Small-Molecule Diversification From Iterated Branching Reaction Pathways
Enabled by DNA-Templated Synthesis
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Abbreviations

BSOCOES bis[2-(succinimidylxycarbonyloxy)-ethyl]sulfone
CAPS 3-(cyclohexylamino)-1-propanesulfonic acid
CPG controlled pore glass
DMF dimethylformamide
DTT dithiothreitol
EDC 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EPPS N-(2-hydroxyethyl)piperazine-N’-3-propanesulfonic acid
MES 2-morpholinoethanesulfonic acid
MOPS 3-morpholinopropanesulfonic acid
s-NHS N-hydroxysulfo succinimide
TEAA triethylammonium acetate

General Experimental Methods

DNA oligonucleotides were synthesized using standard phosphoramidite coupling methods using an Applied Biosystems Expedite oligonucleotide synthesizer. All CPG, reagents, and phosphoramidites were obtained from Glen Research (Sterling, VA). 5’-amino oligonucleotides were synthesized using 5’-Amino-Modifier, 5’-biotinylated oligonucleotides were synthesized using 5’-Biotin Phosphoramidite, 3’-biotinylated oligonucleotides were synthesized using Biotin TEG CPG, 3’-amino oligonucleotides were synthesized using 3’-Amino-Modifier C7 CPG, 3’-thiol oligonucleotides were synthesized using 3’-Thiol-Modifier C3 S-S CPG, and 5’-phospho oligonucleotides were synthesized using Chemical Phosphorylation Reagent II. After synthesis, oligonucleotides were cleaved from the solid phase and deprotected by treatment with 1:1 concentrated ammonia:40% aqueous methyamine for 10 minutes at 65°C. Where necessary, monomethoxytrityl and dimethoxytrityl groups were removed by treatment with 4% aqueous trifluoroacetic acid for 5 minutes at 23°C before quenching with an equal volume of 2 M TEAA, pH 7.0. 5’-phosphates were liberated after detritylation by treatment with 2:1 water:concentrated ammonia for 15 minutes at 23°C. Oligonucleotides were purified by reverse phase HPLC on a C18 stationary phase using a 0.1 M TEAA pH 7.0/acetonitrile gradient and appropriate fractions were lyophilized. Oligonucleotides were quantitated by UV/vis spectrophotometry or densitometry of ethidium bromide-stained gels. Unless otherwise noted, all other chemicals were obtained from Sigma-Aldrich (Milwaukee, WI).

DNA labeling

Reagents 2, 6, 8, 10, 12, 13, 16, 20, 21, Ia’-Va’, IIb’, IIIb’, and Ic’-Vc’: 3.3 µL of a 300 mM aqueous solution of the appropriate small molecule amine (in the case of amino
acids, 300 mM NaOH was added to aid solubility) was added to the appropriate 3’-aminoooligonucleotide in 86.7 µL of 200 mM sodium phosphate buffer, pH 7.2. 10 µL of 100 mM BSOCOES (Pierce, Rockford, IL) in DMF was then added and reactions were agitated for 2 hours, desalted by gel filtration (NAP-5 columns, Amersham Biosciences, Uppsala, Sweden) and purified by reverse phase HPLC. In the case of 16, IVα’ and Va’, the small molecule amine was (2S)-(+)2-amino-6-iodacetamidohexanoic acid (Alexis, San Diego, CA), and prior to desalting, 20 µL of 20 mg/mL sodium diphenylphosphinobenzene-3-sulfonate (TCI America, Portland, OR) in water was added and the reaction mixture was incubated for a further 2 hours before purification. In the case of 20 and Ve’, the oligonucleotide was coupled with Fmoc-diaminopropionate hydrochloride (Novabiochem, San Diego, CA). Deprotection of the amine was achieved by addition of 2 µL of triethylamine prior to lyophilization.

Reagents 4, 18, Ib’, IVb’, and Vb’: The appropriate small molecule acid, EDC, and s-NHS were combined at 150 mM each in 9:1 DMF:water and incubated at 23˚C for 2 hours. Meanwhile, the appropriate 3’-thioooligonucleotide was dissolved in 90 µL 25 mM DTT, 100 mM EPPS pH 8.5 and incubated for 1 hour to reduce the disulfide. The reduced thioooligonucleotide was buffer-exchanged into 200 mM sodium phosphate pH 7.2 using Centri-Sep columns (Princeton Separations, Adelphia, NJ) and 10 µl of the acid/EDC/s-NHS mixture was added. The reaction was incubated for 2 hours at 23˚C, desalted using NAP-5 columns, and purified by reverse phase HPLC.

3’-carboxylate reagents I’-V’: I’-V’ were dissolved in 90 µL 200 mM sodium phosphate. 10 µL of 20 mg/mL succinic anhydride were added, and the reaction mixture was incubated for 1 hr at 23˚C and buffer exchanged into deionized water using Centri-Sep columns. The desalted labeled oligonucleotide was used without purification.

The identities of all oligonucleotide reagents were confirmed by MALDI-TOF mass spectrometry.

**Template assembly**

Templates were assembled from three oligonucleotide cassettes: (i) the 3’ cassette containing a primer-binding site necessary for selections and the first reaction codon; (ii) the internal cassette containing the second reaction codon; and (iii) the 5’ cassette containing a reactive primary amine group, a primer-binding sequence including an EcoRI cleavage site, and the third reaction codon. Oligonucleotides were combined in 50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 µg/mL bovine serum albumin (T4 DNA ligase buffer, New England Biolabs, Beverly, MA) to form each cassette individually at a final concentration of 12 µM. Cassettes were then combined in the relative ratio I:II:III:IV:V 0.75:0.75:1.75:3.5:3.25, and 1/50 volume of T4 DNA ligase (New England Biolabs) was added. The ratio of cassettes was empirically determined to yield a similar amount of each structure in the final product pool. Ligation mixtures were incubated at 16˚C for 12 hours before PAGE purification. DNA was excised from the gel, isolated by the crush and soak method and ethanol precipitated.
DNA-templated chemistry

Figure 1 and 2, conditions a: Templates 1 or I-V were present at 60 nM total; reagents 2, 8, 16, or Ia'-Va' were present at 90 nM total. Reactions were performed in 100 mM MES buffer, 1 M NaCl, 20 mM EDC, 15 mM s-NHS, pH 6.0. Reactions were incubated 12 h at 23°C. A small aliquot was withdrawn for gel analysis, and the reaction mixture was added to 1.05 equivalents of streptavidin-linked magnetic particles (Roche, Indianapolis, IN) prewashed with 5 mL of 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl pH 7.5. The reaction mixture was incubated with the magnetic particles 30 min at 23°C, and the supernatant removed. Beads were washed three times with a reaction volume of deionized water; gel analysis showed that in general, all unreacted template was removed in the first two washes. After washing, product purity was verified by withdrawing a small aliquot of magnetic particles and heating in 1 mM biotin, 10 mM Tris-HCl, 1 mM EDTA pH 7.5 15 minutes at 85°C to elute captured DNA from the beads. The remainder of the magnetic particles was treated with 100 mM CAPS pH 10.0 for 2 h at 37°C to cleave the linker and liberate product templates. Products 3, 9, 17, or Ia-Va were isolated by ethanol precipitation.

Figure 1 and 2, conditions b: Templates 3, 9, 17, or Ia-Va were treated in 1 mL 25 mM DTT, 100 mM EPPS pH 8.5 for 1 hour at 37°C and then desalted by gel filtration (NAP-10 columns, Amersham). Reduced, desalted templates were diluted to a total concentration of 78 nM with 250 mM MOPS, 1.25 M NaCl, pH 7.0 and 117 nM total of reagents 4, 10, 18, or Ib'-Vb'. After 90 min at 23°C, 200 mM EDC and 150 mM s-NHS were added to a final concentration of 20 mM EDC and 15 mM s-NHS, and the reaction was incubated for 12 h at 23°C. 1/200 volume of acetic anhydride was added and the reaction mixture was further incubated 1 h at 23°C. Product isolation was accomplished as above, with the exception that cleavage was effected by treatment with 100 mM CAPS 10 mM 2-mercaptoethanol pH 12.0 for 1 hour at 37°C.

Figure 1 and 2, conditions c: Templates 5, 11, 19, or Ib-Vb were dissolved at a total concentration of 60 nM in a solution containing 100 mM MES, 1 M NaBr, pH 6.0, and 90 nM total reagents 6, 12, 13, 20, 21, or Ic'-Vc'. 1/100 volume of 50 mM Na2PdCl4 preincubated in 100 mM MES, 1 M NaBr, pH 6.0 for 20 minutes was added, and the reaction mixture was incubated at 37°C for 4 hours. 1 M DTT was added to 20 mM final concentration, and the reaction mixture was heated to 85°C for 15 minutes and buffer exchanged (NAP-10) into fresh 125 mM MES, 1.25 M NaBr pH 6.0; reaction volume was adjusted to a total template and reagent concentration of 40 nM and 60 nM, respectively. 200 mM EDC and 150 mM s-NHS were added to final concentrations of 20 mM and 15 mM, respectively, and the reaction mixture was incubated at 23°C for 12 hours. Product isolation was accomplished as above, with the exception that cleavage was effected by treatment with 100 mM CAPS pH 12.0 30 min at 23°C.

Library analysis

Mismatch control: Authentic samples of I-V, Ia-Va, and Ib-Vb were individually generated either by automated oligonucleotide synthesis in the cases of I-V or by DNA-
templated synthesis in the cases of Ia-Va and Ib-Vb. Each was individually treated under conditions identical to those used in the actual library synthesis, but with its complementary reagent oligonucleotide omitted, ethanol precipitated, and analyzed by PAGE.

Template verification: Aliquots of library intermediate pools were withdrawn prior to each step and treated separately with complementary reagents I'-V'. I'-V' were labeled at their 3'-termini with either amines or carboxylates and the appropriately labeled reagent was selected for each pool to ensure reactivity: For I-V and Ib-Va, 3'-carboxylate I'-V' were used; for Ib-Vb, 3'-carboxylate II' and III' and 3'-amino I', IV', and V' were used. In each case, 10 pmol of template pool was combined in 160 µL 100 mM MES, 1 M NaCl, 20 mM EDC, 15 mM s-NHS pH 6.0 with 5 pmol of the appropriate reagent and incubated 12 h at 23°C. Reactions were then ethanol precipitated and analyzed by PAGE.

**MALDI analysis**

Product aliquots were combined with 1.5 equivalents of 3',5'-bisbiotinylated oligonucleotides to reconstitute a double-stranded EcoRI recognition site in 100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 0.025% Triton X-100 pH 7.5 (EcoRI buffer, New England Biolabs). Digestion mixtures were heated to 85°C briefly and allowed to cool slowly to 23°C. 1/50 volume of EcoRI (New England Biolabs) was added, and digests were incubated at 37°C for 12 hours. Digests were added to 1.05 equivalents of streptavidin-linked magnetic particles prewashed with 200 µL of 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl pH 7.5 and incubated for 30 min at 23°C. The supernatant was removed, and the magnetic particles were washed with 50 µL of deionized water. The wash and supernatant were combined and ethanol precipitated. The isolated DNA was then resuspended in 20 µL of 0.1 M TEAA pH 7.0, desalted by ZipTip (Millipore, Billerica, MA), and spotted in a 9:1 40 mg/mL trishydroxyacetophenone/50 mg/mL ammonium citrate matrix in 50% aqueous acetonitrile.

Mass spectra of oligonucleotides containing phosphoranes (17, IVa'/Va', IVb/Vb) gave a side product of mass consistent with loss of the phosphorane group, but it was not fully characterized. This side product arose during the MALDI-TOF mass analysis and was otherwise not present based on the following experiment. Phosphorane-containing DNA, previously observed to contain the spurious species by mass spectrometry but consisting of a single species by HPLC, was treated in 200 mM sodium phosphate pH 8.0 with 2 mg/mL 4-formylbenzoic acid (2 h, 37°C). By HPLC, the conversion was >95% to a single species. The reaction mixture was desalted and spotted onto the MALDI plate as above without other purification, and only the expected cinnamide-containing oligonucleotide was observed in the mass spectrum with no evidence of the unknown side product, suggesting that material maintained Wittig olefination reactivity despite the apparent presence of a species lacking the phosphorane in the mass spectrum.
DNA sequences

DNA sequences used in this work are shown below. Sequences corresponding to the first, second, and third reaction codons are colored green, blue, and red, respectively. EcoRI sites are underlined, and ligation sites for the template assembly are denoted in bold italics in the template and cassette sequences.

Template sequences:

1:  5′H2N-CCATGCGAATTCTCATACCAGTCAGCACACTGTAACGAGTTCTGAGCCCTG

I:  5′H2N-CCATGCGAATTCTATGATGACTGCGGATCTGCTCCTAAAGCTTCCCTAC

II:  5′H2N-CCATGCGAATTCTTACGTTAACAACGGCTTTTCCTCAATCAAGCTTCCCTAC

III:  5′H2N-CCATGCGAATTCTATCCGTTACGCTCTTCTCCCTCAACAGCTTCCCTAC

IV:  5′H2N-CCATGCGAATTCTTATCCGCTAGCTAGCTGTAAGAAAGCTTCCCTAC

V:  5′H2N-CCATGCGAATTCTCAACACAGTGGCTAGCTGCCAGAAAAGCTTCCCTAC

Reagent sequences: Mismatched bases are in lower case.

2, 8, 16:  3′H2N-GGTACTCGTGCAAGA-biotin

4, 10, 18:  3′H2N/HS-GGTACTCGTGACAG-biotin

6, 12, 13, 20, 21:  3′H2N-GGTACAGGTTAGTGGCAG-biotin

mis, steps 1 and 2:  3′H2N/HS-GGTACTCC59CATGA

mis, step 3:  3′H2N-GGTACAGGaAaGcaCAG

Ia′:  3′H2N-GGTAAACAGGGATTT-biotin

IIa′/IIIa′:  3′H2N-GGTAAAGGGTAGTTT-biotin

IVa′/Va′:  3′H2N-GGTAAAGGCACTTCTTT-biotin

Ib′:  3′HS-GGTACACACGCTAACACAGCTACAG-biotin

IIb′/IIIB′:  3′H2N-GGTACAGGCTACAGAAGAG-biotin

IVb′/IVb′:  3′HS-GGTACACAGCTGCTACAGCCAG-biotin

Ic′:  3′H2N-GGTACAGGATGCTTACAG-biotin

IIc′:  3′H2N-GGTACAGATGCTCAGTTAGG-biotin

IIIc′:  3′H2N-GGTACAGATGCTTACAG-biotin

IVc′:  3′H2N-GGTACAGAACAAGGAG-biotin

Vc′:  3′H2N-GGTACAGGATGCTACAG-biotin

Cassette sequences: “P” denotes a 5′-phosphate; sequences denoted by asterisks are ligated in the antisense strand.

I.3:  5′H2N-CCATGCGAATTCTATAGTGAGT

I.3*:  3′-ATCCCATCAATGAC-P

I.2:  5′P-ACCTGCTGAC

I.2*:  3′-ACGCTTACAGAC-P

I.1:  5′P-TTGCTCCTTAAAGCTTCCCTAC

I.1*:  3′-AGGGATT

II.3:  5′H2N-CCATGCGAATTCTACAGGTA

II.3*:  3′-TGAGCATCTCAGGTA

II.2:  5′P-ACCTGCTGCTT

II.2*:  3′-ACCGAAAGAG-P

II.1:  5′P-TGCTCTCCTCAAGCTTCCCTAC

II.1*:  3′-GTAGTTT

III.3:  5′H2N-CCATGCGAATTCTATCCGAT

III.3*:  3′-ATTAGCTAATCTGAC-P

III.2:  5′P-TGCTGCTGCTT

III.2*:  3′-ACCGAAAGAG-P
III.1: 5’P-TCTCCATCAAAGCTTCCAC
III.1*: 3’-GGTAGTTT
IV.3: 5’H2N-CCATGCAGATTCTTGTTCTT
IV.3*: 3’-AACAAGGAAGTC-P
IV.2: 5’P-TCAGCCTAGT
IV.2*: 3’-GGATCGTCAAG-P
IV.1: 5’P-AGTCCGTAGAAAGCTTCCAC
IV.1*: 3’-GCAATTTTT
V.3: 5’H2N-CCATGCAGATTCAACAGT
V.3*: 3’-TGGTTGCAATCTC-P
V.2: 5’P-AGAGCCTAGT
V.2*: 3’-GGATCACTAG-P
V.1: 5’P-AGTCCGTAGAAAGCTTCCAC
V.1*: 3’-GCAATTTTT

EcoRI complement for 1: 3’biotin-GGTACGCTTAAAGTATGG-biotin
EcoRI complement for I-V: 3’biotin-GGTACGCTTAAG-biotin