Synthetic Methods

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

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The use of DNA templates to direct chemical reactions enables the principles of biological evolution to be applied to the discovery of synthetic molecules and new chemical reactions.[1] In addition, the use of DNA hybridization to modulate the effective molarity of DNA-linked reactants allows synthetic molecules to be manipulated in ways that cannot be accessed using traditional synthetic methods.[2] For example, many oligomeric natural products, including proteins, nonribosomal peptides, and polyketides, are biosynthesized in a strictly ordered manner even though all of their constituent building blocks are simultaneously present in the cellular milieu.[3] Nature achieves single-solution ordered multistep synthesis by increasing the effective molarity of specific sets of reactants at precise moments during biosynthesis. Compared to the strategy most frequently used by chemists to execute ordered multistep synthesis—dividing the construction of a molecule into a sequence of isolated reaction steps—nature's single-solution approach is not only remarkably elegant and efficient but also sufficiently selective to obviate the need for protecting groups.

In the absence of enzymes, ordered multistep synthesis in a single solution has proven to be a challenge. The ordered oligomerization of nucleotides on nucleic acid templates[1a,4] has been achieved, but these methods have not allowed the synthesis of non-nucleic acid structures. Tamura and Schimmel[5] have used RNA-templated synthesis to direct peptide-bond formation in an order determined by intrinsic differences in substrate reactivity. Relying on substrate reactivity differences, however, imposes significant constraints on the order of building blocks within the possible products. Even with precisely tuned reactivities, multistep syntheses generally still require sequential additions of reactants to form ordered products. Herein, we report the ordered multistep syntheses of both a triolefin and a tripeptide using DNA-linked substrates of comparable intrinsic reactivity that are simultaneously present in one solution. In both cases, temperature-sensitive variations in DNA secondary structure orchestrate a series of effective molarity changes among four reactants to primarily generate one ordered product out of many possibilities. These results represent two strategies for ordered synthesis in a single solution without the structural constraints imposed by enzymes and can significantly enhance the efficiency and selectivity of multistep DNA-templated synthesis.[6]

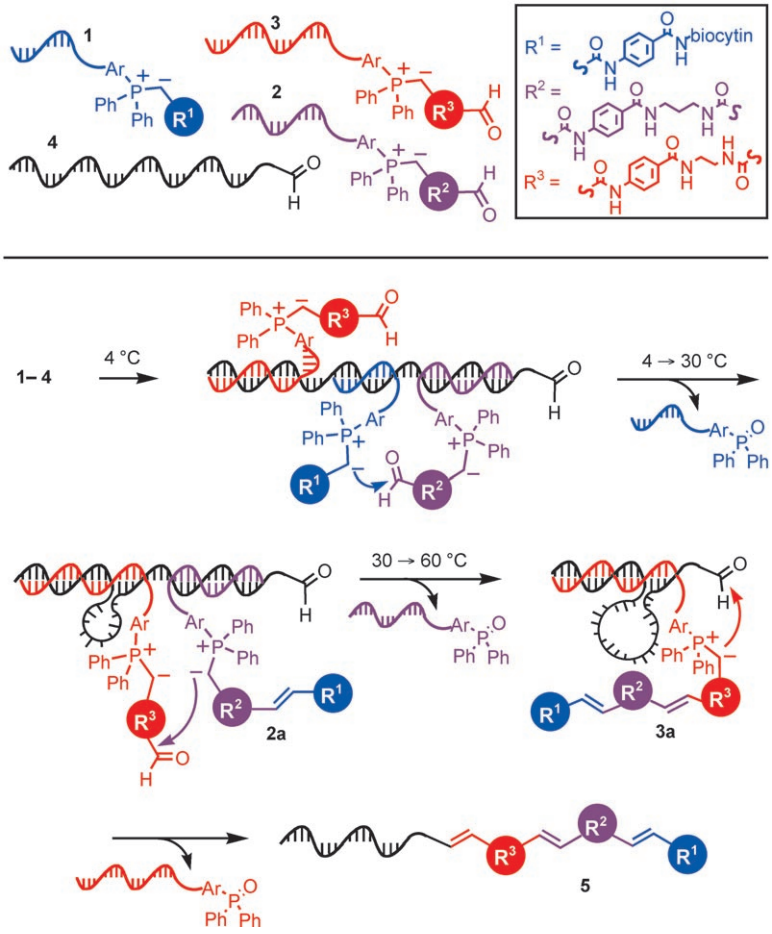

Figure 1. Strategy for the single-solution synthesis of an ordered triolefin. Building blocks are transferred sequentially among phosphorane reagents **1**–**3** before addition to an aldehyde-linked template **4**. The rigidity of double-stranded DNA enforces Wittig olefination regioselectivity. As the reaction temperature is elevated, the DNA secondary structure undergoes sequence-programmed changes that enables the desired Wittig olefination to take place selectively.

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The first strategy (Figure 1) passes synthetic intermediates from site to site on a template in a manner controlled by changes in the DNA secondary structure. Three Wittig olefination substrates (**1**–**3**, with **1** containing a biotin group),[7] each linked to DNA oligonucleotides of varying melting temperature (T_m), were prepared and hybridized to an aldehyde-terminated DNA template **4** at 4 °C. If these substrates were combined at the high concentrations (mM–M) typical of organic synthesis, a complex mixture of many

products would result from their random reaction. At a concentration of 100 nM each, however, only substrates juxtaposed in a productive way by DNA hybridization react at a significant rate.[8] Upon phosphonium deprotonation, seven intermolecular Wittig olefinations were in principle possible among these four reactants. At the lowest temperature (4 °C), however, six of the seven possible Wittig reactions are precluded because their reactants are separated by double-stranded DNA. As we previously demonstrated, the rigidity of double-stranded DNA can enforce the separation of substrates that flank duplex DNA, thus preventing their reaction.[8] At 4 °C, the only phosphorane–aldehyde pair not separated by the duplex DNA was **1** and **2**, which reacted to generate DNA-linked monoolefin **2a**.

As the temperature was elevated to 30 °C, the species with the lowest T_m value (the phosphine oxide of **1**) dissociated from the template, thus allowing reactants **2a** and **3** to react to form diolefin **3a**. When the temperature was increased to 60 °C, the phosphine oxide of **2** dissociated, which enabled the final reaction to take place between **4** and **3a** to generate ordered triolefin **5** (Figure 1).

Following the capture of R^1-linked products with immobilized streptavidin, we obtained triolefin **5** in 24% overall yield (Figure 2a, lanes C and D). As expected, captured products lacking the R^1 group were not detected. Truncated products represented less than 10% of the isolated material, as analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) analysis. To confirm the order of the building blocks in the products, control reactions were performed with reactants lacking aldehyde groups. Removing the aldehyde group from **2** prevented the reaction of **1** with **2** and resulted in a diolefin template-R^3-$R^{2\prime}$ product (Figure 2a, lane B). Removing the aldehyde group from both **3** and **2** prevented all reactions except for the reaction of **3** with template **4** to generate the monoolefin template–$R^{3\prime}$ (Figure 2a, lane A). MALDI-TOF mass-spectroscopic analysis was consistent with the expected product structures (Figure 2b).

We repeated the ordered triolefin synthesis and control reactions using reagents in which the R^2 and R^3 groups were interchanged on **2** and **3**. The resulting products contained the expected R^2-R^3-R^1 building-block order (Figure 2c). Consistent with our reactivity model, control reactions using sequence-mismatched reagents **1c**, **2c**, or **3c** resulted in either no transfer of the biotinylated R^1 group to **4** (using **1c** or **3c**) or in the direct reaction of **1** and **4** (using **2c**; see the Supporting Information). Collectively, these results demonstrate that the order of Wittig olefination in this system is tightly controlled by sequence-programmed changes in the DNA secondary structure and is not determined by intrinsic reactivity differences among the substrates.

Our second target was the synthesis of an ordered tripeptide from a single solution containing three *N*-hydroxysuccinimidyl (NHS) ester activated amino acids. Because the DNA-linked NHS by-product of amine acylation is capable of transesterification with NHS esters (see the Supporting Information), the transfer of building blocks between

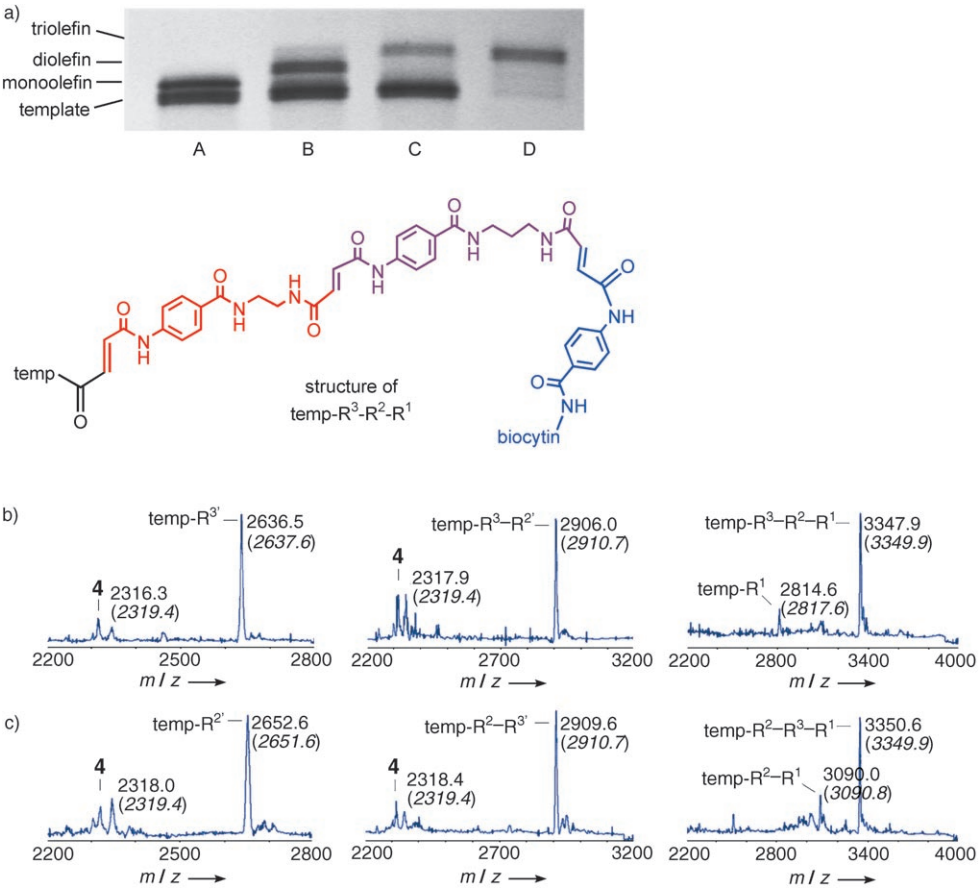

Figure 2. a) Denaturing PAGE analysis of the ordered triolefin synthesis. Conditions: 100 nM of **1**–**4** were hybridized in aqueous 50 mM NaOAc (pH 5.0), 1 M NaCl; treated with 0.1 M *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS; pH 8.0), and 1 M NaCl; and incubated for 1 h at 4 °C, 1 h at 30 °C, and 2 h at 60 °C. The crude reaction mixture is shown in lane C with the streptavidin-captured product in lane D. Control reactions lacking an aldehyde group on both **2** and **3** (lane A) or on **2** only (lane B) were performed under identical conditions to produce either a monoolefin or diolefin, respectively. b) MALDI-TOF mass-spectroscopic data of products from reactions shown in (a). The three spectra correspond, from left to right, to lanes A, B, and D from (a), respectively. c) MALDI-TOF mass-spectroscopic data for the reactions using swapped building blocks (R^3 attached to **2**, and R^2 attached to **3**). Expected masses for (b) and (c) are listed in parentheses; the expected error is ±6 Da. The prime designation ($R^{2\prime}$ and $R^{3\prime}$) in (b) and (c) refers to the forms of these building blocks lacking aldehyde groups (see the text). temp = template **4**.

reagents as in Figure 1 is not possible without significant loss of the desired product through capture by DNA-linked NHS groups. Instead, a different strategy is required that allows three consecutive transfers from three NHS ester reagents to a growing template-linked molecule. We hypothesized that oligonucleotide masks designed to selectively block the hybridization of DNA-linked reagents at certain temperatures could be used to achieve this goal (Figure 3a). Indeed,

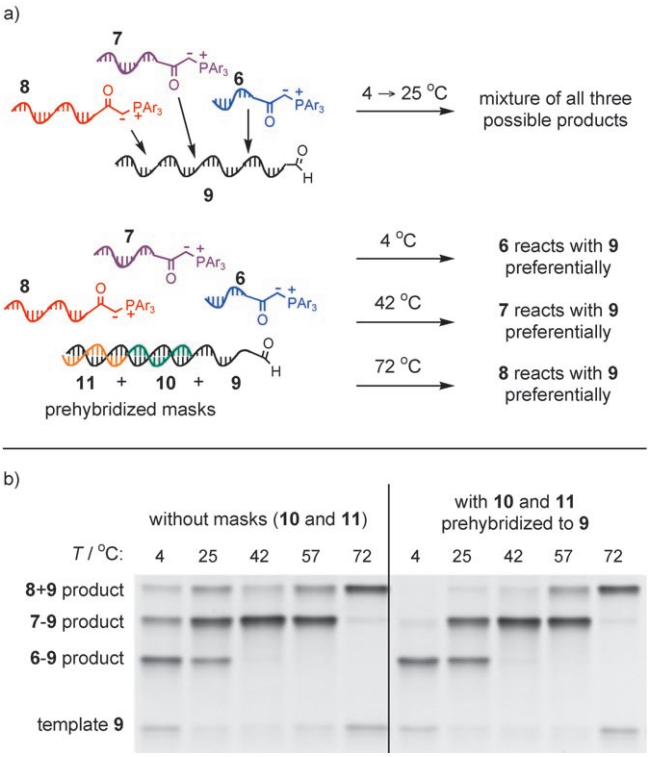

Figure 3. a) Strategy for using oligonucleotide masks **10** and **11** to order the reaction of three reagents **6**–**8** with a template (**9**). When all masks are hybridized (4 °C), only **6** can react. At an intermediate temperature (42 °C), mask **10** is melted from the template allowing **7** to react exclusively. At a high temperature (72 °C), only **8** can react. b) Denaturing PAGE analysis of the reactions in (a). Conditions: 150 nM of **9** (with or without masks **10** and **11** at 225 nM) was incubated at the indicated temperature. Reagents **6**–**8** were added simultaneously to 200 nM each and the reaction was incubated 1 h before analysis.

although a mixture of all possible products was formed when DNA-linked phosphoranes **6**–**8** were added simultaneously to aldehyde-linked template **9**, when masks **10** and **11** were prehybridized to the template before the addition of **6**–**8**, reactivity became strongly dependent on the reaction temperature approaching or exceeding the T_m value of each mask (25–30 °C for **10** and 60–65 °C for **11**; Figure 3b).

Using this approach, we performed a single-solution ordered tripeptide synthesis (Figure 4a). An amine-terminated template **12** was prehybridized with masks **10** and **11** at 4 °C. When NHS ester linked reagents **13**–**15** were combined with the masked template **10** + **11** + **12** at 4 °C for 20 min, only **13** could hybridize to the template and react to generate a monopeptide. As the solution was heated to 37 °C for 20 min, both the expended first reagent and the first mask **10** dissociated from the template, thus exposing the binding site for the second reagent **14**, which then hybridized and reacted to generate a template-linked dipeptide. At the highest temperature (62 °C for 2 h), all masks and expended reagents were melted except for **15**, which reacted to form the ordered tripeptide **16**. Unlike the first strategy in which incomplete reactivity reduces overall yield but does not generate truncated biotinylated by-products, this strategy requires each step to proceed in high yield to generate the tripeptide (and not truncated mono- or dipeptides) as the major product.

Aliquots of the reaction mixture taken after incubations at 4 and 37 °C were quenched with 1M tris(hydroxymethyl)aminomethane (Tris). Analysis by MALDI-TOF-MS (Figure 4b) revealed that **13** reacts exclusively with the template at 4 °C, followed by the reaction of **14** at 37 °C. Biotinylated templates that arise from the reaction of **15** were isolated using streptavidin-linked beads in 45% yield, as determined by denaturing PAGE, and analyzed by MALDI-TOF-MS (Figure 4b). The strongest signal in the purified product mixture is the desired tripeptide **16** with the most significant side product being the dipeptide R^1–R^3 which results from the lack of reaction with **14**. Sequence-mismatched reagents **13b** or **14b** were unable to react in place of **13** or **14** (see the Supporting Information).

When the oligonucleotides linked to the R^1 and R^2 groups were swapped, the order of building-block addition was also switched (R^2 added first at 4 °C, then R^1 added at 37 °C; see the Supporting Information). The biotinylated products of this reaction sequence, including the ordered tripeptide **12**-R^2-R^1-R^3, were isolated in 38% yield after streptavidin purification. For this reaction, the desired tripeptide and truncated side products were resolvable by denaturing PAGE (unlike in the case of **16**), revealing that 55% of the isolated material (21% total overall yield) is the ordered tripeptide (see the Supporting Information). Taken together, these findings indicate that the use of temperature-controlled template masking enables substrates that would normally form a vast mixture of oligomeric products to react in a predominantly ordered manner.

These results represent single-solution ordered multistep syntheses using comparably reactive substrates in the absence of enzymes with only a temperature gradient needed to coordinate the timing of three successive reactions. Both strategies offer faster, higher-yielding routes to multistep DNA-linked products constructed from autocleaving reagents[6] than past examples (indeed, a previous DNA-templated tripeptide synthesis using three discrete steps proceeded in 3% overall yield).[6] In addition to enabling a mode of chemical reactivity that is not possible in a conventional synthesis format, these concepts may also facilitate the design of additional systems that mimic the efficiency and selectivity of organic synthesis in the cell without the structural constraints imposed by enzymes.

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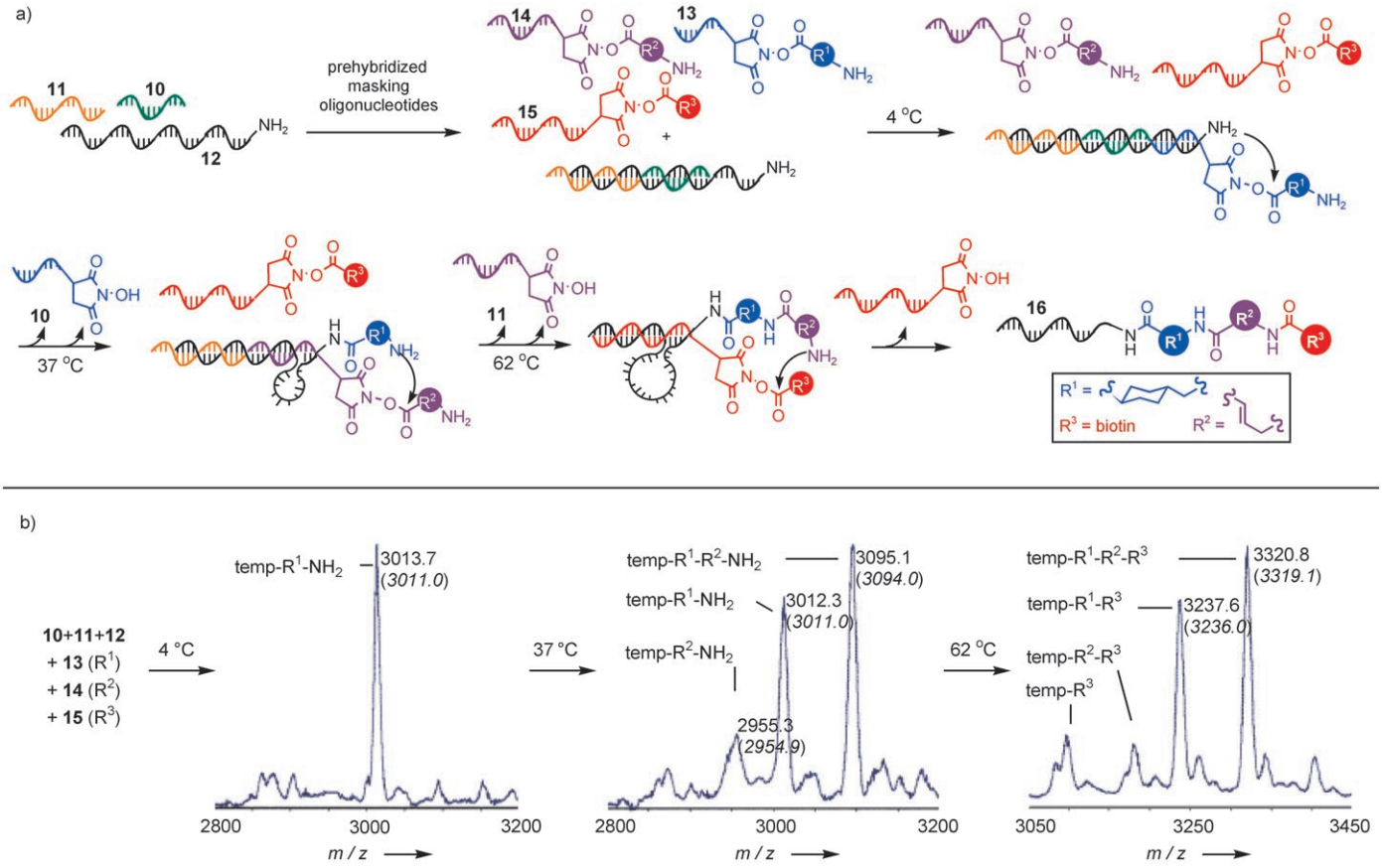

Figure 4. a) Strategy for the single-solution synthesis of an ordered tripeptide using oligonucleotide masks. b) MALDI-TOF mass spectroscopy of each stage of the reaction in (a). Conditions: 200 nM **12** was prehybridized to 1.5 equivalents each of **10** and **11** in 0.2 M 3-(*N*-morpholino)-propanesulfonic acid (MOPS, pH 7.0), 2 M NaCl at 4 °C. Simultaneously, 1.05 equivalents of **13** and **14** and 3.0 equivalents of **15** were added to **10**+**11**+**12**. After dilution caused by the addition of the reagents, the final concentration of solutes in this reaction mixture was 0.1 M MOPS (pH 7.0), 1 M NaCl with 100 nM template **12**. The reaction mixture was incubated at 20 min at 4 °C, 20 min at 37 °C, and 2 h at 62 °C. Reactions were quenched with Tris (after either of the first two steps) or purified with streptavidin-linked beads (after the third step) before analysis. Expected masses for (b) are listed in parentheses; the expected error is ±6 Da. temp = template **12**.

Keywords: biomimetic synthesis · DNA · regioselectivity · synthetic methods · template synthesis

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