Article

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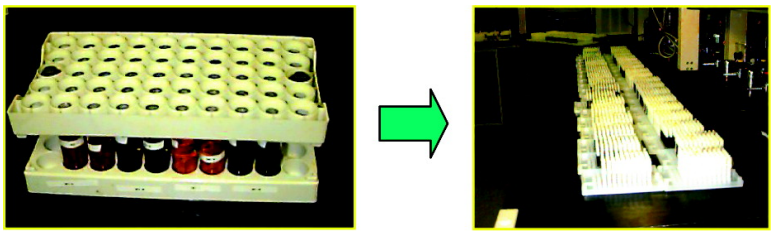

High-Throughput Manual Parallel Synthesis Using SynPhase Crowns and Lanterns

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The high-throughput manual solid-phase parallel synthesis of libraries comprising thousands of discrete samples using pellicular supports (i.e. SynPhase crowns and lanterns) and a suite of novel tools and techniques is described. Key aspects of this approach include the combination of a split-split-split synthesis strategy with spatial encoding to differentiate thousands of crowns, the rapid washing and filtration of up to 48 reaction vessels in parallel, the application of an inexpensive and environmentally friendly technique to remove trifluoroacetic acid from sixteen 96-well plates in parallel, and a high-throughput method for removing cleaved crowns from reusable pin racks. Tens of thousands of discrete samples have been produced in-house using this conceptually and operationally straightforward strategy.

Introduction

The solid-phase synthesis of combinatorial libraries[1] comprising thousands of organic molecules is a powerful tool for ligand identification. A number of strategies have been reported for the efficient production of solid-phase libraries, and these generally fall into two categories: strategies that are designed to afford multiple compounds per physical sample (pools)[2] and those that are designed to afford individual compounds per physical sample (discretes).[3] The relative advantages and disadvantages of pools and discretes are well documented,[4] and we elected to focus our efforts on the solid-phase synthesis of discretes to obviate any deconvolution or decoding steps and to simplify the analysis of the final products. Specifically, we were interested in methods which enabled the weekly production of thousands of discrete samples in multimilligram (>5 mg) quantities. At the time this effort was initiated (1995), these throughput requirements (and cost considerations) eliminated the possibility of using commercially available automation for library production and led us to explore manual methods[5] for solid phase parallel synthesis. Reported herein is an inexpensive and efficient approach to the solid-phase synthesis of thousands of discrete samples using only a multichannel pipet and a suite of novel tools and techniques.[6]

The selection of solid-phase synthesis support can have a significant impact on the techniques employed for library production, particularly those that involve physically handling the support. In this respect, commercially available solid-phase synthesis supports can be broadly classified into two categories: (1) micro- and macroporous resins, which require many microscopic (30−200 μm in diameter) beads for the synthesis of a single compound, and (2) pellicular solid supports, which permit the synthesis of a single compound on a single macroscopic (>3 mm in diameter) grafted polymer. As the original solid support discovered by Merrifield,[7] microporous resins have received the most attention in the synthetic community, and a wide range of supports are commercially available. However, the production of multimilligram quantities of discrete samples via a resin-based approach requires the segregation of 10−50 mg of resin in a format compatible with not only the harsh conditions inherent to multistep chemical syntheses but also the cleavage of the desired material from the solid support into an assay-compatible container (usually a 96-well plate). In 1995, two approaches to resin segregation were known in the literature. The first of these, Houghten's "tea bag" approach,[8] greatly simplified resin handling in solid-phase synthesis but introduced complexity in the cleavage step because the resin in tea bags could not be directly cleaved into 96-well plates. Moreover, the construction of tea bags required additional in-house resource as preloaded tea bags were not commercially available. The second approach[9] to resin segregation utilized 96-well plates equipped with a filter

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Figure 1. SynPhase crowns (left) and lanterns (right).

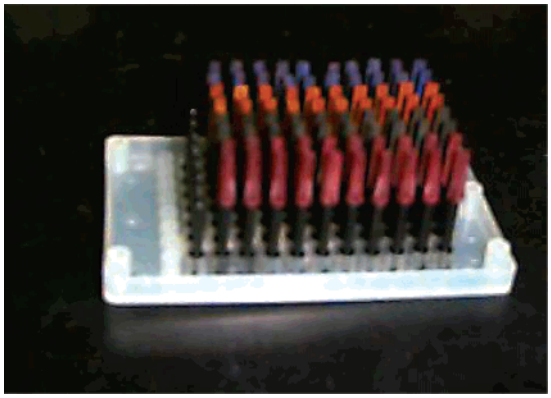
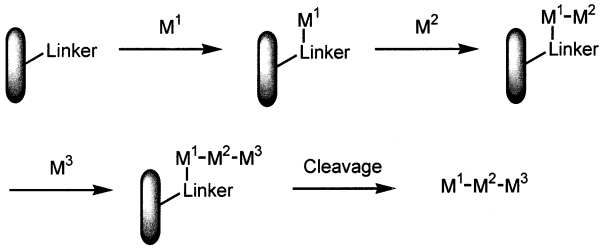


Figure 2. Crowns mounted on a pin rack compatible with 96-well plates.

frit to spatially confine resin to a single well prior to cleavage. This greatly simplified the cleavage step but introduced numerous obstacles in terms of chemical synthesis and resin handling (vide infra). Because neither of these microporous support-based strategies met our needs, we turned our attention to pellicular supports.

The only commercially available pellicular supports are the SynPhase crowns[10] (and recently, SynPhase lanterns[11]) from Mimotopes (formerly Chiron Mimotopes). These supports are shown in Figure 1. Crowns comprise an inert polyethylene core and a surface-grafted reactive polymer (i.e., polystyrene) which is further functionalized with linkers amenable for solid-phase synthesis. In parallel solid-phase synthesis, crowns have three distinct advantages over resin: (1) no "resin segregation" equipment is required because crowns are intrinsically segregated from one another, (2) the loading of a single crown (8−35 μmol, depending upon the graft) is sufficient for the synthesis of multimilligram quantities of material, and (3) the 96-well compatible "pin" format (see Figure 2) allows the direct cleavage of samples from crowns into a 96-well plate. In short, the issue of resin segregation is rendered moot because each crown is large enough to be easily manipulated by hand, and only one crown is required for each target compound.

In recent years, a great deal of progress has been made in resin handling technology, including improved 96-well-compatible reaction blocks and IRORI's "Kan" family of synthesis products.[12] However, the use of crowns for the *manual* synthesis of thousand-member libraries also merits consideration owing to the ease of handling and subsequent cleavage directly into a 96-well format. Moreover, in the absence of an automated sorting system,[13] we have found



Library Size =	M^1	x	M^2	x	M^3
2,480 =	5	x	16	x	31

Figure 3. Generic scheme and dimensions for a hypothetical three-position library.

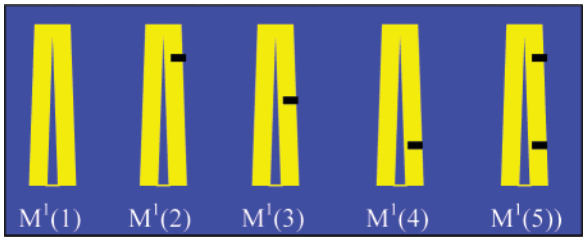


Figure 4. Encoding five crowns via a manual crimping strategy.

that a straightforward split-split-split library production strategy is an efficient means for producing thousands of discretes on crowns.

Experimental Design

Having selected the crown and pin format for the synthesis of large libraries, we next addressed the issue of library dimensions and its effect on the library production strategy. For example, Figure 3 describes the solid-phase synthesis of a hypothetical library with three points of diversity, denoted M^1, M^2, and M^3, and the following dimensions: 5 M^1 × 16 M^2 × 31 M^3 = 2480 discrete samples. The M^1 and M^2 dimensions are the result of an in-house requirement that library samples be submitted as 80 samples in a 96-well plate, with two columns left open for assay controls. With this format requirement, the synthetic efficiency is increased when the product of the M^1 and M^2 monomer sets is a multiple of 80, because it allows the M^3 reaction to be conducted in 80 wells of a 96-well plate containing 80 crowns mounted on pins (Figure 2). M^3 reagents are introduced on a plate by plate basis rather than in a well by well basis, dramatically reducing the complexity of pipetting operations. In the case of the hypothetical library described in Figure 3, each pin rack would contain all 80 combinations of M^1 × M^2 (5 × 16), and 31 pin racks would be required for the M^3 diversity step.

Having established guiding principles for determining the library dimensions, we next considered whether to incorporate a first position manual encoding strategy into our library production process. Each crown has four "vanes" that can easily be modified by "crimping" a notch with a small pair of wire cutters.[14] As shown in Figure 4, the location and number of "crimps" encode the identity of the five M^1 monomers in the aforementioned hypothetical library. Encoding the first monomer set in the library would allow the M^2 monomers to be introduced via pooled reactions, where

Table 1. Effect of Encoding on the Number of Reaction Vessels Required to Synthesize a Hypothetical Library of 2480 Samples

encoding strategy	reaction vessels			total vessels
	M^1	M^2	M^{3a}	
first position	5	16	31	52
none	5	80 (5 × 16)	31	116

[a] In this case, the "reaction vessel" is 80 wells of a 96-well plate containing the same M^3 in every well.

Figure 5. The six-pack synthesis and washing apparatus.

crowns equipped with five different M^1s would be present in each reaction flask. There are obvious throughput advantages to pooled reactions, and as summarized in Table 1, the application of first position encoding to the synthesis of the hypothetical 2480 member library would reduce the total number of reaction vessels from 116 to 52. Unfortunately, manually encoding and decoding thousands of crowns proved to be a painstaking and tedious task, and this prompted us to abandon first-position encoding in favor of an unencoded synthetic strategy. It should be noted that manual encoding is a very effective method for differentiating small numbers (<100) of crowns during chemistry development or monomer rehearsals. An alternative method for the manual encoding of crowns in which the crowns are assembled into a series of "encoded necklaces" has been reported.[15] It is not clear whether the effort required for the assembly and disassembly of these necklaces represents an improvement over crimping when carried out with thousands of crowns.

Manual Library Production on Crowns

To provide additional information about the equipment and crown handling techniques utilized for the manual production of thousands of discretes, the discussion of the specific steps in the process will center on the construction of the hypothetical library of 2480 discrete samples described in Figure 3.

The first step in the synthesis of 2480 discrete samples is the distribution of 496 linker-functionalized crowns into five reaction vessels. This distribution can be accomplished by weight. The average mass of a crown is very consistent, so once one has determined the mass of 100 crowns, it is trivial to weigh five batches of 496 crowns. The crowns are then transferred into five wide-mouth jars wired together in a "six-pack" configuration, as shown in Figure 5. The design of the six-pack merits additional explanation. Initially, we washed crowns in much the same way one would handle

Figure 6. An 8 × 5 array of 40-mL vials in the Wheaton Sandwich.

resin: the crowns were placed into a fritted glass funnel, solvent was poured over the crowns, and the solvent was removed via vacuum filtration. However, it was discovered that one could take advantage of the macroscopic size of crowns and wash them by covering a flask with 2-mm Teflon mesh,[16] introducing solvent through the mesh with a bottle-top dispenser, agitating for 5 min and then simply inverting the flask to drain the solvent. By wiring six bottles together, thousands of crowns can be washed using this simple and short procedure. In practice, we would conduct reactions in the six-pack format with standard bottle caps then replace the caps with 2-mm Teflon mesh caps and wash the crowns nine times (three times with three different solvents), a washing protocol that typically required 45 min to complete for the entire array.

Following the introduction of the first point of diversity (attachment of 5 M^1 monomers to 496 crowns per monomer), it is necessary to redistribute the 5 M^1-equipped crowns into 80 reaction vessels to generate the requisite 80 combinations of 5 M^1 × 16 M^2 monomers. Although the six-pack is well-suited for the first diversity step, 80 reaction vessels would require 14 six-packs, and we sought a more compact solution. From this search emerged the "Wheaton Sandwich" (Figure 6), a 50-vial rack[17] which greatly facilitated the organization and washing of up to 48 separate reactions (two vial positions are occupied by bolts which hold the Sandwich together). For our purposes, each Wheaton Sandwich is organized into an 8 × 5 array,[18] and two Sandwiches are combined to generate an 8 × 10 array and effectively mirror the format of an 80-well plate. As with the six-pack, reactions are conducted in capped vials, and washing is accomplished by removing the caps and covering each vial with Teflon mesh. With the Wheaton Sandwich, the Teflon mesh is introduced by inverting a vial rack equipped with 40 2-mm mesh Teflon disks onto the 40 vials and clamping the two vial racks together with two bolts. The crowns in the 40 vials are washed and drained as a single unit, as shown in Figure 7. Using a bottletop solvent dispenser, we could typically wash the entire array with nine solvents in 45 min.

In the case of the hypothetical 2480 member library, two Wheaton Sandwiches comprise the 5 × 16 array of M^1 and M^2 monomers. The crowns are distributed as follows: starting with the $M^1(1)$ bottle of the six-pack, one transfers 31 crowns into 16 scintillation vials by weight as described previously. Repeating the process for the $M^1(2)-M^1(5)$ bottles of the six-pack affords a 5 × 16 array of vials

Figure 7. Filtering 40 reaction vessels in parallel with the Wheaton Sandwich.

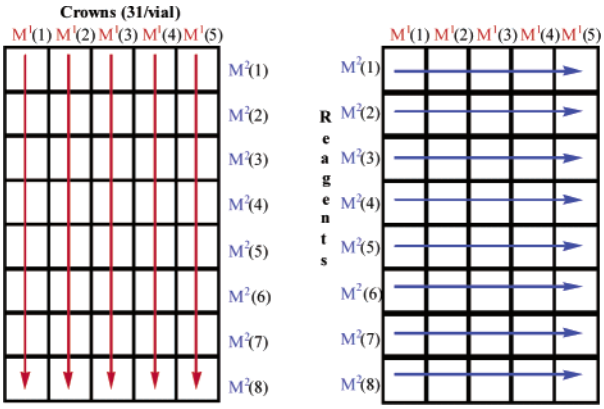


Figure 8. Orthogonal distribution of crowns (distributed vertically) and reagents (horizontally) in a 5 × 8 array to generate 40 $M^1 \times M^2$ combinations.

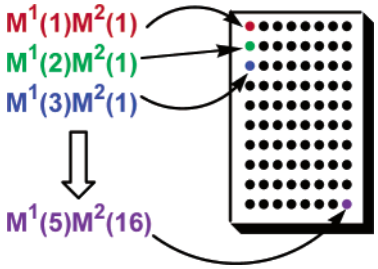


Figure 9. A spatially addressable array of crowns on an 80-pin rack.

containing 31 crowns each. To generate 40 of the 80 different combinations of $M^1 \times M^2$, the reagents corresponding to the $M^2(1)-M^2(8)$ monomers are distributed orthogonally to afford a 5 × 8 array, as shown in Figure 8. To provide the remaining 40 $M^1 \times M^2$ combinations, the second Wheaton Sandwich is handled in a fashion similar to the first, except the reagents corresponding to $M^2(9)-M^2(16)$ are distributed rather than those corresponding to $M^2(1)-M^2(8)$.

To introduce the third and final point of diversity, the crowns are transferred from the Wheaton Sandwich format to the 80-pin plate format in a spatially addressable array as shown in Figure 9. This process of placing crowns onto pins is tedious but efficient: typically, a chemist can "stalk out" 800−1000 crowns per hour. As shown in Figure 10, dozens of pin racks fit on a single benchtop. In the case of the hypothetical library of 2480 samples, ~3 h would be required for one chemist to transfer the 2480 crowns from the 80 vials of the two Wheaton Sandwiches to 31 pin racks containing 80 crowns per rack. The resulting 31 pin racks are identical until they are differentiated by reacting each pin rack with a single M^3 monomer. One obvious advantage to this approach is that it is not necessary to label the pin racks prior to the

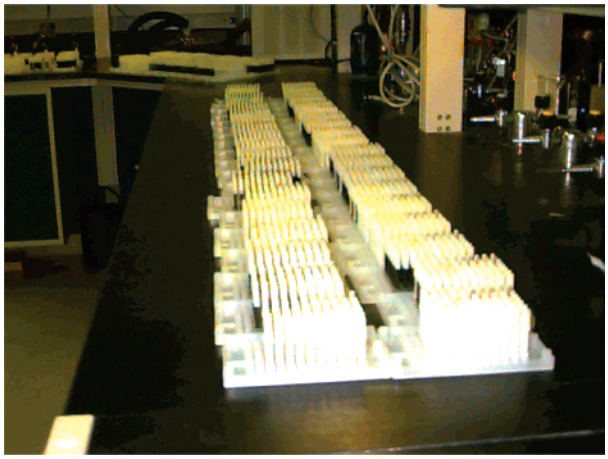


Figure 10. Multiple pin racks equipped with 80 crowns/rack.

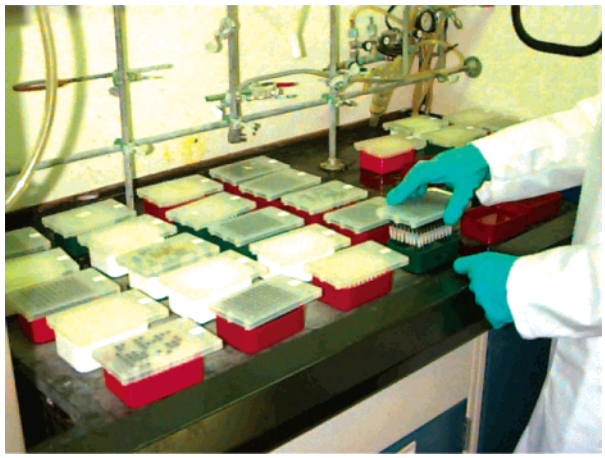


Figure 11. Washing crowns in the pin rack format.

M^3 reaction. In addition, one could generate additional copies of the pin racks and then, as required, react them with a new set of M^3 monomers.

Following the generation of 31 identical pin racks, each of the 31 M^3 monomers is distributed into 80 wells of a 96-well "deep well" plate (one monomer per plate, 31 plates total), and then the 80 crowns representing all 80 combinations of M^1 and M^2 are introduced as a single pin rack. At this point, it is necessary to label the pin racks carefully to identify the M^3 monomer. The crowns are then washed and dried, and the final products are cleaved directly into a 96-well plate. Washing crowns on pin racks is less efficient than washing crowns in the six-pack or Wheaton Sandwich format because each pin rack must be handled separately. As shown in Figure 11, pin racks are placed in plastic "tubs" (available from Mimotopes), and the appropriate washing solvents are

Figure 12. Cleaving samples from crowns mounted on pin racks directly into 96-well plates.

Figure 13. Evaporation of TFA using a high-throughput evaporation system.

introduced. It would take ~2 h to wash 31 pin racks nine times, but the inefficiency of washing crowns on pin racks is readily offset by the efficiency of cleaving the products directly into 96-well plates. Using a pin rack, the transfer of cleaved material into a 96-well plate is significantly more efficient than performing the same operation with resin, because the requisite "filtration step" to remove the solid support from the cleavage cocktail does not require any additional equipment (e.g., a 96-well filter plate or a vacuum manifold). Figure 12 shows a typical cleavage campaign in progress. Relative to resin, the bottleneck in the crown cleavage process shifts from the filtration step to the concentration of the resulting TFA solutions in a high throughput fashion, a situation that is not unfamiliar to many solid-phase chemists.

The volatility and acidity of trifluoroacetic acid make it the reagent of choice for the cleavage of samples from solid supports. Unfortunately, these same properties are generally detrimental to centrifugal concentrators and vacuum pumps. After encountering difficulties with our vacuum-based concentration systems, we developed an economical and environmentally friendly solution to the problem of TFA removal. To wit, we elected to evaporate the TFA with a stream of nitrogen and then capture the highly acidic effluent with a potassium hydroxide bath. This apparatus, which serves as a high-throughput evaporation system, is shown in Figure 13 and comprises a typical "drybox" equipped with four 96-well "Vaccu-Pettes"[19] to direct a stream of nitrogen into each well of four 96-well plates. The exhaust hose was immersed in a base bath (aqueous KOH) to neutralize the TFA. On the basis of the moles of KOH consumed, >90% of the TFA was captured in a typical evaporation. In general, it took ~18 h to evaporate 1 mL of TFA from each well of four 96-well plates, and control experiments established that no "well-to-well" contamination occurred during the evaporation process. In the case of the hypothetical library comprising 2480 samples, four high-throughput evaporation systems would be required to concentrate 16 plates in parallel, and it would take ~2 days to remove the 2.5 L of TFA from 31 96-well plates. Compared to a concentrator, these high throughput evaporation systems represent a very significant cost savings (each system costs less than $1000) and free up our concentrators for more time-sensitive tasks, such as the development of new solid-phase chemistries.

Following the cleavage step, we were faced with the following dilemma: the cleaved crowns were expendable, but the pin racks could be recycled (and they were not inexpensive enough to throw away). To further complicate matters, removing the cleaved crowns by hand often irreparably damaged the pins. This problem was addressed through the design of the "pin reaper", a device that facilitates the high-throughput removal of crowns from pin racks without incurring any damage to the pins. As shown in Figure 14, the pin reaper is a simple device based on the mechanical advantage of the lever. The key to the pin reaper lies in its ability to lock the pins in place on the pin rack while exerting a tremendous upward force on the crowns. Using the pin

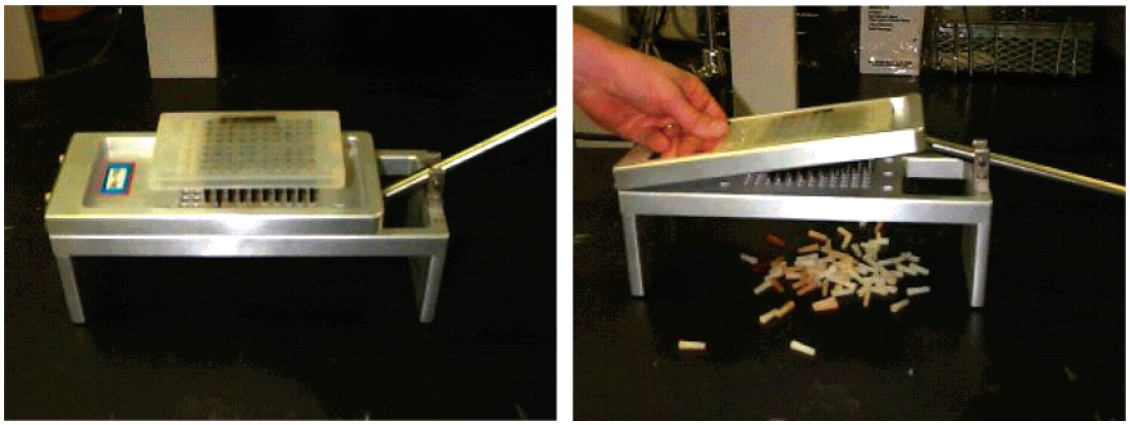
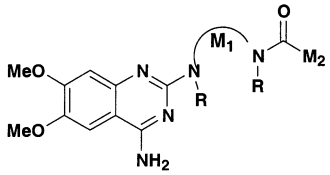
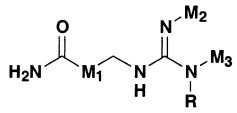
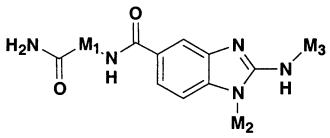
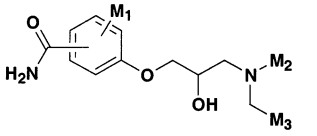
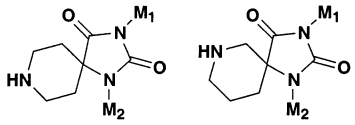
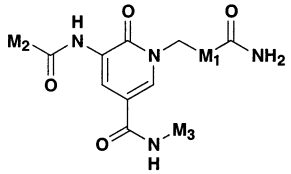
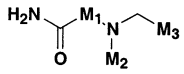


Figure 14. High-throughput crown removal using the pin reaper.

Table 2. Representative Chemotypes Synthesized In-House on Crowns

Entry	Generic Structure	Library Dimensions	Reference
1		20 M_1 x 96 M_2	20
2		5 M_1 x 16 M_2 x 31 M_3	21
3		8 M_1 x 20 M_2 x 20 M_3	22
4		4 M_1 x 20 M_2 x 11 M_3	6
5		2 scaffolds x 40 M_1 x 31 M_2	23
6		4 M_1 x 20 M_2 x 40 M_3	24
7		16 M_1 x 24 M_2 x 24 M_3	25

reaper, it takes <30 s to remove all of the crowns from a pin rack. The crowns can then be discarded, and the pin rack can be recycled. This device has recently been commercialized by Mimotopes.

Results

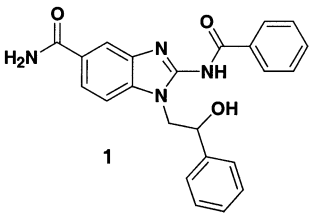
Utilizing the aforementioned split-split-split strategy, our in-house synthesis efforts afforded tens of thousands of discrete samples using minimal automation. Table 2 lists generic structures for representative chemotypes[6,20−25] synthesized with this approach. At the time that these libraries were produced, our synthetic throughput exceeded our analytical capacity (specifically, HPLC for sample purity and mass spectrometry for sample identity) by an order of magnitude. As a result, we were unable to characterize every sample and adopted a strategy for analyzing a representative subset comprising 5−10% of the completed library. Each subset was selected to ensure that every monomer (and certain monomer combinations) was represented in at least one sample in the subset. The criteria we established for the submission of a completed library were as follows: 75% of the representative subset had to exceed 75% purity by HPLC (with UV detection), with confirmation of the molecular ion of the desired product by mass spectrometry (with electrospray detection). All of the libraries listed in Table 2 met or exceeded these criteria. For example, the guanidine library described in entry 2 of Table 2 contained 2480 samples in 31 96-well plates. We selected five samples from each plate in a predefined pattern that sampled each M^1 16 times, each M^2 10 times, each M^3 five times, and all $M^1 \times M^2$ combinations at least once. The resulting 155 samples provided the following analytical data: 94% of the samples afforded the expected MH^+ by mass spectroscopy, 82% of the samples were >75% pure by HPLC with UV detection at 254 nm, and 60% of the samples were >95% pure by HPLC.

Perhaps the best validation of library purity occurs when samples are identified in a biological assay, and resynthesis and purification of these samples confirms their biological activities. For example, the benzimidazole library[22] (Table 2, entry 3) provided multiple samples with nanomolar activity in a neuropeptide Y receptor subtype 5 (NPY-5) SPA-based binding assay.[26] Benzimidazole **1** is a representative potent NPY-5 antagonist and afforded an IC_{50} of 42 nM ($n = 2$) when tested as a crude sample from this library of 3200 discretes. Upon resynthesis and purification of a larger

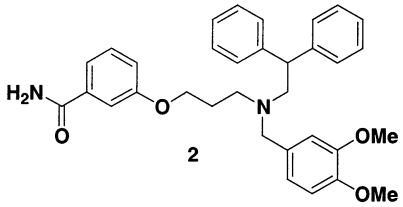
Table 3. Summary of Crown Handling Steps in the Split-Split-Split Synthesis of 2480 Discrete Samples

crown handling step	equipment	time required for completion
first split: 1 vessel → 5 vessels	bulk crowns → 1 six-pack	1 h
second split: 5 vessels → 80 vessels	1 six-pack → 2 Wheaton Sandwiches	2 h
third split: 80 vessels → 2480 vessels	2 Wheaton Sandwiches → 31 pin racks	3 h
cleavage	31 pin racks → 31 96-well plates	3 h (including cleavage)

quantity of **1**,[27] this activity was confirmed (IC_{50} = 52 nM, n = 7). This discovery prompted the synthesis of several follow-up benzimidazole libraries on crowns[22b] and provides strong evidence for the utility of crown-based library production in hit identification. Recently, an LXR agonist (**2**) was identified from the trisubstituted amine library (Table 2, entry 7).[28] Follow-up libraries based on **2** led to the discovery of several potent and selective LXR agonists.



1



2

Summary

As described in the Introduction, crowns compare favorably with resin in manual parallel synthesis applications for a variety of reasons, nearly all of which relate to the technical difficulties faced in resin segregation. Because we have not synthesized the same library on both resin and crowns, it is not possible to directly measure the time savings realized through the use of crowns. However, as outlined in Table 3, it is difficult to imagine a manual resin-handling method which is more efficient than the approach described herein. In particular, the second and third splits in the aforementioned split-split-split synthesis of the 2480 samples would necessitate a great deal of resin handling either by volume (as a slurry) or by weight (as a dry powder). The accuracy of these resin handling techniques is debatable. Furthermore, cleaving samples from resin in a 96-well compatible format requires specialized equipment to prevent leakage of resin, TFA, or both.

It is important to note that all of these comparisons have been conducted in the context of manual parallel synthesis. There are dozens of automation platforms for the production of large libraries of discretes, and many of these approaches have met wide acceptance within the chemical industry. Unfortunately, implementing an automated synthesis system often requires infrastructure and capital resources which are beyond the scope of many chemistry labs. It is our hope that the crown handling equipment and techniques described herein will facilitate the uptake of solid-phase parallel synthesis in resource-constrained settings.

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(27) To 560 Fmoc-protected Rink-equipped MA/DMA macrocrowns (purchased from Mimotopes with a reported loading of 8.0 μmol per crown) in a 500 mL flask was added 300 mL of 30% (v/v) piperidine/DMF, and the mixture was shaken at room temperature for 3 h. The solvent was decanted, and the crowns were washed with DMF (3 × 300 mL), followed by alternating THF (300 mL) and CH_2Cl_2 (300 mL) washes (washing cycle repeated 3×). After air-drying for 1 h, the 560 fmoc-deprotected Rink-amine macrocrowns were suspended in 150 mL of a 0.2 M solution of 4-fluoro-3-nitrobenzoic acid (5.6 g, 30.0 mmol, 6.7 eq/crown) in DMF followed by 150 mL of a DMF solution containing HATU (10.2 g, 27.0 mmol, 6 eq/crown) and DIEA (15.7 mL, 90.0 mmol, 20 eq/crown), and the resulting mixture was gently shaken overnight for 18 h. The solvent was decanted, and the 560 crowns were washed with DMF (3 × 300 mL), followed by alternating THF (300 mL) and CH_2Cl_2 washes (300 mL) (washing cycle repeated 3×). One hundred and twelve of the acylated crowns were placed in a wide-mouth jar containing 60 mL of a 0.1 M solution of 2-amino-1-phenylethanol (0.82 g, 6.0 mmol, 6.7 eq/crown) in DMF, and the suspension was shaken overnight. After 18 h, the 112 crowns were washed with DMF (3 × 60 mL), followed by alternating THF (60 mL) and CH_2Cl_2 (60 mL) washes (3×). The resulting bright yellow crowns were suspended in 60 mL of a 2 M solution of $SnCl_2 \cdot 2H_2O$ (2.7 g, 12.0 mmol, 13.4 eq/crown) in DMF and shaken overnight. After 24 h, the faintly pale-yellow crowns were washed with DMF (3 × 60 mL), 1:3 (v/v) ethylenediamine/DMF (3 × 60 mL) and DMF (3 × 60 mL), followed by alternating THF (60 mL) and CH_2Cl_2 (60 mL) washes (washing cycle repeated 3×). The 112 crowns were air-dried for 1 h, placed in a wide mouth jar, and charged with 50 mL of a 0.2 M solution of benzoylisothiocyanate in 1,2-dichloroethane (DCE) (1.3 mL, 10.0 mmol, 11 eq/crown), followed by 50 mL of a 1.0 M solution of diisopropylcarbodiimide in DCE (7.8 mL, 50.0 mmol, 55 eq/crown). The jar was capped and heated in an oven at 70 °C overnight. After 24 h, the 112 crowns were washed with DMF (2 × 60 mL) and CH_2Cl_2 (4 × 60 mL), followed by alternating THF (60 mL) and CH_2Cl_2 (60 mL) washes (washing cycle repeated 3×). The 112 crowns were treated with 90 mL of 95:5 (v/v) TFA/H_2O for 90 min. The TFA solution was decanted, and the crowns were rinsed with fresh cleavage solution (2 × 15 mL). The combined TFA filtrate was concentrated in vacuo to yield 370 mg (105% crude yield) of **1** as an amber oil. The crude material was 76% pure by reversed-phase HPLC [Waters Delta Pak 5-μm C18 300 column (3.9 × 150 mm), 1.5 mL/min flow rate, 10% CH_3CN/H_2O−90% CH_3CN/H_2O) (0.1% TFA) gradient over 20 min, detection at 254 nm]. This material was triturated with 50 mL hot EtOAc to afford 180 mg (49% overall yield) of **1** as a pale yellow solid. Analytical data for compound **1**: ^{1}H NMR (DMSO-d_6): δ (ppm) 12.85 (s, 1H, 2-NHC(O)), 8.25 (d, J = 7.0 Hz, 2H, NHC(O)-phenyl-2′H), 7.96 (s, 1H, benzimidazole-H-4), 7.94 (bs, 1H, H_2N(CO)) 7.72 (d, J = 9.0 Hz, 1H, benzimidazole-H-6), 7.22−7.53 (m, 1H, H_2N(CO), 9H, ArH), 5.73 (d, J = 4.4 Hz, 1H, −OH), 5.17 (b, 1H, Ar−CH), 4.34−4.37 (m, 2H, N−CH_2). Mass Spectrum (ES) showed MH^+ 401 m/z (base peak). Purity by reversed-phase HPLC = 93%. The yellow solid was dissolved in 3 mL of MeOH containing 2.5 equiv $MeSO_3H$. Addition of ether precipitated 155 mg of **1**·CH_3SO_3H as a white solid (95% pure by reversed-phase HPLC). Analytical data for **1**·CH_3SO_3H: ^{1}H NMR (DMSO-d_6): δ (ppm) 12.85 (s, 1H, 2-NHC(O)), 8.17 (d, J = 6.9 Hz, 2H, NHC(O)-phenyl-2′H), 8.06 (s, 1H, benzimidazole-H-4), 7.81 (d, J = 8.7 Hz, 1H, benzimidazole-H-6), 7.22−7.53 (m, 9H, ArH), 5.16 (t, J = 6.0, Hz, 1H, Ar−CH), 4.54 (b, 2H, N−CH_2), 2.36 (s, 3H, CH_3SO_3).
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