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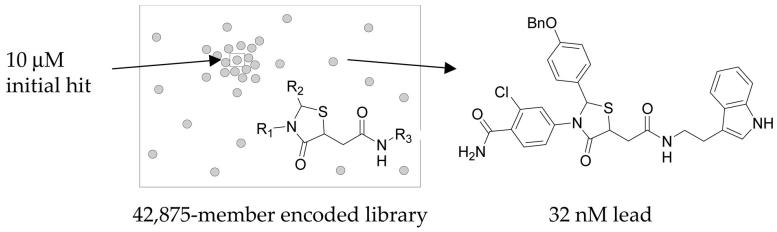

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Agonists of the Follicle Stimulating Hormone Receptor from an Encoded Thiazolidinone Library

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The design, synthesis, characterization, and screening of a large, encoded thiazolidinone library are described. Three sets of 35 building blocks were combined by encoded split−pool synthesis to give a library containing more than 42 000 members. Building block selection was based in part on a novel small molecule follicle stimulating hormone receptor agonist hit and in part for diversity. HPLC/MS techniques were applied at the single-bead level to build confidence in the reliability of library construction. Application of two distinct screening strategies resulted in the identification of compounds with significantly improved potency over the initial hit. This work demonstrates the versatility of encoded libraries for preparing a large number of analogues of a given hit while simultaneously generating a large collection of compounds for screening against other targets.

Introduction

The split−pool strategy for combinatorial library preparation enables the preparation of very large numbers of compounds in a relatively small number of chemical steps.[1−4] The efficiency of this process increases very rapidly as larger sets of building blocks are used. Unfortunately, these attractive numbers for library synthesis are often accompanied by increased difficulty of screening. Compounds are obtained as mixtures, from which active members must be identified by techniques such as deconvolution.[5−7] While a number of successful demonstrations of this technique have been reported,[8,9] the ability to identify individual active components of a mixture can generally be assumed to scale inversely with its size.

Encoding of combinatorial libraries affords a solution to this problem for solid-phase split−pool libraries that result in a single compound on each particle of the insoluble support. Several strategies have been reported for incorporating a code into the support in such a way that the synthetic history of each particle is recorded.[10] Thus, even from a mixture of a very large number of compounds, the identity of a particular compound attached to any individual particle may be deduced.

A key requirement for the usefulness of encoded libraries is that a faithful correspondence exists between a given code and the associated chemical compound. This demands not only that the identity of a cognate molecule can be predicted, but also that the synthetic route has, in fact, produced that compound. Assurance of this integrity may derive from extensive "rehearsal" of conditions for library assembly by parallel synthesis whereby series of individual compounds may be prepared on a sufficiently large scale such that traditional methods of chemical analysis are capable of directly confirming the success of the expected route. It remains, however, highly desirable that the integrity of the actual library be ascertained by analysis of the compounds and codes attached to a sampling of the particles of which the library is composed. This has traditionally been difficult to achieve for libraries prepared on beaded solid supports where only a few hundred picomoles of material are obtained.[11,12]

Encoded libraries are often used in primary (random) screening strategies in which little information is available about the types of compounds that may interact with the target of interest.[13] They have also been applied in situations in which it was desirable to rapidly survey a large number of variations around a structural theme.[14,15] In contrast, parallel synthesis approaches are much more widely employed for optimization of an existing lead. It is usually felt best to generate structure−activity relationships using defined quantities of well-characterized compounds so that accurate measurements of the desired property (e.g., potency in an in vitro assay) will be obtained.[16] This level of detail is impractical for encoded libraries because the compounds are produced in such large numbers and small amounts. On the other hand, large, diverse libraries offer the ability to survey a much wider range of structure types and are less limited to the structure−activity hypotheses that can be explored through smaller, targeted libraries. As a result, encoded libraries may allow entry to novel structural series. A balance

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must be achieved between the quality and the quantity of information obtained.

We have developed synthetic methods for the assembly of solid-supported thiazolidinones.[17] This chemistry is attractive as a route for combinatorial library preparation because it allows incorporation of starting materials for which diverse sets are readily available, namely, amino acids, aldehydes, and amines. We have also demonstrated that this chemistry is compatible with an encoding strategy, whereby an oligomeric "tag" is attached to the solid support concurrent with thiazolidinone assembly.[18] Synthesis and screening of several relatively small libraries by unencoded split−pool approaches led to the identification of a number of thiazolidinones with biological activity.[19] On the basis of these results and previously reported data,[20,21] thiazolidinones appear to embody a useful scaffold for presentation of chemical functionality in a fashion that is compatible with interaction with a diverse range of biological targets and with reasonable drug-like properties.

Among the hits discovered in these studies was compound **1**, which possessed moderate activity as an agonist of follicle stimulating hormone (FSH) by virtue of its ability to stimulate a reporter cell line expressing the FSH receptor.[22] FSH is a 31-kDa heterodimeric glycoprotein, and the discovery of a small-molecule FSH agonist was an unprecedented achievement.[23] An orally active compound of this class could be a useful addition to the portfolio of drugs available for the alleviation of female infertility.[24] The potency of **1** was, however, too weak to be considered as a drug candidate (EC_{50} of 5−10 μM), and initial analoging studies failed to significantly improve on this activity (unpublished data).

Described herein is the assembly and screening of an encoded thiazolidinone library based in part on **1**. A number of the building blocks at each stage of the synthesis were selected to provide analogues of the substituents carried on the thiazolidinone scaffold in **1**, but the majority of building blocks were chosen to generate a diverse compound set for screening against other targets. We will describe the characterization of this library and the identification of FSH agonists with significantly enhanced potency via the use of two efficient screening strategies: "tiered-release" two-stage assay, and single-bead screening in a high-density microtiter plate. This work illustrates the utility of encoded libraries for analoging studies of lead compounds while simultaneously providing a large collection of compounds for random screening against other targets.

Results and Discussion

Preparation of Individual Encoded Thiazolidinone. Encoded beads bearing **1** tethered to TentaGel resin via a photocleavable linker[25] were prepared according to the general scheme shown in Figure 1.[18] Following photorelease, LC/MS of the crude material gave two close-running peaks of the correct mass (1:2 ratio of diastereomeric products) together comprising 95% of the total peak area by HPLC. The tag was also clearly legible on decoding. This served as a revalidation of the chemical orthogonality between thiazolidinone synthesis and the encoding process and also

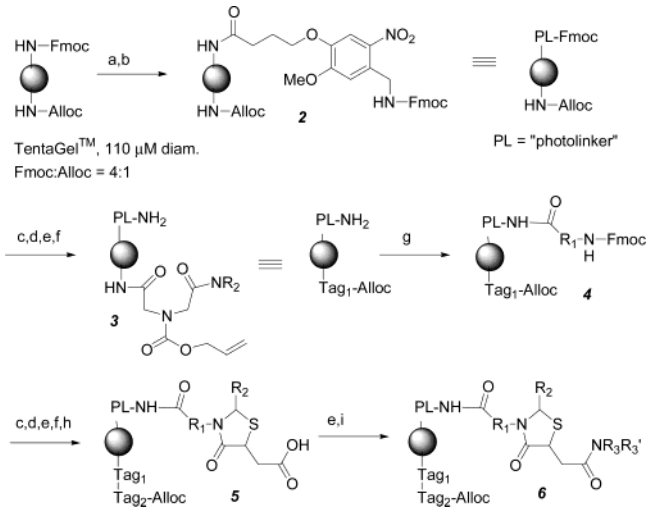

Figure 1. Preparation of encoded thiazolidinones: (a) piperidine, DMF; (b) Fmoc-photolinker, HBTU, DIEA, NMP; (c) $Pd(Ph_3P)_4$, trimethylsilyl azide, TBAF, CH_2Cl_2; (d) *N*-Alloc-iminodiacetic anhydride, DIEA, NMP; (e) pentafluorophenyl trifluoroacetate, pyridine, NMP; (f) amine, NMP; (g) Fmoc-amino acid, HATU, DIEA, NMP; (h) aldehyde, mercaptosuccinic acid, THF, 3-Å molecular sieve, 70 °C, 18 h.

provided beads bearing a photoreleasable compound of known biological activity as a reagent for the development of bead-based assays.

Library Design. As stated in the Introduction, the goals of this work were 2-fold: to optimize FSH-agonist activity by preparing analogues of **1** and to simultaneously prepare a large, encoded, diverse library for primary (random) screening against other targets. It was thus desirable to include building blocks related to those from which **1** was assembled as well as other diverse structures. The number of reagents which could be included was limited by practical rather than chemical concerns, since each of the building block classes to be employed (amino acids, aldehydes, and amines) was available in very large numbers, and prior chemical rehearsal had indicated that a significant number of each class gave good yields of the desired thiazolidinone products. Practical issues included the consideration of the number of reaction vessels which could be conveniently managed simultaneously, the scale of reaction which could be carried out in those vessels (and as a direct consequence, the number of library equivalents[26] that could be prepared), the number of building blocks which could be encoded at each step, and the proposed screening strategy. Affymax has developed synthesis apparatus specifically for split−pool library production with 36 individually addressable vessels. This number was set as the upper limit for the number of building blocks at each step. A modification to the more standard binary encoding scheme allowed us to encode 35 building blocks with five amine tags (by measuring the quantity of each tag, not simply its presence or absence).[27] Using 35 reagents at each step gives a total of 42 875 members in the final library, well within the capability of our synthesis and screening capabilities, and allowed the generation of sufficient diversity to satisfy our goals for the library. Thus, we set out to design and prepare a 35 × 35 × 35-membered thiazolidinone library.

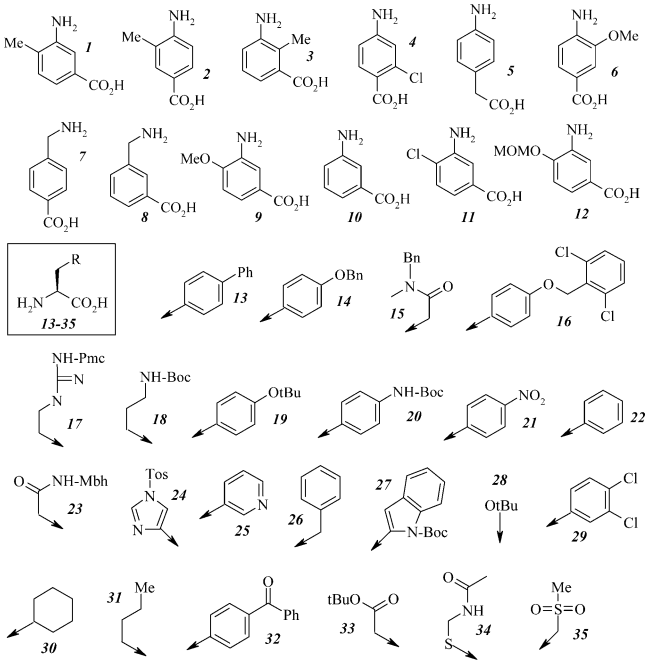

Figure 2. Amino acids (R1).

The selection of specific building blocks was influenced by chemical compatibility based on prior experience in our laboratory, as well as similarity to **1**. Approximately 10 members of each set of building blocks were designed to be analogous (or identical) to the equivalent reagent used in the preparation of **1**, such that ~1000 of the final library members would represent analogues of **1** at all three positions of diversity. The selection of building blocks was subjective, since no substantial knowledge of structure−activity relationships for **1** had been generated in the preliminary work, and computational tools did not play a significant role in our diversity determinations. The selection approach is illustrated by amino acid set R1 (Figure 2). This set contains eight aminobenzoic acids, including 2-chloro-4-aminobenzoic acid *4* from which **1** is derived, and a diverse set of α-amino acids which encompass a range of steric, electronic, and H-bonding options. Similarly, the R2 aldehyde set was composed of substituted benzaldehydes and related aryl aldehydes (including *p*-anisaldehyde from **1**), as well as aldehydes representing wider diversity. Amine set R3 contained 35 aliphatic and arylamines (including tryptamine from **1**) and were designed to broadly explore acceptable diversity at this position. While no attempt has been made to use computer-aided strategies to optimize the structural properties of this library in a *quantitative* sense, the goal was that a diversity relationship *qualitatively* similar to that shown in Figure 3 would be obtained for the overall library.

Library Assembly. Library assembly followed the same procedure as the preparation of individual encoded compounds (Figure 1), with the exception that pool−split operations were employed after the incorporation of each building block/tag pair. A sufficient quantity of differentiated resin **2** was used to provide enough individual beads for at least 50 assays by the techniques described below (more than 170 library equivalents).[26] Monitoring the efficiency of Alloc deprotections and amino acid (R1) couplings was carried out using quantitative Fmoc analysis and indicated successful production of resins **4**{*1*−*35*}.

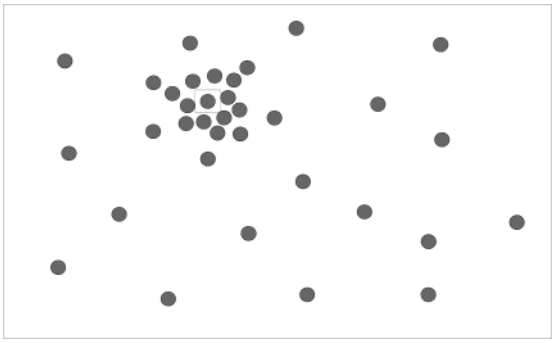

Figure 3. Simultaneous design of diverse and targeted library. The axes represent two hypothetical properties of library members. Building blocks were chosen in part to analogue the existing hit (in box). Thus, a cluster of rather closely related members is seen among the more diverse overall library.

After incorporation of the second tag, thiazolidinone ring formation was achieved by the condensation of each of the 35 aldehydes (R2) and mercaptosuccinic acid with the resin-bound amine to give resins **5**{*1*−*35*,*1*−*35*}.[17] After pooling and splitting for the third and final time, the 35 amines (R3) were incorporated via pentafluorophenyl ester activation of the resin-bound thiazolidinone to give resins **6** as 35 pools of 1225 members each. A third encoding step was not required, since these pools were kept separate.

Library Characterization. Following library assembly, extensive analysis was carried out to ensure that most of the desired members had been made and that hit identification would be possible when an active bead was decoded. These analyses were conducted at the single-bead level, since each pool contained an intractable mixture unsuitable for conventional analysis, but each bead carried, in principle, a single compound. We have previously described methods for this analysis and preliminary data for this library.[28] The process involved photolyzing individual beads and obtaining the mass spectrum of the cleaved material, then retrieving the bead and determining the amine code that it carried. Comparison of the code with the observed mass spectrum indicated the success or failure of synthesis on each bead.[29]

Beads were analyzed at two stages of library assembly: following ring closure (resin **5**) and on completion of the library (resin **6**). In each case, three beads per pool were selected for analysis, for a total of 210 beads (35 pools × 3 beads/pool × 2 resins). As expected, compounds cleaved from resin **5** were found to give good signals by negative-ionization LC/MS, whereas the corresponding samples from resin **6** performed better under positive-ionization conditions. Only eight beads gave poor decoding analysis (i.e., failed to provide unambiguous prediction) for a success rate of 202/210, or 96%. Reasonable results were also obtained in matching the LC/MS data to the code-predicted structure, with 138/202, or 69%, giving the predicted molecular ion as the major observed peak.

Capturing this quality control data prior to screening facilitates the downstream data interpretation, in addition to assessing the overall quality of the library. Thus, if a hit is identified containing a building block with ambiguous quality

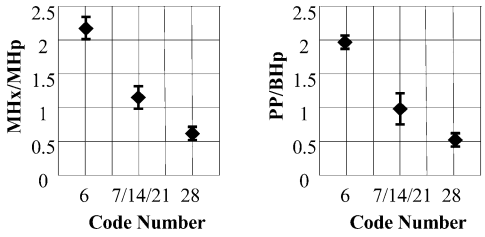

Figure 4. Quantitative coding ratios. Representative data is shown from the first (left) and second (right) tag sets as obtained from individual beads of resins **5** and **6**, respectively. The ratio of HPLC peak areas for two amines from each set is shown for the five codes in which they appear together. Values are the mean of at least three separate beads; error bars are ±1SD.

control data, the data may be regarded with suspicion. Conversely the absence of hits from resins bearing those building blocks should not be interpreted as evidence of the inactivity of those compounds. Failure to observe the predicted molecular ion will result from a problem of any one of the code-predicted building blocks or from a successfully prepared compound with a weak molecular ion. For any particular bead, it is thus not possible to unambiguously identify the cause of failure in analysis, but patterns often emerge when multiple beads are compared. None of the amino acid or amine building blocks resulted in systematic failure, but several of the aldehyde set were problematic. Interestingly, we have learned not to discard those pools identified as ambiguous prior to screening, because many small molecules simply produce weak molecular ions under the conditions we employ in our single-bead mass analyses, and the incremental overhead in screening those pools is minimal.

Evaluation of Quantitative Encoding. As mentioned above, this library made use of a quantitative encoding scheme[30] rather than the more standard binary scheme.[31] Several building blocks were encoded by mixtures of amines that differed only in the ratio of those amines, not their identity. Given the additional complexity of this encoding scheme, the validation of quantitative coding relationships was of particular interest in this study. In the absence of reliable quantitation, codes which differ only by the percentage of amine in the coding mixture may be misassigned more easily than those which contain qualitatively distinct amine mixtures (i.e., binary codes).

Figure 4 illustrates the quality of quantitation obtained by this approach. The left-hand graph in Figure 4 shows representative data from the first code set, corresponding to the amino acid (R1) building block. Methylhexylamine (MHx) and methylheptylamine (MHp) are simultaneously present in 5 of the 35 codes in that set. In 2 of those codes (6 and 28) they are the only amines, in nominal ratios of 2:1 and 1:2, respectively. Codes 7, 14, and 21 contain MHx and MHp together with a third amine that is different in each code. The right-hand graph in Figure 4 shows data from the second code set, choosing representative codes involving dipentylamine (PP) and butylheptylamine (BHp). For all beads sampled in this library, the quantitative ratios could be reliably distinguished. In cases for which the ratio alone was not completely unambiguous, the presence or absence of a third amine always permitted straightforward assignment. Quantitation has proven to be a reliable decoding tool in this library.

The ability to use quantitative codes becomes valuable in the case of extremely large libraries in which the number of distinct coded entities becomes greater than 2^n (where n is the number of available tags). In practice, the number of tags is not, in general, a limiting factor, especially if coding strategies are employed which leave one position uncoded (such as R3 in this library). It is likely that the simplicity of binary coding will make that approach preferred over quantitative approaches in most circumstances.

Library Screening

Assay of Bead-Tethered Positive Control. As described above, **1**, the lead compound against FSH, was a weak, partial agonist, displaying an EC_{50} of 5−10 μM in reporter cell assays. At the outset of screening, we were concerned about our ability to detect compounds with marginal improvements in potency. A rough calculation illustrated the nature of this concern. The beads from which the library was prepared had a loading of ∼200 pmol per bead. Twenty percent of loading is devoted to encoding, and the overall yield of thiazolidinone following library assembly and photorelease may be estimated at 50% for many members. Thus, around 100 pmol of product is likely to be available for assay. In a 96-well plate, with a typical assay volume of 100 μL, this gives an assay concentration of 1 μM, significantly below the EC_{50} of **1**.

The level of FSH agonist activity was measured following photorelease of varying numbers of the beads carrying **1** described earlier. In agreement with the above calculations, at least 10 beads were required to give a reproducible, detectable biological response (data not shown). Thus, at the outset of library screening, it was clear that at least a 10-fold enhancement in activity would be required in order to identify active compounds from single beads in this assay.

Identification of Active Pools by Hit-Rate Analysis. When screening a bead-based, split−pool library, it is desirable to assay sufficient beads to avoid missing rare events, since the selection of beads is largely stochastic. However, there is a penalty for thoroughness in the form of additional assay overhead. Since the current library had maintained the 35 R3 positions as separate sublibraries, it was possible to conduct an initial determination of "hit rate" as a first-pass stratagem to quickly identify active pools. Each of the 35 pools was distributed to one 96-well plate at a density of 30 beads per well, for a total of ∼2.4 library equivalents (30 × 96/1225), calculated to give a 93% chance of screening any particular member at least once. DMSO was added to each well, followed by photolysis for 1 h (sufficient for essentially complete compound release) and assay for FSH agonist activity.

Pool 4 (R3 = tryptamine), which contained the previously identified active compound described above, resulted in no active wells in this screen. Although disappointing, this was not entirely surprising in light of the studies described above with positive control beads. Other pools showed more activity, notably pools 10 (R3 = 2-naphthylmethylamine) and 35 (R3 = 2,3-dimethoxyphenethylamine), which, re-

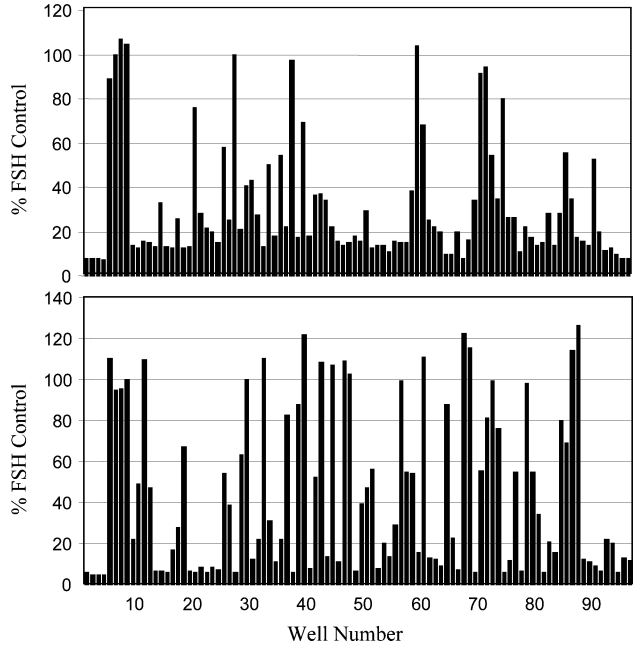

Figure 5. FSH reporter activity at 30 beads/well, full photolysis. Above, ACL1876 pool 10 (R3 = 2-naphthylmethylamine); below, pool 35 (R3 = 2,3-dimethoxyphenethylamine). Wells 1−4 were negative controls (no beads) and wells 5−8 were positive controls (200 pM FSH). The activity of individual wells is calculated as a percentage of the mean of the four positive controls on the same plate.

spectively, contained 15 and 32 wells with more than 50% of the activity of the positive control (200 pM FSH) (see Figure 5). This corresponds to hit rates of 0.5 and 1% for these pools (e.g., in pool 35, around 12 compounds of this arbitrary activity are expected out of 1225 members).

No attempt was made to identify individual active beads in this hit rate determination. Rather, we used these data to focus on the two active pools and to select appropriate strategies to identify active members of those pools. The simplest approach would be to distribute single beads to individual wells and select active beads. However, ~40 96-well plates would be required to screen 3 library equivalents and give a good chance of identifying the most active members. An alternative approach relies on the fact that the library was prepared on a photocleavable linker[25] that allows the release of compound from the bead to be controlled by varying the photolysis time.

Tiered-Release Two-Stage Screen for Identifying Active Beads. A "tiered-release" strategy attempts to release the compound in (at least) two portions (see Figure 6).[32] Initially, a pool of beads is irradiated for one half-life, nominally releasing half of the available compound into solution and leaving the remainder attached to the bead. After assay of the released material, beads from active wells may be individually redistributed to separate wells. Subsequent exhaustive photolysis releases the majority of the material that had remained attached following the initial treatment. Assay of these samples allows the identification of individual beads that had carried active compounds. These beads may then be retrieved and decoded to identify the structure of compounds of interest.

The half-life of release is dependent on many factors, including light intensity, solvent, temperature, and the nature

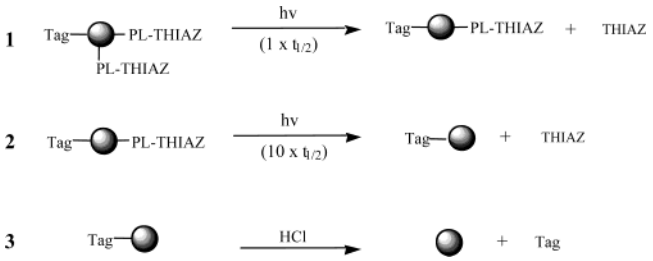

Figure 6. Identifying active beads by tiered release. 1. Batches of 10 beads are exposed to UV lamp for one half-life, assay each well for FSH activity. 2. Redistribute beads from active wells individually, expose beads to further 10 half-lives of UV, assay for FSH activity. 3. Release tag from active beads, analyze, and identify active members.

of the attached compound. However, on the basis of preliminary experiments (data not shown), we felt that this process was sufficiently predictable and that a photolysis time of 2 min (10 mW cm^{-2}, 365 nm) would give a good representation of the majority of library members.

One advantage of the tiered-release strategy is that a relatively small number of individual assays must be run: less than the straightforward single-bead approach by a factor equal to the number of beads per well for the first round. Disadvantages include the potential for both false positives and false negatives due to uneven photolysis. A relatively potent library member may be released more slowly than average; thus, a well containing that member may not be identified as active, and the compound could be discarded. Similarly, a relatively weak compound that is rapidly photoreleased may give rise to potent activity in the first round of screening only to show no activity in the second assay. Even under ideal photorelease conditions, we must be able to detect activity arising from only one-half of the total compound available on a single bead. In the present case, for the active pools identified by hit rate analysis, the potency of the members seems sufficient to overcome this pitfall (Figure 5).

The two active pools from hit-rate analysis were, thus, further screened by tiered release. A single 96-well plate with 10 beads per well was prepared for each pool, giving a total of about three-quarters of a library equivalent (960/1225). These plates were photolyzed for 2 min to release approximately one-half of the compound from each bead, which was then subjected to FSH assay. Pool 35 gave 8 out of 80 wells with at least one-quarter of the activity of the FSH positive control (Figure 7). This hit rate of 8/800 is in good agreement with the 1% activity estimate found in the 30-bead-per-well, total release experiment described above (Figure 5). Pool 10 gave very little activity under these conditions, suggesting that the activity observed in the earlier hit-rate analysis may have been the cumulative effect of low potency compounds.

All beads from the eight active wells found from pool 35 were retrieved and redistributed into individual wells. A total of 85 beads were recovered (pools had from 8 to 12 beads) and photolyzed for 20 min, and the released compound was retested. Six of the eight pools gave one active bead, while one pool had two beads of relatively weak activity, and another pool gave no significant activity (see Figure 8). Some

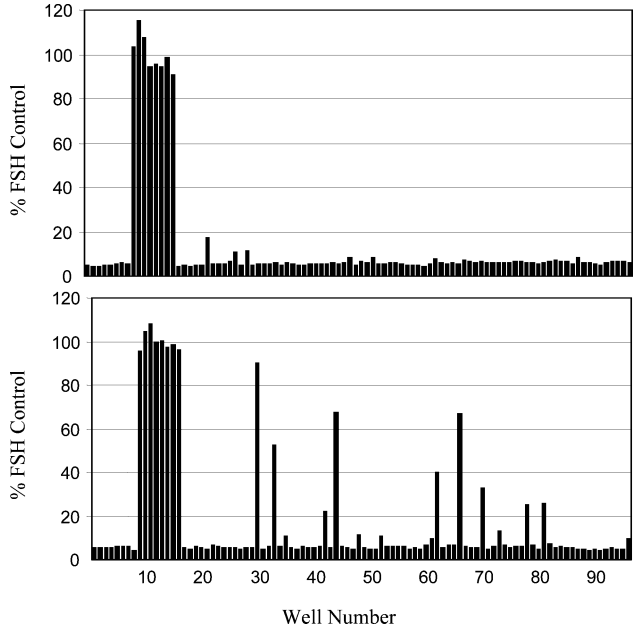

Figure 7. FSH response in first-round tiered-release screening of ACL1876 pools. Upper, pool 10; lower, pool 35. Leftmost eight data points in each graph from wells containing no bead; next eight are FSH positive control (200 pM); remaining wells contain ~10 beads each; 2-min photolysis. The activity of individual wells is calculated as percent of the mean of the positive controls on the same plate.

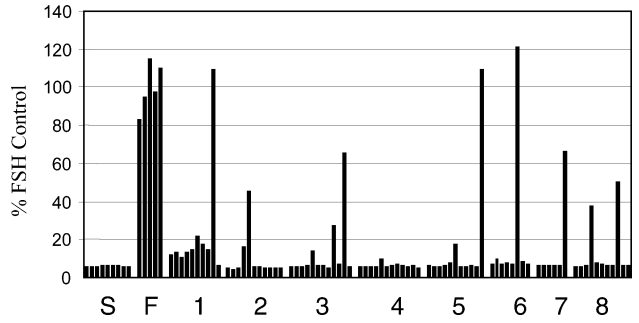

Figure 8. FSH response from single beads from pool 35. Beads from active 10-bead wells individually photolyzed and reassayed. S, solvent; F, FSH (200 pmol); 1−8, sets of beads from eight active wells found in initial release (see Figure 7 and text). The activity of individual wells is calculated as percent of the mean of the FSH wells.

of the active beads gave significantly greater biological response than had been observed in the pools (up to 110% of control), suggesting that the half-life of those compounds to photorelease may, in fact, have been longer than 2 min.

In an attempt to obtain direct analytical support for decoding assignments, individual active beads were photolyzed for a third time (1 h in methanol), and the supernatant was submitted for MS analysis. In many cases, a weak but identifiable molecular ion was observed in the expected molecular weight range, even though the beads had been substantially photolyzed before this step. The beads were again retrieved, and the tags were analyzed. All beads gave clean, unambiguous decodes. In several cases, the predicted structure was confirmed by MS (see Figure 9), including the three most active beads. In the remaining cases, the MS data from the residual compound was too weak to interpret.

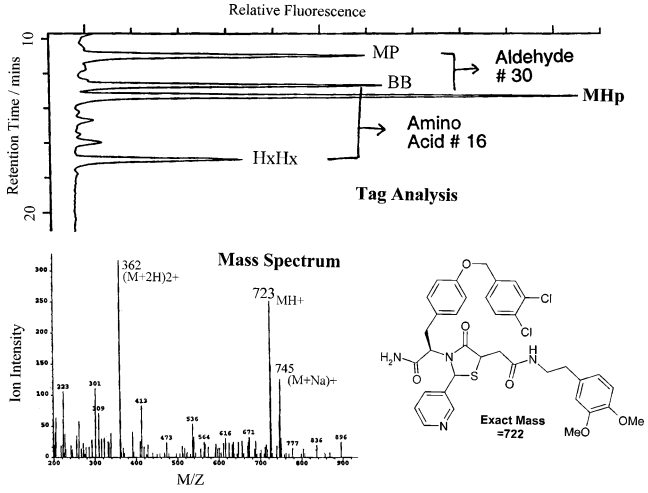

Figure 9. Analysis of bead from tiered-release study. Correlation of Tag/MS analysis. Above, HPLC separation of dansylated tag amines with fluorescent detection; below left, mass spectrum of material obtained from third photolysis; below right, structure and nominal mass of tag-predicted library member.

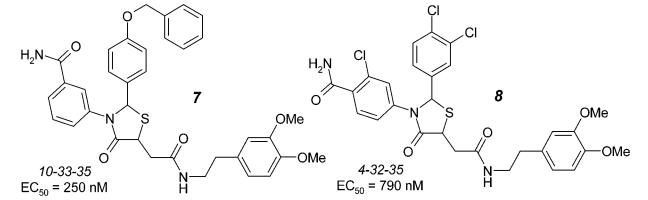

Figure 10. FSH agonists from tiered-release assay. Compounds prepared on the basis of decoding the two most active beads. Building blocks from which each compound was assembled are indicated (R1−R2−R3). The reported activity is for the purified and characterized compound (a racemic mixture of cis diastereomers).

Figure 10 shows the structure of the two most active predicted members.

Single-Bead Screening in High-Density Array. The discovery of compounds **7** and **8** was an important landmark in the project, because their nanomolar potency provided a greatly enhanced starting point for further medicinal chemistry development. In addition, validation of the tiered-release approach with this library was gratifying. However, less than 1 library equivalent had been tested in this study, and therefore, many active members may not have been screened. We hoped that by screening more library equivalents, more hits might be identified, expanding knowledge of structure−activity relationships and helping guide the medicinal chemistry efforts.

The second round of screening was conducted by pursuing an alternative approach using a higher-density screening format. Affymax has developed an integrated screening system based on custom 864-well microtiter plates, robotic workstations, and high-sensitivity CCD imaging systems, as well as even higher density formats for particular applications.[33] The volume of each well in the 864-well plate is around one-tenth of those in a 96-well plate, and assay volumes may be scaled accordingly (in this case, 12 μL vs 100 μL for a 96-well plate assay), with savings in reagents as well as a corresponding increase in the concentration of samples. In addition, the 9-fold greater number of wells in the same footprint as a 96-well plate makes the screening of

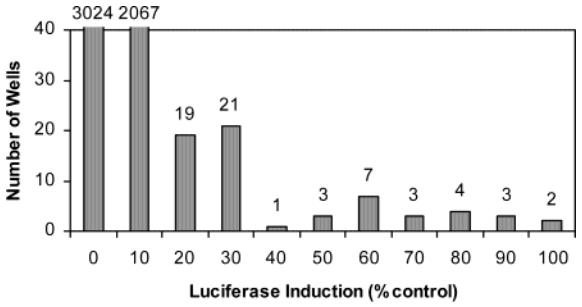

Figure 11. Histogram of 864-well FSH activity; pool 35.

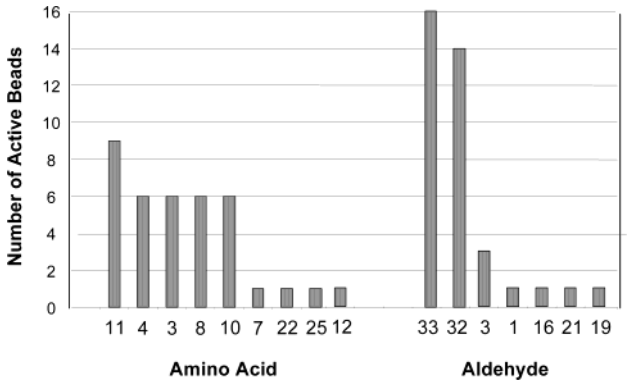

Figure 12. Building blocks found on active, decoded beads; 864-well plate screening of library pool 35. Beads giving a 4-fold signal enhancement over background are included.

large numbers of single beads significantly more practical. For any given pool of this library, screening 3 library equivalents (3775 beads) required 40 96-well plates, but only 5 864-well plates. Thus, it becomes practicable to screen a sufficient number of library equivalents while avoiding the extra steps and assumptions that are inherent to the tiered-release approach, as described above.

Six 864-well plates were distributed with single beads from pool 35 of the library. These beads were suspended in DMSO and photolyzed for 2 h to ensure substantially complete release of photocleavable material that was then diluted into buffer and assayed for FSH activity as above. A small percentage of beads showed interesting activity, with 63 out of more than 5000 wells displaying 2-fold activity over background (Figure 11). Around 80% of these active wells were found to contain a single bead. As in the tiered-release experiment, active beads were retrieved and rephotolyzed in methanol; again, a candidate molecular ion was identified in many cases. Subsequent tag analysis gave the predicted structure of the member attached to that bead. This was always in good agreement with the observed mass spectrum when available. The distribution of building blocks in the decoded active beads is shown in Figure 12 (the structure of aldehyde residues 32 and 33 can be seen in Figure 10).

With the success of the single-bead screen in the 864-well format, we were intrigued whether we could observe sufficient activity from analogues of the original hit **1**, which itself should be present as one of the 1225 compounds in pool 4. Through a series of control reactions, we found that **1** was extremely sensitive to the final TFA treatment applied to the library to remove side-chain deprotection. Fortunately, **1** could be readily prepared and released via photolysis if the TFA step was omitted. Thus, 2 library equivalents of pool 4 were therefore rescreened in 864-well format without acid treatment, and several active beads were observed (Figure 13). The structures of the most active compounds are shown in Figure 14, and data for these and other actives are reported in Table 1.

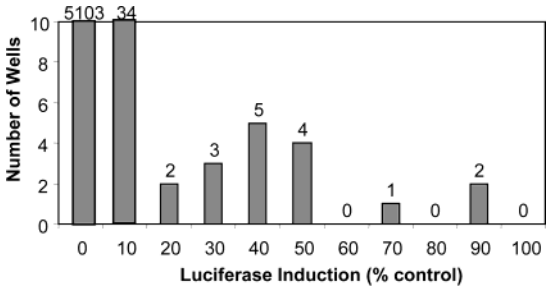

Figure 13. Histogram of 864-well assay; pool 4.

Table 1. FSH Agonist Activity of Library Members

compd	pool no.	pEC_{50}[a]	864-well signal[b]	tiered release[c]	% cis diastereomers[d]
9	4	7.5	24 (2)		3
10	35	7.4	84 (4)		20
11	35	7.0	31 (3)		10
12	4	6.8	77 (4)		45
13	4	6.8	45 (2)		50
7	35	6.6	40 (2)	1	10
8	35	6.6	74 (4)	2	60
14	35	6.1	53 (4)		50
1	4	5.3			35
15	35	5.2		5	50
16	35	5.0		4	40
17	35	n.d.	50 (2)		
18	35	n.d.	62 (1)		
19	4	n.d.	53 (3)		
20	4	n.d.	30 (2)		
21	35	n.d.	44 (2)		
22	35	n.d.	38 (2)		
23	35	n.d.	19 (5)	6	
24	35	n.d.		3	

[a] Compounds are ranked according to the pEC_{50} of the purified racemic mixture of cis diastereomers. [b] Average percent stimulation observed in 864-well single-bead assay (relative to 200 pM FSH) with number of replicate beads bearing that compound in parentheses. [c] The relative rank order of the six most active library members identified in the second round of this screen are shown. [d] Relative amount of the cis diastereomers (see text) observed upon scale-up.

Comparison of Screening Strategies. Table 1 shows the FSH agonist activity of the most active members of this library, which were made as part of the hit validation step. The compounds were prepared by conventional techniques, and the mixture of diastereomers (vide infra) was resolved prior to testing. All the compounds exhibit full efficacy in the reporter assay, with compound **9** showing the greatest potency, with an EC_{50} of 32 nM. A complete discussion of the SAR and medicinal chemistry of these compounds is outside the scope of this article and will be described elsewhere.

Table 1 shows a rather poor correlation between the 864-well, single-bead data and the EC_{50} of purified compounds. Most notably, compound **9**, the single most active library member identified in subsequent work, has rather low activity in the 864-well assay. One factor that appears to play a large part in this discrepancy is the relative amounts of different

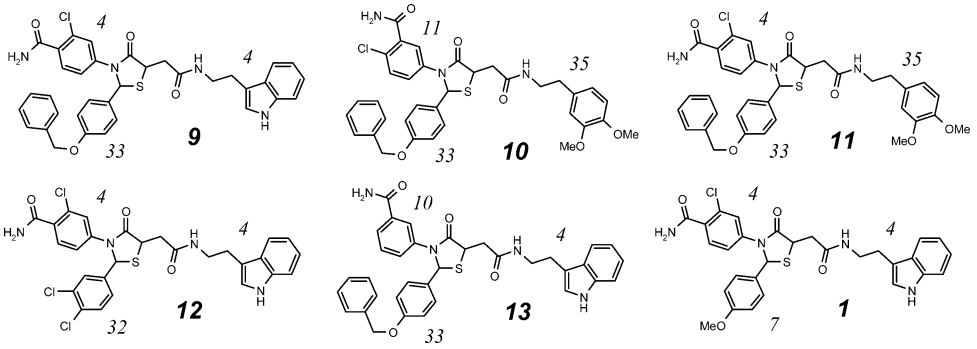

Figure 14. FSH agonists from 864-well array screening. See Table 1 for the activity of single beads bearing these compounds and EC_{50} values for the purified compounds. Compound **1** is included for comparison.

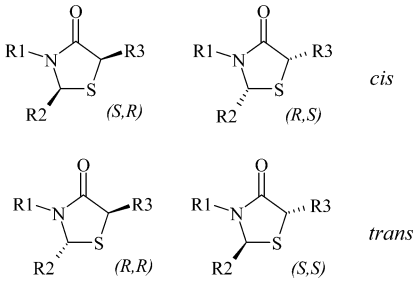

Figure 15. Thiazolidinone diastereomers.

possible stereoisomers formed during thiazolidinone ring formation. One chiral center is derived from the aldehyde unit; the use of racemic mercaptosuccinic acid (the only commercially available form) ensures that four diastereomers are produced: a pair of cis enantiomers (with respect to the 2- and 4-protons of the ring) and a pair of trans enantiomers (see Figure 15). When compounds were prepared on large scale, it was usually possible to separate the pairs of cis and trans isomers by chromatography and assign the structures by NMR. In all cases, the cis racemate was found to have the greater FSH agonist activity, but there was a surprisingly large variability in the relative propotions of cis and trans isomers for the different thiazolidinones. Because it was not possible to measure this ratio in the single-bead work, the observed activity in single-bead screening represents the net activity of the diastereomeric ratio obtained on a particular bead.

A second observation that dictates how the data is used from such single-bead screening is the variability in the biological response from single beads carrying the same library member. Many factors may contribute to this variability, including variation in bead size, uniformity of loading from bead to bead, and slight differences in the amount of reagents (for cleavage and assay) delivered to each well of an 864-well plate. Screening a larger number of library equivalents would lead to the repeated identification of active members and allow a reasonable determination of the true relative activities of each member. However, the purpose of this experiment is primarily to quickly identify hits and progress them into lead compounds. Although we are striving to obtain higher quality data from single-bead studies, the determination of detailed SAR is probably best left to efficient parallel synthesis/purification approaches.

In this work, we have identified many more active beads in the 864-well assay than by tiered-release screening. In addition, some of the most active compounds were apparently missed in the tiered-release approach. These data should not, however, be considered a definitive comparison of the two techniques because the thoroughness of screening was different in the two experiments. Almost six times as many beads were screened in the high-density array format than by tiered release. Screening in 864-well plates (or other high-density array format) is more efficient in reagent use and avoids the pitfalls of tiered release described earlier. However, not all assays can easily be adapted to such small volumes, and reagent deliveries and bead handling are easier in larger well formats such as 96- or 384-well plates. Nevertheless, for the system described here, we have found the higher density format to be an efficient way of rapidly screening this large library.

Summary and Conclusions

The discovery of compounds of rather weak activity is a common phenomenon in library screening, and optimizing that activity can often be difficult and time-consuming. The experiments described here present a strategy for the rapid synthesis and screening of a very large collection of analogues. Many thousands of the 42 875 library members are analogues of the initial lead compound, since several building blocks at each position were chosen because of their similarity (or identity) to those in this compound. The extent of this survey would be extremely difficult to achieve by parallel synthesis approaches, particularly in the absence of strictly additive (i.e., independent) effects of the contributions to potency provided by the different residues. The discovery of a 32 nM agonist from a single round of library design and screening illustrates the value of this strategy for the rapid identification of potent compounds from a weak initial lead.

We have simultaneously prepared a valuable resource for screening against other biological targets of interest. Assay against a single target such as FSH requires only 3 library equivalents for hit rate determination and a further 3−10 equiv for identification of actives (which are expected to be rare events in "random" screening). The quantity of library prepared here is estimated to be sufficient for screening over 50 targets, and additional hits have been identified against several other targets from this library (data not shown). More recent libraries have been prepared on a larger scale to provide a sufficient supply for several hundred screens In addition, the extension of these strategies to a broad range

of chemistries and screening of the resulting libraries against diverse targets is ongoing.

Materials and Methods

General. Reagents and building blocks were obtained from a variety of commercial sources and were used without further purification. Solvents were the purest grade available and were used without further purification. Resin washes were carried out at least three times with a minimum of five volumes of the stated solvent. Quantification of resin-bound Fmoc (9-fluorenylmethoxycarbonyl) was carried out by measuring A_{302} of the supernatant following treatment of a resin sample with piperidine (20% v/v in NMP), as described in ref 15. Photolysis was carried out for the specified time under a UV lamp with intensity at 365 nm of 10 mW cm^{-2}, as measured by a handheld UV detector. HPLC was conducted on Beckman equipment using a 30 cm × 4 mm Vydac C18 column with a gradient of acetonitrile and water (0.1% TFA) and detection at 220 and 254 nm. LC/MS was carried out as described in ref 28.

Orthogonally Protected Bifunctional Resin (2). TentaGel-S−NH_2 resin (polystyrene/poly(ethylene glycol) resin; Rapp Polymere, Tübingen Germany; 110-μm mean diameter, 10.0 g, 0.25 mmol/g, 2.5 mmol) was treated with a solution of Fmoc chloride (5.85 g, 22.5 mmol, 9 equiv), Alloc chloride (allyl-3-oxycarbonyl chloride; 0.26 mL, 2.5 mmol, 1 equiv), and diisopropylethylamine (DIEA; 4.25 mL, 25 mmol, 10 equiv) in *N*-methylpyrrolidine (NMP; 30 mL). After shaking at room temperature for 3 h, the resin was washed well with NMP to give orthogonally protected bifunctional resin with a 4:1 ratio of Fmoc/Alloc determined by quantitative Fmoc analysis, as previously described. After removal of Fmoc (20% v/v in NMP, 20 min, NMP wash), coupling of the Fmoc-photolinker (4-[4-{1-(Fmoc-amino)-ethyl}-2-methoxy-5-nitrophenyoxy]butanoic acid)[25] was carried out as described[18] to give resin **2**.

Encoded, Tethered, Positive Control Beads (6). Fmoc-PL/Alloc resin (**2**; 0.2 g, 0.05 mmol) was placed in a 4-mL filter tube (Alltech Associates). The Alloc protecting group was removed, and the first tag was incorporated using the three-step process described previously.[18] The amine solution used in the final step was a mixture of dibutyl- and dipentylamine (1:1.2 molar ratio; 20% v/v in NMP with respect to total amine). As well as forming the code, this amine mixture removes the Fmoc group. 4-Fmoc-amino-2-chlorobenzoic acid (0.118 g, 0.3 mmol, 6 equiv) and HATU (0.114 g, 0.3 mmol, 6 equiv) were dissolved in NMP (2 mL). DIEA (0.051 mL, 0.3 mmol, 6 equiv) was added, and the solution was shaken for 1 min, then added to the resin. After shaking for 2 h, the resin was drained and washed well with NMP, CH_2Cl_2, methanol, and diethyl ether, then dried for 1 h in vacuo, giving resin **3** (R1 = 4-amino-2-chlorobenzoate residue). The Alloc protecting group was removed, and a second tag was coupled (mixture of dihexyl- and dioctylamines) as described above. The resin was transferred to a screw-cap vial, to which was added THF (0.8 mL), *p*-anisaldehyde (0.095 mL, 0.8 mmol, 20 equiv), 2-mercaptosuccinic acid (0.24 g, 1.6 mmol, 40 equiv), and 3-Å molecular sieves (0.05 g). The mixture was heated at 70 °C for 18 h, releasing the pressure after 5 min and 1 h. After cooling to 25 °C, the resin was returned to a filter tube and washed well with DMF, giving resin **5** (R2 = *p*-MeO−C_6H_4). Equal volumes of pyridine, DMF, and pentafluorophenyl trifluoroacetate (Pfp-Otfa; 0.6 mL each) were added, and the mixture was shaken for 20 min then drained and washed once with DMF. Tryptamine (20% w/v in DMF; 1 mL) was added, and the mixture was shaken for 40 min then drained and washed well with DMF, then with CH_2Cl_2, methanol, and diethyl ether and dried in vacuo to give resin **6** (R3 = tryptamine). A small sample (~2 mg) of the dry resin was placed in a 200-μL glass vial, and a 0.3% solution of ethanolamine in 2-propanol (70 μL) was added. The vial was irradiated by UV lamp for 1 h. Analysis of the resulting solution indicated that **1** was obtained in good purity (C_{18} reversed phase HPLC: 2 peaks eluting at 62 and 63% CH_3-CN respectively; 1:2 ratio; 95% of total area; LC/MS: $[MH^+]$ = 547 observed for main peaks, as predicted for $C_{29}H_{27}ClN_4O_3S$).

Preparation of Encoded Library. Library assembly proceeded in a fashion similar to the route described above for a single encoded thiazolidinone. Differentiated Fmoc-PL/Alloc resin (**2**; 7.5 g, 1.83 mmol) was subjected to Alloc deprotection, reaction with Alloc-iminodiacetic acid and Pfp-Otfa,[18] then split into 35 equal portions (each 0.215 g). Each portion was treated with a solution of the appropriate mixture of secondary amines (20% v/v in NMP) to give encoded resins **3**. The proportions of amines required to provide equimolar ratios were determined as described.[15] For the first set of tags, the relevant reactivities were as follows: dibutyl (100), butylpentyl (95), dipentyl (85);,butylheptyl(75), and dihexyl (65). Fmoc-amino acids were coupled as described above for **1** to give encoded amino acid resins **4**{*1*−*35*}. The incorporation of each amino acid was monitored by ninhydrin test, and the coupling was repeated if necessary. The resin portions were then pooled and mixed, and the second tag was added, again splitting the resin into 35 portions immediately before the amine addition. For this second set of amines, the relevant reactivities were methylpentyl (100), methylhexyl (95), methylheptyl (90), methyldodecyl (40), and ethylbutyl (7.5). Each pool was then subjected to thiazolidinone formation with the appropriate aldehyde, mercaptosuccinic acid, and THF, as described above, to give the doubly encoded resins **5**{*1*−*35*,*1*−*35*}. All resins were thoroughly washed with DMF, CH_2Cl_2, methanol, and diethyl ether, and a small portion (~5 mg) was reserved for analysis before pooling for a second time. The resin was then treated with pyridine, Pfp-OTfa, and DMF (10 mL each) and washed once with DMF before splitting for the third and final time into 35 portions. Addition of the appropriate amine building blocks as 20% solution in DMF, shaking for 1 h, then washing thoroughly with DMF, CH_2Cl_2, methanol, and diethyl ether gave the library **6**{*1*−*35*,*1*−*35*,*1*−*35*} as 35 portions of ~200 mg, each containing 1225 members.

Characterization of Library. Three single beads were randomly sampled from each of the 35 pools following incorporation of aldehyde (resins **5**) and amine (resins **6**). Beads were individually suspended in acetonitrile (60 μL)

in sealed conical 200-μL glass vials and irradiated by UV lamp for 1 h. The supernatant was analyzed by flow injection electrospray mass spectroscopy in negative ion mode for beads **5** and in positive ion mode for beads **6**. Decoding of beads was by LC/MS as described previously.[27]

Preparation of Individual Thiazolidinones. The general procedure was as follows. Compounds were prepared on ArgoGel−Rink−FMOC resin (Argonaut Technologies, Redwood City, CA), typically on a 1-g scale of resin. Coupling of amino acids and subsequent condensation with aldehyde (20 equiv) and mercaptosuccinic acid (40 equiv) was as described previously.[17] The resulting resin-bound carboxylic acid was treated with Pfp-OTfa/pyridine/NMP (1:1:1 v/v; 3 mL) then washed, treated with the appropriate amine (10 equiv), and shaken for 16 h at 20 °C. The resin was washed extensively, then the products were cleaved with 50% trifluoroacetic acid/CH_2Cl_2 for 30 min. The products were purified by reversed-phase preparative HPLC to give racemic mixtures of the cis- and trans- isomers (see Figure 15).

Initial Assay: Determination of "Hit-Rate". Beads were suspended in a neutrally buoyant solution (consisting of trifluoroethanol/water, 15:8 v/v) and dispensed into 96-well plates at ∼30 beads per well, for a total of ∼2800 beads (2.2 library equivalents). Photolysis reagent (5 mM ethanolamine in DMSO; 4 μL) was added to each well, and the plates were photolyzed for 1 h with gentle shaking. After storing overnight at 4 °C in the dark, assay buffer (DMEM/F12 (Gibco, Carlsbad, CA) 1% BSA; 25 μL) was added to each well. A further portion of the same buffer (6 μL) was added to all wells of a separate plate (the "assay plate"). Photolyzed material was transferred from the photolysis plate into the assay plate by multichannel pipet. A solution of FSH (8 nM; 0.5 μL) was added to three wells on each assay plate as a positive control, followed by a suspension of CHO FSHR cells carrying an inducible luciferase reporter (4×10^6 cells/mL; 25 μL). After a 4-h incubation at 37 °C, luciferase detection reagent (LucLite; Packard; 6 μL) was added, and luciferase activity was detected as photon emission (minimum of two 1-min images) using a cryogenically cooled, scientific grade, 512 × 512, back-illuminated, CCD (Photometrics, Tucson,AZ) combined with a camera (Nikkon; 50 mm, f/1.4). The total number of pixel counts for each well was determined with IPLab software (Scanalytics, Fairfax, VA) and further processed with an Excel macro. Pools 10 and 35 showed wells with good activity (see text).

Secondary Assay 1: "Tiered Release". A suspension of beads was distributed into one 96-well plate per pool at 10 beads/well, as described above. Partial release of the compounds was carried out by photolysis for 2 min, as described above. Beads from wells showing significant activity (see text) were retrieved and individually placed into empty wells of a new plate. The remaining resin-bound compound was released and assayed as before. Beads giving rise to significant activity were retrieved and decoded.

Secondary Assay 2: 864-Well Screening. All liquid dispensing steps were performed using a robotic pipeting device developed in-house for custom-molded 864-well plates. After single beads were distributed to individual wells of 864-well plates (using a custom-prepared, automated "bead picker"), photolysis and assay was as described above. Beads in wells with signal greater than 2-fold over the background were retrieved from the photolysis plate and decoded.

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