Articles

Identification of Synthetic Phosphatidylserine Translocases from a Combinatorial Library Prepared by Directed Split-and-Pool Synthesis

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Simple sulfonamide and amide derivatives of tris(2-aminoethyl)amine (Tren) are known to promote the translocation or flip-flop of phosphatidylcholine, but not phosphatidylserine, across bilayer membranes. This paper describes the synthesis of a 300-member, spatially encoded library of Tren derivatives with appended peptide−sulfonamide and peptide−urea arms. The library was synthesized using the Encore method with SynPhase lanterns as the solid support. A high-throughput assay was developed to screen individual members of the library for an ability to translocate a fluorescent NBD derivative of phosphatidylserine across vesicle membranes. Several lead compounds were identified, and one was synthesized independently to confirm its high phosphatidylserine translocation activity.

Introduction

The distribution of phospholipids across mammalian plasma membranes is not symmetric. For example, the choline-containing polar lipids sphingomyelin (SM) and phosphatidylcholine (PC) are found primarily in the membrane outer monolayer, whereas the inner monolayer is enriched with the ammonium-containing phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) (Chart 1).[1] This transmembrane asymmetry is maintained by the concerted action of phospholipid translocase enzymes that vary in lipid specificity, energy requirements, and direction of translocation.[2] The asymmetry controls important cellular processes, such as signaling, fusion, blood coagulation, and apoptosis. In particular, the appearance of phosphatidylserine (PS) in the outer monolayer correlates with programmed cell death and subsequent cell clearance by phagocytosis. We are attempting to develop chemical methods that scramble the transmembrane distribution of PS and expose it on the cell surface. The main barrier to phospholipid translocation (or flip-flop) is passage of the polar phospholipid headgroups through the lipophilic interior of the membrane (Figure 1). Recently, our group has identified several synthetic low-molecular-weight organic compounds that can associate with the headgroups and facilitate membrane translocation. Our first-generation sulfonamide, **1**, enhances the inward translocation of zwitter-
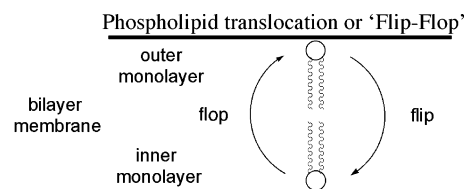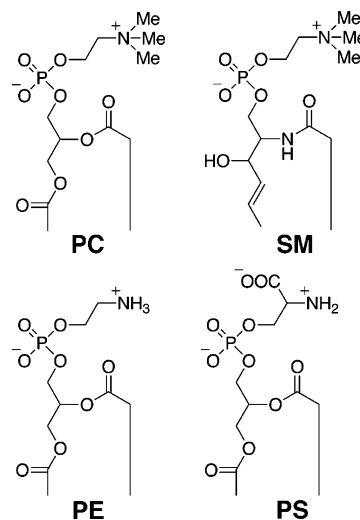

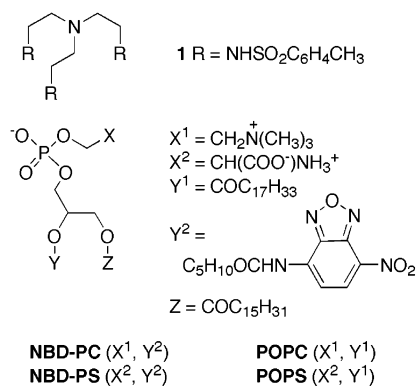**Figure 1.** Phospholipid translocation, or 'flip-flop'.

Chart 1

ionic phosphocholine (PC) but not anionic PS (Scheme 1).[3,4] The synthetic translocase works by forming a lipophilic, hydrogen-bonded complex with the polar PC headgroup, which allows subsequent PC diffusion through the lipophilic interior of the membrane. We have also described some

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Scheme 1

1 R = NHSO$_2$C$_6$H$_4$CH$_3$

X^1 = CH$_2$N$^+$(CH$_3$)$_3$
X^2 = CH(COO$^-$)NH$_3^+$
Y^1 = COC$_{17}$H$_{33}$

Y^2 =

C$_5$H$_{10}$OCHN — NO$_2$

Z = COC$_{15}$H$_{31}$

NBD-PC (X^1, Y^2)   POPC (X^1, Y^1)
NBD-PS (X^2, Y^2)   POPS (X^2, Y^1)

highly active second-generation translocases whose structures are based on a steroidal scaffold.[5] One cationic structure exhibited useful PS translocase activity, but surprisingly, some closely related structures were inactive.[5b] With the limited amount of data available, we concluded that the rational design of synthetic PS translocases was a considerable challenge.[6] A successful PS translocase appears to require a delicate balance of complementary structural shape and multitopic charge, as well as an appropriate amount of amphiphilicity.

To accelerate the acquisition of structure/activity data, we decided to synthesize and screen a large number of PS translocase candidates. This approach required the development of a high-throughput flip-flop assay as well as selection of a suitable method for library synthesis. We chose to employ a one-bead—one-compound method to produce the chemical library.[7] More specifically, we devised a directed split-and-pool synthesis of 300 compounds using pellicular solid supports. A significant advantage with this approach resides in the controlled distribution of compounds, the chemical history of each support being known, which is in contrast to the random split-and-pool approach in which the distribution of compounds is driven by statistical probabilities and the identity of the compounds is lost. Several solid-phase supports and handling technologies have been developed to accommodate split-and-pool library synthesis.[8–12] To briefly summarize, Houghten introduced resin beads in meshed polypropylene packets (T-bags),[8] and workers at IRORI developed meshed cans (MacroKan, MicroKan, and Nano-Kan for 100, 30, and 8 mg of resin, respectively) to allow robot handling.[9] Of relevance to this study are the versatile SynPhase lanterns that have been developed and commercialized by Mimotopes Pty. Ltd.[10,11] The directed split-and-pool method using lanterns as solid supports is well-suited to our purpose, because a moderate amount of sample (2–5 mg) is needed for the PS translocation assay. The library was prepared using the Encore method, a straightforward and inexpensive way to conduct directed split-and-pool synthesis.[13,14] Tracking the chemical history is based on a combination of three different encoding techniques: necklace coding is employed for the first combinatorial step, color-coding for the second step, and reaction vessel coding in the third combinatorial step. Consequently, this method is referred to as the Encore technique (*en*coding by a *n*ecklace, *co*lor, and *re*action vessel). The linear sequence
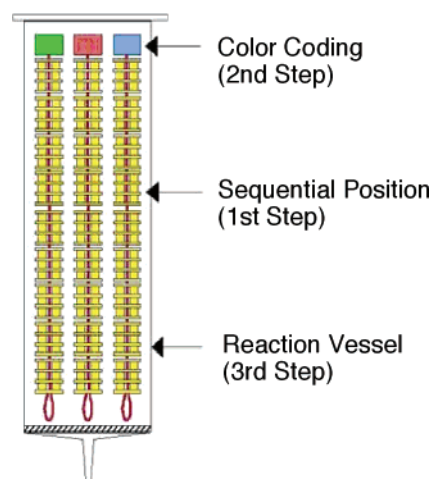
**Figure 2.** Coding of lanterns on a Lapis tool.

of lanterns is assembled on the Lapis tool (*la*ntern *pi*cking and *s*tringing tool), which allows the user to integrate the formation of linear sequences after the first combinatorial step. (Figure 2).

In addition to the synthesis and characterization of the chemical library, we describe the library screening using a high-throughput fluorescent assay to detect PS translocation ability. The screening uncovered a number of active lead structures, and one of them was synthesized by an independent solution-state method to confirm its high translocation activity.

Results & Discussion

Library Design and Synthesis. With compound **1** as the initial lead structure, we decided to use tris(2-aminoethyl)-amine (Tren) as a relatively simple three-armed scaffold that can be anchored to a solid phase, leaving two arms available for construction of a suitable hydrogen binding pocket. Although the acyclic Tren scaffold appears at first glance to be quite flexible, our previous X-ray and NMR studies have shown that its conformational freedom is actually quite restricted, and in particular, its amide derivatives have a significant degree of desirable preorganization.[4] As described in Scheme 2, our library design utilized 5 different L-amino acids (AA^1) in the first step, 10 L-amino acids (AA^2) in the second step, and 6 capping groups (Cap, three sulfonamides and three ureas) in the third combinatorial step. After attaching a 4-nitrophenyl carbonate methylphenoxybutyrate linker to aminomethylated SynPhase polystyrene lanterns, the conjugated lanterns **2** were reacted with 15 molar equiv of Tren in DMF to afford lantern-bound Tren **3** (Scheme 3). The Tren-functionalized lanterns, **3**, were then elaborated by standard Fmoc solid-phase peptide synthesis methods using DIC and HOBt as coupling agents. Finally, the double-stranded lanterns, **5**, were deprotected and capped with arylsulfonyl chlorides or aryl isocyanates to give sulfonamides (chemset **1**) and ureas (chemset **2**), respectively. In each case, the amino acid side chains were protected with acid-labile groups, which allowed the concomitant deprotection of the side chains and cleavage of target compounds from the lanterns with 50% TFA in DCM.

Scheme 2

AA^1 : Gly, Phe, Pro, Thr, Lys
AA^2: Gly, Phe, Pro, Thr, Lys, Ile, Asp, Ala, Leu, Glu

Cap :
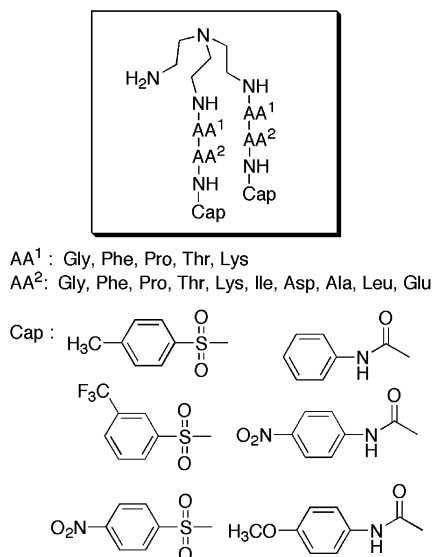
Library Characterization. The entire 300-member library was analyzed after cleavage from the lanterns by LC/MS. The sulfonamide-capped sublibrary **7** (chemset **1** with 150 members) showed the presence of all desired compounds with an average crude product purity of 72% (based on integration of HPLC traces at 235 nm). The gravimetric yields for cleaved sulfonamide products calculated from the initial loading ranged from 20 to 45% (see Supporting Information). In some cases, a minor impurity, **8**, arising from cross-linking of the Tren to the resin was also observed by LC/MS in 0−15% yield. Unexpectedly, treatment of **5**, after Fmoc removal, with arylisocyanates (chemset **2**) did not produce the corresponding urea version of **6**; instead,
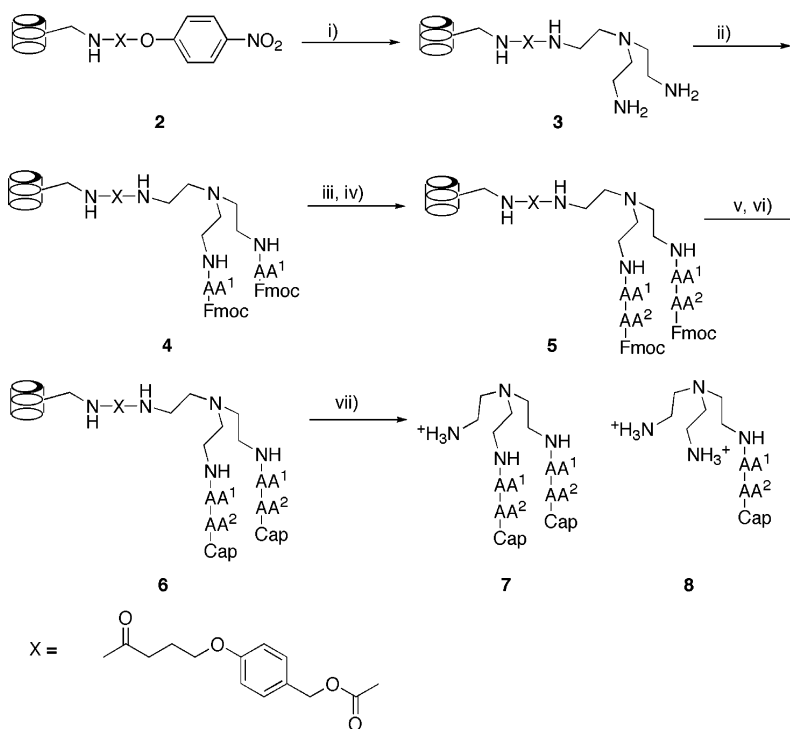
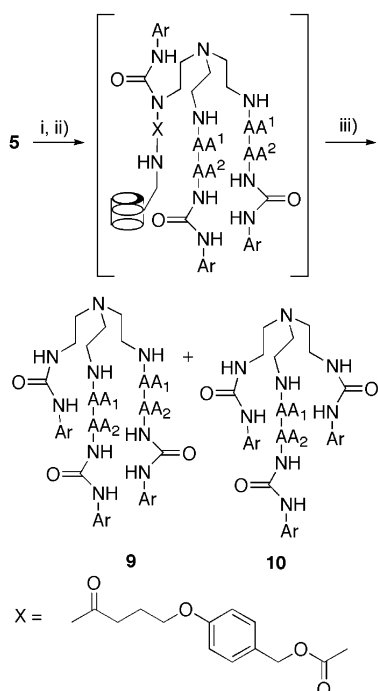overacylation of the anchoring nitrogen produced the tris−ureas, **9**, as the major product after cleavage from the lanterns (Scheme 4). The anchoring carbamate nitrogen is apparently less hindered and more nucleophilic than other amide nitrogens in the peptide backbone. To the best of our knowledge, there are no previous reports of *N*-acylurea formation by carbamate overacylation in solid-phase organic synthesis; however, overacylation of glycine nitrogens in peptide synthesis has been documented.[15] The average purity of sublibrary **9** in the crude product obtained after cleavage from the lanterns was 55%. The major contamination was byproduct, **10**, arising from cross-linking of Tren, which was observed in amounts up to 15%. One of the members of sublibrary **9**, with AA^1 = Gly and AA^2 = Phe, was synthesized independently by the pathway shown in Scheme 5. Mono-protected Tren derivative **11** was prepared using a previously reported procedure.[4] Reaction of **11** with Boc-Phe-Gly−OH, **12**, using EDC as the coupling reagent afforded **13** in 71% yield. Deprotection of **13** using TFA and subsequent reaction with 4-nitrophenylisocyanate gave the tris−urea **14**, which was shown by LC/MS to be identical to the corresponding member of sublibrary **9** (chemset **2**) prepared in Scheme 4.
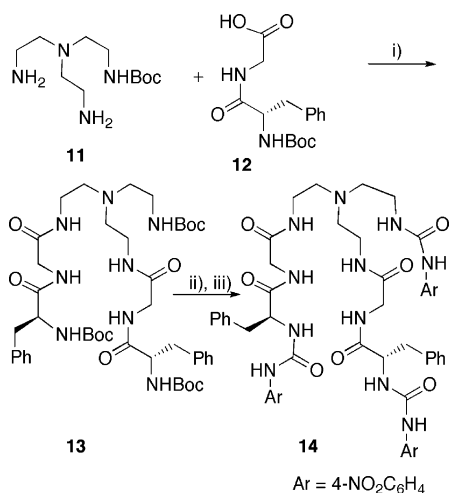
High-Throughput Screening. After cleavage from the lanterns, the crude, individual library compounds were air-dried, redissolved in methanol, and tested for translocation activity (no library replicates were prepared). The high-throughput screening for phospholipid translocation employed a modified NBD/dithionite quenching assay that uses as a probe phospholipid containing a fluorescent NBD group in one of its acyl chains.[3−6,16] Exo-labeled vesicles were prepared by addition of a small aliquot of NBD−PS (2 mol %

Scheme 3

(i) 15 equiv TREN; (ii) Fmoc-AA^1−OH, DIC, HOBt; (iii) piperidine/DMF (20:80); (iv) Fmoc-AA^2−OH, DIC, HOBt; (v) piperidine/DMF (20:80); (vi) Et$_3$N, arylsulfonyl chloride/arylisocyanate; (vii) 50% TFA in DCM.

Scheme 4

$$X =$$

(i) Piperidine/DMF (20:80); (ii) ArNCO; (iii) 30% TFA in DCM.

Scheme 5

Ar = 4-NO$_2$C$_6$H$_4$

(i) EDC, HOBt, CH$_3$CN, 71%; (ii) TFA; (iii) 4-nitrophenylisocyanate, 23%.

of total phospholipid) in ethanol to a suspension of unlabeled POPC/cholesterol (7:3) vesicles (phospholipid structures are shown in Scheme 1). The NBD–PS readily inserts into the vesicle outer monolayer. Upon treatment with sodium dithionite, the NBD fluorescence is quenched due to reduction of the NBD nitro group. Vesicle membranes are effectively impermeable to dithionite; therefore, only NBD–PS located in the outer leaflet is chemically quenched. The fraction of probe located in the outer monolayer can be determined from the drop in fluorescence intensity when a portion of the vesicles is subjected to dithionite quenching. The translocation measurements were conducted in 5 mM TES/100 mM NaCl buffer at pH 7.4 and 25 °C with 100-nm unilamellar vesicles prepared by membrane extrusion. The inward translocation of NBD–PS across vesicle membranes induced by each library member is shown in Figure 3.
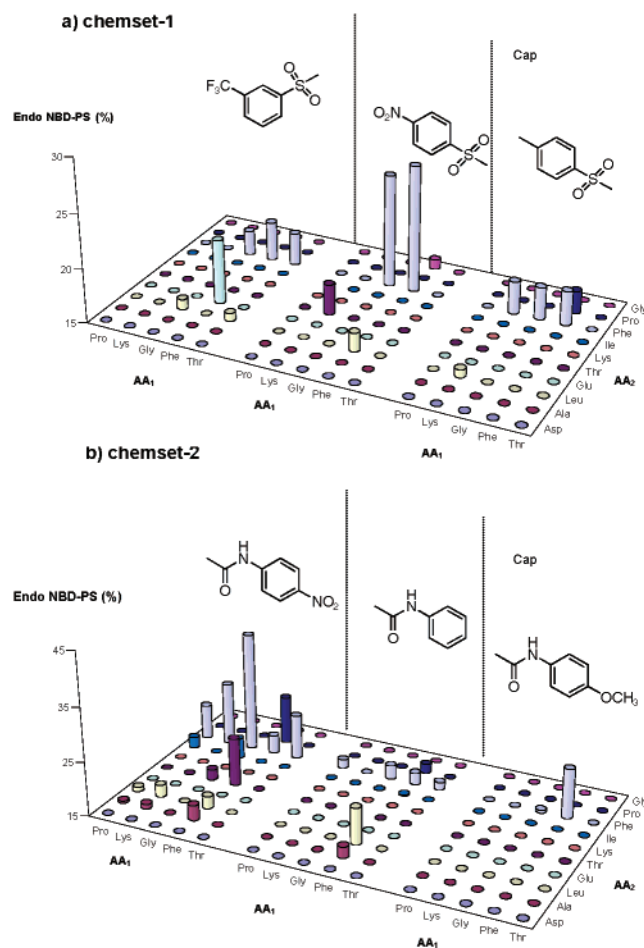

Figure 3. Inward translocation of 2 mol % NBD–PS across POPC/cholesterol (7:3) vesicles (25 μM) in the presence of 40 μM translocase candidates at pH 7.4 and 25 °C. Endo NBD–PS % is the amount of NBD–PS that was translocated into the vesicles at $t = 60$ min after addition of translocase candidate. (a) Chemset **1** describes the 150 sulfonamide versions of structure **7**; (b) chemset **2** describes the 150 versions of tris–urea **9**.

Inspection of the data in Figure 3 shows that compounds containing glycine as AA^1, and L-phenylalanine as AA^2 were generally active as PS translocases. A few selected active compounds were resynthesized in order to independently confirm the activity. Within chemset **1**, three library members were resynthesized in solution, namely, **15** [(Tos-Phe-Gly)$_2$Tren], **16** [(Nosyl-Phe-Gly)$_2$Tren], and **17** [(Tos-Gly-Phe)$_2$Tren]. After purification, the three compounds were evaluated for PS-translocation activity, and as shown in Figure 4, the order of PS translocation abilities was about the same as that observed in the screen, that is, **16** > **15** > **17**. In the case of chemset **2**, the crude sample of tris–urea derivative **14**, obtained after cleavage from the lantern, exhibited the highest PS-translocation activity (42% of the NBD–PS was translocated into the vesicles). Unfortunately, a pure sample of **14**, prepared by independent synthesis, was found to be insoluble in water, which prevented a reliable assessment of translocation ability. Nonetheless, the core Tren–urea structure was considered a useful lead, and derivatives with improved solubilities were prepared in a subsequent structure/activity study and shown to have very high PS-translocation activity.[17]
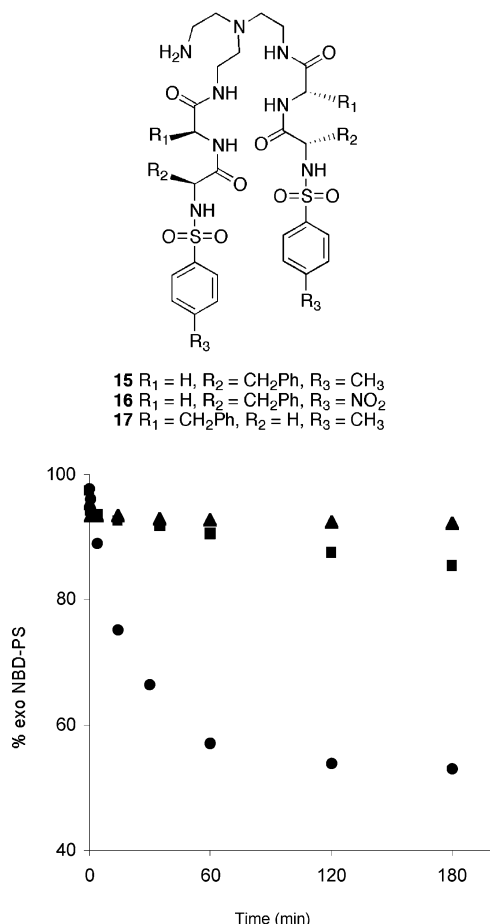
15 R₁ = H, R₂ = CH₂Ph, R₃ = CH₃
16 R₁ = H, R₂ = CH₂Ph, R₃ = NO₂
17 R₁ = CH₂Ph, R₂ = H, R₃ = CH₃

Figure 4. Inward translocation of NBD−PS (2 mol %) across POPC/cholesterol (7:3) vesicles (25 μM) treated with 37.5 μM **15** (■), **16** (●), and **17** (▲). The system progresses from an initial value of 100% exo NBD−PS to an equilibrium value of around 55% exo NBD−PS.

In summary, we have employed the Encore method with SynPhase lanterns as the solid support to successfully construct a spatially encoded, 300-member combinatorial library of PS translocase candidates. The Encore method is a relatively cheap and straightforward way of conducting manual, directed split-and-pool synthesis of chemical libraries with fewer than 1000 members. Several lead compounds were identified, and one in particular (**16**) was independently resynthesized, purified, and shown to possess impressive PS translocation activity (**16** increases the rate of PS translocation >100 times over background). Mechanistic studies are ongoing but the precedence of our previous work strongly suggests that these synthetic PS scramblases work by forming hydrogen-bonded, charge-neutral complexes with the PS headgroup.[3−6] Studies are also underway to evaluate if synthetic PS translocases have desirable biological activities. In any event, this study illustrates how combinatorial chemistry can be used as a method to rapidly generate a large amount of structure/activity data that can be subsequently used to design and construct next-generation compounds with improved properties.[17] The structures of lead compounds (e.g., **14** and **16**) appear to be quite flexible, and it is not obvious that they would have been discovered by rational design methods.

Experimental Section

The following abbreviations are used: DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; HOBT, 1-hydroxybenzotriazole; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TFA, trifluoroacetic acid; TREN, tris(2-aminoethyl)amine; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

Materials and Methods. All solvents and DIC were purchased from Aldrich and were used without further purification. Protected amino acids, *p*-nitrophenyl carbonate Wang resin, and HOBt were obtained from Novabiochem. PS-AMM lanterns were obtained from Mimotopes.[11] The Encore synthesizer was obtained from Torviq.[14]

1. Standard Fmoc-Deprotection Protocol. Fmoc-deprotection steps were carried out by immersing lanterns in a mixture of DMF and piperidine (80:20) for 4−6 h.

2. Standard Washing Protocol. Washing steps after coupling or deprotection were performed by dipping the lanterns in DMF (3 × 10 min), DMF/DCM (1:1) (2 × 10 min), and DCM (2 × 10 min). The lanterns were allowed to air-dry for 10 min.

Synthesis of the library. The library was synthesized using 5 and 10 amino acids in the first and the second combinatorial steps, respectively, and 6 capping groups in the third combinatorial step, totaling 300 compounds.

1. Combinatorial Step 1. Five DMF solutions (50 mL) containing each of the different amino acids (Fmoc-AA¹−OH, Scheme 2) (4.1 mmol), HOBt (4.1 mmol), and DIC (4.1 mmol) were prepared. Sixty lanterns were immersed in each of the coupling solutions and allowed to react overnight at room temperature. The reaction mixture was decanted, and the lanterns were washed and deprotected following the standard washing and deprotection protocol.

2. Stringing Lanterns on Lapis Tools. Sixty lanterns from one reaction vessel were arranged into an array of 6 rows and 10 columns using the arraying tool and were transferred into the shafts of the magazine.[14] This procedure was repeated for the lanterns from all reaction vessels, thus creating linear sequences of five lanterns in each shaft. Lanterns were then stringed on 60 color-coded Lapis tools. Ten different colors were used, six Lapis tools for each color. Lapis tools tagged by the same color were placed into one reaction vessel for the next combinatorial step.

3. Combinatorial Step 2. Ten DMF/DCM, 1:1 (40 mL) solutions, each containing one of the Fmoc-protected amino acids (Fmoc-AA²-OH, Scheme 3, 2.97 mmol), HOBt (2.97 mmol), DIC (2.97 mol) were prepared, and six Lapis tools (each containing five different lanterns) coded with same color were allowed to react in each solution overnight at room temperature. Standard washing and deprotection protocol was followed.

4. Reshuffling of Lapis Tools. Ten Lapis tools with different color tags were pooled into each of six reaction vessels for the third combinatorial step.

5. Combinatorial Step 3. Three solutions of arylsulfonyl chlorides (4.1 mmol) and Et₃N (4.1 mmol) in DMF/DCM, 1:1 (50 mL), and three arylisocyanates (4.1 mmol, Scheme-3) in DMF/DCM, 1:1 (50 mL), were prepared and reacted with lanterns for 8 and 12 h, respectively. The reaction

solution was decanted, and the lanterns were washed consecutively with DMF (3 × 10 min), DMF/DCM, 1:1 (3 × 10 min), and DCM (2 × 10 min) and allowed to air-dry.

6. Dispensing of Lanterns for Cleavage. Lapis tools with lanterns were placed into the shafts of the dispensing tool, with 10 Lapis tools from one reaction vessel into one row. The Lapis tools were removed, and the lanterns were dispensed into five 96-well plates, 60 lanterns per plate.

7. Cleavage. The lanterns were treated with 2 mL of 50% TFA in DCM for 2 h. The cleavage solution was allowed to evaporate, and the released material was dissolved in methanol.

Analysis of Library Members; LC/MS Analyses. After cleavage from the lanterns, all 300 library members were prepared as methanol samples. The LC/MS system consisted of a Waters Alliance 2690 HPLC coupled to a Micromass Platform II spectrometer (electrospray ionization mode, ESI$^+$). All analyses were carried out using an XTerra MS C$_{18}$, 3.5-μm, 2.1 × 30 mm reversed-phase column (Waters, www.waters.com), with integration of the LC traces at 235 nm. A flow rate of 750 μL/min and a gradient of 5−95% B over 7 min was used (eluent A, water/1.0% formic acid; eluent B, acetonitrile). Positive ion electrospray mass spectra were acquired at a solvent flow rate of 350 μL/min using a splitter. Nitrogen was used as both the nebulizing gas and the drying gas.

Synthesis of Individual Compounds; Synthesis of 13. Boc-protected Tren, **11**[4] (0.308 g, 1.25 mmol), Boc-Phe-Gly−OH, **12** (0.806 g, 2.5 mmol), DIC (0.315 g, 2.5 mmol), and HOBt (0.34 g, 2.5 mmol) in 50 mL of DMF were allowed to stir for 12 h at room temperature. Removal of solvent and aqueous workup gave crude **13**, which was chromatographed on silica using DCM/MeOH (95:5) as eluent. Yield 0.600 g (71%); ^{1}H NMR (300 MHz, CD$_3$OD) δ 7.22 (m, 10H), 4.4 (m, 2H), 4.15 (m, 2H), 3.60 (m, 2H), 3.42 (m, 2H), 3.1 (m, 8H), 2.45 (m, 6H), 1.38 (s, 27H); FAB-MS m/z 855 [M + H]$^+$.

Synthesis of 14. Compound **13** was reacted with 30% TFA in DCM (30 mL), and TLC showed consumption of starting material after 2 h. After removal of solvents, the crude residue (0.450 g, 91%, 0.8 mmol) was dissolved in chloroform, and 4-nitrophenylisocyanate (0.400 g, 2.43 mmol) was added at ice-bath temperature. The reaction mixture was warmed to room temperature and allowed to stir for 20 h. Removal of the solvent and chromatography (SiO$_2$, DCM/MeOH, 90:10) gave compound **14** (0.200 g, 23%). ^{1}H NMR (300 MHz, CD$_3$OD) δ 8.08 (d, 4H, J = 9.0 Hz), 8.05 (d, 2H, J = 9.0 Hz), 7.50 (m, 6H), 7.28 (m, 10H), 4.54 (m, 2H), 3.88 (d, 4H, J = 3.0 Hz), 3.15−3.26 (m, 6H), 3.00−3.05 (m, 2H), 2.56 (m, 6H); ^{13}C NMR (75 MHz, CD$_3$OD) δ 175.3, 171.7, 157.2, 156.8, 147.9, 147.3, 143.4, 142.9, 138.0, 132.5, 130.7, 129.8, 128.2, 126.0, 118.7, 118.5, 57.1, 55.0, 54.9, 44.0, 39.1, 39.0 (some signals obscured by solvent); ESI-MS m/z 1048 [M + H]$^+$.

Synthesis of 15. 4-Nitrophenyl carbonate Wang resin (0.70 g, loading 0.69 mmol/g) was placed in a peptide synthesis vessel and allowed to stand for 30 min in 2 mL of DMF. The DMF was drained, and the resin was treated with a solution of Tren (0.30 g, 2.05 mmol) in DMF (3 mL) for 8 h. A typical washing protocol was DMF (2 mL × 3) for 5 min, DMF/DCM (2 mL × 3) for 5 min, and DMF (2 mL × 2) for 5 min. After washing the reaction with a freshly prepared solution of Fmoc-Gly−OH (0.071 g, 0.24 mmol), DIC (0.030 g, 0.24 mmol), and HOBt (0.032 g, 0.24 mmol) in DMF (3 mL) was performed for 10 h. Washing and deprotection with 2 mL of 20% piperidine in DMF for 2 h was carried out. A 3-mL solution of Fmoc-Phe−OH (0.093 g, 0.24 mmol), DIC (0.030 g, 0.24 mmol), and HOBt (0.032 g, 0.24 mmol) in DMF/DCM (2:1) was added to the resin and allowed to react overnight. The washing and deprotection steps were carried out as described above. The dipeptide resin was treated with a solution of p-toluenesufonyl chloride (0.046 g, 0.24 mmol) in the presence of triethylamine (0.024 g, 0.024 mmol) in DCM (3 mL) for 10 h. After washing with DMF (3 mL × 3); DMF/DCM, 1:1 (3 mL × 3); and DCM (3 mL × 3), cleavage from the support was achieved using 50% TFA in DCM (3 mL) for 4 h. The residue was chromatographed on silica gel using DCM/methanol (90:10) as eluent to afford a colorless oil (0.025 g, 60.9%): ^{1}H NMR (300 MHz, CD$_3$OD) δ 7.47 (d, 4H, J = 9.0 Hz), 7.18 (d, 4H, J = 9.0 Hz), 7.13 (m, 6H), 7.01 (m, 4H), 3.85 (m, 4H), 3.44 (m, 2H), 3.35 (m, 2H), 3.20 (m, 2H), 2.99 (m, 4H), 2.67 (m, 6H), 2.38 (s, 6H); ^{13}C NMR (75 MHz, CD$_3$-OD) δ 174.7, 171.9, 145.1, 139.0, 137.7, 130.8, 130.3, 129.6, 128.1, 127.9, 60.1, 55.3, 52.8, 48.6, 44.2, 39.3, 39.0, 21.4; HRMS (FAB$^+$) m/z 863.3554 found, 863.3584 calcd for C$_{42}$H$_{54}$N$_8$O$_8$S$_2$.

Synthesis of 16. Compound **16** was synthesized using p-nitrophenyl carbonate Wang resin following the procedure described for **15**, except that in the last step, 4-nitrobezene-sulfonyl chloride was used (0.053 g, 0.024 mmol): ^{1}H NMR (300 MHz, CD$_3$OD) δ 8.16 (d, 4H, J = 9.0 Hz), 7.78 (d, 4H, J = 9.0 Hz), 7.09 (m, 10H), 4.05 (m, 2H), 3.97 (ABq, 4H), 3.37 (m, 6H), 3.06 (m, 4H), 2.77 (m, 6H); ^{13}C NMR (75 MHz, CD$_3$OD) δ 174.5, 171.7, 151.3, 146.9, 137.8, 130.4, 129.5, 129.2, 127.8, 125.3, 60.3, 55.4, 52.6, 49.9, 44.0, 39.1, 38.8; HRMS (FAB$^+$) m/z 926.3082 found, 926.3051 calcd for C$_{40}$H$_{49}$N$_{10}$O$_{12}$S$_2$.

Vesicle Preparation. Lipids dissolved in chloroform (POPC/cholesterol, 7:3) were rotary-evaporated and dried under vacuum for at least 1 h. Hydration was performed at room temperature with an appropriate amount of 5 mM TES and 100 mM NaCl, pH 7.4 (TES buffer). Multilamellar vesicles (25-μM) were generated using a Vortex mixer; use of a Pyrex glass bead ensured complete lipid removal from the flask wall. The multilamellar vesicles were extruded to form large unilamellar vesicles with a hand-held Basic LiposoFast device. The vesicles were extruded 29 times through a 19-mm polycarbonate Nucleopore filter with 100-nm-diameter pores.

Inward NBD-PS Translocation Assay. The inward NBD-lipid translocation experiments were performed with some minor modifications to the procedure described previously.[3−6,16] The modifications were made to allow rapid analysis of samples in a 96-well plate. Exo-labeled vesicles were generated upon addition of an ethanolic solution of NBD−PS to a 20-mL solution of unlabeled vesicles (2 mol % of

NBD−PS in 25 μM unlabeled lipid) at room temperature. The vesicle solution was stirred gently for 5 min to ensure complete incorporation of the added NBD−PS in the outer monolayer of the vesicle. Excitation was set at 470 nm, and fluorescence emission was measured at 530 nm using a 515-nm filter. The POPC/cholesterol (7:3) vesicles (25 μM) in TES buffer vesicles were pipetted into a 96-well microtiter plate (black, polystyrene) with 200 μL/well. After addition of 10 μL/well of translocase solution (0.2 mM in methanol, 10 μM final concentration in each well), the plate was incubated on a shaker at 25 °C for 1 h. Average fluorescence (F_i) for 30 acquisitions was determined with a microtiter plate fluorescence reader (Molecular Devices Spectra Max Gemini XS). To each well was added 12 μL of dithionite solution in 1 M Tris buffer, at pH 10. After incubation at 25 °C for 1 min, the fluorescence emission from each well was read again (F_f). The amount of probe located in the outer monolayer was calculated according to the following equation

$$\% \text{ endo probe} = 100 - [(F_i - F_f)/F_i] \times 100$$

The assay for each library member was conducted three times and averaged.

Supporting Information Available. Table of yields and purities of crude products after cleavage from the lanterns, selected spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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