active compounds in cytoblot [23] assays. However, upon closer inspection, most of these 'active' wells held more than one synthesis bead, and thus contained a mixture of compounds. Some of the apparent activities were ephemeral, presumably a non-specific effect resulting from high concentrations of the compound mixtures. As it is necessary to decode and re-synthesize each compound in order to confirm a positive in an assay, or to test lower concentrations in order to determine the potency of a compound, these steps presented an insuperable bottleneck to our early approach.

From this experience, we concluded that efficient screening requires a library realization platform that produces sufficient compound per bead to perform many hundreds of assays; i.e. a minimum of 50 nmol of small molecule per synthesis bead (Fig. 1). To this end, we have developed 500–600 μm PS macrobeads for diversity-oriented syntheses that yield ~100 nmol of synthetic compound per macrobead [24]. Since the majority of these compounds will not show biological activity, we believe that generating unique small molecules on this scale is not only cost-effective, but also more environmentally sound and conscious of future storage requirements. Moreover, due to the substantially larger size of the PS macrobeads, they can be arrayed reliably and efficiently (~5 min per plate) into individual wells of standard 384-well assay plates [14,16]. The overall process is affordable and should be accessible to many laboratories. The use of these macrobeads in split-pool diversity-oriented syntheses is a key element of our chemical genetics technology platform.

2. Results and discussion

2.1. Development of a robust bead/linker system

At the outset, we desired a bead/linker combination amenable to the widest array of reactions used in modern organic synthesis [24]. Beyond chemical stability requirements, two other criteria were addressed regarding our bead/linker: (1) library starting materials (building blocks (BBs)) should be loaded onto the support with high efficiency, and (2) the final small molecule products should be quantitatively released from the support. Applying a similar rationale used in target-oriented synthesis [25], we chose a silicon-based linker as the solution [26,27]. The final parameter of the bead/linker equation was the size and nature of the polymer support; 500–600 μm, lightly cross-linked (1% divinylbenzene), PS macrobeads appeared to be the only support commercially available with the physical capacity to deliver ≥50 nmol/bead. Moreover, it was the most physically robust support in our hands (see below), in comparison to either high-capacity ArgoPore™ (Argonaut Technologies) or TentaGel [18] resins.

The synthesis of the bead/linker system was adapted from the protocol of Woolard et al. [26] and is shown in Fig. 2 (see below). Hydroboration of allylsilane **1**, followed by a Suzuki cross-coupling with bromine-functionalized PS macrobeads (**3**) yielded diisopropylalkyl silicon-functionalized support **4** containing ~200 nmol of Si/bead. This support can be stored indefinitely and used in an 'off-the-shelf' fashion. Activation is achieved through treatment with excess triflic acid, generating a silyl triflate-functionalized support. The silyl triflate can be trapped efficiently with primary, secondary, and phenolic alcohols in the presence of excess 2,6-lutidine as the support loading protocol [24]. Once library synthesis is complete, quantitative cleavage is achieved by treatment of the PS macrobeads with hydrogen fluoride/pyridine (HF/py), followed

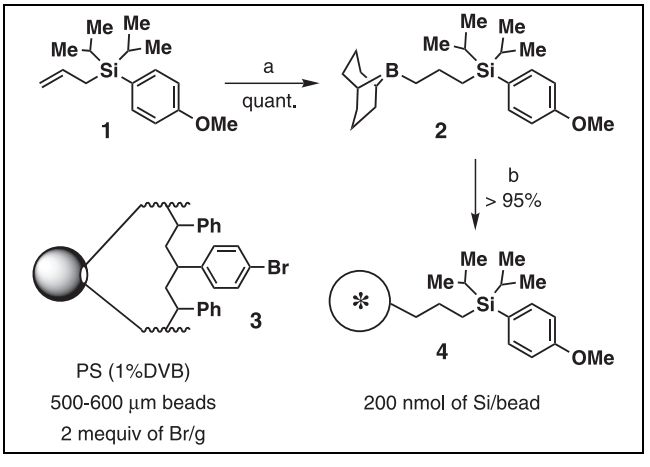

Fig. 2. Synthesis of the PS macrobead/linker system. Reagents and conditions: (a) 9-borabicyclo[3.3.1]nonane (1 eq.), THF, 25°C, 3 h; (b) 5 mol% $Pd(PPh_3)_4$, NaOH (2 eq.), **3** (0.6 eq.), THF, reflux, 36 h.

by quenching with methoxytrimethylsilane (TMSOMe) or ethoxytrimethylsilane. This procedure yields the released small molecule and volatile by-products, the latter being removed easily in vacuo. After drying, the resulting compound can be eluted from the bead by repetitive washing with either acetonitrile (CH_3CN) or dimethylformamide (DMF). The wash solution can be transferred away from the bead for stock solution generation elsewhere [16].

2.2. *Maintenance of bead integrity throughout solid-phase synthesis*

While the use of sequences of tandem organic reactions can efficiently generate complex molecules in diversity-oriented syntheses [8], we have observed that successive organic transformations, coupled with rigorous bead washing between reactions, can damage the PS macrobeads. Our technology platform (Fig. 1), however, demands that we isolate one physically intact bead per well prior to compound cleavage for several reasons. First, fragments of beads yield weaker compound stock solutions after bead arraying, cleavage, and resuspension. Second, the possibility of isolating more than one fragment per well allows for stock solution contamination and the concomitant incorrect decoding of that well. To avoid these problems, we have developed a set of standard practices for bead handling during library synthesis and encoding that dramatically minimize the possibility of bead breakage.

In general, we have found that the less we handle the solid supports physically, either by submission to chemical reactions, washing, or drying, the less bead breakage we observe. This reinforces the importance of an effective planning algorithm for diversity-oriented syntheses. Short reaction sequences yielding complex and diverse compounds not only ensure that positives can be re-synthesized readily, but also promote the integrity of the beads. In order to quantify bead integrity, we used population size distribution measurements (obtained by light obscuration) to monitor the shift of the average particle size in a sample of beads (data not shown). We first observed that the PS macrobeads were fragile when swollen in organic solvents. Since the use of solvents and drying are required in library synthesis, we assessed several solvent, drying, and agitation conditions. Even though certain chemical transformations appear to cause more bead breakage than others, we did not include different chemical reactions as experimental variables in our studies because we did not want to limit the types of chemistry utilized in library synthesis.

As evidence that even the most simple and gentle handling induces damage, supports swollen in dichloromethane (CH_2Cl_2) and drained seven times, followed by overnight air drying resulted in a shift to a smaller average size distribution. As an example of extreme damage, beads were subjected to swelling in tetrahydrofuran (THF) (45 min), followed by treatment with methanol (MeOH) (45 min) and 360° rotation. The beads were then rapidly dried via lyophilization, and the whole process was repeated seven times. These supports show even more extensive damage and a greater degree of bead fragmentation. The 'best practices' we extrapolated from these experiments include light agitation from a wrist-action shaker, followed by blowing N_2 over the resin (30 min), and final drying under high vacuum conditions from any organic solvent. While a shift in average size still exists, these conditions minimize fragmentation and are suitable for library syntheses, as judged by our ability to array one intact bead per well after library synthesis (see below) [14,16].

2.3. *An optimized binary encoding protocol for 500–600 μm PS macrobeads*

To facilitate compound identification following split-pool syntheses, compound encoding strategies [28] have been developed that allow the identity of the compounds to be inferred post-synthesis [29]. Most often, the chemical reaction and BB used to synthesize each compound are encoded. Thus, the synthetic history of the compound is encoded rather than its direct chemical identity. While numerous encoding techniques have been reported [28], including spatial [30], chemical [21,22,31,32], spectrometric

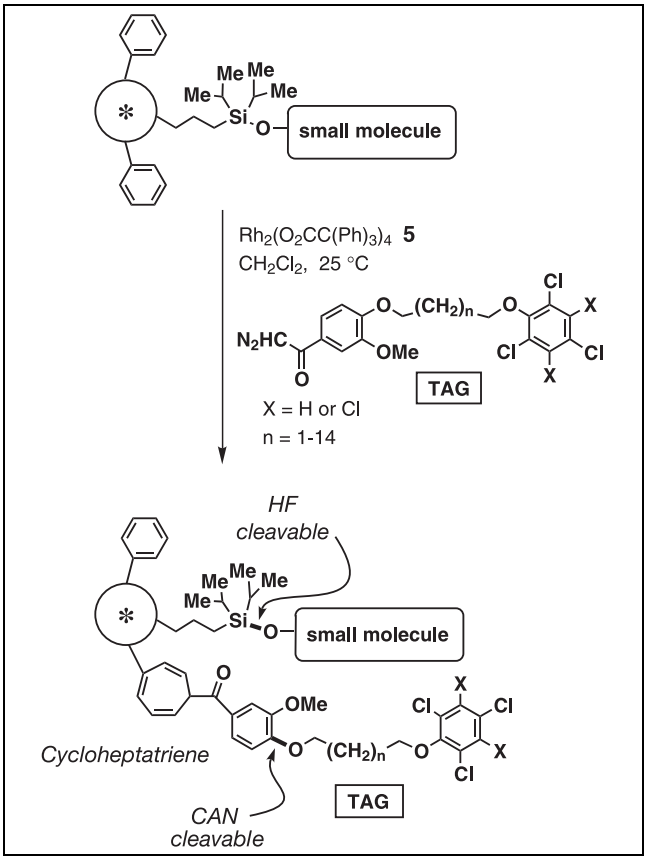

Fig. 3. General reaction protocol for encoding PS macrobeads with diazoketone chloroaromatic tags. The chemical bonds broken upon compound cleavage and tag cleavage are highlighted in bold.

[33,34], electronic [12,13,35], and physical methods [36], we chose to use the chemical encoding strategy introduced by Still and co-workers [21] because it is amenable to the synthesis of very large libraries (i.e. on the order of millions), operationally straightforward, and relatively inexpensive. This method uses chloroaromatic, diazoketone tags that are detectable at sub-picomolar levels by electron capture gas chromatography (GC/ECD) [22]. The tags are introduced via a $Rh_2(O_2CC(Ph)_3)_4$ (**5**)-catalyzed acylcarbene insertion into the aromatic rings of the PS solid support to yield cycloheptatrienes (Fig. 3) [37,38]. The carbene can insert indiscriminately into both the PS support and the compound bound to the support. However, it has been postulated that the carbene inserts predominantly into the support due to the greater proportion of the support relative to compound [22]. Furthermore, the acylcarbene insertion reaction is in direct competition with the more favored carbene homodimerization reaction; therefore, the supports' tagging level is quite low relative to the bound compound (on average 1%). This inefficient tagging reaction is compensated by the exquisite sensitivity of the GC/ECD for haloaromatic functionality.

The chloroaromatic portion of the tags is oxidatively labile and can be readily cleaved from the support with ceric ammonium nitrate (CAN) to yield free alcohols [39]. The tag cleavage protocol is orthogonal to our HF/py compound cleavage strategy and to the majority of our library synthesis steps (i.e. oxidative reaction conditions are preferentially avoided). After silylation with *N*,*O*-bis-(trimethylsilyl)acetimide (BSA), an aliquot of the tag ethers can be injected directly onto a GC/ECD for analysis. Each tag silyl ether has a characteristic GC/ECD retention time.

Numerous groups have prepared combinatorial libraries encoded with the chloroaromatic tags [29]. However, none of these libraries was synthesized on polymeric supports analogous to the high-capacity PS macrobeads we have selected (see above). To date, the majority of the libraries encoded with chloroaromatic tags [21] have been prepared on 80–100 μm TentaGel resin [18] which has markedly different physical properties from those of the large, hydrophobic PS support [40]. Preliminary experiments on 500–600 μm PS macrobeads quickly revealed that previously reported encoding and decoding procedures for 80–100 μm TentaGel were not transferable simply by scaling the procedures relative to bead size [17,41]. It was not surprising that these decoding protocols were not applicable to our hydrophobic PS support because these procedures were carried out in predominantly aqueous CAN solutions, amenable to hydrophilic TentaGel beads. Therefore, we had to develop an optimal binary encoding and decoding strategy for 500–600 μm PS macrobeads to address these issues and, importantly, enable our technology platform. Moreover, we chose to develop protocols that were as straightforward as possible, lending themselves to automation in the future.

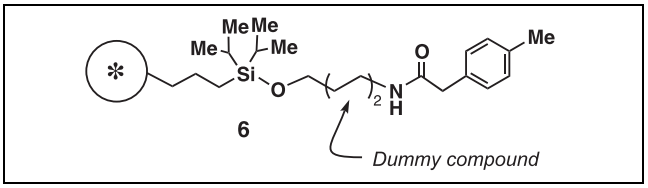

Fig. 4. Encoding test support **6** employed in all encoding optimization experiments.

2.4. Optimization of the encoding procedure

In order to mimic conditions during actual library encoding steps, we used an encoding test support (**6**) on 500–600 μm PS macrobeads to which a 'dummy compound' was bound (Fig. 4). *N*-(5-Hydroxy-pentyl)-4-methyl-benzamide [42] was chosen as the 'dummy compound' because it: (1) could be loaded onto the support easily through the primary alcohol moiety, (2) had low volatility, thereby expediting cleavage and analyses of small compound samples, and (3) had structural elements that would allow us to study if the carbene was indiscriminately inserting into the compound [43] versus the polymer support (see below). Finally, we decided to encode the support (**6**) with four tags in each encoding optimization experiment (Fig. 3): Tag C3Cl3 (X = H, $n = 1$), Tag C3Cl5 (X = Cl, $n = 1$), Tag C9Cl5 (X = Cl, $n = 7$), and Tag C16Cl5 (X = Cl, $n = 14$). These four tags were selected because they had GC retention times that spanned the full window of the ~12 min GC chromatogram.

In general, an encoding step requires the addition of solutions of both tag and rhodium catalyst **5** to the polymer support. As the PS macrobeads swell very well in CH_2Cl_2, a property necessary for good reaction kinetics on solid phase, CH_2Cl_2 was our solvent of choice for the encoding reactions. For each reaction condition we examined, 10 beads were subjected to the reaction condition and decoded in parallel (data averaging over 10 beads was essential due to the relatively wide size distribution of the PS macrobeads (500–600 μm)), and the resulting picomolar data for each tag on the 10 beads were averaged. While we were concerned initially with bead breakage during the tagging reactions (see above), we found that gentle tumbling during the reaction was essential for maximum tag incorporation; in the end, our encoding procedure was essentially non-destructive to the beads. Next, we systematically increased the tag and rhodium catalyst **5** concentrations from concentrations previously used on TentaGel resin [17] (2 nmol tag/bead, 40 pmol catalyst/bead) to concentrations approximately 10- and 50-fold higher for tag and catalyst, respectively (20 nmol tag/bead, 2 nmol catalyst/bead). Altering the concentration of either the tag or catalyst by three orders of magnitude about these conditions demonstrated that our initial increased tag and catalyst concentrations were superior.

The next experimental condition we perturbed was the

order of addition of tags and catalyst **5** (Table 1, entry 1a–e). Test support **6** was pre-soaked in a solution of tag or catalyst for varying amounts of time, after which catalyst **5** or tag, respectively, was added to the encoding reaction. Analysis of the relative GC peak area for each tag (post-bead decoding) indicated that pre-soaking the support in tag solution for 45 min prior to the addition of the rhodium catalyst **5** solution was optimal.

We next turned to the optimization of the overall encoding reaction time (Table 1, entry 2a–c). A time course experiment was run over 24 h, before which each support sample was pre-soaked in tag solution for 45 min. Reactions were quenched at specific times throughout the time course by addition of heptylamine to the reaction mixture, which deactivated the rhodium catalyst **5** [21]. The amount of tag covalently bound to the beads increased steadily over the course of the first 16 h, but after this point, there was no significant further tag incorporation. From this point forward, we conducted all of our encoding reactions for 16 h, after a 45 min pre-soak period with tag solution.

2.5. Compound integrity after the encoding procedure

The selectivity of the carbene insertion reaction for the 'dummy compound' on test support and the polymeric support itself warrants discussion. We were concerned that the tags were predominantly inserting into the 'dummy compound', instead of the support, when we observed that the tag peaks in the GC traces for macrobeads decoded before compound cleavage were dramatically stronger than those for macrobeads decoded after compound cleavage (Table 1, entry 3a–c). Since compound cleavage will precede bead decoding routinely in our technology process (Fig. 1), compound was cleaved from the beads prior to decoding in all of our optimization studies thereafter. To examine the degree of tag insertion into a compound relative to the PS macrobeads, we carried out experiments with the 'dummy compound' (Fig. 4). A portion of test support **6** was treated with HF/py to effect cleavage of the 'dummy compound' and to generate an unadulterated compound sample, before any encoding took place. The remainder of the support sample was encoded using our optimized protocol, and then treated with HF/py to release the compound. The two samples were characterized by ^{1}H nuclear magnetic resonance (NMR) and tandem liquid chromatography/mass spectroscopy (LC/MS), and both techniques failed to show any detectable tag incorporation into the compound (Fig. 5). Further studies have shown that the stock solutions derived from single macrobeads encoded with the described encoding protocol remain analytically pure ($>99.9\%$), and we expect that any trace impurities introduced via tag insertion into the compound should not compromise a 'one-bead, one-stock solution' approach.

2.6. Optimization of the decoding procedure

With an optimized encoding procedure in hand, we

Table 1
Encoding optimization data

Entry	Conditions[a]	Tag C3Cl3 (pmol)	Tag C3Cl5 (pmol)	Tag C9Cl5 (pmol)	Tag C16Cl5 (pmol)
1a	No pre-soak[b,c]	0.16	0.31	0.02	–[d]
1b	Pre-soak tags[e] (15 min)	0.19	0.26	0.03	–
1c	Pre-soak tags (45 min)	0.30	0.42	0.17	0.02
1d	Pre-soak cat. **5**[e] (15 min)	0.11	0.03	0.02	–
1e	Pre-soak cat. **5** (45 min)	0.22	0.07	0.05	–
2a	10 h reaction[f,g]	1.42	1.54	1.71	0.68
2b	16 h reaction	2.84	3.19	3.99	2.38
2c	24 h reaction	2.83	2.72	3.71	2.25
3a	Decode bead w/compound[c,h,i]	10.29	8.89	9.83	7.29
3b	Decode bead w/out compound[c,h,j]	0.39	0.48	0.37	0.23
3c	Decode stock solution[c,h,k]	33.82	23.00	40.52	5.91

[a]Each data point is an average of 10 identical experiments with individual beads of encoding test support **6**. All reactions conducted in CH_2Cl_2. Concentrations of total tag and rhodium catalyst **5** added = 20 nmol tag/bead and 2 nmol/bead, respectively. Picomolar amounts of tags cleaved from individual beads determined by integration of GC/ECD spectra relative to a calibration curve.

[b]Catalyst **5** and tags added simultaneously; 2 h reaction time at 25°C.

[c]Bead decoding protocol (per bead) = suspend bead in 5 μl CAN (0.25 M, 1:1 THF/H_2O) and 8 μl decane; 2 h reaction time at 25°C; sonication for 1 min; decane layer removed and 1 μl BSA added; 3 μl injected for GC/ECD analysis.

[d]Tag not detectable by GC.

[e]Catalyst **5** (or tag) was added after the initial pre-soak; 2 h reaction time at 25°C.

[f]Macrobeads were pre-soaked with all four tags for 45 min prior to addition of catalyst **5**. Reactions were quenched by the addition of 5 μl heptylamine.

[g]Optimized bead decoding protocol used (see text): 0.24 M CAN (5:1 THF/H_2O), 21 h, 37°C, 1 min sonication, 1:1 BSA/decane.

[h]Bead/stock solution encoded with unoptimized conditions.

[i]Decode prior to compound cleavage with HF/py.

[j]Decode after compound cleavage with HF/py.

[k]Subject all of the dried compound eluted from one macrobead post-cleavage to bead decoding conditions.

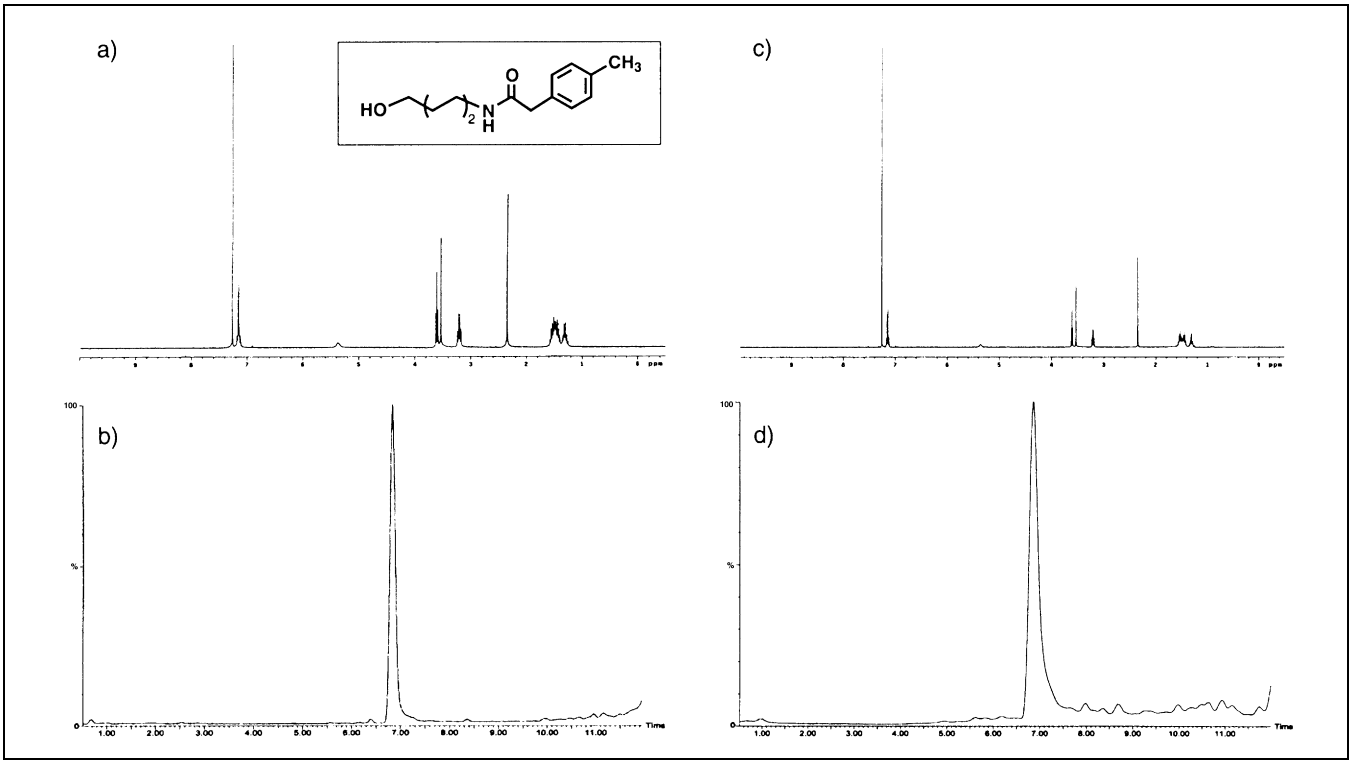

Fig. 5. Compound integrity studies. ^{1}H NMR (400 MHz, $CDCl_3$) and LC traces of the 'dummy compound', *N*-(5-hydroxy-pentyl)-4-methyl-benzamide, cleaved from ~20 mg test support **6** before (a, b) and after (c, d) the encoding reaction. The MS (APCI) spectra for the main peak in the LC traces corresponded to the molecular ion of the model compound (M+H = 236). No molecular ions or fragments corresponding to tag insertion products (or with diagnostic chlorine isotope splitting patterns) were observed in the MS spectrum of the model compound after the encoding reaction.

turned to the optimization of the bead decoding protocol. Support (**6**) was encoded using the protocol above to generate a decoding test support (**7**), from which the 'dummy compound' was cleaved (Fig. 6a). Our initial decoding protocol, devised by scaling up from previous procedures for 90 μm TentaGel [17], involved multiple steps: (1) placing individual beads in glass autosampler inserts, (2) adding 5 μl of a 0.25 M CAN solution in 1:1 THF/H_2O, (3) layering onto this 8 μl of anhydrous decane, (4) allowing the reaction to proceed for 2 h at room temperature, (5) sonicating the samples for 1 min, and finally, (6) manually removing the decane layer from the insert to a fresh glass autosampler insert, followed by silylation with BSA and injection on the GC/ECD (Fig. 6b). The PS macrobeads float near the interface of the CAN solution and decane layers. The CAN cleaves the hydrophobic chloroaromatic tags from the macrobeads, which then partition into the decane layer. Optimization of this multifaceted protocol required the careful scrutiny of each step.

We chose not to alter the volumes of decane (8 μl) and CAN solution (5 μl) initially because these volumes are easy to manipulate either manually or in a potential automated format using a liquid-handling robot. First, we observed that longer decoding reaction times allowed for more complete tag cleavage from the beads, and we selected 21 h as the optimal reaction time (Table 2, entry 1a–d). Second, we observed that increasing the reaction temperature significantly improved the efficiency of tag cleavage (Table 2, entry 2a–c). While more tag was released at 60°C relative to lower temperatures, we decided that 37°C was a judicious selection because of the decreased proba-

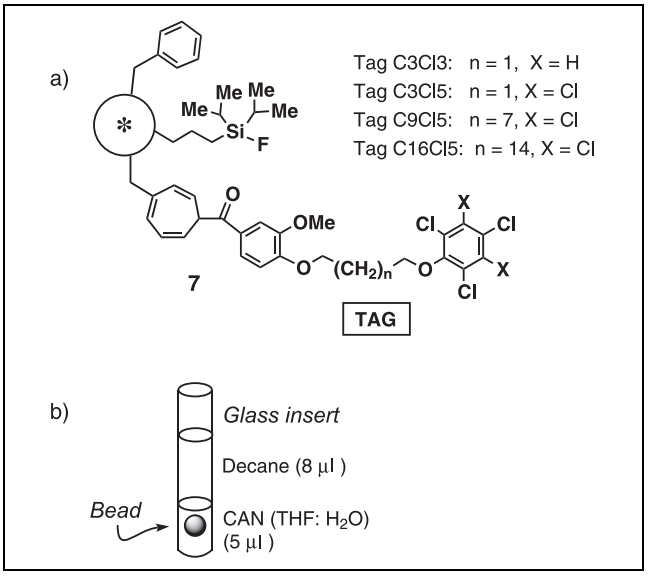

Fig. 6. Decoding optimization studies. a: Structure of the decoding test support **7** encoded with four tags. b: Schematic of the glass autosampler insert used as a tag cleavage vessel. The biphasic tag cleavage cocktail consists of a lower aqueous CAN solution and an upper decane layer (see text).

Table 2
Decoding optimization data

Entry	Conditions[a]	Tag C3Cl3 (pmol)	Tag C3Cl5 (pmol)	Tag C9Cl5 (pmol)	Tag C16Cl5 (pmol)
1a	40 min reaction[b]	0.54	0.87	0.24	0.03
1b	2 h reaction	0.57	0.69	0.33	0.03
1c	8 h reaction	0.93	1.53	1.59	0.09
1d	21 h reaction	0.84	1.68	1.44	0.12
2a	25°C reaction[c,d]	0.57	0.69	0.33	0.03
2b	37°C reaction[e]	1.23	1.14	1.02	0.06
2c	60°C reaction[f]	1.59	2.04	1.87	0.18
3a	0.4 M CAN, 1:1 THF/H_2O[g]	1.47	1.83	0.87	0.09
3b	0.25 M CAN, 1:1 THF/H_2O	1.41	1.95	1.17	0.18
3c	0.24 M CAN, 5:1 THF/H_2O	1.92	2.34	2.67	0.21
3d	0.16 M CAN, 9:1 THF/H_2O	1.62	2.22	2.85	0.27
3e	0.09 M CAN, 19:1 THF/H_2O	0.44	1.68	2.16	0.12
4a	No sonication[h,i]	0.43	0.44	0.19	0.03
4b	1 min sonication	0.56	0.68	0.33	0.04
4c	10 min sonication	0.78	0.89	0.40	0.05
4d	30 min sonication	0.77	0.94	0.41	0.06
5a	Silylate w/100% BSA[j]	0.90	1.04	1.01	0.21
5b	Silylate w/50% BSA[k]	1.42	1.54	1.71	0.68
5c	Silylate w/20% BSA	0.90	0.79	1.13	0.29
5d	Silylate w/10% BSA	0.74	0.62	1.00	0.32
5e	Silylate w/1% BSA	0.25	0.20	0.08	0

[a]Each data point is an average of 10 identical experiments with individual beads of decoding test support **7**. Five μl CAN solution and 8 μl decane were used per bead in all decoding reactions. Dry, degassed THF and double-distilled H_2O were used in CAN solutions. Reactions were performed in conical autosampler glass inserts in vials sealed with Parafilm. Picomolar amounts of tags cleaved from individual beads were determined by integration of GC/ECD spectra relative to a calibration curve.
[b]Reaction series conditions: 0.25 M CAN (1:1 THF/H_2O), 25°C, 1 min sonication, 1:1 BSA:decane.
[c]Reaction series conditions: 0.25 M CAN (1:1 THF/H_2O), 2 h, 1 min sonication, 1:1 BSA:decane.
[d]Inserts were stored at room temperature.
[e]Inserts were placed in a 37°C incubator.
[f]Inserts were placed in a 60°C oven.
[g]Reaction series conditions: 21 h, 37°C, 1 min sonication, 1:1 BSA:decane.
[h]Prior to removal of the decane solution.
[i]Reaction series conditions: 0.25 M CAN (1:1 THF/H_2O), 2 h, 25°C, 1:1 BSA:decane.
[j]Reaction series conditions: 0.24 M CAN (5:1 THF/H_2O), 21 h, 37°C, 1 min sonication. Beads encoded for 10 h were used in place of test support **7** (encoded for 16 h). Cleaved tag alcohols were silylated prior to GC analyses.
[k]Solutions in decane.

bility of losing solvent by evaporation, a concern when working with microliter volumes. Furthermore, placing large numbers of sealed samples in a 37°C laboratory incubator was operationally straightforward.

We next examined the effect of CAN solution concentration and solvent composition on the efficiency of the decoding procedure (Table 2, entry 3a–e). Since H_2O is required as a co-solvent in the CAN cleavage mechanism [39], we used other polar organic solvents in place of THF to investigate the optimal aqueous solvent mixture for tag cleavage. Notably, THF proved to be the best co-solvent; in fact, as we increased the ratio of THF:H_2O, we saw a dramatic increase in tag cleavage. We attributed this to the markedly improved swelling of copolymer beads in THF relative to H_2O, and therefore, better reaction kinetics within the polymer matrix. At ratios of THF:H_2O higher than 5:1, CAN began to precipitate out of 0.24 M solutions. Lower CAN concentrations were investigated at higher THF:H_2O solvent ratios, but no significant improvements were observed, and precipitation of CAN remained a problem. This suggested that the cleavage reaction was dependent on bead swelling and total CAN concentration. Ultimately, we selected the 5:1 THF:H_2O, 0.24 M CAN solution as the optimal solution for tag cleavage. Other apolar solvent layers were not investigated because the hydrophobicity and low volatility of decane were optimal for sequestering the chloroaromatic alcohols and for sample handling, respectively.

We investigated whether a brief sonication period was necessary at the end of the decoding reaction (Table 2, entry 4a–d). At the outset, we believed this procedure would dislodge the cleaved tag alcohols entrained in the polymer bead and allow them to escape and migrate to the decane layer. Also, the heating induced by sonication could push the CAN oxidation further towards completion. In fact, we observed a reduction in the amount of tag observed if we omitted the sonication step completely. Sonication times up to 30 min were studied, and a 1–10 min sonication period was chosen as the optimal post-decoding reaction procedure. Finally, we observed that sily-

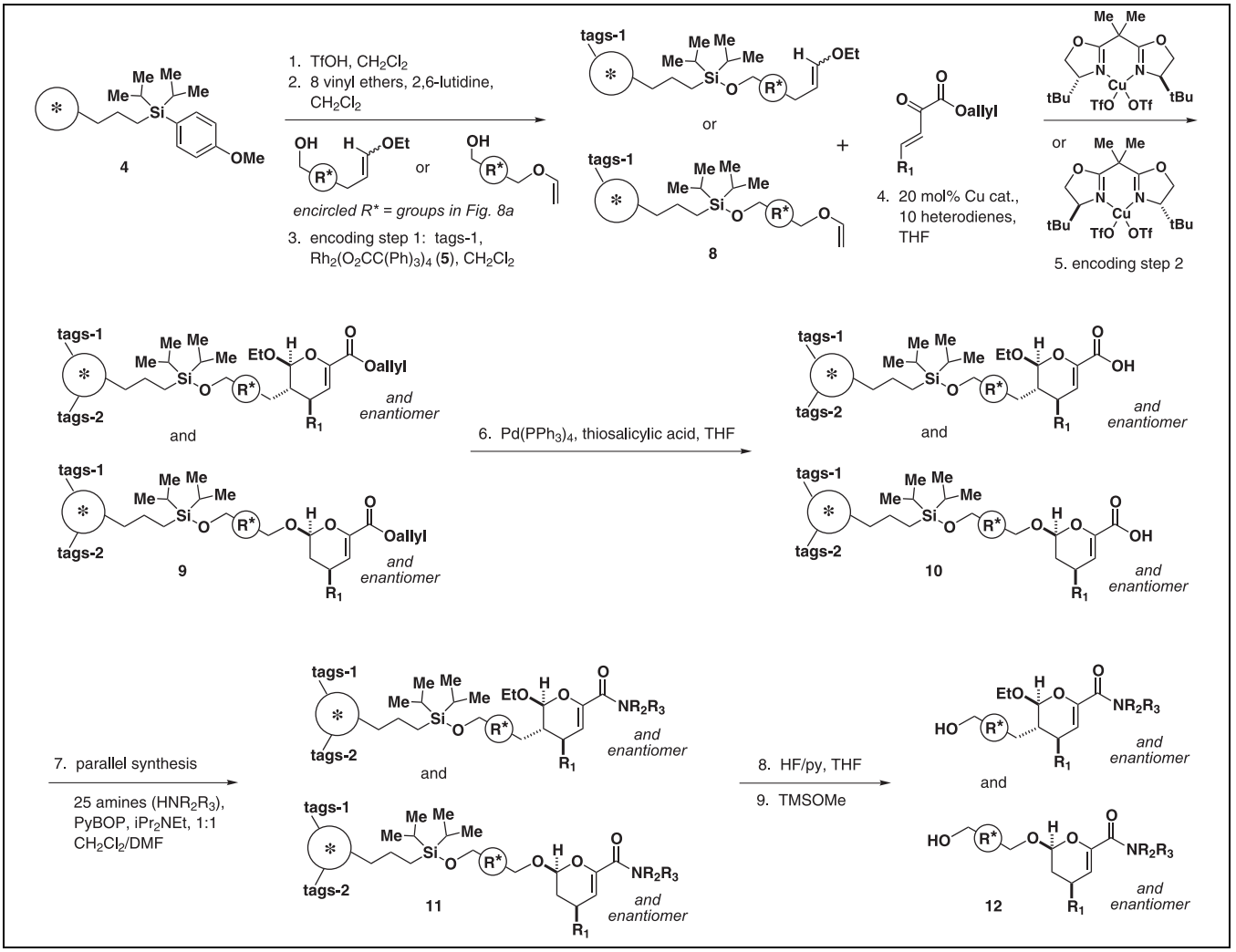

Fig. 7. Synthesis of the 4320-member dihydropyrancarboxamide library (**12**). All reactions were carried out at room temperature. The three sets of library BBs are shown in Fig. 8.

lating the tag alcohol solutions from each bead with 1 μl of a 1:1 BSA:decane solution gave the most reliable and strongest GC traces (Table 2, entry 5a–e). This hydrocarbon solution of BSA does not hydrolyze readily in open air and could be amenable to a liquid-handling robot in an automated decoding process. Of note, in a direct comparison with a recently published decoding protocol [41], our final, optimized bead decoding procedure delivered consistently larger values of cleaved tag (0.02 vs. 2 pmol of each tag) per PS macrobead, thus confirming the value of these optimization studies for 500–600 μm PS macrobeads.

2.7. Decoding directly from compound stock solutions cleaved from individual beads

While the integrity of a compound attached to a macrobead is not adversely affected by the chemical encoding process (see above), we have found that sufficient tag is inserted into the small molecule itself to decode the compound reliably by subjecting a fraction (1–5%) of its corresponding stock solution to our optimized decoding protocol [43]. (Attempts to determine the structure of the tag-inserted compound derived from the 'dummy compound' have been unsuccessful so far. However, we postulate that an aromatic ring and/or N-H insertion reaction occurred.) The stronger and more reproducible traces obtained via 'stock solution decoding' are, in part, due to the better reaction kinetics observed in the homogeneous CAN cleavage reaction of the tags from the stock solution in comparison to the heterogeneous cleavage reaction from the macrobead. This new decoding method is a key advance for the 'one-bead, one-stock solution' platform because it should: (1) expedite compound structure elucidation and (2) minimize storage requirements as the macrobeads can be discarded after compound cleavage and elution. Furthermore, stock solution decoding should facilitate the global decoding of entire split-pool libraries through an automated process.

Stock solution decoding provides several other practical advantages over bead decoding. For example, decoding

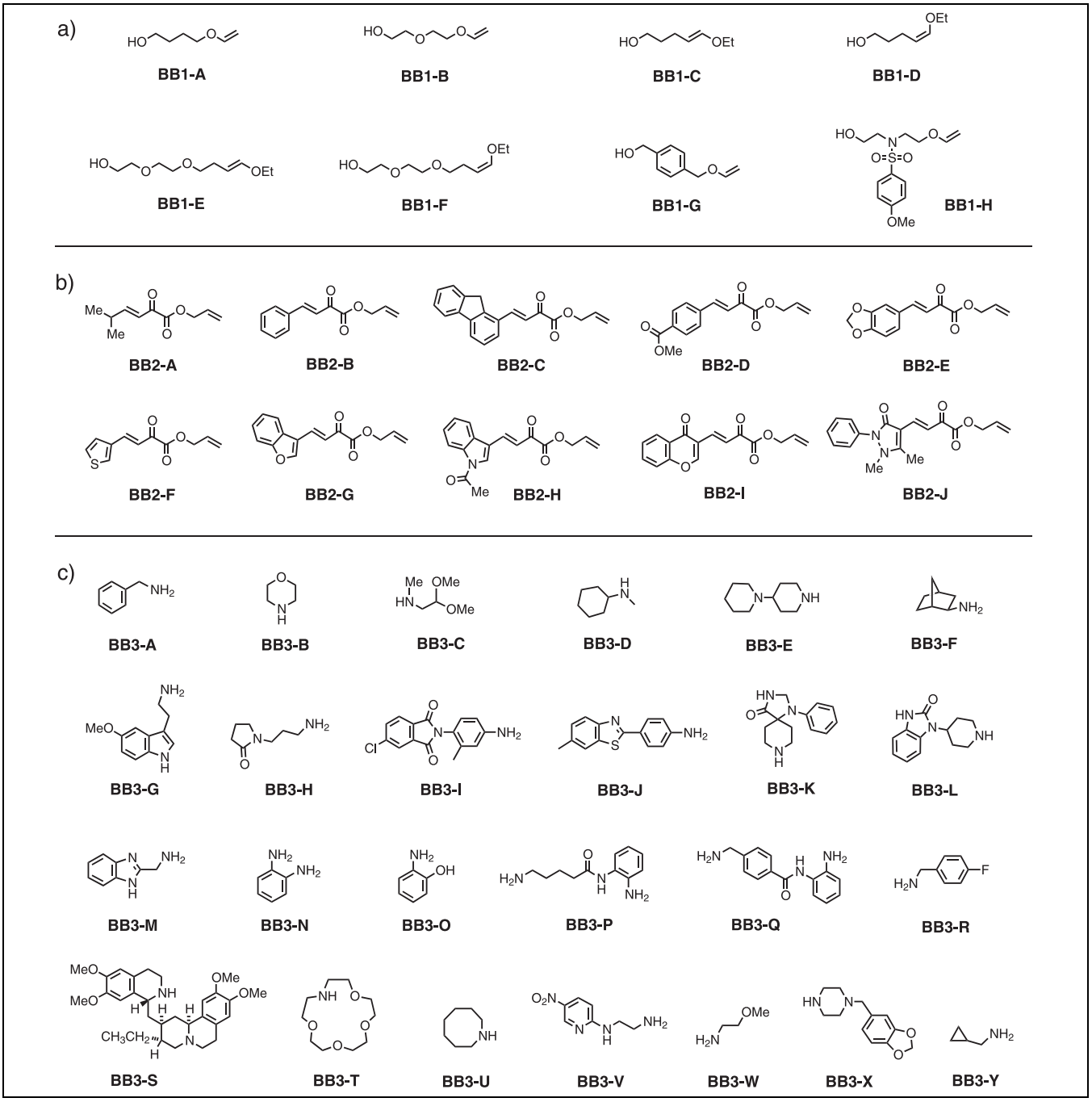

Fig. 8. Three sets of BBs used in the synthesis of library **12**: (a) eight vinyl ethers, (b) 10 heterodienes, and (c) 25 amines.

from the stock solutions allows multiple samples to be prepared for GC/ECD decoding if necessary, as opposed to having only one attempt with bead decoding. Stock solution decoding is especially useful in instances when identification of a compound via MS is not possible, e.g. when a library includes several members of the same molecular weight (structural isomers, enantiomers, or diastereomers), or when a compound does not ionize well. If a GC/ECD chromatogram is difficult to interpret or if the macrobead has been lost (a reality in solid-phase synthesis, most frequently due to static electricity), the ability to decode from a fraction of the bead's respective stock solution is an excellent alternative for the elucidation of the compound's chemical history. In our opinion, these features make the stock solution decoding method a dramatic improvement over standard bead decoding, and on-going work is directed at the implementation of a fully automated protocol for stock solution decoding, utilizing the same liquid-handling robots described in the following paper in this issue.

Table 3
Binary codes for encoding BB1 of library **12**

Entry	Tag C4Cl3	Tag C6Cl3	Tag C3Cl5	Tag C4Cl5
BB1-A	1	0	0	0
BB1-B	0	1	0	0
BB1-C	0	0	1	0
BB1-D	0	0	0	1
BB1-E	1	1	0	0
BB1-F	1	0	1	0
BB1-G	1	0	0	1
BB1-H	0	1	1	0

2.8. Process validation: encoded, enantioselective diversity-oriented synthesis and partial decoding of a 4320-member library prepared on 500–600 μm PS macrobeads

In order to test both the PS macrobeads and the encoding/decoding protocol detailed above in an actual library synthesis, an encoded, split-pool library of 4320 dihydropyrancarboxamides (**12**) was synthesized featuring an *R*- or *S*-bis(oxazoline)copper (II) triflate-catalyzed heterocycloaddition reaction [44,45] as a key diversity-generating step (Fig. 7). A detailed description of the pathway development phase of this research has been reported elsewhere [15]. The three sets of BBs used in the library synthesis are shown in Fig. 8. Three chemical steps were central to the library synthesis: (1) loading of eight vinyl ethers onto the PS beads (**4**), (2) enantioselective cycloaddition with 10 β,γ-unsaturated ketoesters, followed by allyl ester deprotection, and (3) PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate)-mediated coupling [46] to 25 different amines to yield support-bound dihydropyrancarboxamides (**11**).

Reaction step and BB encoding were carried out twice in the library synthesis: first, after loading the eight vinyl ethers, and second, after the cycloaddition reaction with 10 heterodienes. The tags and binary codes used for each BB are shown in Tables 3 and 4. In the cycloaddition reaction, one set of beads (10 portions) was treated with the (*S*)-Cu (II) catalyst, and the other 10 portions were treated with the (*R*)-catalyst (Fig. 7). From this step onwards, these two groups of enantiomers were kept separate even though they were encoded for each enantiomer of the catalyst used. The subsequent reactions were carried out in parallel so that spatial decoding could be performed had the chemical encoding failed. The 25 final amide pools were kept separate to reduce the number of chemical encoding steps. This library synthesis resulted in 54 (27×2 enantiomers) separate portions of solid supports (**11**) containing, theoretically, three copies of 4320 stereochemically and structurally distinct compounds (**12**). Finally, as our macrobead handling 'best practices' were observed throughout the library synthesis (see above), the majority of the library supports remained intact ($>90\%$). In order to test the integrity of our optimized library encoding/decoding protocol, 108 macrobeads from the library (theoretically 2.5% of the total library compounds) were arrayed into tubes and treated with HF/py, followed by TMSOMe to release the compounds (**12**) from the beads. The residue isolated from each bead was dissolved in CH_3CN and transferred to individual glass autosampler inserts to provide arrayed stock solutions of small molecules. A fraction of each of these stock solutions was subjected to LC/MS analysis, and the corresponding macrobeads were submitted to our optimized decoding protocol to compare the two results (Fig. 9).

Decoding consisted of deriving the identities of BBs

Table 4
Binary codes for encoding BB2 of library **12** in heterocycloadditions catalyzed by either *R*- or *S*-bis(oxazoline)copper (II) triflate

Entry	Catalyst	Tag C5Cl5	Tag C6Cl5	Tag C7Cl5	Tag C8Cl5	Tag C9Cl5
BB2-A	*S*	1	0	0	0	0
BB2-B		0	1	0	0	0
BB2-C		0	0	1	0	0
BB2-D		0	0	0	1	0
BB2-E		0	0	0	0	1
BB2-F		1	1	0	0	0
BB2-G		1	0	1	0	0
BB2-H		1	0	0	1	0
BB2-I		1	0	0	0	1
BB2-J		0	1	1	0	0
BB2-A	*R*	0	1	0	1	0
BB2-B		0	1	0	0	1
BB2-C		0	0	1	1	0
BB2-D		0	0	1	0	1
BB2-E		0	0	0	1	1
BB2-F		1	1	1	0	0
BB2-G		1	1	0	1	0
BB2-H		1	1	0	0	1
BB2-I		1	0	1	1	0
BB2-J		1	0	1	0	1

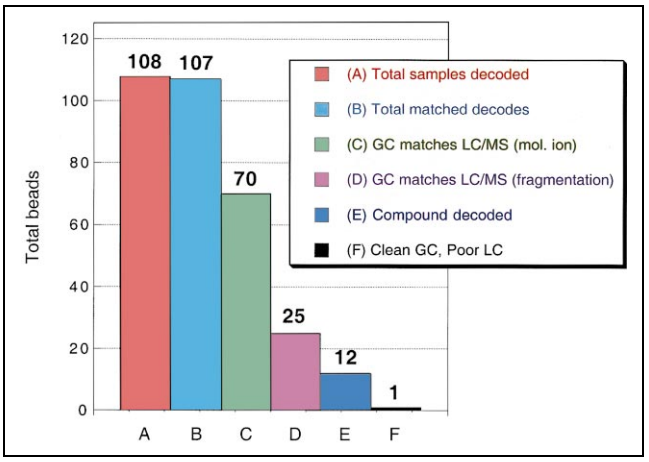

Fig. 9. Partial decoding of dihydropyrancarboxamide library **12**. Graph of the observed complementarity between the GC and MS decoding of 108 beads from library **12**.

1 and 2 by GC tag analysis, adding their combined molecular weight to that of the amine corresponding to the pool of supports from which the macrobead was taken, and comparing this composite mass to the mass observed experimentally by APCI/MS. The structural data obtained via GC decoding were in complete agreement with the MS data obtained from the compounds' stock solutions (Fig. 9) for 107 of the 108 samples. Seventy of the 108 macrobeads (65%) yielded GC traces that decoded for a compound with a molecular ion identical to that expected based on the MS data. Twenty-five macrobeads (23%) showed GC traces that decoded for a compound whose molecular ion corresponded to a fragment of the proposed structure. Direct stock solution decoding (see above), using the optimized decoding protocol on a fraction ($\sim$5%) of the stock solutions generated from individual macrobeads, was carried out successfully to identify the structures of the 12 remaining samples [43]. The structures assigned to 25 of the samples and representative GC, LC, and MS traces for a single macrobead are shown in Figs. 10 and 11, respectively.

The successful synthesis and partial decoding of library **12** validate not only our binary encoding/decoding protocol, but also the entire synthesis platform as a reliable procedure for the generation of encoded split-pool libraries. The use of stock solution decoding further enables this platform as it simplifies the elucidation of structures of 'hits' from assays and lends itself to future automation. Four libraries currently underway in our laboratory are being encoded using this protocol, and preliminary data

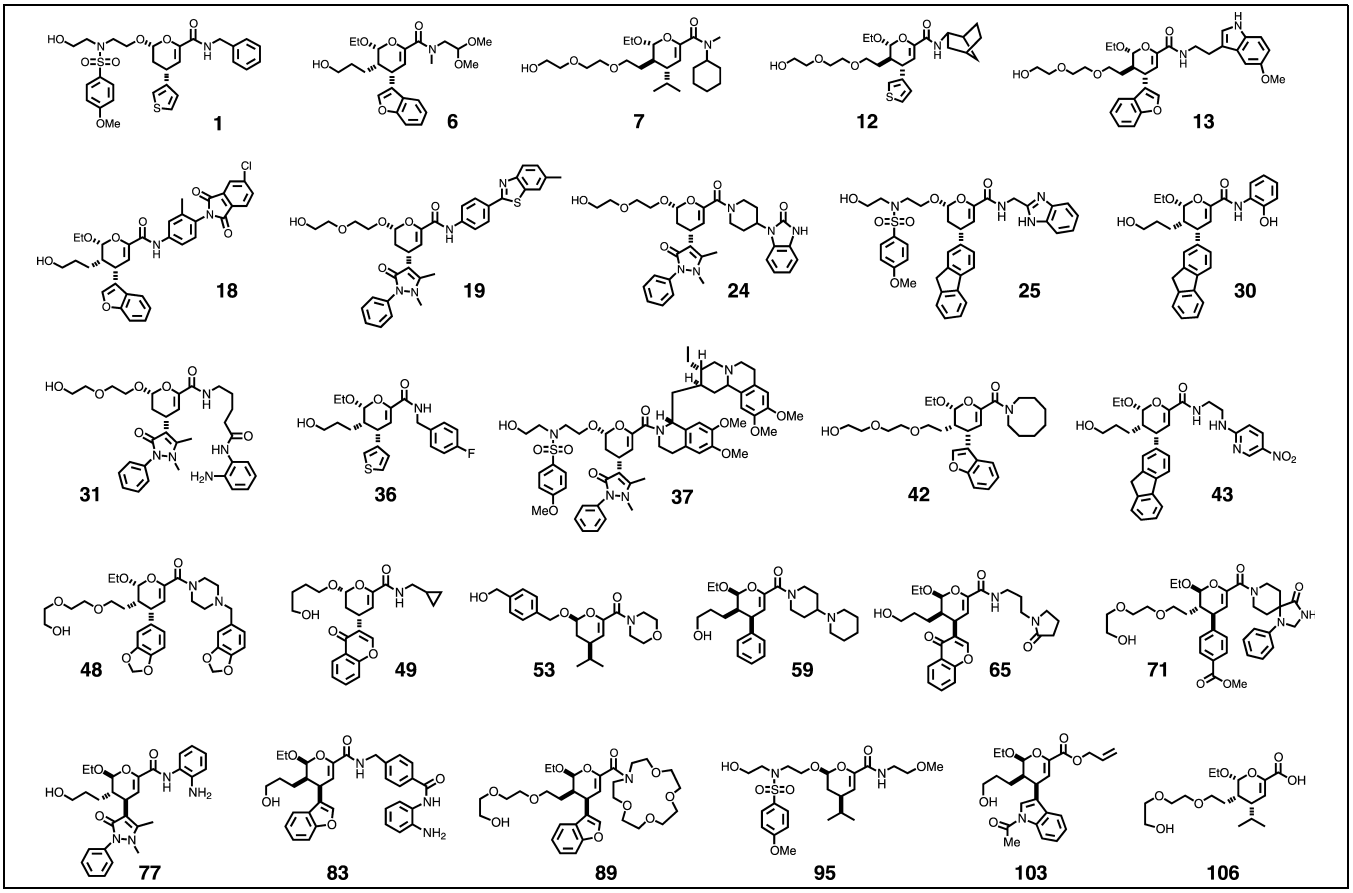

Fig. 10. Structures of 25 representative compounds from the 108 beads decoded of library **12**. Numbers in bold refer to bead number. All structures show agreement between their GC and MS decoding data.

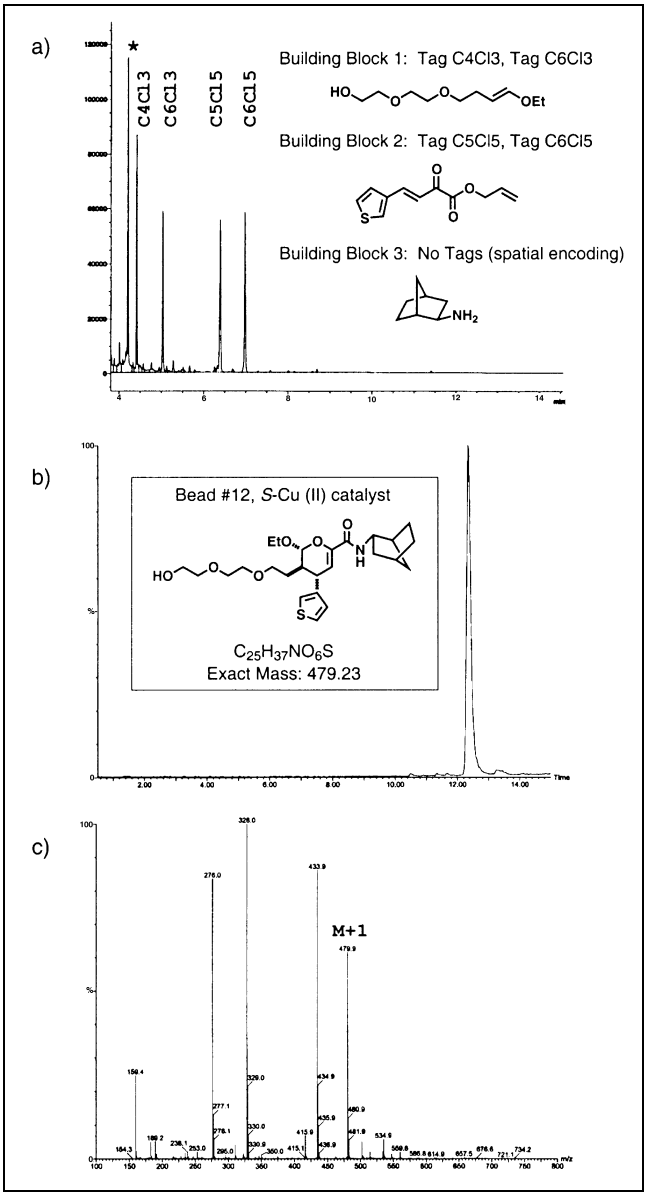

Fig. 11. Representative example of GC (a) and LC/MS (b, c) bead decoding. The GC/ECD chromatogram (a) for bead #12 decodes for a library compound with an exact mass identical to that obtained by MS (c) of the compound cleaved from that bead (APCI, observed mass = 479.9 (M+1)). The starred peak (*) in the GC chromatogram (a) is an impurity frequently present with the electrophoric tags.

suggest that our encoding protocol is tolerant of the diverse chemical transformations contained in these four pathways.

3. Significance

We have outlined the first phase of the development of a technology platform aimed at advancing chemical genetics. The platform consists of, in part, a robust bead/linker system based on 500–600 μm PS macrobeads, a diversity-oriented synthesis planning algorithm for library design, and an optimized procedure for library encoding and decoding. The successful synthesis of an encoded split-pool library (**12**) using this platform validates the approach. The synthesis platform uses commercially available reagents and straightforward synthetic procedures; therefore, we believe it could be readily established in other laboratories. This work lays the foundation for the second phase of platform development, where the members of libraries are distributed on a per bead basis into multiwell assay plates, submitted to automated cleavage, and resuspended to generate plates of pure, arrayed stock solutions, as described in the following paper in this issue [16]. The individual stock solutions originating from single macrobeads have been found to be sufficient for hundreds of phenotypic assays (forward chemical genetics) and thousands of protein-binding assays (reverse chemical genetics) before a need for re-synthesis (Fig. 1).

4. Materials and methods

4.1. General synthetic methods

General reagents were obtained from Aldrich Chemical Co., Acros, or J.T. Baker and used without further purification. Reaction solvents (THF, diethyl ether, DMF, toluene, and CH_2Cl_2) were obtained from J.T. Baker (high-performance liquid chromatography (HPLC) grade) and purified by passage through two solvent columns prior to use [47]. Diisopropylethylamine and 2,6-lutidene were distilled from calcium hydride; MeOH was distilled from magnesium methoxide. Brominated PS beads (bead diameter = 500–600 μm; two mequiv/g) were obtained from Polymer Labs, Inc. and derivatized with the silyl ether linker according to the published procedure [24]. Diazoketone chloroaromatic tags and $Rh_2(O_2CC(Ph)_3)_4$ catalyst (**5**) were purchased from Pharmacopeia, Inc. and TCI, respectively, and used without further purification. BSA was purchased in 1 ml sealed glass ampoules from Pierce Chemical Co. and used immediately after opening.

4.2. Solid-phase reactions

Small-scale solid-phase reactions (5–10 mg resin) were performed in 500 μl polypropylene Eppendorf tubes with mixing provided by a Vortex Genie-2 vortexer fitted with a 60 microtube insert. Medium-scale solid-phase reactions (20–500 mg resin) were performed in 2 ml fritted polypropylene Bio-Spin® chromatography columns (Bio-Rad) or 10 ml fritted polypropylene PD-10 columns (Pharmacia Biotech) with 360° rotation on a Barnstead-Thermolyne Labquake™ shaker. Large-scale solid-phase reactions (> 500 mg resin) were performed in silanized 50 or 100 ml fritted glass tubes equipped for vacuum filtration and N_2 bubbling. The tubes were silanized by treatment with 20% dichlorodimethylsilane/CH_2Cl_2 for 15 min, MeOH for 15 min, followed by oven heating at 120°C for at least 2 h. After small-scale reactions, resin samples were transferred to 2 ml Bio-Spin® columns. Resin samples in polypropylene columns were washed on a Vac-Man® Laboratory Vacuum Manifold (Promega) fitted with nylon three-way stopcocks (Bio-Rad). Resin samples in glass

tubes were washed in the tubes with alternating periods of N_2 bubbling and vacuum draining. The following standard wash procedure was used: 3×THF, 3×DMF, 3×THF, 3×CH_2Cl_2. For compound cleavage, resin samples were transferred via spatula to 500 μl Eppendorf tubes and suspended in Ar-degassed HPLC grade THF, followed by pyridine and HF/py (Aldrich, HF (70%)/py (30%)) in a ratio of 90:5:5 (e.g. ~150 μl total volume for 10 mg of macrobeads). Samples were then sealed with Parafilm and gently agitated on a vortexer for 90 min. TMSOMe was added (e.g. ~150 μl for 10 mg of macrobeads), and the samples were sealed with Parafilm and placed on a vortexer for an additional 30 min. The supernatant fluid was removed, transferred to another Eppendorf tube, and concentrated in vacuo.

4.3. Purification and analysis

HPLC was performed on a Nest Group Hypersil C18 100 Å 3 μM, 4.6 mm×6 cm column using a flow rate of 3 ml/min and a 4 min gradient of 0–99.9% CH_3CN in H_2O/0.1% trifluoroacetic acid, constant 0.1% MeOH with diode array UV detection. NMR spectra were recorded on Varian Inova 500 MHz and 400 MHz instruments. Mass spectra were obtained on Jeol AX-505H or SX-102A mass spectrometers by electron impact ionization, chemical ionization with ammonia, or fast atom bombardment ionization with glycerol or 3-nitrobenzyl alcohol/sodium iodide matrices. LC/MS data were obtained on a Micromass Platform LCZ mass spectrometer in APCI mode attached to a Waters 2690 HPLC system. LC/MS chromatography was performed on a Waters Symmetry C18 3.5 μM, 2.1 mm×50 mm column using a flow rate of 0.4 ml/min and a 10 min gradient of 15–100% CH_3CN in H_2O, constant 0.1% formic acid with 200–450 nm detection on a Waters 996 photodiode array detector. GC/ECD data were obtained on a Hewlett Packard 6890 gas chromatograph fitted with a 7683 series injector and autosampler, split-splitless inlet, μ-ECD detector, and a J&W DB1 15 m×0.25 mm×0.25 μm column. (Gradient start temperature: 110°C; hold 1 min, ramp 45°C/min to 250°C, hold 2 min, ramp 15°C/min to 325°C, hold 2 min. Flow rate: constant flow, 1 ml/min. Inlet was purged at 1 min with flow rate 60 ml/min, reduced to 20 ml/min at 2 min).

4.4. Synthesis of the 'dummy compound' (*N*-(5-hydroxy-pentyl)-4-methyl-benzamide)

N-(5-Hydroxy-pentyl)-4-methyl-benzamide was prepared via a standard 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride-mediated coupling between 5-amino-1-pentanol and *p*-tolylacetic acid in CH_2Cl_2 in the presence of triethylamine and 4-(dimethylamino)pyridine [42].

4.5. Representative bead encoding procedure

Twenty dry macrobeads (~3 mg solid supports) were placed in a 700 μl Eppendorf tube. A fresh 8.4 mM (in each tag) solution in dry CH_2Cl_2 was prepared in an oven-dried, Teflon-capped glass vial, and 50 μl of this tag solution was added to the Eppendorf tube. The tube was agitated for 45 min at room temperature on a tabletop orbital shaker. A 4.4 mg/ml solution of the catalyst, $Rh_2(O_2CC(Ph)_3)_4$ (**5**), in dry CH_2Cl_2 was prepared under Ar in an oven-dried, Teflon-capped glass vial, and 50 μl of the catalyst solution was added to the resin. The Eppendorf tube was agitated for 16 h (overnight) at room temperature. The supports were then washed in a 1 ml Bio-Rad tube 2×15 min CH_2Cl_2, 16 h (overnight) THF, 2×15 min THF, and 2×15 min CH_2Cl_2. Afterwards, the supports were dried under house vacuum for ~15 min before proceeding to either another synthesis step or compound cleavage (as described above).

4.6. Representative bead decoding procedure

Macrobeads were dried under house vacuum for at least 1 h prior to decoding. A 0.24 M solution of CAN in 5:1 THF/H_2O was prepared (132 mg CAN/0.83 ml dry, degassed THF+0.17 ml double-distilled H_2O) in an oven-dried vial. (Note: this solution should be prepared immediately before use.) A single macrobead was placed in a glass autosampler sample insert, and 5 μl of the CAN solution were added to the glass autosampler insert, followed by 8 μl of dry decane. The insert was centrifuged in a microfuge to separate the two layers before placing it into an autosampler vial which was capped tightly. The vial was sealed with Parafilm and heated at 37°C for 21 h (in a standard laboratory incubator). After cooling the vial to room temperature, the glass insert was removed from the autosampler vial, and it was sonicated for 1–10 min. Once again, the insert was centrifuged to ensure separation of the two layers. Using a 200 μl Pipetman equipped with a gel-loading tip, the top decane layer was removed and transferred to a new GC autosampler glass insert. (After heating overnight, the CAN layer will be colorless, so caution must be used to avoid contamination of the decane layer with CAN in transfer.) A 1:1 BSA/decane solution in an oven-dried vial was prepared. (Note: this solution should be prepared immediately before use.) 1.0 μl of this BSA solution was added to the decane layer in the GC insert which was then spun down in a microfuge for 30–40 s to ensure efficient mixing of the BSA solution with the sample. The insert was placed in an autosampler vial, capped tightly, and stored at 0°C until GC analysis.

4.7. Supplementary material

Bead stability studies, graphs of encoding/decoding optimization data, and details of the partial decoding of dihydropyrancarboxamide library **12** can be found at: http://slsiris.harvard.edu/home/research_results.html.

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