



Supporting Information

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## Ordered Multistep Synthesis in a Single Solution Directed by DNA Templates

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### General Synthesis and Analysis Methods

DNA oligonucleotides were synthesized on a PerSeptive Biosystems Expedite 8090 DNA synthesizer using standard phosphoramidite protocols and purified by reverse-phase HPLC using a triethylammonium acetate (TEAA)/CH<sub>3</sub>CN gradient. Modified phosphoramidites and CPG for DNA synthesis were purchased from Glen Research. The 5'-amino modified oligonucleotides were synthesized using the 5'-Amino Modifier 5 Phosphoramidite. The 3'-amino modified oligonucleotides were synthesized using 3'-Amino-Modifier C7 CPG 500. The 3'-thiol modified oligonucleotides were synthesized using 3'-Thiol-Modifier C3 S-S CPG. The 5'-thiol modified oligonucleotides were synthesized using 5'-Thiol Modifier C6. The doubly biotinylated (3' and 5'-biotin modified) oligonucleotides were synthesized using 3'-BiotinTEG-CPG to install the 3'-biotin group and 5'-Biotin Phosphoramidite to install the 5'-biotin group. Oligonucleotides were quantitated by UV and all modified DNAs and reagents were characterized by MALDI-TOF mass spectroscopy. Reaction products, including multistep reaction sequences, were also characterized by MALDI-TOF mass spectroscopy as described below. Reaction yields were characterized by denaturing polyacrylamide gel electrophoresis (PAGE) followed by ethidium bromide staining, UV visualization, and CCD-based densitometry. All chemicals, unless otherwise noted, were purchased from Sigma-Aldrich.

### Ordered Triolefin Sequence

#### *Oligonucleotide Sequences*

Reagent **1** and **1b**: 5'- CGACTGTGA-NH<sub>2</sub>

Mismatched **1c**: 5'- CTCTGGTGA-NH<sub>2</sub>

Reagent **2** and **2b**: 5'-H<sub>2</sub>N-GGACAACATGTG

Mismatched **2c**: 5'-H<sub>2</sub>N-GGTGACAATCTG

Reagent **3**, **3b**, and **3d**: 5'- GGGGCTGACGGGCTATCGCTTGTGA-NH<sub>2</sub>

Mismatched **3c**: 5'- GGGTCCGTCCGCCAATCTCTCGTGA-NH<sub>2</sub>

Template **4**: 5'-H<sub>2</sub>N-TCACATGTTGTCCATCACAGTCGTAGCGATAGCCCGTCAGCCCC

Complementary oligonucleotide for restriction digestion and MALDI analysis of products linked to template **4**: 5'- CTGTGATGGACAACATGTGA

#### *Preparation of Phosphorane Reagents 1, 2, and 3*

Reagent oligonucleotides were synthesized as described above on CPG resin with either a 3'-amino modification (for **1**, **3**) or a 5'-amino modification (for **2**). Oligonucleotides with 3'-amino termini were treated with piperidine:DMF (20:80) to deprotect the amino group; 5'-amino modified oligonucleotides were synthesized without the terminal MMT group then washed with DIPEA in DMF. To CPG resin linked to these oligonucleotides was added 20 mg N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; ~100 μmol) and 30 mg 4-(diphenylphosphino)benzoic acid (~100 μmol) in 500 μL dry DMF with 50 μL DIPEA. The mixture was incubated at 37 °C for > 4h. The beads were washed with DMF, deprotected and

cleaved in 1:1 concentrated ammonium hydroxide:aqueous methyl amine supplemented with 4 mg/ml tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at 65 °C for 10 min, purified by reverse-phase HPLC, and lyophilized.

The phosphine-linked oligonucleotides were redissolved in 0.2 M sodium phosphate buffer, pH 7.2, and combined with 2 mg/mL N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB, Pierce) for 5 min before the addition of an appropriate amine-containing reagent. For **1**, this reagent is biocytin (Bachem) which was added in slight excess relative to the SIAB linker and reacted for 90 min at 25 °C.

For **2/2b** and **3/3b**, the additional reagent is a tartrate-modified amine, prepared as follows. A diamine (100  $\mu$ mol, ethylenediamine for R<sup>3</sup>, 1,3-diaminopropane for R<sup>2</sup>, 1,8-diaminooctane for a control substrate below) in CH<sub>2</sub>Cl<sub>2</sub> was added dropwise to a solution of 33 mg diacetyl tartaric anhydride (150  $\mu$ mol) in 1 mL CH<sub>2</sub>Cl<sub>2</sub>. After 1 h at 25 °C, 1 N NaOH was added and the reaction stirred for 1 h to fully cleave the acetate protecting groups. HCl was added to neutralize the solution and the aqueous layer was recovered and concentrated *in vacuo* (for 1,8-diaminooctane, the organic layer was recovered instead). One-tenth of this crude reaction, dissolved in 1:1 DMF:0.2 M sodium phosphate, pH 7.2, was added to the oligonucleotide/SIAB mixture described above and reacted for 90 min after which 1  $\mu$ L of acetic anhydride was added.

For all reagents, the reactions were desalted by gel filtration using Sephadex G-25 and purified by reverse-phase HPLC. All phosphorane reagents were characterized by MALDI-TOF mass spectroscopy. The tartrate-modified reagents are **2b** and **3b** respectively; aldehydes **2** and **3** were made from **2b** and **3b** by NaIO<sub>4</sub> oxidation immediately prior to use.

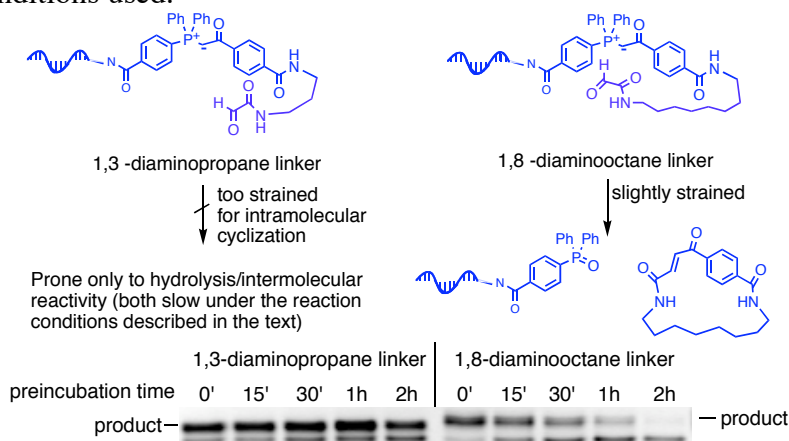
#### *Preparation of Aldehyde Template 4*

The template was synthesized with a 5'-amino modification (MMT off) and then washed with DIPEA in DMF to deprotonate the amines. Diacetyl tartaric anhydride (21.6 mg, 100  $\mu$ mol), HOBt (15.3 mg, 100  $\mu$ mol, NovaBiochem), and tryptamine (16.0 mg, 100  $\mu$ mol) were mixed together in 400  $\mu$ L dry DMF for 1 h. A solution of 20.6 mg 1,3-dicyclohexylcarbodiimide (100  $\mu$ mol) in 100  $\mu$ L DMF was added and the resulting solution was incubated at 25 °C for 30 min. The solution was centrifuged and the supernatant added to the CPG beads. After 2 h, the beads were washed with DMF, deprotected and cleaved from the support with 1:1 ammonium hydroxide:methyl amine for 10 min at 65 °C, and purified by reverse-phase HPLC. Following lyophilization, the collected tartrate-modified template was redissolved in 0.05 M NaOAc, pH 5.0, and oxidized using 50 mM NaIO<sub>4</sub> for 45 min. The reaction was desalted by gel filtration using Sephadex G-25 and purified by reverse-phase HPLC to yield the aldehyde template **4**, as verified by MALDI-TOF mass spectroscopy.

#### *Stability of Phosphorane Reagents in Solution*

Previous work<sup>[1]</sup> has shown that intramolecular cyclizations between DNA-linked ylides and aldehydes are possible, forming macrocyclic fumaramides. To demonstrate that the phosphorane reagents used for this ordered synthesis could not cyclize intramolecularly and were stable to the reaction conditions, control phosphorane reagents were made (as described above) that could directly react with an aldehyde-linked template<sup>[2]</sup>. The reagents used either a 1,3-diaminopropane or a 1,8-diaminooctane linker between the ylide and aldehyde groups as shown (Figure S1). The reagents were oxidized with NaIO<sub>4</sub> in 50 mM NaOAc, pH 5.0 and added to 0.1M TAPS buffer pH 8.0 with 1 M NaCl (150 nM reagent concentration). The aldehyde

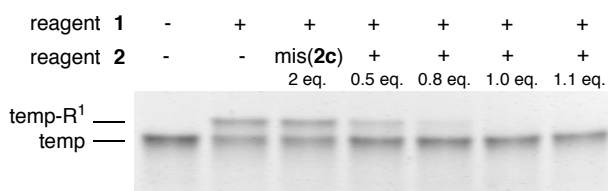
template (**4**) was added to these reactions either immediately or after 15 min, 30 min, 1 h, or 2 h. The reactions were precipitated with ethanol and analyzed by denaturing PAGE. The product yield for the 1,3-diaminopropane linker did not noticeably decrease after as much as 2 h of preincubation; however, the product for the 1,8-diaminooctane essentially disappeared after 2 h (Figure S1). These results suggest that the reagents themselves are stable in solution except when intramolecular cyclization is possible. While some hydrolysis of the ylide was observed, particularly at higher temperatures, the building blocks for the triolefination sequence are stable to the reaction conditions used.



**Figure S1:** Stability of the phosphorane reagents. An intramolecular cyclization is only possible for the long octane linker and the reactivity of this reagent noticeably decreases with preincubation in pH 8.0 buffer; the reagent with the shorter propane linker maintains most of its reactivity even after 2 h at 25 °C.

### Testing the Reactivity of Reagent **1** in the Presence or Absence of **2**

To allow the one-step purification of the desired triolefin **5**, the system was designed with a purifiable group (biocytin) attached to **1** so that only a product that has undergone three successive Wittig olefinations would link  $R^1$  to the template (the truncated products template- $R^3$  and template- $R^3-R^2$  as well as an unreacted template would lack biotin). This system requires that **2**, when present in the reaction, prevents the direct reaction of **1** (and transfer of  $R^1$ ) to the template **4**. Reactions were performed using the multistep conditions (1 h at 4 °C, 1 h at 30 °C, 2 h at 60 °C) with varying equivalents of **2** added to **4** (100 nM) in 0.1 M TAPS, pH 8.0 before adding **1** (200 nM, 2 equiv.) to the reaction. Control reactions were performed that lacked **2** entirely or that replaced **2** with a mismatched reagent **2c** (200 nM, 2 equiv.) that cannot anneal to the template. The reactions were precipitated with ethanol and analyzed by denaturing PAGE. The results show that a single equivalent of **2** effectively blocks the direct reaction of **1** with **4** (Figure S2).



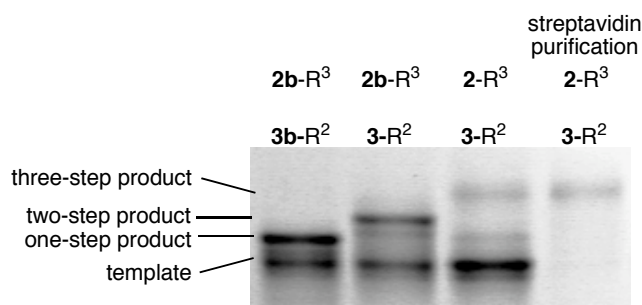
**Figure S2:** Reaction of **1** directly to **4**. While the transfer of  $R^1$  to the template **4** is observed in the absence of **2** and with substoichiometric quantities of **2**, once 1.0 equiv. of **2** relative to **4** are present, the direct transfer of  $R^1$  to **4** is no longer observed under the multistep reaction conditions (1 h at 4 °C, 1 h at 30 °C, 2 h at 60 °C).



### Three-Step Ordered Wittig Olefination

The three-step reaction was set up in an identical manner as the two-step reaction described above but with **3** instead of **3d** and both **2** and **3** oxidized for 30 min with 1.5 mM NaIO<sub>4</sub>. The template (**4**) was hybridized with **1**, **2**, and **3** at pH 5.0, 4 °C for 10 min. To start the reaction, 0.1 M TAPS pH 8.0, 1 M NaCl was added. In the final mixture, all reagents were at concentrations of 100 nM. To facilitate analysis, control reactions were also performed (see the main text) that use tartrates **2b**, or **2b** and **3b**, in place of their oxidized counterparts to prevent the first step or both the first two steps from occurring. Reactions proceeded for 1 h at 4 °C, 1 h at 30 °C, and 2 h at 60 °C before ethanol precipitation. Aliquots of the final reaction mixture were purified using streptavidin-agarose (Novagen) as described above and analyzed by denaturing PAGE (Figure 2a). Because of the potential of **2** and **3** to react with each other and cyclize if **2** fails to react with **1**, there is no observed diolefin side product in lane C of Figure 2a and more unmodified template is observed compared to lanes A or B despite equivalent amounts of starting material.

Reagents were also synthesized that swapped the building blocks R<sup>2</sup> and R<sup>3</sup> on **2** and **3**. Reactions were run under identical conditions and then analyzed by PAGE. The R<sup>2</sup>-R<sup>3</sup>-R<sup>1</sup> ordered triolefin product was generated in 15% overall yield (Figure S4).



**Figure S4:** PAGE of ordered triolefin synthesis to generate the R<sup>2</sup>-R<sup>3</sup>-R<sup>1</sup> product (similar to Figure 2a, but using reagents with swapped small-molecule building blocks).

### MALDI-TOF Mass Spectroscopy of Reaction Products

The samples for the control reactions with unoxidized reagents lacking aldehyde groups (to produce mono- and di-olefins) were prepared for MALDI-TOF as follows. The reaction was annealed (70 °C for 5 min, 55 °C for 12 min, then 37 °C) to a doubly biotinylated complementary oligonucleotide (3 equiv.), captured with streptavidin-agarose, washed with TAPS buffer, pH 8.0, 1 M NaCl several times to remove **1**, **2**, and **3** from the template, and eluted in 95% formamide, 10 mM EDTA before ethanol precipitation. The pellets are redissolved in NEBuffer 4 (New England Biolabs) and annealed as above before adding BSA and *Nla*III to cleave the template to leave a 7-base sequence suitable for MALDI. After 4 h, the enzyme was denatured and the digestion products captured with streptavidin-agarose. The supernatant and first H<sub>2</sub>O wash were precipitated with ethanol and subjected to MALDI analysis.

The three-step reaction was prepared for MALDI-TOF as follows. The reaction was precipitated with ethanol, redissolved in NEBuffer 4 (New England Biolabs) and 3 equiv. of a nonbiotinylated DNA complement was added. The solution was annealed as above and then BSA and *Nla*III were added for restriction digestion. Following denaturation and streptavidin-agarose purification, the sample was washed several times with H<sub>2</sub>O and eluted with 95% formamide, 10 mM EDTA before precipitation with ethanol. Only biotinylated templates (with R<sup>1</sup>) remain in the analyzed sample.

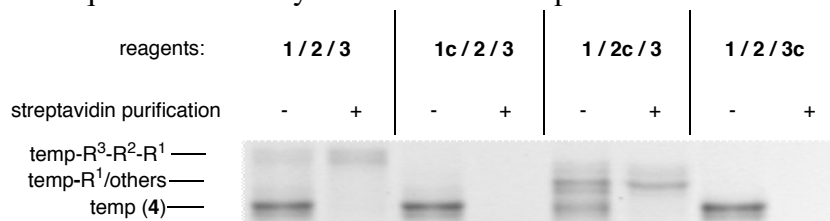
Samples were redissolved in 0.1 M TEAA, purified using Zip-Tips (Millipore) and spotted on a MALDI plate in a 9:1 matrix of 40 mg/mL 2,4,6-trihydroxyacetophenone (THAP, Fluka) in 1:1 ACN: ddH<sub>2</sub>O to 50 mg/mL ammonium citrate (dibasic) in ddH<sub>2</sub>O. The resulting spectra are presented in Figs. 2b and 2c. The predicted and observed masses for all possible products are presented in Table S1. Because of potential ionization differences between product species, the relative heights of peaks in the included MALDI spectra may not be representative of the relative amounts of individual species in the product mixtures.

Species	Predicted Mass	Observed Mass
Template-Aldehyde (from <b>4</b> )	2302.42 (2319.4 for hydrated form)	(2316.3 to 2318.4) ± 6 (Fig. 2b, 2c; left and middle)
Template-R <sup>3'</sup>	2637.55	2636.5 ± 6 (Fig. 2b, left)
Template-R <sup>2'</sup>	2651.55	2652.6 ± 6 (Fig. 2c, left)
Template-R <sup>1</sup>	2817.64	2814.6 ± 6 (Fig. 2b, right)
Template-R <sup>3</sup> -R <sup>2'</sup>	2910.66	2906.0 ± 6 (Fig. 2b, middle)
Template-R <sup>2</sup> -R <sup>3'</sup>		2909.6 ± 6 (Fig. 2c, middle)
Template-R <sup>3</sup> -R <sup>1</sup>	3076.74	Not observed
Template-R <sup>2</sup> -R <sup>1</sup>	3090.75	3090.0 ± 6 (Fig. 2c, right)
Template-R <sup>3</sup> -R <sup>2</sup> -R <sup>1</sup>	3349.85	3347.9 ± 6 (Fig. 2b, right)
Template-R <sup>2</sup> -R <sup>3</sup> -R <sup>1</sup>		3350.6 ± 6 (Fig. 2c, right)

**Table S1:** Summary of predicted and observed masses by MALDI-TOF mass spectroscopy of olefination products. The template (**4**) has been digested to a 7-mer prior to analysis by *Hla*III. The prime designation (R<sup>2'</sup> and R<sup>3'</sup>) refers to the unoxidized tartrate form of these building blocks from **2b** or **3b**.

### Mismatch Triolefin Controls

Reagents **1c**, **2c**, **3c** were prepared identically to **1**, **2**, and **3** but with scrambled oligonucleotide sequences that cannot anneal to template **4**. The three-step sequence described above was performed three additional times with one of each of the mismatch reagents (**1c**, **2c**, or **3c**) replacing one of the original reagents (**1**, **2**, or **3**, respectively). The reactions were analyzed directly as well as after streptavidin purification by denaturing PAGE. For **1c**, reagent **2** and **3** (each with an ylide and aldehyde) can react twice with each other to release their attached substrates by cyclization, thereby transferring no material and no R<sup>1</sup> to the template. For **2c**, reagent **1** and **3** can react directly with **4** and a one-step biotinylation product was recovered. For **3c**, no material was transferred to the template **4** (Figure S5). All of these observations are consistent with the sequence selectivity of these DNA-templated reactions.



**Figure S5:** Mismatch controls of three-step sequence. The matched three-step sequence is shown in the two leftmost lanes with the remaining lanes containing the three mismatch controls. For the mismatch controls, only with mismatched reagent **2c** is any biotinylated material transferred to the template, and this product corresponds to R<sup>1</sup> adding directly. Using mismatch reagents **1c** or **3c** lead to no modified templates. These results demonstrate that all three reagents must be capable of hybridizing to the template to produce the multistep product.

### DNA-Linked NHS Interferes with Reagent Transfer

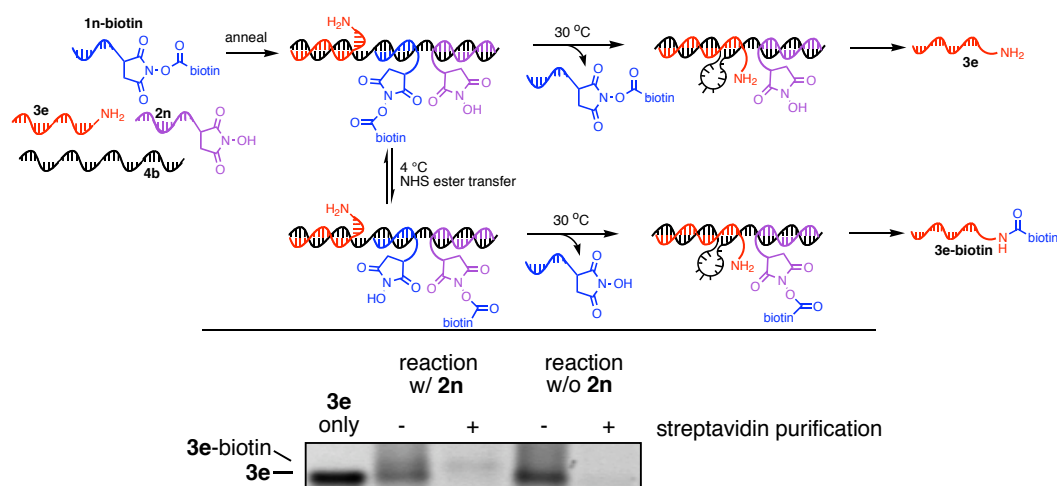
To test whether the strategy in Figure 1 could be extended to the production of a tripeptide using NHS-linked DNAs, we synthesized NHS-linked oligonucleotide reagents **1n** and **2n** using the same DNA sequence as those for **1** and **2** but with 3' and 5' thiol modifications respectively.

The protected thiol oligonucleotides were purified following DNA synthesis using reverse-phase HPLC. For the 3' thiol (**1n**), the DMT group was deprotected in 3% TFA and precipitated with ethanol. To deprotect the thiol group, 100 mM DTT, pH 8.5 was added at 25 °C for 30 min. The reaction was desalted by gel filtration using Sephadex G-25 and added directly to 250  $\mu$ L of a 40 mg/mL solution of N-hydroxymaleimide in 0.5 M MOPS, pH 7.5. After 30 min, the reaction was concentrated *in vacuo*, desalted by gel filtration, and purified by reverse-phase HPLC to generate a 3' NHS-linked oligonucleotide. For the 5' thiol (**2n**), the oligonucleotide was redissolved in 200  $\mu$ L 0.1 M TEAA, pH 7.0. 75  $\mu$ L of 1 M AgNO<sub>3</sub> was added and after 30 min, 75  $\mu$ L of 1.33 M DTT was added. The solution was centrifuged and the supernatant collected. The pellet was washed with 150  $\mu$ L 0.1 M TEAA and, after centrifugation, the supernatant was collected again. The combined supernatants were desalted by gel filtration using Sephadex G-25 and added directly to 250  $\mu$ L of a 40 mg/mL solution of N-hydroxymaleimide in 0.5 M MOPS, pH 7.5. After 30 min, the reaction was concentrated *in vacuo*, desalted by gel filtration, and purified by reverse-phase HPLC to generate a 5' NHS-linked oligonucleotide.

Biotin was activated as an NHS-ester on **1n** by adding ~0.5 mg of biotin in DMF to .2 mg of EDC (total volume ~ 50  $\mu$ L). After 20 min, 20  $\mu$ L of this mixture was added directly to an aliquot of NHS-linked oligonucleotide in 80  $\mu$ L 0.1 M MES pH 6.0. After 5 min, the reaction was desalted by gel filtration using Sephadex G-25 and purified by reverse-phase HPLC. By adding 1% TFA to the collected material in TEAA:CH<sub>3</sub>CN before lyophilization, the NHS ester-linked DNA could be recovered in pure form (without the addition of TFA, hydrolysis of the NHS ester occurs under the lyophilization conditions).

To test the ability of NHS to attack and capture NHS-linked esters, an experiment was performed with unmodified DNA **4b** (same sequence as **4**), 3'-amine modified **3e** (same sequence as **3**), and **1n-biotin** and **2n** as shown below (Figure S6). Species **1n-biotin**, **2n**, **3e**, and **4b** were annealed together at 100 nM in 0.1 M MOPS, pH 7.0, 1 M NaCl, 4 °C. The reaction was run for 20 min at 4 °C and 20 min at 30 °C before being precipitated with ethanol or purified by streptavidin-agarose beads as described above. A control reaction excluding **2n** was also performed.

Denaturing PAGE analysis of the reactions demonstrated that biotin can be transferred from **1n** to **3e** only in the presence of **2n**; this strongly suggests that the NHS group on **2n** can reversibly capture proximal NHS-linked esters (Figure S6). A tripeptide synthesis using the method in Figure 1 would be problematic as consumed reagents (DNA-linked NHS groups) would sometimes capture growing product molecules due to this reversible transfer of esters between NHS groups.



**Figure S6:** Demonstration of transfer of an NHS ester onto DNA-linked NHS. Because of the potential for the NHS group to serve as a nucleophile and attack an NHS ester, the ordered synthesis of a tripeptide cannot be performed efficiently using this transfer scheme. The reaction with NHS-linked DNA **2n** can transfer biotin from **1n** to **3e** but no transfer is seen when **2n** is excluded.

### Test of Template Masking as a Means of Controlling Reactivity

#### Oligonucleotide Sequences Used

Reagent **6**: 5'-CATGAGAAC-NH<sub>2</sub>

Reagent **7**: 5'-CTGTGATGGACCAGAAC-NH<sub>2</sub>

Reagent **8**: 5'-CTGACGGGCTATCGCTACGAAGAAC-NH<sub>2</sub>

Template **9**: 5'-H<sub>2</sub>N-GTTCTCATGGTCCATCACAGTCGTAGCGATAGCCCGTCAG

Mask **10**: 5'-TGTGATGG

Mask **11**: 5'-ACGGGCTATCGCTACG

To demonstrate that oligonucleotide masks such as **10** and **11** can be used to control the reactivity of DNA-linked reagents, we synthesized three DNA-linked phosphoranes (**6**, **7**, **8**) as well as an aldehyde-linked template **9** as previously described<sup>[21]</sup>. The template **9** (at 150 nM) and masks **10** and **11** (at 225 nM) were preannealed in 0.1 M TAPS, pH 8.0, 1 M NaCl, and then transferred to 4 °C, 25 °C, 42 °C, 57 °C, or 72 °C. An equimolar mixture (200 nM in each reagent after addition) of the three phosphorane reagents (**6**, **7**, and **8**) was added and the mixture reacted for 1 h before ethanol precipitation. Identical reactions were performed that excluded masks **10** and **11**. Denaturing PAGE analysis of the reactions demonstrated that, while all three reagents can react at low temperatures without **10** and **11**, only **6** reacts well in the presence of the masks at low temperatures. As temperature increases, the reactivity of **7** is restored and predominates at 42 °C. At the highest temperature (72 °C), only **8** can anneal and react (Figure 3).

### Ordered Tripeptide Sequence

#### Oligonucleotide Sequences Used

Template **12**: 5'-H<sub>2</sub>N-GTTCTCATGGTCCATCACAGTCGTAGCGATAGCCCGTCAG

Reagent **13**: 5'-CATGAGAAC-SH

Mismatched **13b**: 5'-GAACAGAAC-SH

Reagent **14**: 5'-CTGTGATGGACCAGAAC-SH

Mismatched **14b**: 5'-CTGCAAAGACGCAGAAC-SH

Reagent **15**: 5' - CTGACGGGCTATCGCTACGAAGAAC-SH

Complementary oligonucleotide for restriction digestion and MALDI analysis of products linked to template **12**: 5' - CTGTGATGGACCATGAGAAC

Template **12** requires no further modifications and was purified directly using reverse-phase HPLC.

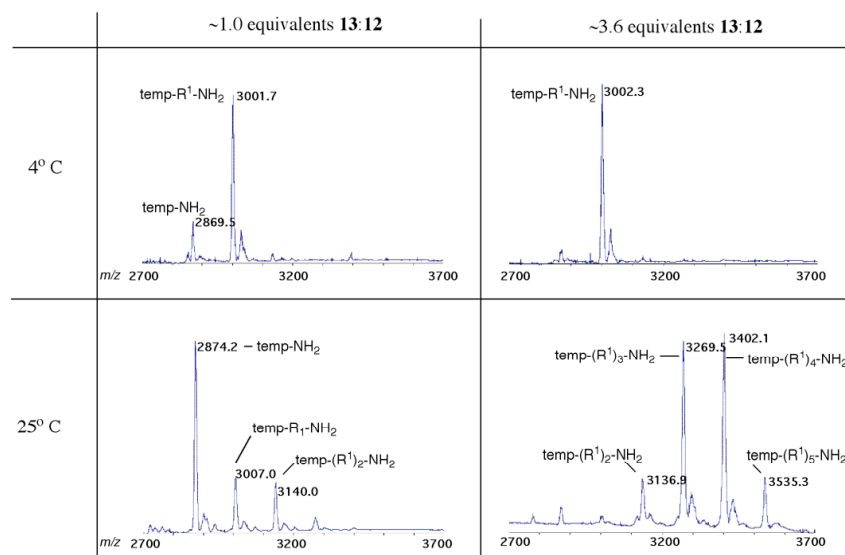
#### *NHS Ester Reagent Preparation*

NHS-linked DNAs were prepared as described above. NVOC-labeled amino acids were prepared using a previously described protocol.<sup>[3]</sup> Briefly, to a mixture of the amino acid and Na<sub>2</sub>CO<sub>3</sub> in H<sub>2</sub>O, an equimolar amount of 4,5-dimethoxy-2-nitrobenzyl chloroformate was added in dioxane. After 1 h at 25 °C, the reaction was quenched with NaHSO<sub>4</sub> and extracted using ethyl acetate. The crude reactions were used directly for labeling of DNA. Amino-terminated oligonucleotides conjugated to the amino acids (using EDC/NHS coupling)<sup>[2]</sup> and purified by reverse-phase HPLC were characterized by MALDI-TOF. Exposure of these oligonucleotides to long wavelength (365 nm) UV light for 30 min resulted in quantitative deprotection as followed by MALDI-TOF.

DNA-linked NHS esters were synthesized as described above using ~0.5 mg of either biotin (for **15**) or the crude NVOC-amino acids derived from 4-transaminocrotonic acid (for **14**) or trans-4-(aminomethyl)-cyclohexanecarboxylic acid (for **13**); coupling yields for these reagents ranged from 50-95%. To prepare unprotected DNA-linked NHS esters **13** and **14**, the NVOC-protected NHS ester reagents were dissolved in 50 mM NaOAc pH 5.0, 1 M NaCl and exposed to 365 nm UV light for 30 min at 4 °C using a hand-lamp. The deprotections proceeded in 90-100% yield; to obtain pure **13** and **14**, the reagents were repurified by reverse-phase HPLC and lyophilized (with 1% TFA).

#### *Reactivity of DNA-Linked NHS Ester Reagents:*

To test the reactivity of the amino acid-linked reagents **13** and **14**, model reactions were performed by preannealing template **12** (100 nM) and masks **10** and **11** (150 nM) and then adding either ~1.0 equiv. or ~3.6 equiv. of **13** in 0.1 M MOPS pH 7.0, 1 M NaCl at both 4 °C and 25 °C for 1 h. The reactions were quenched by the addition of 1 M Tris, pH 8.0 and precipitated with ethanol. The reactions were prepared for MALDI as described above for the Wittig olefination products using a doubly-biotinylated complementary oligonucleotide and analyzed. The MALDI spectra are shown below (Figure S7). As long as the temperature is significantly lower than the T<sub>m</sub> of the reagent, exchange of the reagents does not occur and only a single addition of the amino acid to **12** is seen even with excess reagent. However, at temperatures near the T<sub>m</sub>, exchange of the reagents allows for multiple additions of the same amino acid. For the purposes of an ordered synthesis, only a single equivalent of the building block is desired so low temperature (non-exchanging) conditions are used. This experiment was repeated for reagent **14** and multiple additions were observed after 1 h at and above 42 °C but a single addition of R<sup>2</sup>, even with ≥ 3 equiv. of **14**, is seen at or below 37 °C.



**Figure S7:** Reactivity of NHS-linked amino acid reagents as analyzed by MALDI-TOF. Different numbers of equivalents of **13** were added to **12** (with **10** and **11**) at either 4 °C or 25 °C. At temperatures near the melting temperature of the reagent, exchange of the oligonucleotides leads to multiple additions of a single reagent to the template. At temperatures much lower than  $T_m$ , the reagent, once hybridized, remains in a stable duplex. As a result, even in the presence of an excess of reagent only a single addition of  $R^1$  is observed.

### Three-Step Ordered Tripeptide Synthesis

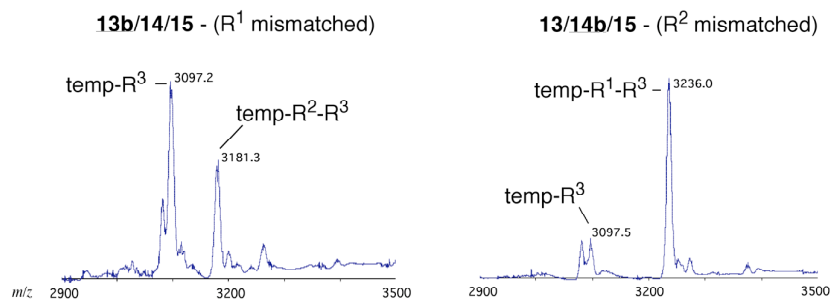
Reagents **13**, **14**, and **15** were prepared as described, redissolved after lyophilization in 50 mM NaOAc, 1 M NaCl, desalted by gel filtration using Sephadex G-25, and quantitated by UV. Template **12** and masks **10**, **11** (1.5 equiv. relative to template) are annealed together at 4 °C in 0.2 M MOPS, pH 7.0, 2 M NaCl. **13** (1.05 eq), **14** (1.05 eq), **15** (3 equiv.) were added to the reaction mixture and reacted at 4 °C for 20 min, 37 °C for 20 min, 62 °C for 2 h. After dilution caused by the addition of the reagents, the final concentration of solutes in this reaction was 0.1 M MOPS, pH 7.0, 1 M NaCl with 100 nM template **12**. Following the 4 °C and 37 °C steps, aliquots of the reaction were quenched by the addition of 1 M Tris, pH 8.0. The final reaction mixture was isolated by streptavidin-linked bead capture and quantitated by denaturing PAGE to give 45% yield of recovered biotinylated template (this yield includes the tripeptide product as well as all truncated byproducts containing biotin). The quenched aliquots and final reaction were prepared for MALDI as previously described with the results shown in Figure 4b. A summary of expected masses of DNA-linked peptide products is provided below (Table S2). Because of potential ionization differences between product species, the relative heights of peaks in the included MALDI spectra may not be representative of the relative amounts of individual species in the product mixtures.

Species	Predicted Mass	Observed Mass
Template-NH <sub>3</sub> (from <b>12</b> )	2871.86	(2870.5 to 2873.2) ± 6 (Fig. 4b, S9; left and middle)
Template-R <sup>2</sup> -NH <sub>3</sub>	2954.90	(2954.5 to 2955.3) ± 6 (Fig. 4b, middle; Fig. S9, left and middle)
Template-R <sup>1</sup> -NH <sub>3</sub>	3010.96	(3009.4 to 3013.7) ± 6 (Fig. 4b, left and middle; Fig. S9, middle)
Template-R <sup>1</sup> -R <sup>2</sup> -NH <sub>3</sub>	3094.00	3095.1 ± 6 (Fig. 4b, middle)
Template-R <sup>2</sup> -R <sup>1</sup> -NH <sub>3</sub>	3094.00	3092.4 ± 6 (Fig. S9, middle)
Template-R <sup>3</sup>	3096.95	(3097.2 to 3097.5) ± 6 (Fig. 4b and S9, right; Fig. S8)
Template-R <sup>2</sup> -R <sup>3</sup>	3179.96	(3179.9 to 3181.4) ± 6 (Fig. 4b and S9, right; Fig. S8, left)
Template-R <sup>1</sup> -R <sup>3</sup>	3236.02	(3236.0 to 3237.6) ± 6 (Fig. 4b, S8, S9; right)
Template-R <sup>1</sup> -R <sup>2</sup> -R <sup>3</sup>	3319.06	3320.8 ± 6 (Fig. 4b, right)
Template-R <sup>2</sup> -R <sup>1</sup> -R <sup>3</sup>	3319.06	3319.0 ± 6 (Fig. S9, right)

**Table S2:** Summary of predicted masses for expected peptide products (in increasing order). The template (**12**) has been digested by *Hla*III to a 9-mer prior to analysis.

### Mismatch Tripeptide Controls

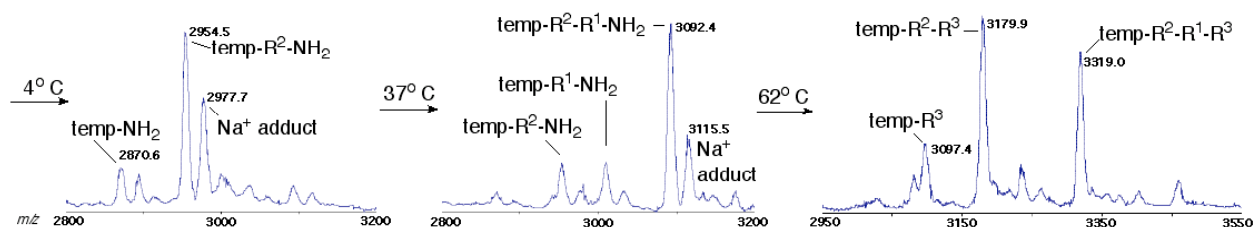
Reagents **13b** and **14b** were prepared in an identical manner as **13** and **14** except that the DNA sequence was scrambled to prevent hybridization. The three-step reaction was performed as above with **13b/14/15** or **13/14b/15** and then analyzed by MALDI. In each case, the building block attached to the mismatched reagent is not incorporated in the product (Figure S8).



**Figure S8:** MALDI-TOF of three-step reactions with mismatched reagents. No incorporation of the building block on the mismatched reagent is detected for either reaction.

### Three-Step Tripeptide Sequence with Swapped Building Block Order

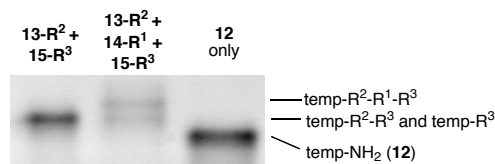
Variants of **13** and **14** (**13-R<sup>2</sup>** and **14-R<sup>1</sup>**) were prepared that attached R<sup>2</sup> to **13** and R<sup>1</sup> to **14**. The three-step sequence was performed as above (with aliquots quenched after the 4 °C and 37 °C steps) and isolated after streptavidin purification in 38% yield as determined by denaturing PAGE. MALDI-TOF revealed that the order of addition of building blocks is now R<sup>2</sup> at 4 °C and R<sup>1</sup> at 37 °C (Figure S9), indicating that the DNA sequence (and not the reactants themselves) determines the order of addition of substrates in this system.



**Figure S9:** MALDI-TOF of the three-step sequence with the building blocks on **13** and **14** swapped. Just as with the sequence shown in Figure 2b, the building block on **13** (in this case  $R^2$ ) adds first at 4 °C and then the building block on **14** (in this case  $R^1$ ) adds at 37 °C. Differences in MALDI ionization may lead to the lower overall signal for the tripeptide product relative to the truncated dipeptide for the final product mixture.

### Quantitation of the Tripeptide in the Three-Step Sequence

While the two major products ( $R^1$ - $R^2$ - $R^3$  and  $R^1$ - $R^3$ ) of the three-step sequence in Figure 4a could not be resolved by denaturing PAGE, the products of the  $R^2$ - $R^1$ - $R^3$  sequence were separable. Denaturing PAGE was performed on the streptavidin-purified products of a three-step reaction including **13**- $R^2$ , **14**- $R^1$ , and **15** as well as a control reaction with just **13**- $R^2$  and **15** that can produce only the dipeptide  $R^2$ - $R^3$  and mono-peptide  $R^3$ . While the **13**- $R^2$ /**15** reaction runs as a single band, the **13**- $R^2$ /**14**- $R^1$ /**15** product runs as two bands representing the major tripeptide product and the truncated products lacking  $R^1$ . From quantitation of these bands, ~55% of the final isolated material is the tripeptide (Figure S10). We expect a similar overall purity for the  $R^1$ - $R^2$ - $R^3$  sequence but, as stated above, cannot resolve the products of this reaction for a direct quantitation.



**Figure S10:** PAGE analysis of  $R^2$ - $R^1$ - $R^3$  tripeptide sequence. While the reaction that excludes the second reagent (**14**- $R^1$ ) runs as a single band, containing both  $temp$ - $R^2$ - $R^3$  and  $temp$ - $R^3$ , the reaction with all three reagents runs as two bands with the upper band representing the tripeptide product with  $R^1$ . Based on densitometry analysis, the tripeptide represents 55% of the products in the final isolated mixture.

### Supporting Information References:

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- [2] Z. J. Gartner, M. W. Kanan, D. R. Liu, *Angew. Chem. Int. Ed.* **2002**, *123*, 1796-1800.
- [3] S. A. Robertson, J. A. Ellman, P. G. Schultz, *J. Am. Chem. Soc.* **1991**, *113*, 2722-2729.