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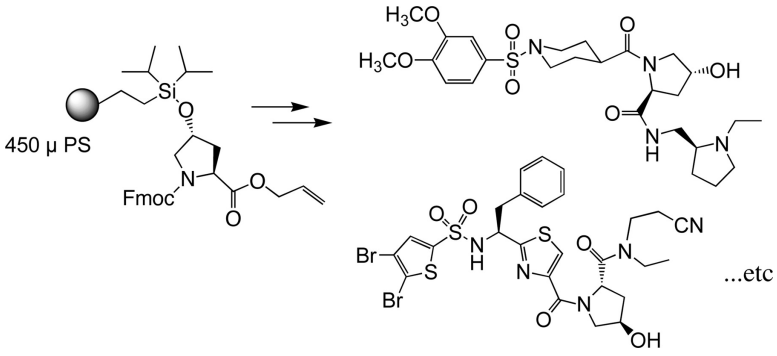

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One-Bead-One-Compound Library of End-Capped Dipeptides and Deconvolution by Microflow NMR.

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As part of our program to identify novel small molecules with interesting biological activity, we have designed and synthesized a library of end-capped dipeptides with an emphasis on compound diversity, complexity, and membrane permeability. An ∼1500-member library was synthesized manually on large polystyrene beads using the mix-and-split method. The final compounds were cleaved into 384-well plates to generate individual stock solutions for input into high-throughput biological screens. Individual compounds were decoded using a combination of mass spectrometry and microflow NMR spectroscopy. In principle, this approach to deconvolution obviates the need for complicated binary encoding−decoding strategies for one-bead-one-compound libraries.

Introduction

The identification of small-molecule probes for biology has accelerated with the advent of high-throughput screening (HTS) methods and their applicability to a wide range of biological assays. The term "diversity-oriented synthesis" has been applied to the process of generating large, diverse collections of small molecules as input into high-throughput biological screens.[1] Diversity-oriented syntheses often incorporate features found in bioactive natural products[2], such as complexity and cell permeability, to maximize the chances of identifying active compounds in target-based or phenotypic screens. The traditional strategy of permuting the R-groups on a constant core scaffold has been augmented by strategies that introduce variation in the scaffold itself.[3] Bifurcating reaction pathways that produce multiple scaffolds[3−7] and stereochemical outcomes[8] have introduced the possibility of greatly enhancing chemical diversity in relatively small synthetic libraries.

Backbone-modified cyclic and linear peptides represent a diverse class of natural and synthetic compounds that often show potent biological activity. Although peptides are often associated with poor bioavailability, modifications such as cyclization, N-alkylation, the presence of non-α- and D-amino acids, and nonpeptidic N- and C-terminal capping groups serve to improve bioavailability, placing such compounds within the realm of druglike small molecules. These modifications improve cell permeability[9] and increase resistance to proteolytic degradation.[10] In addition, geometric variation in the peptide backbone can have dramatic conformational effects, facilitating exploration of "chemical diversity space" beyond what is achievable with normal peptide sequences.

We have designed a library of linear, end-capped peptides using non-α-amino acid linking elements to serve as input into biological screens. This library was designed to maximize diversity and complexity within the constraints of synthetic efficiency, with a particular emphasis on membrane permeability among the final library members. Here, we report the synthesis of a 1470-member, one-bead-one-compound library of end-capped carboxamides and sulfonamides on polystyrene macrobeads and demonstrate the use of mass spectrometry and microflow NMR spectroscopy to decode the final cleaved products.

Results

We used hydroxyproline as a trifunctional linker[11] and loaded it onto polystyrene macrobeads (400−450-μm diameter, ∼7500 beads/g) via an all-carbon diisopropylsilyl ether linkage.[12] Five geometrically unique Fmoc-protected amino acid building blocks including the null group (**1**{*0*−*4*}) were incorporated into the library using standard coupling conditions (Schemes 1 and 2). We were limited to non-α-amino acids at this position since α-amino acids rapidly reacted to form diketopiperazine upon Fmoc deprotection (data not shown). Amino acid **1**{*1*} was synthesized by analogy with published methods,[13] and amino acids **1**{*2*} and **1**{*3*} were synthesized by reductive amination of the corresponding aldehydes with methylamine, followed by Fmoc addition.

Monomer rehearsals were performed for each of the building blocks on model compounds to identify conditions that would ensure high purity for the largest number of building blocks. For coupling of amino acids **1**{*1*−*4*}, the best coupling conditions were found to be HBTU/DIPEA in DMF. The next set of monomer rehearsals were performed using compound **2**{*4*} as the model dipeptide. We evaluated several conditions for sulfonamide formation and found that addition of 15 equiv of sulfonyl chloride, 15 equiv of DIPEA, and 15 equiv of pyridine gave >95% purity for the 12 sulfonyl chlorides in Scheme 3. The carboxylic acid capping groups were coupled most efficiently with HBTU and DIPEA

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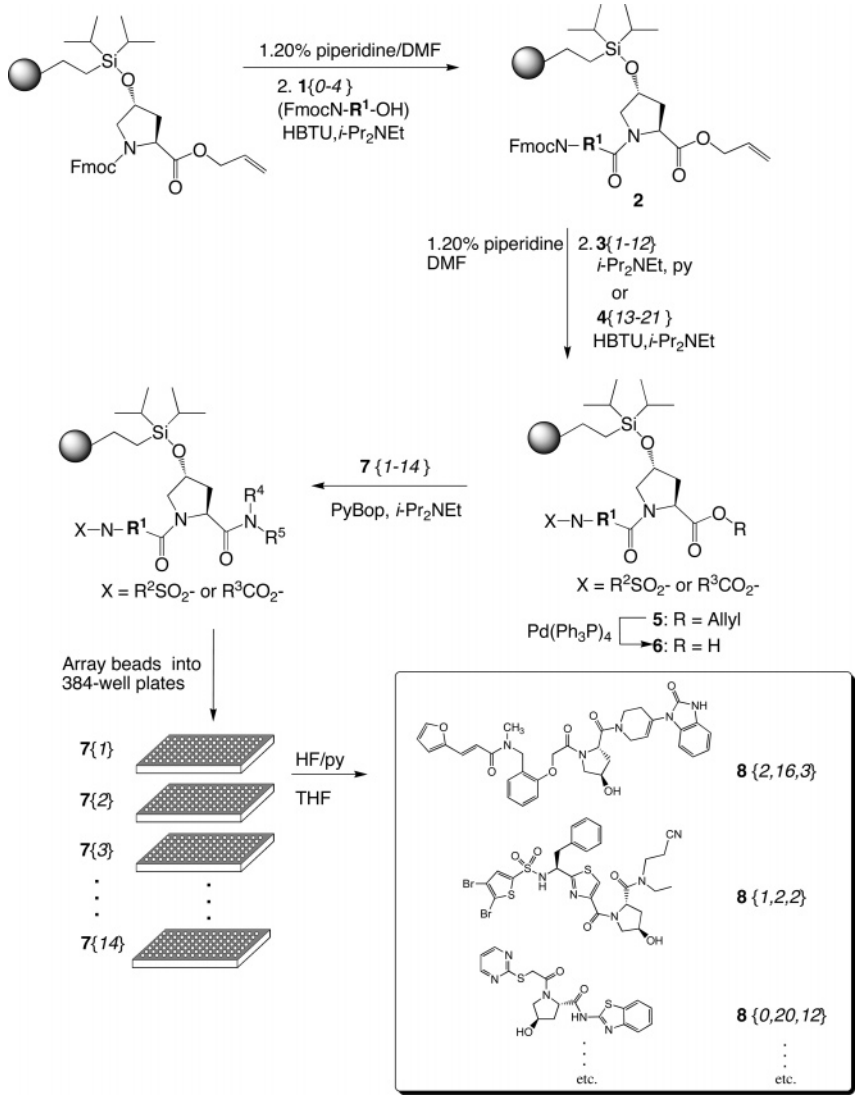
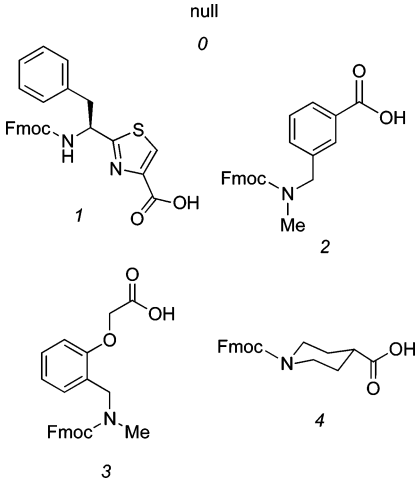
Scheme 1. Library Synthesis Starting with *trans*-Hydroxyproline-Functionalized Polystyrene Resin[a]



[a] Examples of library members are shown in box.

Scheme 2. Amino Acid Monomers **1**{*0*−*4*}



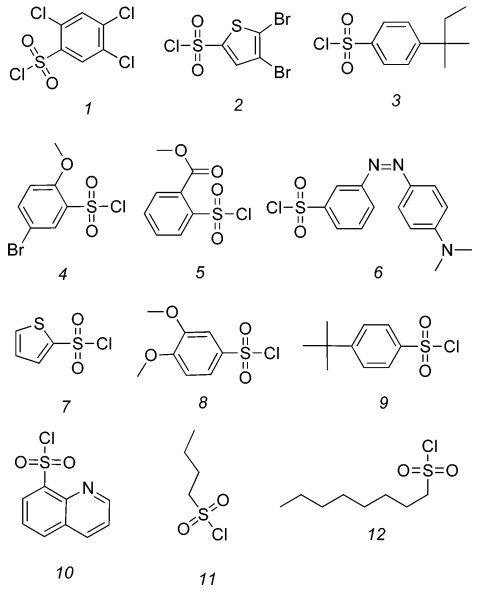
in DMF. Twelve sulfonyl chlorides (Scheme 3) and 9 carboxylic acids (Scheme 4) were thus selected for inclusion in the final library synthesis.

Next, the allyl ester was deprotected using Pd(0), and the resulting carboxylic acid was capped with a set of structurally diverse amines. The set of amines in Scheme 5 gave >95% when coupled to model compound **6**{*4,9*}. Interestingly, many arylamines coupled efficiently, even under standard peptide coupling conditions. We were thus poised to synthesize a split−pool library based on the selected building blocks.

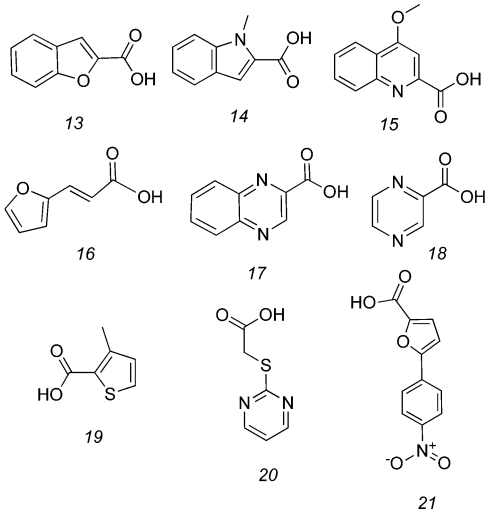
The library was synthesized using the mix-and-split method.[14] The same conditions were used for each building block as determined in the monomer rehearsals. After addition of amines **7**{*1*−*14*}, the beads were dried under vacuum and arrayed into 384-well plates with one bead per well. The beads were kept segregated according to the final monomer to facilitate compound identification by mass spectrometry. After cleavage with HF/pyridine and quenching with TMSOEt, the plates were evaporated, and the resulting compounds were taken up into DMSO at 30 μL per well. Analysis of 75 randomly selected beads (5% of the library) by LC/MS gave an average purity of 94% based on LC/MS using light scattering detection (see Supporting Information).

Microflow NMR probes are now commercially available that allow good quality ^{1}H spectra to be obtained from as little ∼10 μg of material. We selected a well at random from

Scheme 3. Sulfonyl Chlorides **3**{*1*−*12*} Incorporated as N-Terminal Capping Groups

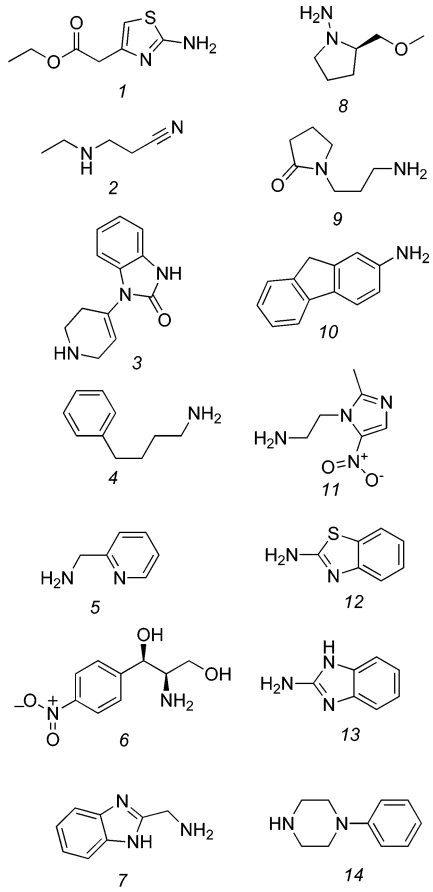


Scheme 4. Carboxylic Acids **4**{*13*−*21*} Incorporated as N-Terminal Capping Groups



the library and analyzed one-third of the sample by LC/MS (Figure 1). The mass of 685.3 was unique to compound **9**. The remainder of the DMSO stock solution was evaporated to dryness, and the resulting residue was taken up into CD_3OD (15 μL). The solution was injected into an NMR microflow probe (Protasis), and ^{1}H NMR data were acquired for 30 min. Compound **9** was resynthesized on a 40-μmol scale, purified by preparative HPLC, and analyzed by ^{1}H NMR spectroscopy using a 0.7-mL sample in a standard probe. The microflow spectrum obtained from the single bead was identical to that obtained from the resynthesized material (Figure 1). The vinylic region showed three signals corresponding to the vinyl hydrogen on the C-terminal capping group. We hypothesized that this heterogeneity was due to the presence of amide bond rotamers that equilibrate slowly relative to the NMR time scale. High-temperature NMR spectra of the resynthesized material confirmed this because the vinylic signals coalesced into a single resonance above 55 °C (see Supporting Information).

Scheme 5. Amines **6**{*1*−*14*} Incorporated as C-Terminal Capping Groups



Conclusion

We have synthesized a one-bead-one-compound library using simple amide and sulfonamide bond-forming chemistry. We have also demonstrated the ability to use microflow NMR in combination with mass spectrometry to decode cleaved library products. This suggests an attractive alternative to chemical encoding and decoding strategies, such as the binary tags developed by Still and co-workers.[15,16] NMR and LC/MS decoding not only obviates costly and tedious encoding schemes, but it also offers the possibility of identifying library product(s) directly rather than reporting only the reaction history of a particular bead. The recent report of an automated microflow NMR system[17] suggests that whole mix-and-split libraries can be decoded and structurally characterized using a combination of LC/MS and NMR spectroscopy without the need for chemical encoding during library synthesis.

Experimental Section

Fmoc-*trans*-Hyp-OAll Ester. In a 250-mL round-bottom flask equipped with a stir bar, Fmoc-*trans*-Hyp-OH (5 g, 14.1 mmol) was suspended in neat allyl bromide (61.6 mL, 707.4 mmol). To this suspension, *N*,*N*-diisopropylethylamine (DIPEA; 11.5 mL, 69.3 mmol) was added. The mixture was heated to reflux, and a small amount of DMF was added to aid dissolution of the protected amino acid. After refluxing for 2 h, the reaction was judged to be complete by TLC in 3% MeOH/CH_2Cl_2. The reaction mixture was allowed to cool

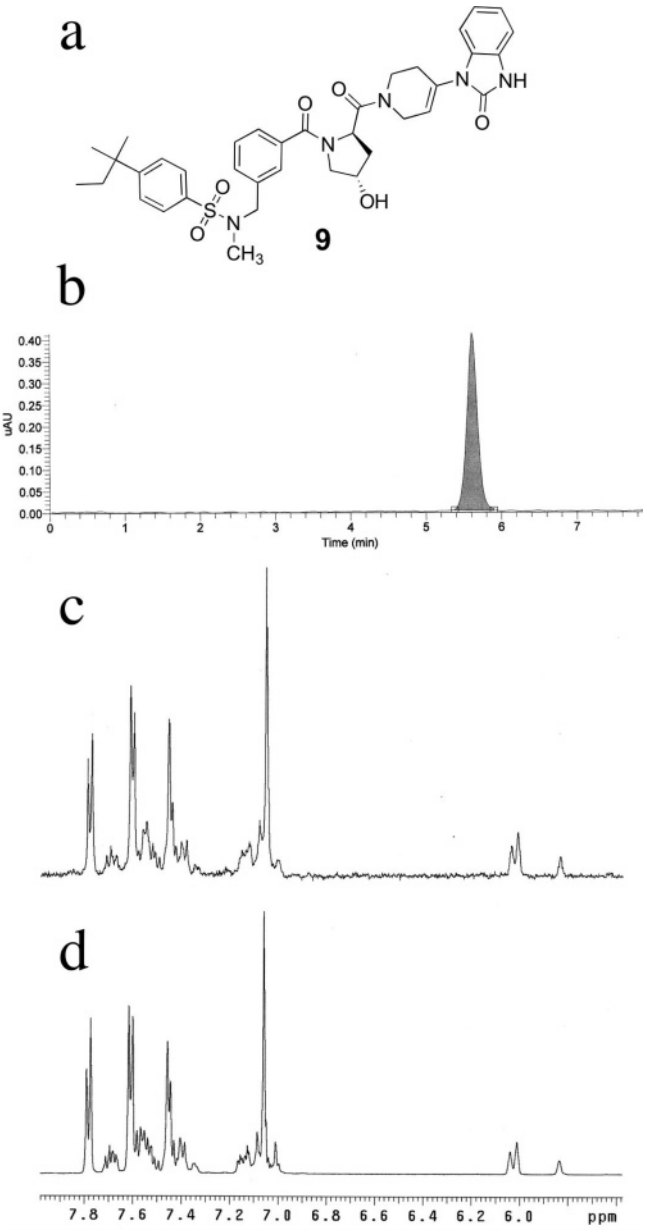


Figure 1. (a) Structure of compound **9**, derived from well 3-F09, deduced from mass spectrometry, microcapillary NMR, and large scale resynthesis. (b) Light-scattering HPLC analysis of well 3-F09. One-third of the stock solution was injected. (c) Aromatic and vinylic region of the ^{1}H NMR spectrum from 2/3 of the stock solution from well 3-F09 using microcapillary probe. (d) Same region of ^{1}H NMR spectrum from large-scale resynthesis of **9** after HPLC purification.

to room temperature, diluted with ethyl acetate, and then washed with saturated sodium chloride three times. The organic layer was dried over $MgSO_4$, filtered, and evaporated to yield a viscous, amber oil. The crude product was diluted with 3% methanol in dichloromethane and purified by isocratic column chromatography in the same solvent system, and 5.47 g of the allyl ester was obtained as a yellowish oil (98%). ^{1}H NMR ($CDCl_3$) δ 2.13 (p, 1H; J = 3.3 Hz), 2.33 (t, J = 3.3 Hz, 2H), 2.40 (s, 1H), 3.56 (d, J = 6.7 Hz, 1H), 3.76 (d, 1H), 4.22 (d, 1H), 4.35 (m, 1H), 4.60 (d, 2H), 4.66 (d, 2H), 5.27 (m, 2H), 5.88 (m, 1H), 7.31 (t, J = 7.3 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.61 (m, 2H), 7.77 (d, J = 7.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 39.5, 47.2, 55.4, 58.0, 65.9, 67.9, 70.3, 119.0, 120.0, 125.2, 127.2, 127.8, 131.7, 141.3, 143.8, 155.1, 172.3. LR-MS (ES) m/z 394.3 [M + H^+], calcd for $C_{23}H_{24}NO_5$: 394.2].

Fmoc-Phe-Thz-OH (1{*1*}).[13] ^{1}H NMR (THF-d_8) δ 8.16 (s, 1H), 7.77 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.5 Hz, 1H), 7.56 (d, J = 7.0 Hz, 1H), 7.45 (d, J = 9.0 Hz, 1H), 7.34 (t, J = 7.5 Hz, 2H), 7.26−7.20 (m, 4H), 7.15−7.12 (brt, 1H), 5.26 (m, 1H), 4.33 (m, 2H), 4.15 (t, J = 7 Hz, 1H), 3.50 (m, 1H), 3.13 (m, 1H). ^{13}C NMR (THF-d_8) 183.2, 175.5,162.5, 156.8, 145.3, 138.9, 130.4, 128.3, 127.9, 127.4, 126.1, 120.7, 92.6, 55.8, 48.5, 41.5. LR-MS (ES) m/z 471.3 [M + H^+], calcd for $C_{23}H_{24}NO_5$: 470.1].

Fmoc-*N*-methyl-2-aminomethylphenyoxy Acetic Acid (1{*2*}**).** In a 100-mL round-bottom flask, 3 g (16.6 mmol) of 3-carboxybenzaldehyde was dissolved in 25 mL of methanol and cooled to 0°C. A 43-mL (50 mmol, 3 equiv) portion of a 40% aqueous solution of methylamine was added, followed by 311 mg (8.3 mmol, 0.5 equiv) of $NaBH_4$. After stirring for 3 h, the solution was evaporated to dryness using a rotary evaporator. The residue was taken up in 50 mL of a 20:20:10 mixture of methanol, ethyl acetate, and DIPEA, and the solution was evaporated to dryness. The resulting solid was dissolved in 15 mL of *N*,*N*-dimethylformamide (DMF) with 3 mL of water and 1.16 mL of DIPEA. To this solution was added 7.15 g (21.6 mmol, 1.25 equiv) of Fmoc-OSu. After 3 h, the solution was partitioned between ethyl acetate with 10% hexane (200 mL) and citric acid buffer (300 mL, 0.1 M, pH = 4.5). The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to yield a white solid. The crude product was purified by flash chromatography with 95:4:1 CH_2Cl_2/MeOH/HOAc. Acetic acid was removed from the purified compound by repeated evaporation from heptane, followed by recrystallization from hot hexanes/ethyl acetate to yield 4.1 g (9.79 mmol, 59%) of a white powder. ^{1}H NMR δ 2.82 (s, 3H), 4.23 (br t, 1H), 4.46 (d, J = 6.1 Hz), 4.59 (s, 2H), 4.62 (s, 2H), 6.82 (d, J = 7.3 Hz, 1H), 7.04 (m, 1H), 7.23−7.44 (m, 6H), 7.58 (br d, 2H), 7.78 (d, J = 7.2 Hz, 2H). ^{13}C NMR (DMSO-d_6) δ (two rotamers in 1:1 ratio) 170.8, 156.5, 156.3, 156.1 (2C), 144.6, 141.5 (2C), 128.9 (2C), 128.5, 128.3 (2C), 127.7, 120.8, 112.3, 67.3, 67.1, 47.6, 47.5, 47.4, 47.2, 35.5, 34.6. HR-MS ESI m/z [M + H^+] = calc. 418.1651, found 418.1649 (0.5 ppm).

Fmoc-*N*-methyl-3-aminomethylbenzoic Acid (1{*3*}). In a 50-mL round-bottom flask, 1 g (6.66 mmol) of 3-carboxybenzaldehyde was dissolved in 10 mL of methanol and cooled to 0°C. A 17.2-mL (20 mmol, 3 equiv) portion of a 40% aqueous solution of methylamine was added, followed by 126 mg (3.33 mmol, 0.5 equiv) of $NaBH_4$. After stirring for 3 h, the solution was evaporated to dryness using a rotary evaporator. The residue was taken up in 50 mL of a 20:20:10 mixture of methanol, ethyl acetate, and DIPEA, and the solution was evaporated to dryness. The resulting solid was dissolved in 15 mL of *N*,*N*-dimethylformamide (DMF) with 3 mL of water and 1.16 mL of DIPEA. To this solution was added 2.24 g (6.66 mmol, 1 equiv) of Fmoc-OSu. After 3 h, the solution was partitioned between ethyl acetate with 10% hexane (100 mL) and citric acid buffer (200 mL, 0.1 M, pH = 4.5). The organic layer was washed with brine, dried

over Na_2SO_4, and evaporated to yield a white solid. The crude product was recrystallized from hot hexanes/ethyl acetate to yield 1.6 g (4.1 mmol, 62%) of a white powder. ^{1}H NMR δ (two rotamers in 1:1 ratio) 8.06 (br m, 1H), 8.02 (s, 0.5 H), 7.91 (s, 0.5H), 7.95 (d, J = 6.5 Hz, 1H), 7.73 (d, J = 6.5 Hz, 1H), 7.65 (d, J = 6.5 Hz, 1H), 7.50 (br m, 2H), 7.43−7.35 (br m, 4H), 7.19 (br d, 1H), 4.59 (s, 2H), 4.54 (d, J = 6.5 Hz, 1H), 4.40 (s, 1H), 4.32 (br t, 0.5H), 4.26 (br t, 0.5H), 2.94 (s, 1.5 H), 2.90 (s, 1.5H). ^{13}C δ (two rotamers in 1:1 ratio) 171.8, 157.1, 144.2, 141.6, 138.3, 133.4, 132.7, 130.0, 129.6, 129.2, 129.1, 127.9, 127.3, 125.3, 125.0, 120.2, 67.9, 67.7, 52.6, 52.2, 47.6, 34.9, 34.0. HR-MS ESI m/z [M + H$^+$] = calc. 388.1549, found 388.1543 (−1.4 ppm).

Loading Beads with Fmoc-Hyp-OAll. 4-Methyoxyphenyltriisopropylsilane polystyrene resin (3.59 g, 400−450-μ diameter, 1.43 mmol/g, provided as a gift from John Tallarico and prepared according to ref 10) was dried overnight over P_2O_5 and then added to a dry 500-mL round-bottom flask. The beads were swelled in dry DCM for 30 min, and the excess DCM was removed using a slightly crimped 20-gauge cannula. A solution of CF_3SO_3H (5 g) in DCM (87 mL) was prepared and added to the beads via cannula, at which the beads became a deep red color. After 40 min, the solution was decanted via cannula and rinsed once with a minimum amount of dry DCM. A solution of Fmoc-Hyp-OAll (3.03 g, 1.1 equiv, dried by evaporation from toluene) and 2,6-lutidine (4.8 mL, 8 equiv) in 20 mL of dry DCM were added via cannula, and the beads were gently agitated on a shaker overnight at room temperature. The resin was treated with dry ethanol for 2 h to block any remaining silyl triflate, followed by exhaustive rinsing with DCM; DMF; DCM; and finally, with acetonitrile, then dried under vacuum. Fmoc quantitation by deprotection with piperidine (ϵ_{290} = 8300 M^{-1} cm^{-1}) provided a loading value for the resin of 0.49 mmol/g.

Library Synthesis. The beads were swelled in DCM for at least 20 min previous to all reactions. A 1.5-g portion of beads loaded with the Fmoc-Hyp-OAll linker (loading 0.49 mmol/g) was deprotected with 20% piperidine in DMF for 1 h, then rinsed extensively with DCM, DMF, then DCM. The beads were divided into five pools, four of which were then coupled with (5 equiv, 0.4 M) amino acids **1**{*1*−*4*} in the presence of HBTU (4 equiv, 0.3 M) and DIPEA (10 equiv, 0.8 M) in anhydrous DMF. The fifth pool was set aside as the null residue. Completion of the coupling reaction was checked by cleaving 3−7 beads from each sample and submitting the cleaved product to LC/MS analysis. The beads were drained and washed with DCM and DMF. The five pools of beads were then recombined to yield chemset **2**. The Fmoc group was deprotected (20% piperidine in DMF, 1 h), and the beads were washed (DCM/DMF) and divided into 21 pools for capping the free amine with either sulfonyl chlorides **3**{*1*−*12*} or carboxylic acids **4**{*13*−*21*}. Fifteen equivalents (0.53 M) of each sulfonyl chloride, pyridine, and DIPEA were dissolved in anhydrous 1:2 DCM/THF and agitated for 20 h. For the carboxylic acids, 25 equiv (0.87 M) of each carboxylic acid, 25 equiv (0.87 M) of HBTU, and 38 equiv (1.33 M) of DIPEA were mixed in anhydrous DMF and agitated with the beads overnight. The beads were then washed with DCM/DMF/DCM rinses and recombined to yield chemset **5**. The beads were added to a solution of 37:2:1 $CHCl_3$/AcOH/*N*-methylmorpholine (v/v/v) under argon for 30 min, and 4 equiv of $Pd(Ph_3P)_4$ was added to the solution, which remained agitating under Ar for 3 h. The beads were then rinsed with 0.5% DIPEA and 0.5% sodium diethyldithiocarbamate trihydrate in DMF, followed by a DCM/DMF/DCM rinse, yielding the deprotected carboxylic acid. The resin **7** was divided into 21 pools, and 25 equiv (0.8 M) of each amine (**6**{*1*−*14*}), 10 equiv (0.32 M) of PyBop, 15 equiv (0.48 M) of DIPEA, and 10 equiv (0.48 M) of HOAt were added to each pool in anhydrous DMF and allowed to react for 20 h under agitation. The beads were then washed and kept separated into these 14 pools to facilitate deconvolution by LC/MS.

Library Arraying and Compound Cleavage. The resin **7** from each of the 14 amine pools (**6**{*1*−*14*}) was arrayed into polypropylene (Genetix) 384-well plates using a home-made vacuum bead distribution manifold.[16] Cleavage from the resin was accomplished by adding a solution of 5% HF−pyridine in THF (via multichannel pipet) for 1 h, followed by addition of an equal amount of ethoxytrimethlysilane to quench the cleavage reaction. The solvent was evaporated by allowing the plates to stand in a fume hood for 2 days. Residues in each well were taken up into 30 μL of DMSO, and the plates were stored frozen at −20 °C until needed.

Microflow NMR. A 7-μL portion of the stock solution from plate 3, well F09, was diluted with 30 μL of CH_3CN and analyzed by LC/MS. The remaining ∼14 μL of the well was placed in a 0.5-mL polypropylene tube and evaporated in a vacuum desiccator over P_2O_5 overnight. The residue was dissolved in 15 μL of CD_3OD and injected into a Protasis/MRM MicroFlow NMR Probe. Spectra were run at 500 MHz on a Varian UNITY+500 spectrometer using a spectral width of 6515 Hz. Fifteen hundred transients were acquired at 1.3 s per transient. Transmitter presaturation of the water resonance was performed at 4.8 ppm.

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Supporting Information Available. LC/MS traces of individual library members and percent purity based on light scattering integration. NMR spectra of resynthesized 3-F09 and single-bead microflow NMR spectrum of same compound. High-temperature NMR spectrum of resynthesized 3-F09 in DMSO-d_6. This information available via the Internet at http://pubs.acs.org.

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