Supporting Online Material

Abbreviations

AMA (1:1 aqueous methylamine (40% in water):aqueous ammonium hydroxide (30% in water))
BOC (*t*-butyloxycarbonyl)
BSOCOES (*bis*[2-(succinimidooxycarbonyloxy)ethyl]sulfone)
CAPS (3-(cyclohexylamino)-1-propanesulfonic acid)
CPG (controlled-pore glass)
DCM (dichloromethane)
DIPEA (diisopropylethylamine)
DMF (dimethylformamide)
DTT (dithiothreitol)
EDC (1-[3[(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride)
EDTA (ethylenediamine tetraacetic acid)
FMOC (9-fluorenylmethyloxycarbonyl)
HBTU (*O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate)
HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HOBt (*N*-hydroxylbenzotriazole)
HPA (3-hydroxypicolinic acid)
MES (*N*-morpholinoethane sulfonic acid)
NHS (N-hydroxysuccinimide)
PBS (phosphate-buffered saline)
SIA (*N*-succinimidyl iodoacetate)
sNHS (N-hydroxysulfosuccinimide)
TBE (tris-borate-EDTA)
TEAA (triethylammonium acetate)
THAP (2,4,6-trihydroxyacetophenone)

General Experimental Methods

DNA oligonucleotides were synthesized using standard automated solid-phase phosphoramidite coupling methods. The following modified phosphoramidites and CPG beads (all from Glen Research) were used to install chemical functionality: 5'-Amino-Modifier-5 for 5' amino-functionalized oligonucleotides; 3'-Amino-Modifier C7 CPG for 3' amino-functionalized oligonucleotides; 3'-Thiol-Modifier C3 S-S CPG for 3' thiol-terminated oligonucleotides; 5'-Biotin Phosphoramidite for 5' biotinylated oligonucleotides; and BiotinTEG CPG for 3' biotinylated oligonucleotides. Oligonucleotide concentrations were determined by densitometry of SYBR green-stained DNA and also by UV spectroscopy. Unless otherwise specified, all reactions were carried out at 25 °C. Oligonucleotides were purified by reverse-phase HPLC using a C18 stationary phase and an acetonitrile/100 mM aqueous triethylammonium acetate gradient. Functionalized DNA-linked reagents and templates were characterized by HPLC and by MALDI-TOF mass spectrometry using a hydroxypicolinic acid/ammonium citrate matrix (error for oligonucleotide-linked species estimated at 0.2%)

Template and Reagent Syntheses (Compounds 1-4)

*Synthesis of Templates (**1**) (for R = tryptamine)*

Sixty-four of the 65 templates within the 65-membered template library were prepared by split-pool oligonucleotide synthesis. The common 3' PCR primer binding sequences and four step 3 codons were synthesized separately and the CPG beads linked to these partial template oligonucleotides was pooled. The mixed beads were divided equally among four new syntheses of the step 2 codons. The beads were pooled again and divided before the synthesis of the four step 1 codons, common 5' PCR primer binding sites, and coupling to the 5' amino modifier (see above). The lysine derivative was installed at the 5' amino terminus of the pooled fully protected, CPG-linked modified oligonucleotides by addition of a solution containing Fmoc-Lys(MMT)-OH (50 μmol, Novabiochem), HOBt, (50 μmol), HBTU (45 μmol), DIPEA (115 μmol) in 1.2 mL dry DMF and agitation for 10 h. Unreacted amine was capped with a

mixture of acetic anhydride/THF/1-methylimidizole/pyridine. The beads were washed with DMF and acetonitrile. The MMT group was removed by washing with 3% trichloroacetic acid in DCM until the supernatant was colorless. A separate solution of tryptamine (200 μmol) and diacetyl tartaric anhydride (200 μmol) in 600 μL dry DMF was agitated for 1 h. The reaction was combined with HBTU (180 μmol), HOBt (200 μmol), DIPEA (460 μmol), and the resulting solution was added to the CPG beads. The resulting brown solution was agitated 2.5 h, washed, and dried under reduced pressure. Treatment for 1 h with AMA at 55 °C fully deprotected the oligonucleotide and liberated the 5' modified templates from the support. Templates **1a**-**1e** were prepared individually in an analogous manner. To complete the preparation of the 65-membered template library, the 64-membered template library was combined with **1e** such that each template in the resulting mixture was approximately equimolar.

*Synthesis of Templates (**1**) (for R = methylamine)*

The NHS ester of (-) O,O'-dibenzoyl-(L)-tartaric acid mono(dimethylamide) was prepared by combining (-) O,O'-dibenzoyl-(L)-tartaric acid mono(dimethylamide) (1 mmol, Fluka) with EDC (1.5 mmol) and NHS (1.1 mmol) in 10 mL DCM. The mixture was stirred under nitrogen for 3 h, diluted with 15 mL DCM, extracted with saturated aqueous $NaHCO_3$, saturated aqueous $NaHSO_4$, and saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to yield 289 mg of crude white crystalline product (58% yield). The lysine derivative was installed onto fully protected, CPG-linked DNA containing the 5' amino modifier by addition of a solution of Fmoc-Lys(MMT)-OH (50 μmol), HOBt, (50 μmol), HBTU (45 μmol), and DIPEA (115 μmol) in 1.2 mL dry DMF and agitation for 10 h. Unreacted amine was capped with a mixture of acetic anhydride/THF/1-methylimidizole/pyridine. The FMOC group was removed from the lysine α-amine using three consecutive washes with 20% piperidine/80% DMF (5 min agitation per wash). A 4-pentenoyl group was installed onto the lysine α-amine by treatment with a solution of 4-pentenoic anhydride (50 μmol), HOBt, (50 μmol), HBTU (45 μmol), DIPEA (115 μmol) in 1.2 mL dry DMF under agitation for 4 h. The beads were washed with DMF and acetonitrile. The lysine ε-amine MMT group was removed by washing with 3% trichloroacetic acid in DCM until the supernatant was colorless. Treatment for 1 h with AMA at 55 ºC fully deprotected the oligonucleotides and liberated the 5' modified templates from the support, while simultaneously converting the dimethylamide to the methylamide. After drying *in vacuo*, the deprotected templates were desalted by gel filtration (NAP-10 column, Pharmacia) and ethanol precipitation. The resulting templates were resuspended in 175 μL of 140 mM HEPES (pH 8.5), and reacted with 25 μL of O,O'-dibenzoyl-(L)-tartaric acid mono(dimethylamide) NHS ester solution in dry DMF (70 mM final concentration of NHS ester) under agitation for 4 h. The mixture was purified by gel filtration and reverse phase HPLC, lyophilized, ethanol precipitated, and incubated at 55 ºC in AMA for 1 h to remove the benzoyl groups. The debenzoylated templates were dried *in vacuo,* resuspended in 9:1 water:oxidizing solution (20 mM I_2, pyridine, THF, water, Glen Research) to remove the 4-pentenoyl group of the lysine α-amine, and purified via reverse-phase HPLC.

*Synthesis of Reagents **2** and **3***

In a typical reaction, the appropriate 3' amino-terminated oligonucleotide (Fig. 3A) was dissolved in 150 μL of 750 mM aqueous HEPES, pH 8.5, then added rapidly and with sonication to a mixture of amino acid (15 μmol), DMF (30 μL), and BSOCOES (10 μmol). The resulting mixture was allowed to react an additional 40 min with mixing, after which it was desalted by gel filtration (NAP-5 column, Pharmacia) and purified by reverse phase HPLC (see above). Yields of functionalized oligonucleotide reagents were typically 50-80% depending on the identity of the amino acid.

*Synthesis of Reagents **4***

In a typical reaction, 25 μL of 1 M amino acid in 1 M phosphate buffer (pH 7.2) was combined with 5 μL of 200 mg/mL SIA in DMF and agitated for 10 min. The 3'-4-(diphenylphosphanyl)benzoic acid-amide linked oligonucleotide (synthesis described previously in Ref. 5) dissolved in 50-100 μL water was added and the resulting mixture was agitated for 1 h. The resulting mixture was desalted by gel filtration and purified by reverse phase HPLC. Yields of functionalized oligonucleotide reagents were typically between 50-80%, with the phosphine oxide being the predominant side product (removed during HPLC).

*Synthesis of the Phenylsulfonamide Amino Acid Used to Prepare Reagent **2e***
(L)-Z-Lys-OBz (1 mmol, Bachem, phenyl sulfonate salt with ε-ammonium group) was combined with N-Boc-protected (L)-phenyl glycine (1.1 mmol, Novabiochem), EDC (1.1 mmol), HOBt, (1.1 mmol), and DIPEA (2.2 mmol) in 20 mL DCM and stirred under nitrogen for 12 h. The solution was extracted three times with saturated aqueous $NaHSO_4$, three times with saturated aqueous Na_2CO_3, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to yield 489 mg of the crude N-Boc-protected dipeptide (80% crude yield). The crude product was characterized by electrospray mass spectrometry (theoretical mass = 603.7, observed mass = 603.6±0.6). The Boc group was removed by dissolving the crude dipeptide (0.77 mmol) in 10 mL neat TFA and stirring for 20 m. The reaction was quenched with saturated $NaHCO_3$ and extracted three times with DCM; the pooled organic fractions were dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to yield 403 mg of crude dipeptide product containing a free amino terminus (electrospray mass spectrometric analysis: theoretical mass = 503.6; observed mass = 503.4±0.5). The dipeptide (389 mg, 0.77 mmol) was dissolved in 25 mL DCM and combined with 4-carboxybenzenesulfonamide (0.85 mmol), EDC (0.85 mmol), HOBt (0.85 mmol), and DIPEA (1.7 mmol). The solution was stirred under nitrogen for 12 h, diluted with 25 mL DCM, extracted twice with saturated aqueous $NaHCO_3$ and once with saturated aqueous Na_2HSO_4, filtered, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude material was purified by silica gel flash chromatography using 95% DCM/5% MeOH and concentrated *in vacuo* to yield 202 mg of purified dipeptide-linked sulfonamide (38% yield for two steps). ES-MS: theoretical = 686.2, found = 686.6±0.7. The dipeptide-linked sulfonamide (202 mg, 0.29 mmol) was dissolved in 25 mL of 4.4% formic acid in methanol and added to 100 mg of Pd^0 (finely divided black powder) to effect benzyl ester and benzyl carbamate deprotection. The reaction was stirred 2 h, filtered, and concentrated *in vacuo* to yield 89 mg (66% yield) of the amino acid portion of **2e** (Fig. 3A). Electrospray mass spectrometric analysis: theoretical mass = 462.5; observed mass = 462.3±0.4. The preparation of **2e** using this material proceeded as described above.

DNA-Templated Syntheses

General Step 1, Step 2, and Step 3 DTS Reaction Conditions
Step 1 conditions: Templates were added to a final concentration of 60 nM each and reagents (**2**) were added to a final concentration of 90 nM each to a solution containing 0.1 M MES (pH 6.0), 1 M NaCl, 20 mM EDC, and 15 mM sNHS and reacted 4 h at 25 ºC.

Step 2 conditions: Templates were added to a final concentration of 60 nM each and reagents (**3**) were added to a final concentration of 90 nM each to a solution containing 0.1 M MES (pH 6.0), 1 M NaCl, 20 mM EDC, and 15 mM sNHS and reacted 4 h at 37 ºC.

Step 3 conditions: Templates were added to a final concentration of 24 nM each and reagents (**4**) were added to a final concentration of 36 nM each to a solution containing 0.1 M MES (pH 6.0), 1 M NaCl, 20 mM EDC, and 15 mM sNHS and reacted 4 h at 37 ºC.

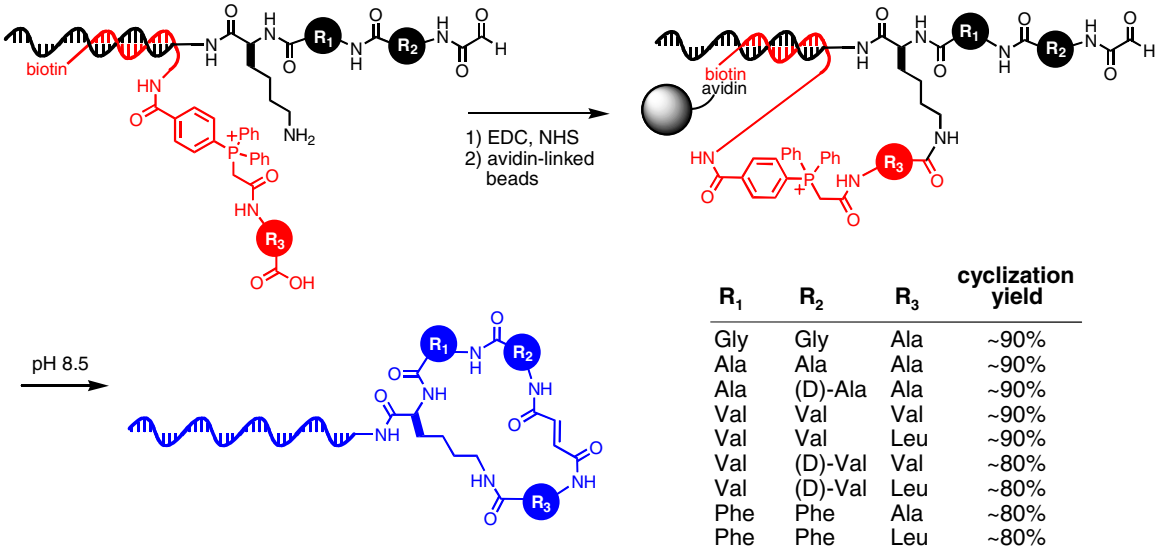
*Synthesis of **8a***
The conditions for each DNA-templated step are listed above. Covalent intermediates (prior to reagent oligonucleotide linker cleavage) were purified by capture with streptavidin-linked magnetic beads (Roche). The beads were washed four times with water to remove unreacted template. Cleavage of the sulfone linker and elution of amino acylated template products was effected by treating the beads with one volume of solution containing 100 mM CAPS (pH 11.8) and 1 mM EDTA at 37 ºC for 2 h. For step 3, the washed beads were first treated with 50 mM $NaIO_4$, 500 mM NaOAc (pH 3.7) for 1 min to oxidatively cleave the diol to the glyoxalamide, and were then washed once with 500 mM NaOAc (pH 3.7), and once with 25 mM NaOAc (pH 3.7) to remove residual periodate. Cyclization was then induced by resuspending the beads in 0.1 M HEPES (pH 8.5) with 1 M NaCl and agitating for 2 h. The final product was collected in the supernatant and in four rinses of the beads with water.

*Synthesis of **8a**-**8d** (4-Membered DNA-Templated Macrocycle Library)*
DTS reaction conditions and product purification protocols were similar to those given for the synthesis of **8a**, except that mixtures of templates **1a**-**1d** were used instead of template **1a** alone, and mixtures of reagents **2a**-**2d**, **3a**-**3d**, and **4a**-**4d** were used instead of reagents **2a**, **3a**, or **4a** alone.

Synthesis of the 65-Membered DNA-Templated Macrocycle Library
Library synthesis proceeded as described for the synthesis of **8a**-**8d**, except that the template library of 65 sequences (see above for preparation details) was used instead of templates **1a**-**1d**. The codons in Fig. 3A were chosen computationally to ensure that at least five mismatches exist between any two codons, minimizing the possibility of undesired reagent-template annealing.

Macrocyclization Studies

To evaluate the efficiency and generality of the Wittig macrocyclization reaction, we prepared 11 precyclized substrates related to the branched intermediates (**7**) by a combination of conventional template functionalization and DNA-templated synthesis (Fig. S1). Each of the macrocylization substrates was immobilized using streptavidin-linked magnetic beads, oxidized with sodium periodate, and subjected to the pH 8.5 macrocyclization conditions described above. The supernatants and bead-associated species were recovered separately, and the latter were eluted from beads by incubation with 95% formamide containing 1 mM biotin and 1 mM EDTA at 95 °C for 5 min. Denaturing PAGE analysis of the supernatant and formerly bead-associated species was used to estimate macrocyclization efficiencies. For each macrocyclization substrate, a control reaction lacking sodium periodate oxidation but otherwise processed identically was used to verify that in the absence of Wittig olefination, no significant elution of material from avidin-linked beads takes place under these conditions.



R_1	R_2	R_3	cyclization yield
Gly	Gly	Ala	~90%
Ala	Ala	Ala	~90%
Ala	(D)-Ala	Ala	~90%
Val	Val	Val	~90%
Val	Val	Leu	~90%
Val	(D)-Val	Val	~80%
Val	(D)-Val	Leu	~80%
Phe	Phe	Ala	~80%
Phe	Phe	Leu	~80%
GABA	GABA	β-Ala	~80%
Phe	Phe	Phe	~60%

Fig. S1. Eleven macrocyclization substrates related to **7** (described in the table) were prepared using conventional and DNA-templated synthesis. The efficiencies of each substrate for Wittig olefination-mediated macrocyclization were evaluated upon exposure of the streptavidin bead-captured substrates to pH 8.5 buffer as described above.

Characterization of DNA-Templated Synthetic Products

Restriction Digestion and MALDI-TOF Mass Spectrometric Analysis
DTS product (0.5-10 pmol) from step 1, step 2, or step 3 (cyclized) was hybridized with 12.5 pmol of digestion oligonucleotides (5'-BGXXXXXXGTAGACATGCB-3', where B designates the 5'- or 3'-biotinylated groups described in the General Experimental Methods section, and XXXXXX denotes the

six-base anticodon sequence for the step 1 reagents) in 10-30 μL of NEB 4 buffer (New England Biolabs). Restriction endonuclease *Hpy*8I (5 U, Fermentas), which cleaves the sequence 5'-GTNNAC-3', was added to effect template cleavage after the seventh nucleotide, and the digestion reaction was incubated for 1 h at 37 ºC. The digestion oligonucleotide byproducts were removed by binding to streptavidin-linked magnetic beads and the DNA-templated small molecule products together with seven template nucleotides (5'-(small molecule)-GCATGTC-3') were collected from the supernatant and desalted using Zip-Tips (Millipore). The sample was eluted directly onto a MALDI analysis plate using 1 μL of 8:1 (50 mg/mL THAP in 1:1 water:acetonitrile):(50 mg/mL ammonium citrate in water). Data was collected on a Voyager DE MALDI-TOF mass spectrometer operated in negative ion (M-H) reflector mode.

Denaturing PAGE Analysis (e.g., Figs. 2C and 3C)
DTS products (1-10 pmol) from step 1 (before and after linker cleavage), step 2 (before and after linker cleavage), or step 3 (before and after cyclization) were loaded onto a 15% denaturing polyacrylamide gel containing 8 M urea (BioRad) and subjected to electrophoresis at 300 V. Gels were stained in aqueous TBE buffer containing SYBR Green (Molecular Probes). Reaction yields were quantitated by UV visualization of stained gels and densitometry of product and starting material bands using a Stratagene Eagle Eye II densitometer. Yield calculations assumed that species in denaturing gels stain with comparable intensity per nucleotide.

DNA-Templated Thiol Addition to Macrocyclic Fumaramide Products
The DNA-linked thiol addition reagent in the disulfide form 5'-HO-GTAGACATGC–S-S$(CH_2)_3$OH was incubated in a solution containing 100 mM DTT and 100 mM HEPES (pH 8.5) for 4 h to reduce the disulfide. The reduced thiol reagent was desalted by gel filtration, and 20 pmol was added to 0.5 pmol macrocyclic fumaramide-linked template in 100 μL of 100 mM HEPES (pH 8.5) with 1 M NaCl. The reaction was allowed to proceed 72 h prior to analysis by denaturing PAGE. Control reactions under identical conditions with templates lacking fumaramide groups did not generate higher molecular weight products.

Selection of the DNA-Templated Macrocycle Library for Carbonic Anhydrase Affinity
A solution containing 2 mg/mL bovine carbonic anhydrase (CA, Sigma) in PBS buffer was incubated with NHS-activated Sepharose 4 Fast Flow beads (Pharmacia) according to the manufacturer's protocol. The resulting CA-linked beads were washed extensively with buffer containing 10 mM Tris (pH 7.4) and 500 mM NaCl (buffer A) and then with buffer containing 10 mM Tris (pH 7.4) and 100 mM NaCl (buffer B). Washed CA-linked beads (40 μL) were combined with 100 fmol of the 65-membered macrocycle library (**8**) and shaken at 4 °C for 1 h. The mixture was washed three times with buffer A (300 μL total) and one time with buffer B (100 μL), then resuspended in 30 μL buffer B. A small aliquot of the bead/buffer mixture (1 μL) was removed as the sample after one round of selection before the remaining beads were combined with 70 μL of 6 M guanidinium HCl containing 100 mM EDTA. The mixture was incubated at 90 °C for 20 min. The eluant was filtered away from the beads and exchanged into buffer B using a CENTRI-SEP spin column (Princeton Separations). Fresh CA-linked Sepharose (40 μL) was added to the resulting library and a second round of selection was carried out as described above.

The first round aliquot (1 μL) and the second round material (30 μL), along with the pre-selected library (20 fmol), were amplified for 22 cycles by PCR using the following primers: 5'-GCAGTACCAAGCATGTCTAC and 5'-CCTGACTACACTCGAGGTGT. The amplified material was buffer exchanged into 1 mM Tris (pH 8.5) using CENTRI-SEP spin columns and digested with *Nla*III (New England Biolabs), a restriction endonuclease that uniquely cuts the template encoding the phenyl sulfonamide (**1e**). The digested mixtures were subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination and densitometry.

Characterization of Stereochemistry of Large-Scale, Non-DNA-Templated Reaction Products

Stereochemical Integrity of Amino-Acid Derived Chiral Centers After Steps 1 and 2

To study the stereochemical purity of amino acid-derived chiral centers after steps 1 and 2 of the library synthesis, a dipeptide ((L)-Gln–(L)-Ala-NH_2) analogous to macrocycle intermediates **5** and **6** was synthesized on multimilligram scale by amine acylation under reaction conditions (400 mM aqueous MES buffer, pH 6.0, 1 M NaCl, 100 mM EDC, 100 mM sNHS, 100 mM of N-protected amino acid building block) that mimic as closely as possible those used in steps 1 and 2 (Fig. S2). As standards for comparison, we also prepared by conventional solid-phase peptide synthesis both authentic diastereomers of the dipeptide containing (L) and (D) stereochemistries at the newly coupled amino acid: (L)-Gln–(L)-Ala-NH_2 and (D)-Gln–(L)-Ala-NH_2. These diastereomeric standards could be well resolved by analytical reverse-phase HPLC (see data below) following treatment with diphenic anhydride to increase hydrophobicity.

To test the possible effects of the basic sulfone linker cleavage conditions (used after step 1 and step 2) on the stereochemical purity of both the N-protected dipeptide and the deprotected dipeptide, the crude dipeptide (analogous to intermediate **6**) generated from the above amine acylation was exposed to sulfone linker cleavage conditions identical to those used after step 1 and step 2 during macrocycle library synthesis (aqueous 100 mM CAPS pH 11.8, 1 mM EDTA, 37 °C, 2 h). The N-terminal protecting group (an allyl carbamate) was then removed using $Pd(PPh_3)_4$ (1.0 eq, in 8 mL 37:2:1 $CHCl_3$:AcOH:/4-methylmorpholine), and the deprotected products were again exposed to sulfone linker cleavage conditions (aqueous 100 mM CAPS pH 11.8, 1 mM EDTA, 37 °C, 2 h). The resulting material was analyzed by electrospray mass spectrometry (expected mass of dipeptide = 216.12 D; observed mass of dipeptide = 216.10 D) and by HPLC as described below (Fig. S2).

The dipeptide products and standards were treated with 100 mM diphenic anhydride in 200 µL 250 mM HEPES buffer, pH 8.5, and 112 mM NHS to increase hydrophobicity prior to comparison by analytical HPLC. Analytical reverse-phase HPLC (5 min of 10% MeCN + 90% aqueous 0.1% TFA, then 60 min of 10% to 30% gradient MeCN in aqueous 0.1% TFA) revealed that 7 97% of the dipeptide product generated under step 1/step 2 conditions was identical to that of the authentic dipeptide containing the original (L)-(L) building-block stereochemistries (Fig. S2 and data shown below), with a minor peak representing 3% of the product matching the (D)-(L) dipeptide. These studies show that the reaction conditions in steps 1 and 2 generate the desired amine acylation products and do not significantly compromise side-chain stereochemistry of the resulting products.

retention time of authentic (L)-Gln–(L)-Ala-NH_2 dipeptide = 48.6 min
retention time of authentic (D)-Gln–(L)-Ala-NH_2 dipeptide = 45.8 min
retention time of dipeptide synthesized under step 1/step 2 conditions = 48.6 min (7 97%) + 45.8 min (3%)

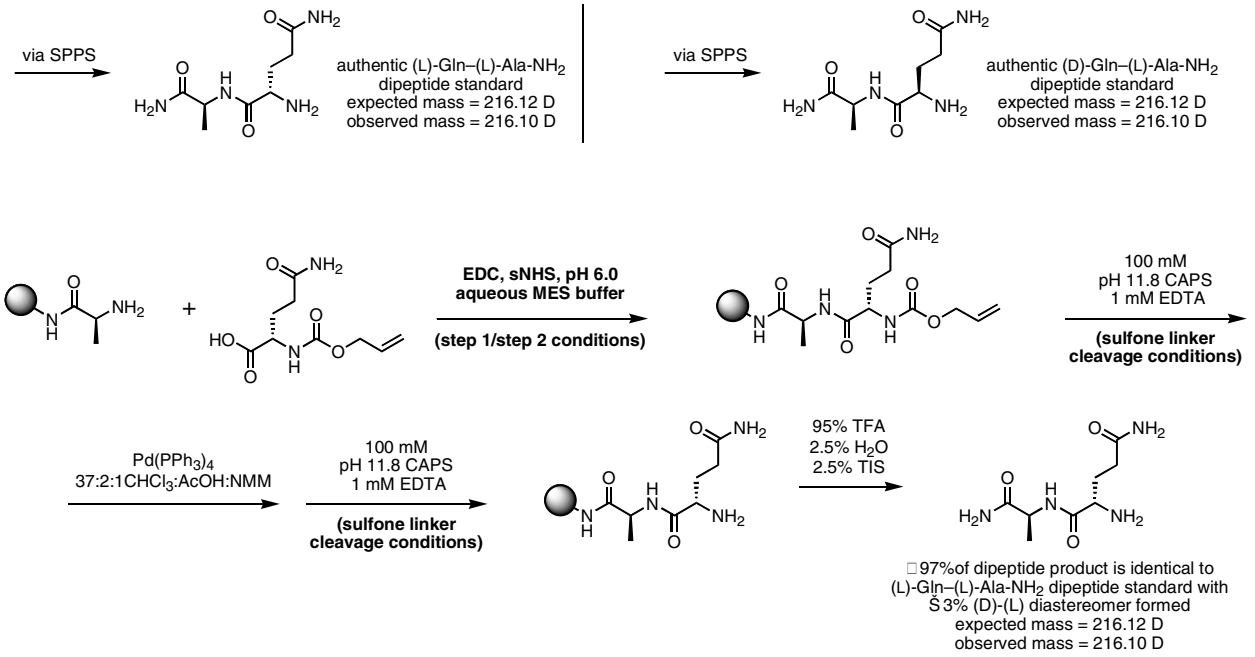


Fig. S2. Step 1 and step 2 reaction and linker cleavage conditions do not significantly compromise the stereochemical purity of the products.

Stereochemical Integrity of Amino-Acid Derived Chiral Centers After Step 3

Because the phosphorane-containing building block in step 3 differs from the building blocks used in steps 1 and 2, we performed a separate set of experiments to assess the stereochemical purity of amino acid-derived chiral centers after step 3 of the library synthesis. A tripeptide (sulfo-Ph_3PCH_2CO-(L)-NorLeu–(L)-Thr-NH_2) analogous to macrocycle intermediate **7** was synthesized on multimilligram scale by amine acylation under reaction conditions (400 mM aqueous MES buffer, pH 6.0, 1 M NaCl, 100 mM EDC, 100 mM sNHS, 100 mM of N-protected amino acid building block) that mimic as closely as possible those used in step 3 (Fig. S3). As standards for comparison, we also prepared by conventional solid-phase peptide synthesis both authentic diastereomers of the tripeptide containing (L) and (D) stereochemistries at the newly coupled amino acid: sulfo-Ph_3PCH_2CO-(L)-NorLeu–(L)-Thr-NH_2 and sulfo-Ph_3PCH_2CO-(D)-NorLeu–(L)-Thr-NH_2 (Fig. S3). These diastereomeric standards could be distinguished by analytical reverse-phase HPLC (see data below).

The crude tripeptide arising from amine acylation containing the phosphonium group (analogous to intermediate **7**) was deprotected with treatment with 95% TFA and analyzed by electrospray mass spectrometry (expected mass of tripeptide = 614.20 D; observed mass of tripeptide = 614.20 D) and by HPLC (Fig. S3). Comparison with the diastereomeric tripeptide standards by analytical reverse-phase HPLC (5 min of 10% MeCN + 90% aqueous 0.1% TFA, then 60 min of 10% to 30% gradient MeCN in aqueous 0.1% TFA) revealed that 7 95% of the tripeptide generated under step 3 conditions was identical to that of the authentic tripeptide containing the original (L)-(L) building-block stereochemistries (Fig. S3, and data shown below), with a minor product peak (5%) co-eluting with the (D)-(L) diastereomer. These studies show that the reaction conditions in step 3 generate the desired product structures and do not significantly compromise the side-chain stereochemistry of the resulting products.

retention time of authentic sulfo-Ph_3PCH_2CO-(L)-NorLeu–(L)-Thr-NH_2 tripeptide = 57.9 m
retention time of authentic sulfo-Ph_3PCH_2CO-(D)-NorLeu–(L)-Thr-NH_2 tripeptide = 60.3 m
retention time of the tripeptide synthesized under step 3 conditions = 57.9 m (7 95%) + 60.3 m (5%)

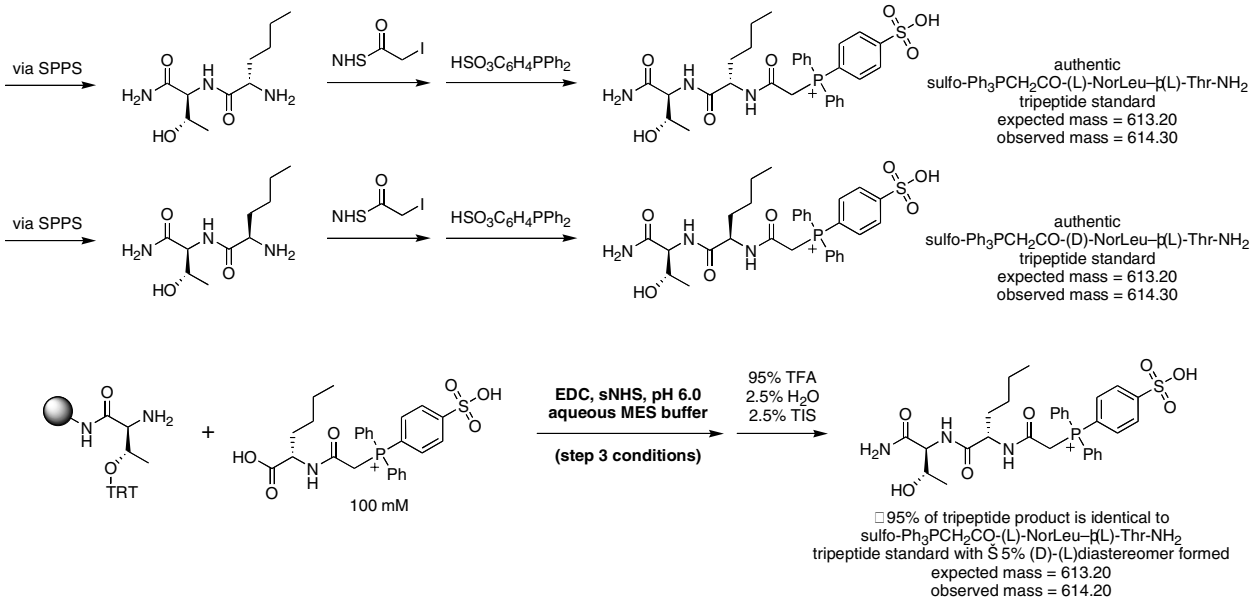


Fig. S3. Step 3 reaction conditions do not significantly compromise the stereochemical integrity of products.

Stereochemistry of the Alkene Arising from Wittig Macrocyclization

We also synthesized a non-DNA-linked macrocyclization precursor chosen from among the precyclized library structures (**7**) by conventional solid-phase synthesis techniques using Rink amide PEGA resin (Novabiochem) on a multimilligram scale suitable for NMR analysis of macrocyclization products (Fig. S4). The precursor was characterized by HPLC and electrospray mass spectrometry (expected masses of monomethyl and dimethyl amide precursors = 902.3 and 916.3 D; observed masses = 902.2 and 916.3). We exposed this precursor to macrocyclization conditions (aqueous 100 mM HEPES, pH 8.5, 25 mM $NaIO_4$) that mimic those used in the library macrocyclization step. The resulting material was purified by reverse-phase HPLC as the predominant new product peak, characterized by proton NMR, 2D COSY NMR, HPLC, and electrospray mass spectrometry, and found to be a non-DNA-linked version of an actual macrocycle present in the 65-membered library (macrocycle **8** using building blocks **2c**, **3d**, and **4c**, expected mass = 454.2; observed mass = 454.3). Proton NMR and homonuclear COSY analysis was consistent with the formation of the expected macrocycle containing the *trans* alkene (vinyl-vinyl proton coupling ^3J = 16 Hz, Fig. S5). These studies confirm the structure of the expected macrocycle, and suggest that the known preference of stabilized phosphoranes such as **7** to generate *trans*-alkenes during the Wittig olefination (see Ref. 12) can still apply in the context of macrocycles of the form **8**.

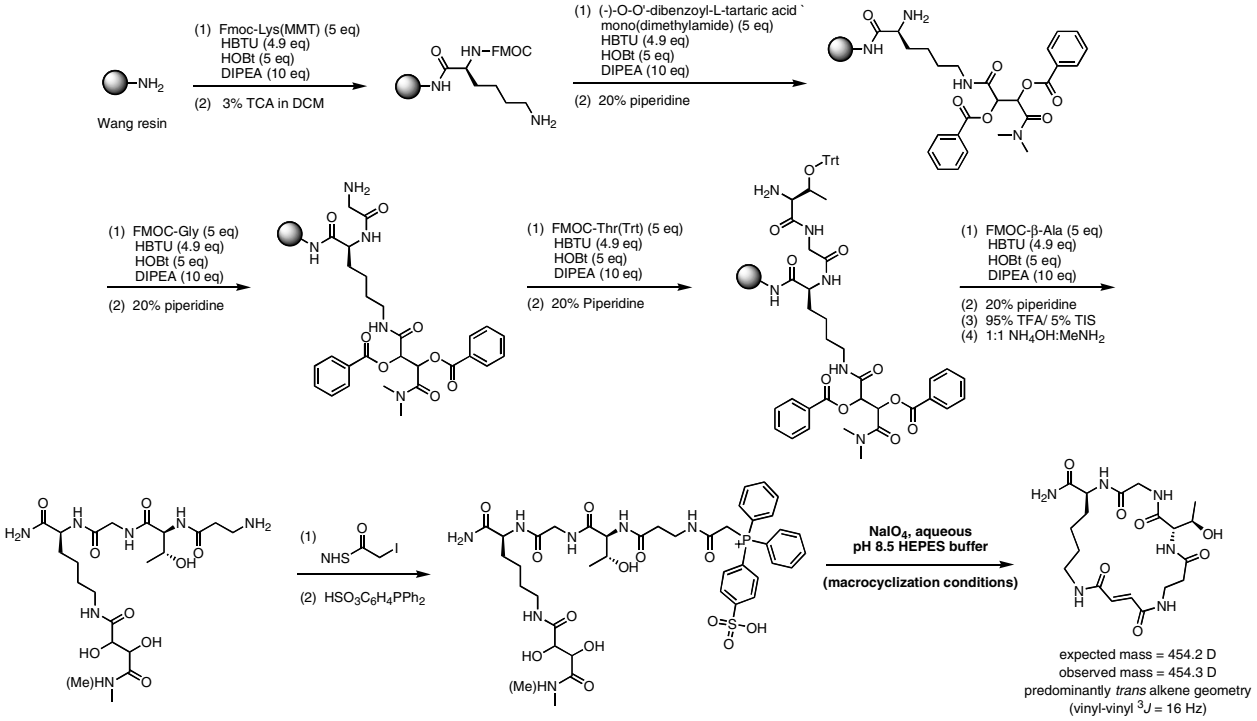


Fig. S4. Large-scale macrocyclization of a non-DNA-linked library member under conditions used in the library synthesis generates the *trans*-alkene-containing macrocycle.

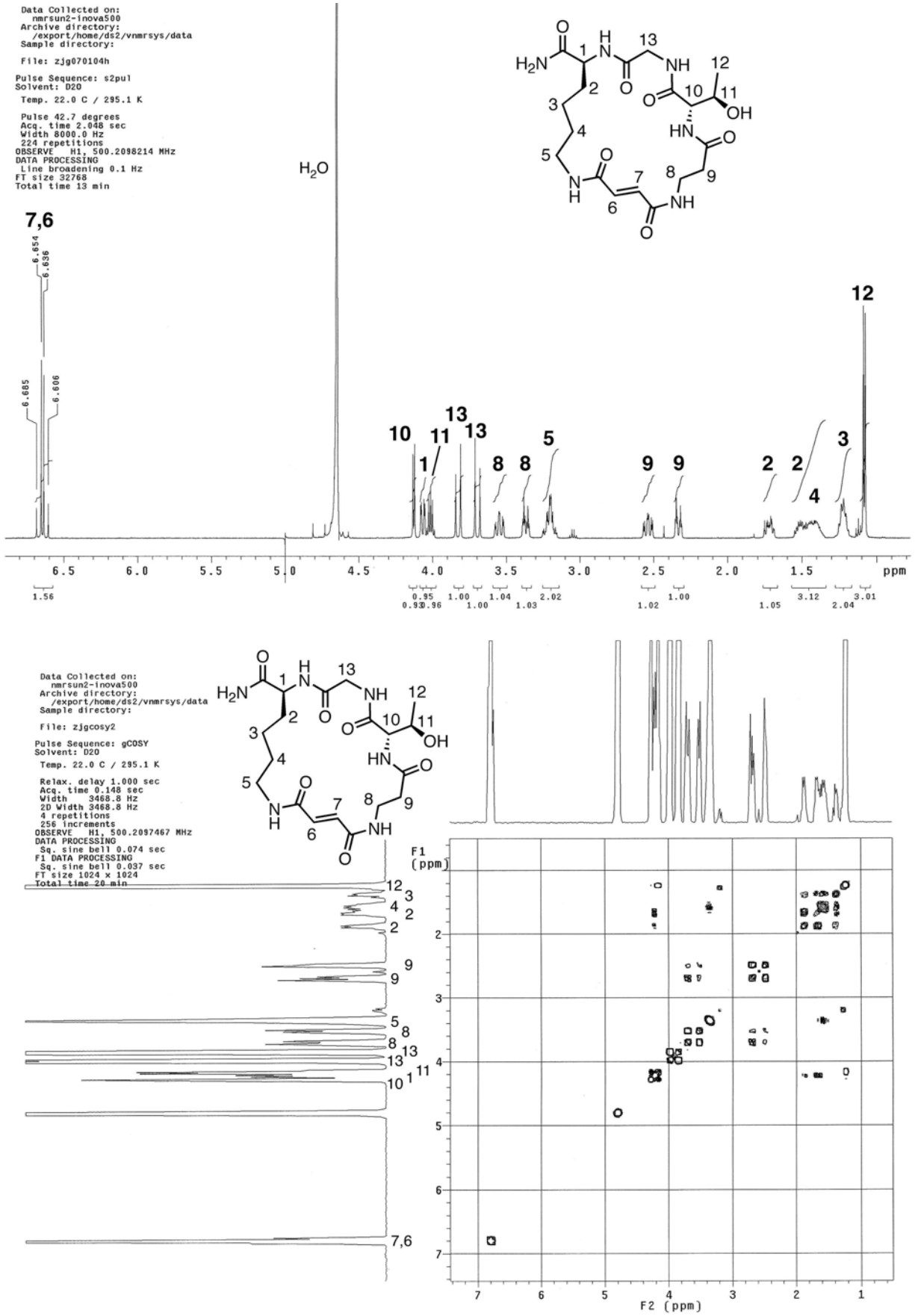


Fig. S5. Proton NMR and homonuclear COSY NMR spectra of the macrocycle generated in Fig. S4.