Discovery of a Novel Synthetic Phosphatase from a Bead-Bound Combinatorial Library

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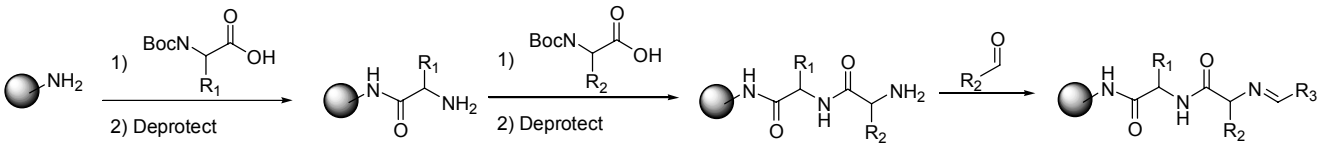
Experimental Section

General. All solid phase reactions were carried out in Biospin columns fitted with a frit and plastic stopcock from Bio-Rad. The reactions were stirred on a LabQuake rotator from Fisher Scientific. ^{1}H NMR spectra were recorded on a Bruker (400 MHz) spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard ($CDCl_3$: 7.24 ppm). Data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), and coupling constants (Hz). Microanalysis was performed by Robertson Microlit. Analytical gas-liquid chromatography (GLC) was performed on a Hewlett-Packard 6890 Series chromatograph using a μ-electron capture detector and a Hewlett-Packard 10091A-120 (Ultra 1) column with helium as the carrier gas.

All Boc-protected amino acids were purchased from Chem Impex, Synthetech, or Aldrich. Gadolinium nitrate was purchased as the hydrate from Fisher Scientific. The naphthol AS-MX phosphate, fast red and buffer solution were purchased as a kit from Pierce. The Tentagel-NH_2 resin was purchased from Bio-Rad. The photocleavable tags were made according to literature precedent.[1]

Library Synthesis.



Scheme 1

Representative procedure for library synthesis.

A. HATU Activated Coupling Reaction for the First Amino Acid Position. 262 mg of Tentagel-Macrobead resin (0.25 mmol/g) is placed in a Biospin fritted column with a stopcock and swollen in DMF for 20 minutes. The solvent is drained and diisopropylethylamine (57 μL, 5 eq), then a Boc-protected amino acid (2.5 eq) is added. Finally HATU (50 mg, 2 eq) is added and the mixture is rotated for 3 hrs. The solution is drained and the beads rinsed with a succession of DMF, methylene chloride, methanol, and DMF for 20 minutes. The coupling is repeated to ensure complete reaction of the polymer bead. Completion is checked by the Kaiser test.[2]

B. Boc Deprotection. The beads are washed with methylene chloride for 20 minutes. 4 mL of a solution of 9:1 trifluoroacetic acid (TFA) : methylene chloride is added to the beads and allowed to rotate for 20 minutes. The solution is drained and the beads quickly rinsed with methylene chloride. This step is repeated once more to ensure complete deprotection. The beads are then neutralized with a 20% solution of triethylamine in methylene chloride for 20 minutes. Finally, the beads are washed with DMF, methylene chloride, methanol and DMF for 20 minutes. The beads are then encoded for the monomer attached.[1] The resin is then pooled, ready for splitting.

C. Resin Splitting. The beads are placed in a 50 mL plastic tube and diluted with DMF to a total volume of 28 mL. Nitrogen gas is bubbled through the mixture both to ensure proper mixing of the beads and to create a heterogeneous "solution" of beads. The gas in bubbled through using a glass pipette with the tip placed 1 mm from the bottom of the tube. Using an Eppendorf Pipetteman, 1 mL portions are distributed to 14 Biospin columns. The bead solution is rediluted to 28 mL and the procedure repeated. The entire solution is then distributed to all the reaction vessels after the third dilution.

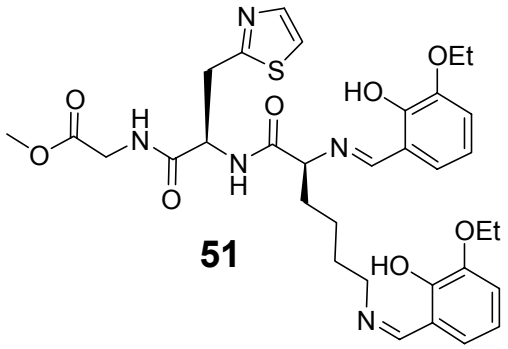
D. Schiff Base Formation. The beads are swollen in a 10:3 DMF: trimethylorthoformate solution and then 4 eq of the aldehyde are added. The beads are rotated for 4 hrs, then they are washed with DMF, methylene chloride and DMF for 20 minutes. The aldehyde is coupled a second time overnight to ensure complete reaction to Schiff base formation. The beads are washed again.

Assay Procedure

Into a Biospin column was added 260 mg (~5 copies of the library, 0.066 mmol active sites) library beads. The beads were rotated in THF for 20 minutes. A 0.1M solution of $Gd(NO_3)_3 \cdot 6H_2O$ in THF was then added to the column and allowed to complex for 4 hrs. The beads were then washed with THF and then buffer from the Pierce Fast Red TR/AS-MX kit. The beads were then rotated in fresh buffer for 1 hr to remove any uncomplexed gadolinium. Then 1.0 mL (0.013 mmol) of the naphthol AS-MX solution from the kit was added to the beads along with 1.5 mL of buffer solution. The vessel was rotated for 20 min and then the beads were washed twice for 5 min in clean buffer to remove any unreacted phosphate. The beads were then treated with a solution of 21 mg (0.013 mmol) Fast Red TR salt in 2.5 mL of buffer and rotated for 20 minutes. The beads were then washed with the buffer twice for 5 min each. The beads were then placed in a petri dish to spread them out and the colored beads were selected and decoded.

Decoding the bead. The selected beads were washed with DMF, methylene chloride and methanol, then rotated in DMF for 30 min. The beads were then washed twice in hexanes for 20 min each. Each bead was placed in a capillary tube with 4 μL pure decane and the capillary flame sealed. The capillary was then placed under a Blak-Ray long wave UV lamp for 1 hr. An aliquot of 1.5 μL of this solution was then mixed with 3 μL of bis(trimethylsilyl)trifluoroacetamide. After 5 min, 1 μL of this solution was injected on the GC and the resulting peaks were analyzed to identify the library member.

Confirmation of Library Hits



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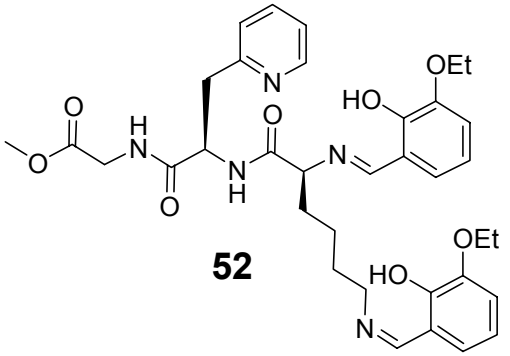

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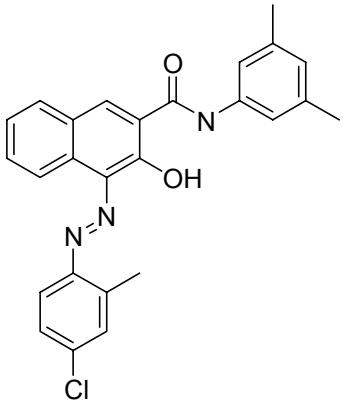
In order to confirm the two promising ligands identified from the library, they were synthesized wiht Wang resin preloaded with Fmoc-glycine, using the same procedure outlined above. Following literature precedent, the two ligands were cleaved to afford their adduct shown above, **51** and **52**.[3] For **51**, LR ES-MS ([M+H]) 667.9, [M+Na] 689.8, [M+K] 705.8. For **52**, ([M+H]) 661.9, [M+Na] 683.9, [M+K] 699.8.

Studies on the Importance of the Ligand Functionality

All the ligands tested were synthesized via the same procedure outlined above. For the ligands that differentiated between the side chain and backbone amino groups of the lysine monomer, distinct protecting groups were used such that one of the amino groups was protected during Schiff base formation and deprotected afterwards.

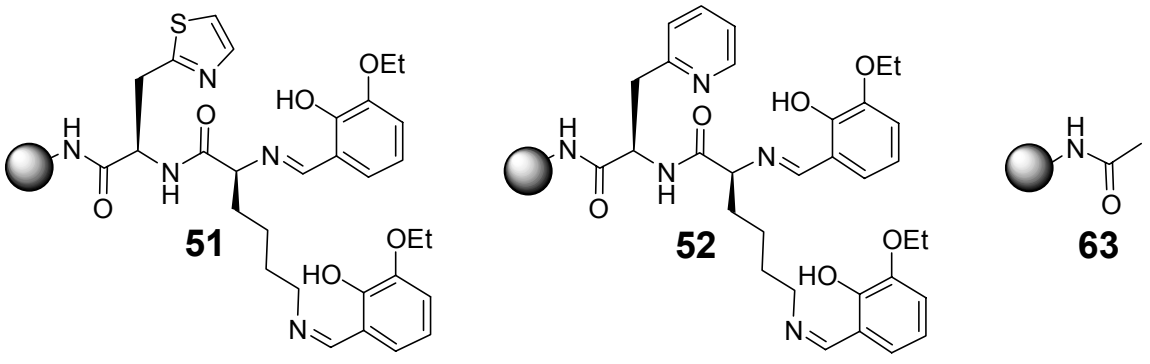
Acetate capped beads. The resin was placed in a Biospin column and swollen with methylene chloride. A mixture of 450:68:90 methylene chloride : acetic anhydride : triethylamine by volume was added to the vessel along with a small crystal of dimethylaminopyridine (DMAP). The vessel was rotated 4 hrs, washed with DMF, methylene chloride methanol and then DMF for 20 min. Completion was checked using the Kaiser test.

These ligands were assayed the same as described for the library.



4-(4-chloro-2-methyl)phenylazo-3-hydroxy-[2]naphthoic acid-3,5-dimethylanilide. In a 10 mL round bottom flask was added 100 mg (0.344 mmol) of naphthol AS-MX phosphate and 265 mg (1.03 mmol) of Fast Red TR salt in 2.5 mL of pyridine. The reaction was stirred overnight and the solvent removed under reduced pressure. The residue was purified via flash chromatography using 10% methanol in $CHCl_3$ as the eluent. ^{1}H NMR (400 MHz) δ 11.50 (1H, brs), 9.18 (1H, s), 8.53 (1 H, d, J=8.0 Hz), 8.23 (1H, d, J=8.0 Hz), 8.09 (1H, d, J=8.8), 7.83 (1H, d, J=7.2 Hz), 7.69 (1H, t, J=7.4 Hz), 7.40 (1H, dd, J=2.0, 8.4 Hz), 7.34 (1H, d, J=2.0 Hz), 7.12 (1H, brd, J=8.4 Hz), 7.10 (1H, brs), 2.56 (3H, s), 2.47 (3H, s), 2.37 (3H, s). HR-MS [M+H$^+$] 444.1455. Anal. Calc'd for $C_{26}H_{22}ClN_3O_2$: C 70.34; H 5.00; N 9.47 Found : C 70.43; H 4.98; N 9.37.

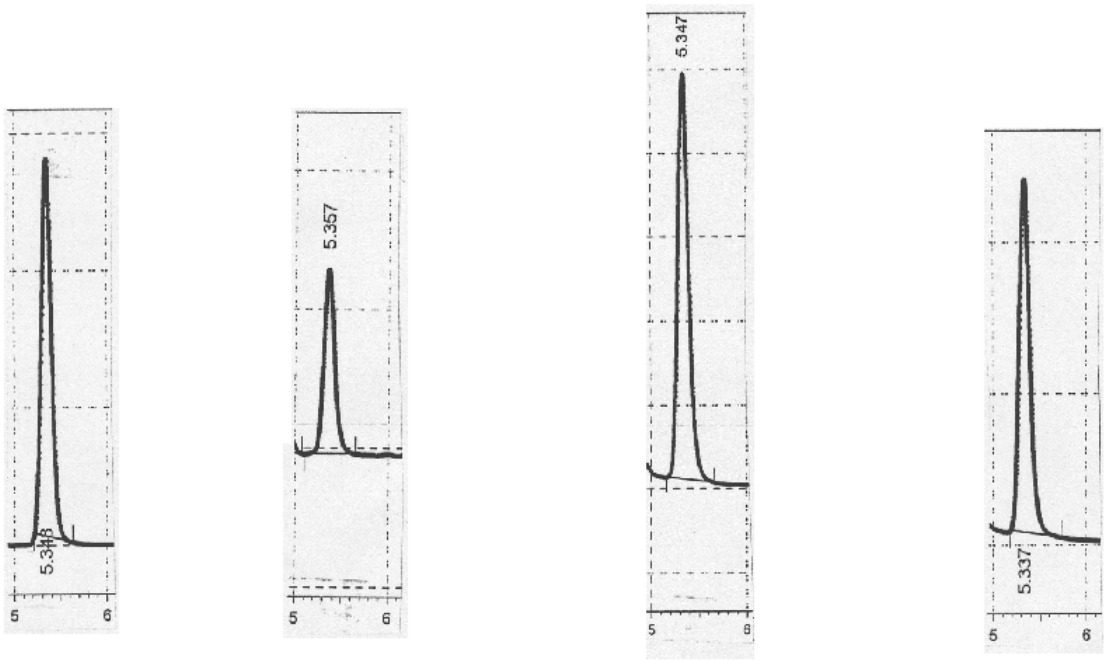
Kinetic Studies of the Identified Ligands.



10 mg (~0.002 mmol) each of **3**, **4**, and **5** are placed in separate reaction vessels. The beads are complexed with a 0.1M solution of $Gd(NO_3)_3 \cdot 6H_2O$ for 4 hrs. The beads are then washed with the

buffer for 1 hr. Next, 150 µL (0.002 mmol) of the Naphthol AS-MX solution is added. The vessels are rotated for 10 minutes and quickly washed twice with the buffer for 5 min and then treated with 3 mg of Fast Red TR salt in 1 mL of buffer for 20 minutes. The beads are then washed twice with the buffer and rotated in 0.25 mL of chloroform to remove the deposited adduct. The solution was then collected and the beads washed with another 0.5 mL $CHCl_3$. The solution was analyzed via HPLC using a LiChrospher column with a flow rate of 0.8 mL/min, eluting with 30% $CHCl_3$ in hexanes.

Amount of 50 released from catalytic reaction:



	Authentic **50**	**From 63**	**From 51**	**From 52**
Area	N/A	9182	92339	36996

[1]W.C. Still et al., *Proc. Natl. Acad. Sci*., USA 1993, 90, 10922.
[2] E. Kaiser et al., *Anal. Biochem.*, 1970, 34, 595.
[3] Snapper and Hoveyda et al., *J. Amer. Chem. Soc.*, 2000, 122, 2657.