

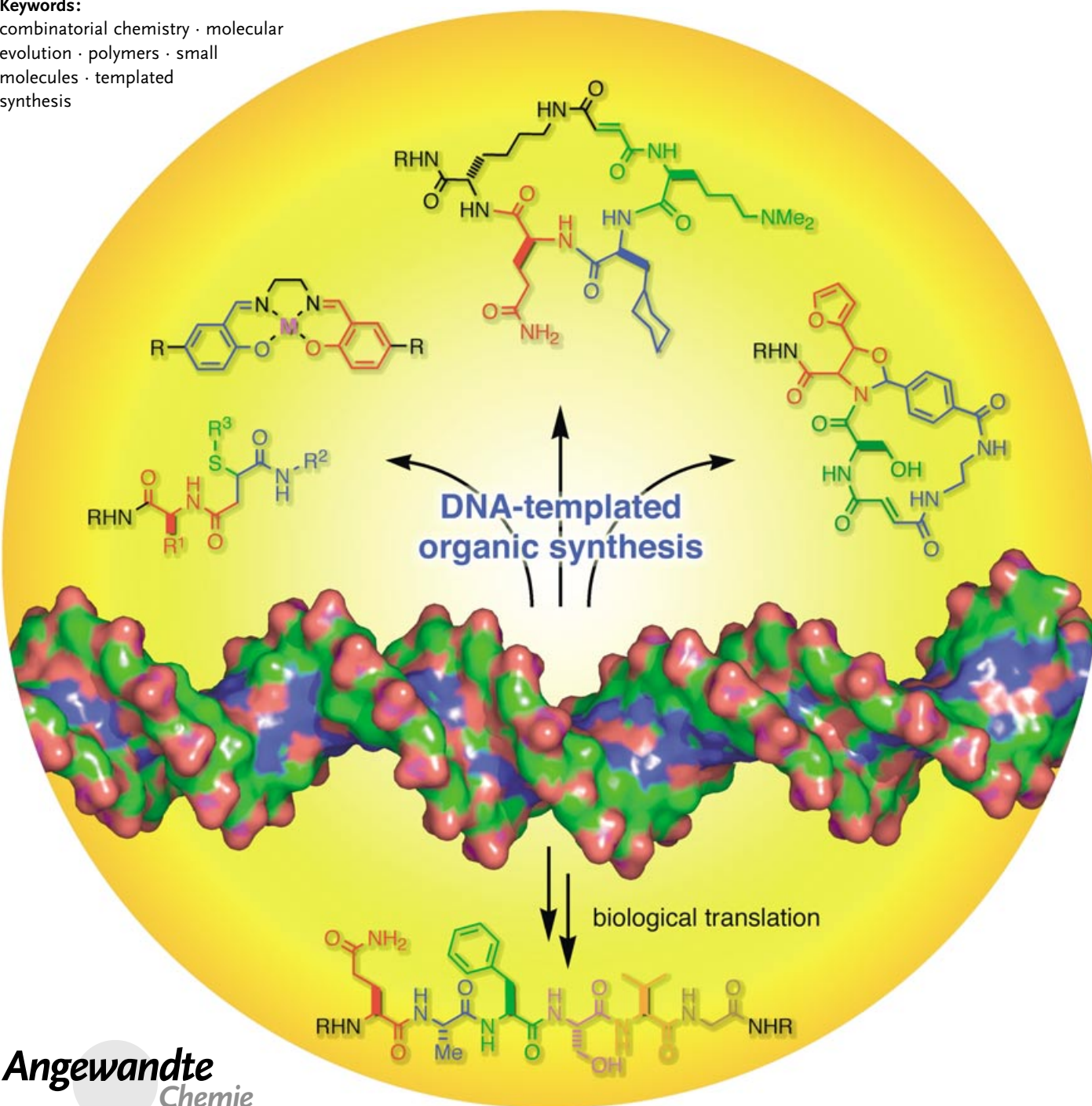
## Synthetic Methods

# DNA-Templated Organic Synthesis: Nature's Strategy for Controlling Chemical Reactivity Applied to Synthetic Molecules\*\*

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**Keywords:**

combinatorial chemistry · molecular evolution · polymers · small molecules · templated synthesis



Angewandte  
Chemie

*In contrast to the approach commonly taken by chemists, nature controls chemical reactivity by modulating the effective molarity of highly dilute reactants through macromolecule-templated synthesis. Nature's approach enables complex mixtures in a single solution to react with efficiencies and selectivities that cannot be achieved in conventional laboratory synthesis. DNA-templated organic synthesis (DTS) is emerging as a surprisingly general way to control the reactivity of synthetic molecules by using nature's effective-molarity-based approach. Recent developments have expanded the scope and capabilities of DTS from its origins as a model of prebiotic nucleic acid replication to its current ability to translate DNA sequences into complex small-molecule and polymer products of multistep organic synthesis. An understanding of fundamental principles underlying DTS has played an important role in these developments. Early applications of DTS include nucleic acid sensing, small-molecule discovery, and reaction discovery with the help of translation, selection, and amplification methods previously available only to biological molecules.*

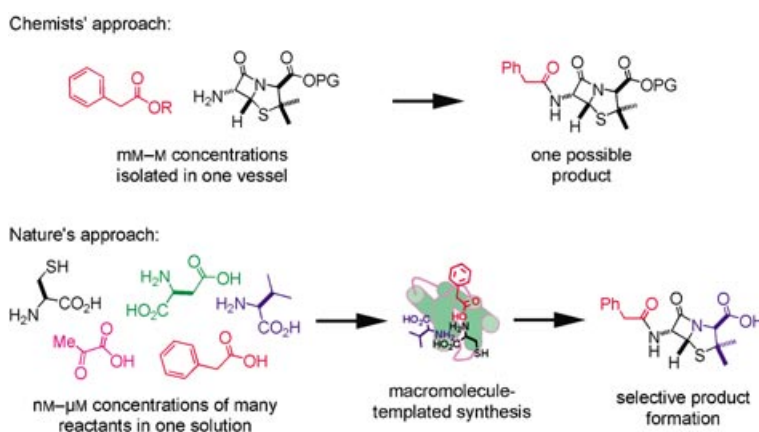
## 1. Introduction

The control of chemical reactivity is a ubiquitous and central challenge of the natural sciences. Chemists typically control reactivity by combining a specific set of reactants in one solution at high concentrations (typically mM to M). In contrast, nature controls chemical reactivity through a fundamentally different approach (Figure 1) in which thousands of reactants share a single solution but are present at concentrations too low (typically nM to  $\mu$ M) to allow random intermolecular reactions. The reactivities of these molecules are directed by macromolecules that template the synthesis of necessary products by modulating the effective molarity of reactive groups and by providing catalytic functionality (Figure 2 shows several examples). Nature's use of effective molarity to direct chemical reactivity enables biological reactions to take place efficiently at absolute concentrations that are much lower than those required to promote efficient laboratory synthesis and with specificities that cannot be achieved with conventional synthetic methods.

Among nature's effective-molarity-based approaches to controlling reactivity, nucleic acid templated synthesis plays a central role in fundamental biological processes, including the replication of genetic information, the transcription of DNA into RNA, and the translation of RNA into proteins. During ribosomal protein biosynthesis, nucleic acid templated reactions effect the translation of a replicable information carrier into a structure that exhibits functional properties beyond that of the information carrier. This translation enables the expanded functional potential of proteins to be combined with the powerful and unique features of nucleic acids

## From the Contents

1. Introduction	4849
2. The Reaction Scope of DNA-Templated Synthesis	4850
3. Expanding the Synthetic Capabilities of DNA-Templated Synthesis	4854
4. DNA-Templated Polymerization	4858
5. Toward a Physical Organic Understanding of DNA-Templated Synthesis	4860
6. Applications of DNA-Templated Synthesis	4863
7. Summary and Outlook	4867
8. Abbreviations	4868



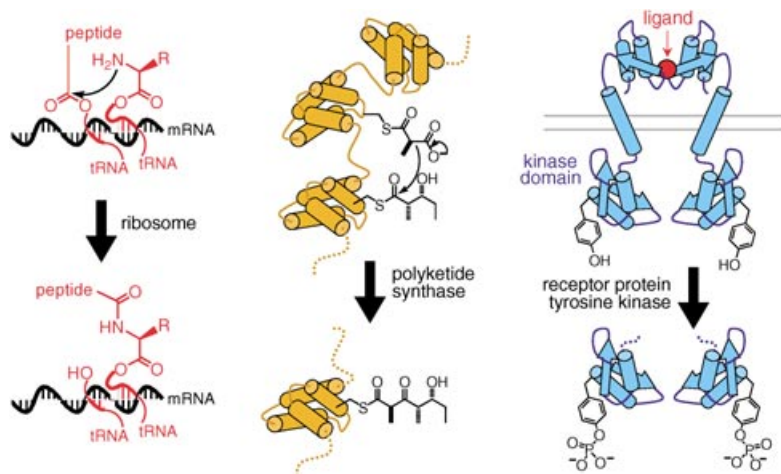
**Figure 1.** Two approaches to controlling chemical reactivity.

including amplifiability, inheritability, and the ability to be diversified. The extent to which primitive versions of these processes may have been present in a prebiotic era is widely debated,<sup>[1–12]</sup> but most models of the precell world include some form of template-directed synthesis.<sup>[1,2,13–26]</sup>

In addition to playing a prominent role in biology, nucleic acid templated synthesis has also captured the imagination of chemists. The earliest attempts to apply nucleic acid tem-

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[\*\*] Section 8 of this article contains a list of abbreviations.



**Figure 2.** Examples of effective-molarity-based control of bond formation and bond breakage in biological systems.

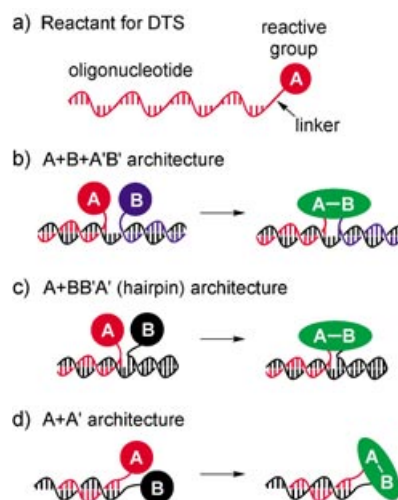
plated synthesis to nonbiological reactants used DNA or RNA hybridization to accelerate the formation of phosphodiester bonds or other structural mimics of the nucleic acid backbone.<sup>[1,14,24–41]</sup> More recently, researchers have discovered the ability of DNA-templated organic synthesis to direct the creation of structures unrelated to the nucleic acid backbone.<sup>[42–48]</sup> A growing understanding of the simple but powerful principles underlying DTS has rapidly expanded its synthetic capabilities and has also led to emerging chemical and biological applications, including nucleic acid sensing,<sup>[27–30,49–60]</sup> sequence-specific DNA modification,<sup>[61–80]</sup> and the creation and evaluation of libraries of synthetic molecules.<sup>[44,47,81,82]</sup>

Herein we describe representative early examples of nucleic acid templated synthesis and more recent developments that have enabled DNA templates to be translated into increasingly sophisticated and diverse synthetic molecules. We then analyze our current understanding of key aspects of DTS, describe applications that have emerged from this understanding, and highlight remaining challenges in using DTS to apply nature's strategy for controlling chemical reactivity to molecules that can only be accessed through laboratory synthesis.

## 2. The Reaction Scope of DNA-Templated Synthesis

A reactant for DTS consists of three components (Figure 3 a): 1) a DNA oligonucleotide that modulates the effective molarity of the reactants but is otherwise a bystander, 2) a reactive group that participates in the DNA-templated chemical reaction, and 3) a linker connecting the first two components. When two DTS reactants with complementary oligonucleotides undergo DNA hybridization, their reactive groups are confined to the same region in space, increasing their effective concentration.

The extent to which the effective molarity of DNA-linked reactive groups increases upon DNA hybridization could depend in principle on several factors. First, the absolute concentration of the reactants is critical. For a DNA-templated reaction to proceed with a high ratio of templated to nontemplated product formation, reactants must be sufficiently dilute (typically nM to  $\mu$ M) to preclude significant random intermolecular reactions, yet sufficiently concentrated to enable complementary



**Figure 3.** a) The three components of a reactant for DTS. b)–d) Template architectures for DTS. A/B and A'/B' refer to reactants containing complementary oligonucleotides, and + symbols indicate separate molecules.



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oligonucleotides to hybridize efficiently. Second, the precision with which reactive groups are aligned into a DNA-like conformation could influence the increase in effective molarity upon DNA hybridization. It is conceivable, for example, that only those reactions that proceed through transition states consistent with the conformation of duplex DNA may be suitable for DTS. Recent studies have evaluated the importance of each of these factors and revealed the reaction scope of DTS. Additional factors influencing the effective molarity of reactive groups in DTS are analyzed in Section 3.

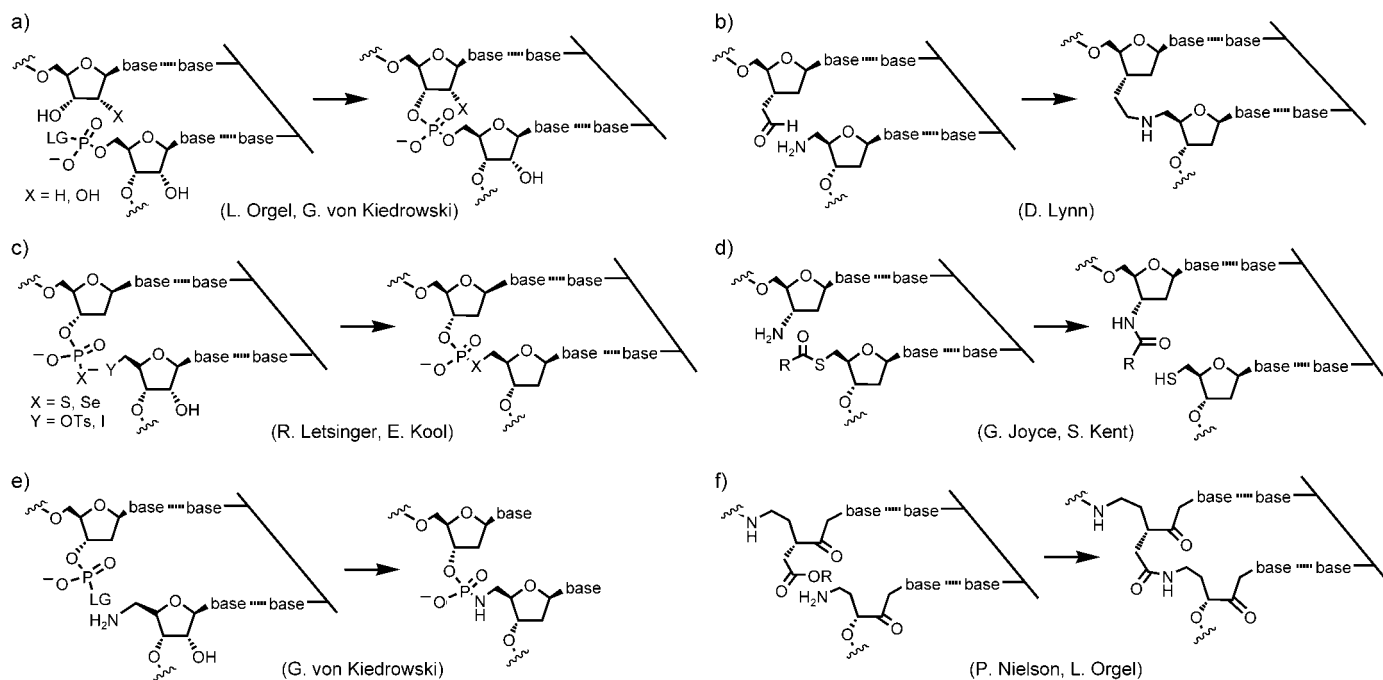
### 2.1. Nucleic Acid templated Synthesis of Nucleic Acids and Nucleic Acid Analogues

Nucleic acid templated syntheses prior to the current decade predominantly used DNA or RNA templates to mediate ligation reactions that generate oligomers of DNA, RNA, or structural analogues of nucleic acids (Figure 4).<sup>[1,14,24–41,70,83,84]</sup> Since there are several excellent articles<sup>[1,31,37,42,61]</sup> on the DTS of nucleic acids and their analogues, we summarize only a few key examples below. In these cases, the reactive groups were usually functionalities already present in the oligonucleotides or oligonucleotide analogues, and linkers were often absent. The template architecture used to support these DNA-templated reactions most frequently placed the site of reaction at the center of a nicked DNA duplex (Figure 3b). The reactive groups in these examples mimic the structure of the DNA backbone during product formation.

The first report of a nucleic acid templated nucleotide ligation was the observation of Naylor and Gilham in 1966<sup>[13]</sup> that a poly(A) template could direct the formation of a native

phosphodiester bond between the carbodiimide-activated 5' phosphate of (pT)<sub>6</sub> and the 3' hydroxy group of a second (pT)<sub>6</sub> molecule (5% yield). Several examples of DNA- or RNA-templated oligonucleotide syntheses have since been reported (Figure 4), including Orgel's pioneering work on nucleic acid templated phosphodiester formation between 2-methylimidazole-activated nucleic acid monomers and oligomers (Figure 4a),<sup>[1,85–87]</sup> Nielson's and Orgel's RNA-templated amide formation between PNA oligomers (Figure 4f),<sup>[24]</sup> Joyce's DNA-templated peptide–DNA conjugation (Figure 4d),<sup>[84]</sup> von Kiedrowski's carbodiimide-activated DNA coupling<sup>[88]</sup> and amplification of phosphoramidate-containing DNA (Figure 4e),<sup>[14]</sup> Lynn's DNA-templated reductive amination and amide formation between modified DNA oligomers (Figure 4b),<sup>[31–39,83,84]</sup> Eschenmoser's nucleic acid templated TNA ligations,<sup>[89–91]</sup> and Letsinger's and Kool's DNA- and RNA-templated phosphothioester and phosphoselenoester formation (Figure 4c).<sup>[26–30,40,41]</sup> Oligonucleotide analogues have also served as templates for nucleotide ligation reactions. Orgel and co-workers used HNA, a non-natural nucleic acid containing a hexose sugar (see Figure 16), as a template for the ligation of RNA monomers through activated phosphate coupling,<sup>[92]</sup> while Eschenmoser and co-workers have shown that nonnatural pyranosyl-RNA can template the coupling of complementary pyranosyl-RNA tetramers through phosphotransesterification with 2',3'-cyclic phosphates.<sup>[93]</sup>

In addition to analogues of the phosphoribose backbone, products that mimic the structure of stacked nucleic acid aromatic bases have also been generated by DTS (Figure 5). Photoinduced [2+2] cycloaddition, typically involving the C5–C6 double bond of pyrimidines, has served as the most common reaction for the DTS of base analogues. One of the



**Figure 4.** Representative DNA-templated syntheses of oligonucleotide analogues.<sup>[1,14,24–41]</sup> LG: leaving group.

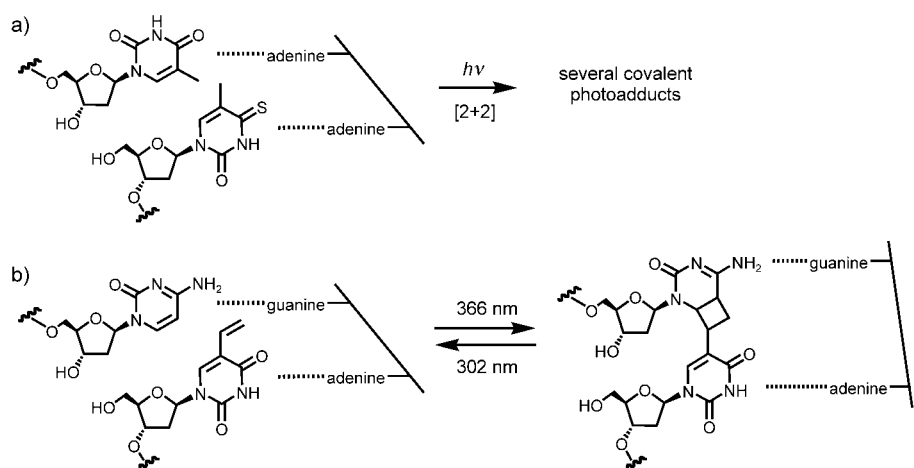


Figure 5. DNA-templated photoinduced [2+2] cycloaddition reactions.<sup>[94–101]</sup>

first examples was the DNA-templated formation of a thymine dimer by irradiation at  $> 290$  nm described by Lewis and Hanawalt.<sup>[94]</sup> DNA-templated photoligations between thymidine and 4-thiothymidine have also been reported (Figure 5 a).<sup>[95]</sup> Other photoreactive groups used in DNA-templated [2+2] cycloaddition reactions include coumarins,<sup>[96]</sup> psoralens,<sup>[97]</sup> and stilbenes.<sup>[98–100]</sup> Recently, Fujimoto, Saito, and co-workers described a reversible DNA-templated photoligation—photocleavage mediated by [2+2] cycloaddition between adjacent pyrimidine bases, one of them modified with a 5-vinyl group (Figure 5 b).<sup>[101]</sup>

The products of the templated nucleotide ligation reactions described above are structurally similar to the nucleic acid backbone and typically preserve the six-bond spacing between nucleotide units or the relative disposition of adjacent aromatic bases. An implicit assumption underlying these studies is that a DNA-templated reaction proceeds efficiently when the DNA-linked reactive groups are positioned adjacently and the transition state of the reaction is similar to the structure of native DNA.

## 2.2. DNA-Templated Synthesis of Products Unrelated to the DNA Backbone

While structural mimicry of the DNA backbone may maximize the effective concentration of the template-organized reactants, it severely constrains the structural diversity and potential properties of products generated by nucleic acid templated reactions. The use of DTS to synthesize structures not necessarily resembling nucleic acids is therefore of special interest and has been a major focus of research in the field of template-directed synthesis since 2001.

Our group probed the structural requirements of DTS by studying DNA-templated reactions that generate products unrelated to the DNA backbone.<sup>[44]</sup> A series of conjugate addition and substitution reactions

between a variety of nucleophilic and electrophilic groups (Figure 6) were found to proceed efficiently at absolute reactant concentrations of  $60$  nM.<sup>[44]</sup> In contrast, products were not formed when the sequences of reactant oligonucleotides were mismatched (noncomplementary). These findings established that the effective molarity of two reactive groups linked to one DNA double helix can be sufficiently high that their alignment into a DNA-like conformation is not needed to achieve useful reaction rates.<sup>[44]</sup> This conclusion is consistent with simple geometric models of effective molarity. For example, confining two reactive groups to  $< 10$  Å separation—achievable by conjugating them to the 5' and 3' ends of

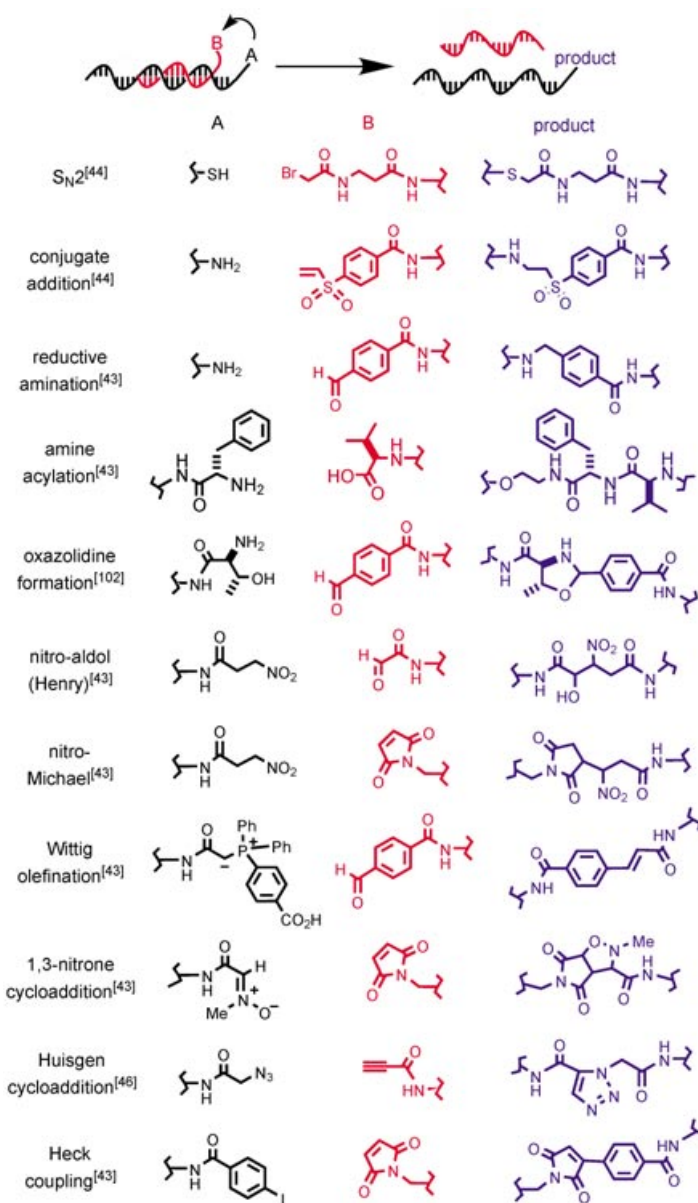


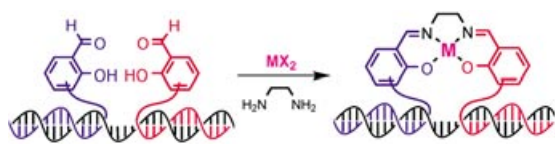
Figure 6. DNA-templated reactions that generate products not resembling nucleotides.<sup>[43,44,46,102]</sup>

hybridized oligonucleotides—can correspond to an effective molarity of  $> 1\text{M}$ .

We also compared the ability of two distinct DNA template architectures to mediate DTS. Both a hairpin template architecture (A+BB'A', a closed form of the A+B+A'B' architecture that enables products to remain covalently linked to templates, see Figure 3c) and a linear A+A' template architecture (Figure 3d) were found to mediate efficient product formation.<sup>[44]</sup> The A+A' architecture is especially attractive because the corresponding reactants are the simplest to prepare. Furthermore, the oligonucleotide portion of the A+A' architecture is less likely to influence the outcome of a DTS beyond simple modulation of the effective molarity compared with a hairpin or A+B+A'B' arrangement in which the reaction site is flanked on both sides by DNA (see Section 5.3).

Following the discovery that DNA mimicry is not a requirement for efficient DTS, our group extended the reaction scope of DTS to include many types of reactions, the majority of which were not previously known to take place in a nucleic acid templated format.<sup>[43,44]</sup> Conjugate additions of thiols and amines to maleimides and vinyl sulfones,  $\text{S}_{\text{N}}2$  reactions, amine acylation, reductive amination,<sup>[43,44]</sup>  $\text{Cu}^{\text{I}}$ -mediated Huisgen cycloaddition,<sup>[46]</sup> and oxazolidine formation<sup>[102]</sup> were found to proceed efficiently and sequence specifically with a DTS format using the A+A' template architecture (Figure 6).<sup>[43]</sup> Several useful carbon–carbon bond formation reactions were also successfully transitioned into a DTS format, including the nitro-aldol addition (Henry reaction), nitro-Michael addition, Wittig olefination, Heck coupling, and 1,3-dipolar nitron cycloaddition (Figure 6).<sup>[43,44]</sup> These transformations included the first carbon–carbon bond forming reactions other than photoinduced cycloaddition that are templated by a nucleic acid. The Pd-mediated Heck coupling was the first example of a DNA-templated organometallic reaction. Czapinski and Sheppard reported the DTS of metallosalens (Figure 7).<sup>[45]</sup> Two salicylaldehyde-linked DNA strands were brought together by a complementary DNA template in the A+B+A'B' architecture. Metallosalen formation occurred in the presence of ethylenediamine and  $\text{Ni}^{2+}$  or  $\text{Mn}^{2+}$ . Gothelf, Brown, and co-workers recently applied this reaction to the DNA-templated assembly of linear and branched conjugate structures (see Section 3.3).<sup>[103]</sup>

Collectively, these studies have conclusively demonstrated that DTS can maintain sequence-specific control over the effective molarity even when the structures of



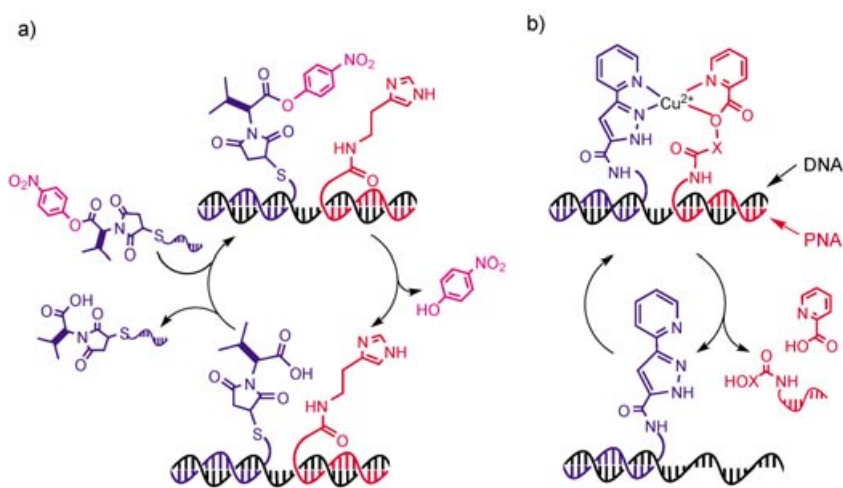
**Figure 7.** DNA-templated assembly of metallosalen–DNA conjugates ( $\text{M} = \text{Ni}^{2+}$  or  $\text{Mn}^{2+}$ ).

reactants and products are unrelated to that of nucleic acids. The array of reactions now known to be compatible with DTS, while modest compared with the compendium of conventional synthetic transformations developed over the past two centuries, is sufficiently broad to enable the synthesis of complex and diverse synthetic structures programmed entirely by a strand of DNA (see Sections 3.2 and 3.3).

### 2.3. DNA-Templated Functional Group Transformations

The examples described above used DNA hybridization to mediate the coupling of two DNA-linked reactive groups. While coupling reactions are especially useful for building complexity into synthetic molecules, functional group transformations are also important components of organic synthesis. A few DNA-templated functional group transformations have recently emerged.

Ma and Taylor used a 5'-imidazole-linked DNA oligonucleotide and the A+B+A'B' architecture for the DNA-templated hydrolysis of a 3'-*p*-nitrophenyl ester linked oligonucleotide (Figure 8a).<sup>[49]</sup> The initial product of the templated reaction, an imidazolyl amide linked at both ends to DNA, undergoes rapid hydrolysis to generate the free



**Figure 8.** DNA-templated functional group transformations.<sup>[49,104]</sup> X in (b):  $\text{OCH}_2\text{CH}_2$ .

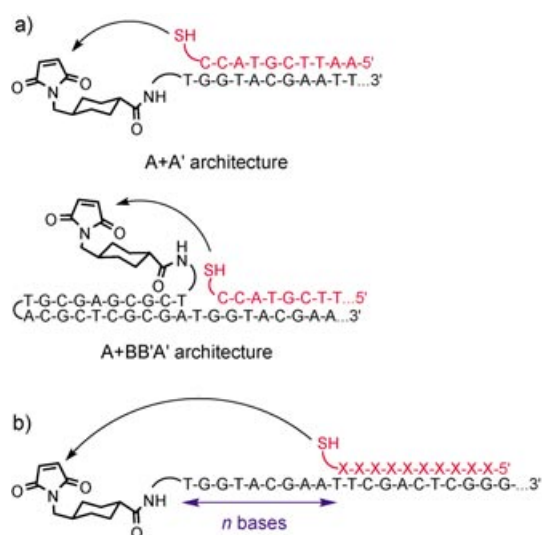
carboxylic acid. The net outcome of this reaction is the DNA-templated functional group transformation of a *p*-nitrophenyl ester into a carboxylic acid. Ma and Taylor demonstrated that the template can dissociate from the product-linked DNA strand after ester hydrolysis and can participate in additional rounds of catalysis with other ester-linked oligonucleotides. Brunner, Kraemer, and co-workers recently developed a conceptually related DNA-templated functional group transformation that uses DNA templates to mediate a  $\text{Cu}^{2+}$ -catalyzed aryl ester cleavage (Figure 8b).<sup>[104]</sup> In this first example of templated catalysis involving DNA-linked metal complexes, DNA-linked aryl esters are transformed into alcohols.

### 3. Expanding the Synthetic Capabilities of DNA-Templated Synthesis

Together with the above efforts to broaden the reaction scope of nucleic acid templated synthesis, several recent insights and developments have significantly enhanced the synthetic capabilities of DTS. These findings include 1) DTS between reactive groups separated by long distances, 2) multistep DTS in which the product of a DNA-templated reaction is manipulated to serve as the starting material for a subsequent DNA-templated step, 3) the design of template architectures that increase the types of reactions which can be performed in a DNA-templated format, 4) synthesis templated by double-stranded DNA, and 5) new modes of controlling reactivity made possible by DTS that cannot be achieved with conventional synthetic methods.

#### 3.1. Distance-Independent DNA-Templated Synthesis

The ability of DNA hybridization to direct the synthesis of molecules that do not mimic the DNA backbone suggests that functional group adjacency may not be necessary for efficient DTS. Our group evaluated the efficiency of simple DNA-templated conjugate addition and nucleophilic substitution reactions as a function of the number of intervening single-stranded template bases between hybridized reactive groups (Figure 9).<sup>[44]</sup> Surprisingly, for both reactions tested, apparent second-order rate constants of product formation did not significantly change when the distance between hybridized reactive groups was varied from one to thirty bases (Figure 9). Reactions exhibiting this behavior were designated “distance-independent”. Replacement of the intervening single-stranded DNA bases with a variety of DNA analogues or with duplex DNA demonstrated that efficient long-distance templated synthesis requires a flexible intervening region, but



**Figure 9.** Distance-independent DNA-templated synthesis. a) Two distinct architectures that can support distance-independent DTS. b) A DTS reaction exhibits distance independence if the rates of product formation are comparable for a range of values of *n*.<sup>[43,44]</sup>

does not require a backbone structure specific to DNA. A significant fraction of the DNA-templated reactions studied by our group to date have demonstrated at least some distance independence.<sup>[43,44]</sup>

Distance-independent DTS is initially puzzling in light of both the expected decrease in effective molarity as a function of distance and the notorious difficulty of forming macrocycles,<sup>[105,106]</sup> but is in part explained by the ability of DNA hybridization to elevate the effective molarity to the point that bond formation for some reactions is no longer rate determining. Indeed, subsequent kinetic studies revealed that DNA hybridization, rather than covalent bond formation between reactive groups, is rate determining in distance-independent DTS.<sup>[44]</sup> Additional factors contributing to efficient long-distance DTS are discussed in Section 5.1.

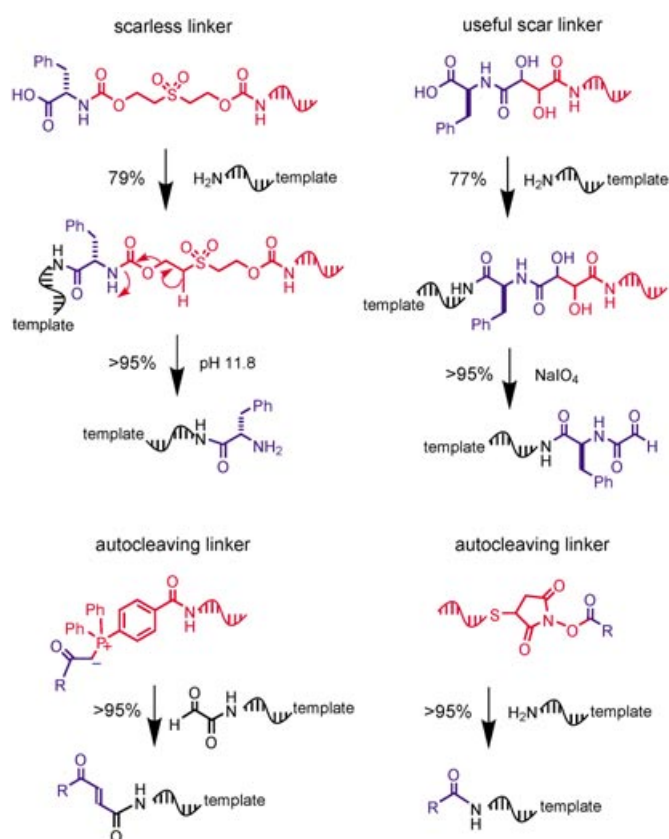
#### 3.2. Multistep DNA-Templated Synthesis

Synthetic molecules of useful complexity typically must be generated through multistep synthesis. The discovery of distance-independent DTS was an important advance toward the DNA-templated construction of complex synthetic structures because it raised the possibility of using a single DNA template to direct multiple chemical reactions on progressively elaborated products.

Our group achieved this goal by developing a series of linker and purification strategies that enable the product of a DNA-templated reaction to undergo subsequent DNA-templated steps. The major challenges were to develop general solutions for separating the DNA portion of a DTS reagent from the synthetic product after DNA-templated coupling has taken place (Figure 10), and to develop methods appropriate for pmol-scale aqueous synthesis that enable the products of DNA-templated reactions to be purified away from unreacted templates and reagents.

Integrating the resulting developments, we used DNA templates containing three 10-base coding regions to direct three sequential steps of two different multistep DNA-templated synthetic sequences.<sup>[47]</sup> Both a nonnatural tripeptide generated from three successive DNA-templated amine acylation reactions (Figure 11 a) and a branched thioether generated from an amine acylation–Wittig olefination–conjugate addition series of DNA-templated reactions (Figure 11 b) were prepared. These studies are the first examples of translating DNA through a multistep reaction sequence into synthetic small-molecule products.

Following these syntheses, the development of additional DNA-templated reactions, linker strategies, and template architectures (see Section 3.3) has enabled the multistep DTS of increasingly sophisticated structures. For example, we used recently developed DNA-templated oxazolidine formation, a new thioester-based linker, and the second-generation template architectures described in Section 3.3 to translate DNA templates into monocyclic and macro-bicyclic *N*-acyloxazolidines (see Figure 13).<sup>[102]</sup> While the first products of multistep DTS are modest in complexity compared with many targets of conventional organic synthesis, these initial examples already suggest that sufficient complexity and structural diversity can



**Figure 10.** Three linker strategies for DNA-templated synthesis.<sup>[47]</sup> Cleavage of a “useful scar linker” generates a functional group that serves as a substrate in subsequent steps. A “scarless linker” is cleaved without introducing additional unwanted functionality. An “autocleaving linker” is cleaved as a natural consequence of the reaction.

be generated to yield DNA-templated compounds with interesting biological or chemical properties.

### 3.3. New Template Architectures for DNA-Templated Synthesis

The DNA-templated reactions described above use one of three template architectures (Figure 3):  $A+A'$ ,  $A+B+A'B'$ , or the hairpin form of the latter ( $A+BB'A'$ ). The predictability of DNA secondary structures suggests the possibility of rationally designing additional template architectures that further expand the synthetic capabilities of DTS.

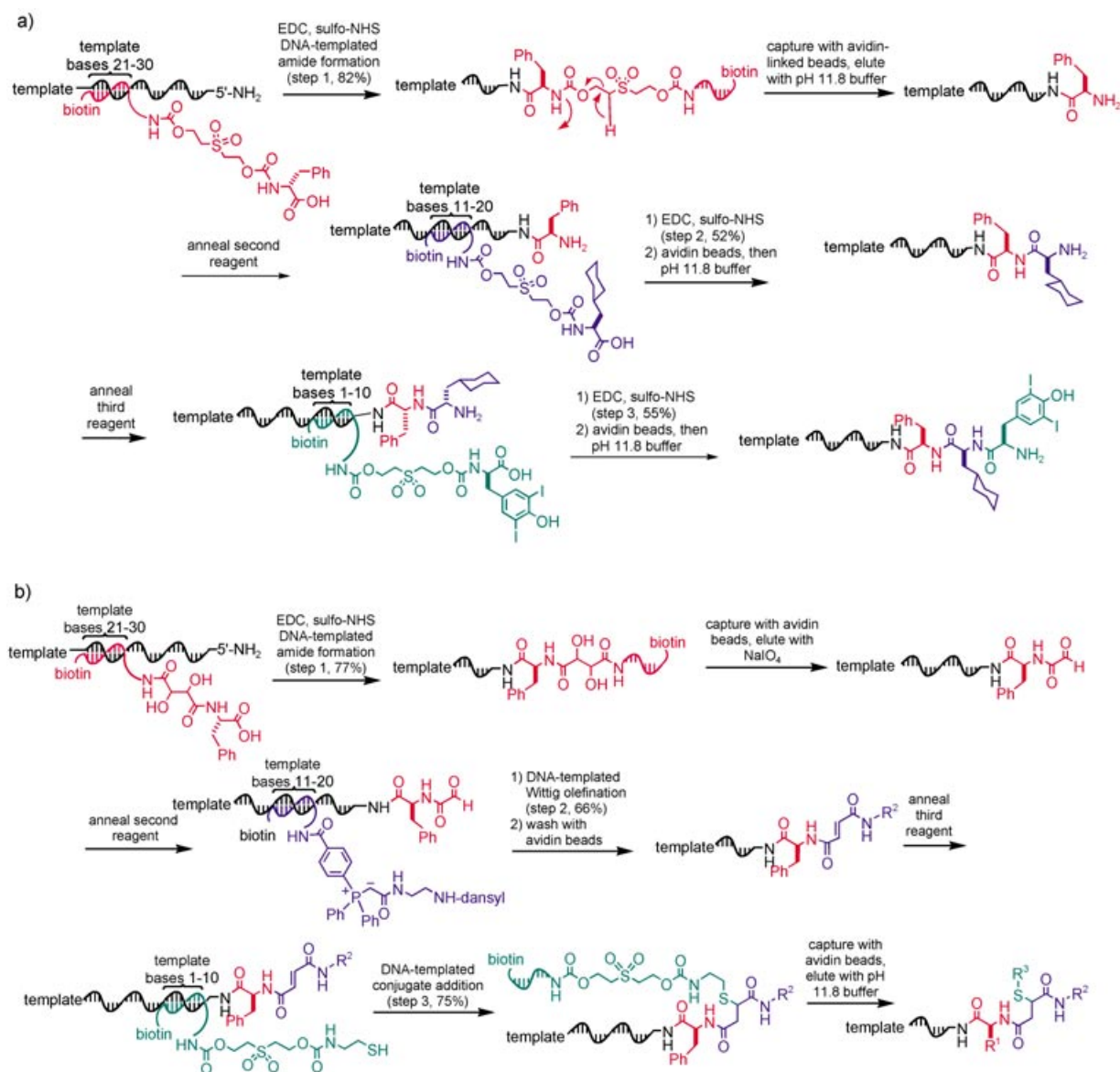
The distance dependence of some DNA-templated reactions (for example, nitron–olefin dipolar cycloaddition or reductive amination reactions) limits their use in multistep DTS because each of the three template architectures listed above can accommodate at most one distance-dependent reaction (by using the template bases closest to the reactive group). Our group developed a new template architecture that enables normally distance-dependent reactions to proceed efficiently when encoded by template regions far from the reactive group. Distance dependence was overcome by using three to five constant bases at the reactive end of the template to complement a small number of constant bases at

the reactive end of the DNA-linked reagent (Figure 12).<sup>[46]</sup> This arrangement, the omega ( $\Omega$ ) architecture, made efficient distance-dependent reactions possible even when they were encoded by bases far from the reactive end of the template. Importantly, sequence specificity is preserved in the  $\Omega$  architecture despite the presence of invariant complementary bases near the reactive groups because the favorable energetics of hybridizing the constant bases barely offset the entropic penalty of ordering the template bases separating the reactive groups (Figure 12a).<sup>[46]</sup> In principle, any DNA-templated reaction can be encoded anywhere along a template of length comparable to those studied (up to  $\sim 40$  bases) by using the  $\Omega$  architecture.

A second template architecture developed in our group allows three reactive groups to undergo a DNA-templated reaction together in a single step.<sup>[46]</sup> The efficient reaction of three groups in a single location on a DNA template is difficult in the  $A+A'$ ,  $A+B+A'B'$ , or  $A+BB'A'$  template architectures because the rigidity of duplex DNA is known to inhibit DTS between reactive groups separated by double-stranded template–reagent complexes (Figure 12b).<sup>[44]</sup> Relocating the reactive group from the end of the template to the non-Watson–Crick face of a nucleotide in the middle of the template enables two DNA-templated reactions involving three reactive groups to take place in a single DTS step (Figure 12a,c). This “T” architecture was used to generate a cinnamide in one step through DNA-templated substitution reaction and Wittig olefination of DNA-linked phosphane,  $\alpha$ -iodoamide, and aldehyde groups. In a second example, we used the T architecture to synthesize a triazolylalanine from DNA-linked amine, alkyne, and azide groups through amine acylation and  $\text{Cu}^I$ -mediated Huisgen cycloaddition (Figure 12c).<sup>[46]</sup> As some DNA polymerases used in PCR tolerate template appendages on the non-Watson–Crick face of nucleotides,<sup>[107]</sup> the complete information within a T architecture template could be amplified by PCR.

These two second-generation template architectures were essential components of recent multistep DNA-templated syntheses of monocyclic and bicyclic *N*-acyloxazolidines (Figure 13).<sup>[102]</sup> Beginning with an amine-linked T template, we used an  $\Omega$  architecture-assisted long-distance DNA-templated amine acylation to generate T-linked amino alcohols. In the second step, DNA-templated oxazolidine formation was effected by recruiting DNA-linked aldehydes to the 3' arm of the amino alcohol linked T templates. The instability of the resulting oxazolidines required that the final reaction, the oxazolidine N acylation, takes place in the same step as the oxazolidine formation. The N acylation was therefore directed by the 5' arm of the T template. Linker and purification strategies, involving sulfone and thioester cleavage and biotin-based affinity capture and release, provided the DNA-linked *N*-acyloxazolidine in Figure 13a.<sup>[102]</sup> A modified version of this synthesis was also implemented; it uses sulfone, phosphane, and diol linkers and ends with a Wittig macrocyclization, providing the bicyclic *N*-acyloxazolidine shown in Figure 13b.<sup>[102]</sup>

Eckardt, von Kiedrowski, and co-workers recently achieved the DNA-templated formation of three hydrazone groups simultaneously by combining a branched Y-shaped



**Figure 11.** Multistep DNA-templated synthesis of a) a synthetic tripeptide and b) a branched thioether. Only one of the possible thiol addition regioisomers is shown in (b). R<sup>1</sup>: CH<sub>2</sub>Ph; R<sup>2</sup>: (CH<sub>2</sub>)<sub>2</sub>NH-dansyl; R<sup>3</sup>: (CH<sub>2</sub>)<sub>2</sub>NH<sub>2</sub>; dansyl: 5-(dimethylamino)naphthalene-1-sulfonyl.<sup>[47]</sup>

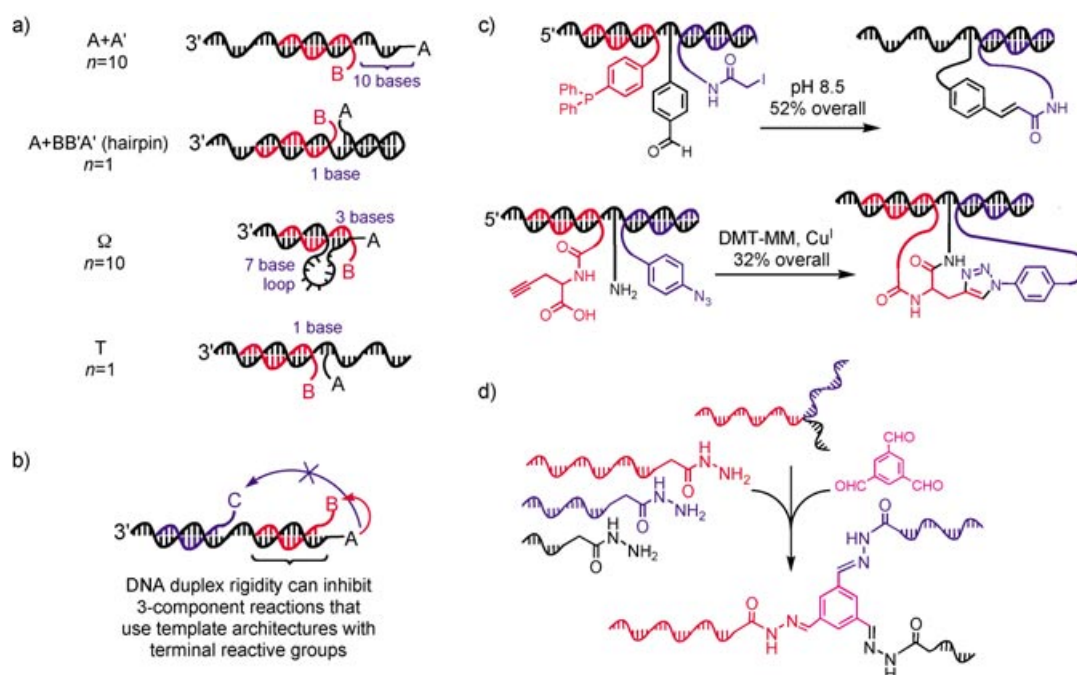
DNA template with three complementary hydrazide-linked oligonucleotides and free trimesaldehyde (Figure 12d).<sup>[108]</sup> The branched nature of the template was copied into the Y-shaped product, demonstrating the nucleic acid templated replication of nonlinear connectivity. The complete sequence information and connectivity within a branched template, however, cannot easily be copied using polymerase-based reactions such as PCR and therefore such a template may be better suited for the replication of branched structures than for applications that require decoding of complete template information (see Section 6). The Y template architecture was also used by Gothelf, Brown, and co-workers to assemble branched conjugated polyenes linked by metallosalen groups.<sup>[103]</sup>

The six template architectures described above (A+A', A+B+A'B', A+BB'A', hairpin, Ω, T, and Y) are important developments in DTS because they expand the arrangements

of template sequences and reactive groups that can lead to efficient DNA-templated product formation. In some cases,<sup>[102]</sup> the synthesis of a target molecule is only possible with a particular template architecture. The feasibility of generating novel DNA architectures in a predictable manner<sup>[109–118]</sup> suggests that increasingly sophisticated template architectures will continue to expand the synthetic capabilities of DTS.

### 3.4. Synthesis Templated by Double-Stranded DNA

The examples described above all use single-stranded templates to bind complementary oligonucleotides linked to reactive groups by Watson–Crick pairing. Double-stranded DNA can also serve as a template for DTS by using either the major or the minor groove to bind reactants.<sup>[119,120]</sup> Luebke



**Figure 12.** Architectures for DNA-templated synthesis. a) Representative examples of  $A+A'$ ,  $A+BB'A'$  (hairpin),  $\Omega$ , and  $T$  architectures. b) Duplex template regions can preclude multiple DNA-templated reactions on a single template in one step. c) Two DNA-templated reactions on a single template in one solution mediated by the  $T$  architecture.<sup>[46]</sup> d) A Y-shaped template mediates tris-hydrazone formation.<sup>[108]</sup>

and Dervan reported duplex-DNA-templated 3',5'-phosphodiester formation between two DNA oligomers designed to bind adjacently in the major groove of a double-stranded template through Hoogsteen base pairing.<sup>[119]</sup> The resulting triplex DNA product differs from the products of DNA-templated nucleic acid synthesis described in Section 2.1 in that the sequence of the third strand is neither identical nor complementary (in a Watson–Crick sense) with that of the template.

Li and Nicolaou developed a self-replicating system that uses both double- and single-stranded DNA to template phosphodiester formation (Figure 14a).<sup>[15]</sup> An  $A+A'$  double helix templated the synthesis of a third strand through triplex formation. Because  $A$  was a palindromic sequence, this third-strand product was identical to  $A$ . The newly synthesized  $A$  then dissociated from the  $A+A'$  duplex and templated the formation of its complement ( $A'$ ) from two smaller oligonucleotides to provide a second-generation  $A+A'$  duplex that is ready to enter the next round of replication.<sup>[15]</sup> This cycle requires that replicating sequences be palindromic for the third-strand product to be identical to one of the two duplex strands. As with all triplex-based systems, these approaches are limited to homopurine:homopyrimidine templates.

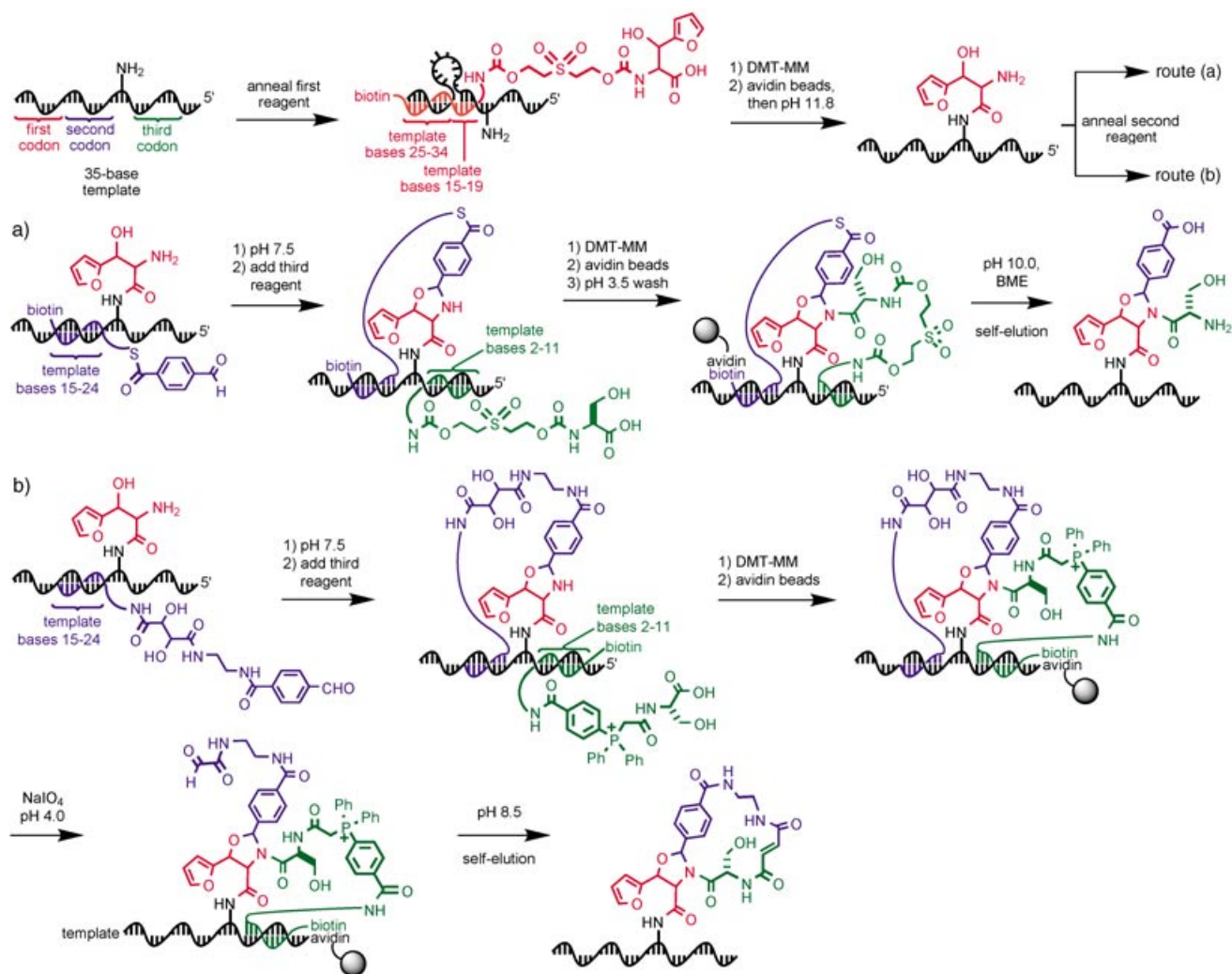
A duplex-DNA-templated synthesis mediated by minor-groove rather than major-groove binding was recently reported by Poulin-Kerstien and Dervan.<sup>[120]</sup> Hairpin polyamides containing  $N$ -methylpyrrole and  $N$ -methylimidazole groups are known to bind to duplex DNA in the minor groove sequence specifically.<sup>[121]</sup> When conjugated to azide and alkyne functionalities, two adjacent hairpin polyamides undergo duplex-DNA-templated Huisgen cycloaddition<sup>[122–126]</sup> to provide a branched polyamide that spans both

minor-groove binding sites and shows greater affinity than either of the polyamide reactants (Figure 14b). The reaction exhibits strong distance dependence, consistent with the rigidity of duplex templates<sup>[44]</sup> compared with the flexibility of single-stranded DNA that can enable distance-independent DTS.<sup>[44]</sup> This distance dependence may prove useful in the self-assembly of small molecules that target double-stranded DNA sequence specifically since both the spacing between binding sites and their sequences must be optimal for efficient coupling.

### 3.5. New Modes of Controlling Reactivity Enabled by DNA-Templated Synthesis

The use of effective molarity to direct chemical reactions enables nature to control reactivity in ways that are not possible in conventional laboratory synthesis. Primary amino groups, for example, undergo amine acylation during peptide biosynthesis, form imines during biosynthetic aldol reactions, and serve as leaving groups during ammonia lyase catalyzed eliminations—all in the same solution and in a substrate-specific manner. In contrast, under conventional synthetic conditions, amine acylation, imine formation, and amine elimination reactions cannot simultaneously take place in a controlled manner without the spatial separation of each set of reactants.

DTS enables synthetic molecules containing functional groups of similar reactivity to also undergo multiple, otherwise incompatible reactions in the same solution. We demonstrated this mode of controlling reactivity by performing (in one solution) three reactions of maleimides (amine



**Figure 13.** Translation of DNA into *N*-acyloxazolidines. Route (a): mutistep DNA-templated synthesis of a monocyclic *N*-acylated oxazolidine; route (b): multistep DNA-templated synthesis of a bicyclic *N*-acylated oxazolidine.<sup>[102]</sup> BME: 2-sulfanylethanol.

addition, thiol addition, and nitro-Michael addition) which generated exclusively three sequence-programmed products out of nine possible products.<sup>[126]</sup> Similarly, two aldehyde coupling reactions (reductive amination and Wittig olefination) were performed in one solution, and three amine reactions (amine acylation, reductive amination, and maleimide addition) were also performed in a separate single solution to afford only the desired DNA-templated products.<sup>[126]</sup> Finally, all six of the above reactions were performed simultaneously by combining twelve DNA-linked reactive groups in a single solution (Figure 15). Even though the combination of these reactants in a conventional synthesis would lead to the formation of at least 28 possible products, the DNA-templated reactions exclusively generated the six sequence-programmed products shown in Figure 15.<sup>[126]</sup>

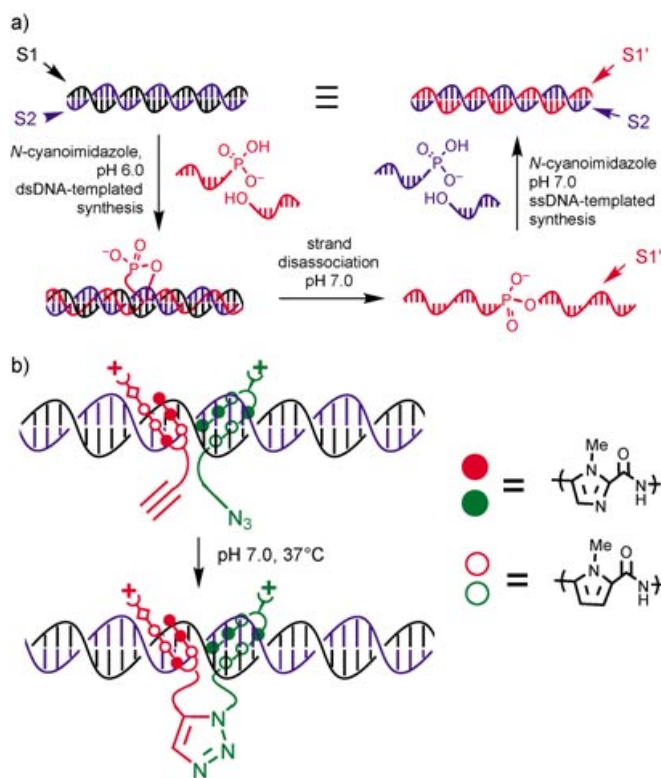
These findings also suggest that DTS may enable the diversification of synthetic small-molecule libraries in a single solution by using different reaction types without the efforts or constraints associated with spatial separation. This strategy in principle can achieve some of the goals of recent diversity-oriented library syntheses (most notably, the work of

Schreiber and co-workers to introduce skeletal diversity into small-molecule libraries<sup>[127]</sup>, but without the requirement of pre-encoding skeletal information within substrate groups. As with any DTS strategy, however, reactions used in this approach must be compatible with the mildly electrophilic and mildly nucleophilic groups present in DNA, and all non-DNA-linked reactants must be mutually compatible.

Finally, it has been recently shown (see the *Note Added in Proof* at the end of this article) that DTS enables heterocoupling reactions to take place between substrates that preferentially homocouple under conventional synthesis conditions. Exclusive heterocoupling is possible in a DNA-templated format because the effective molarity of the heterocoupling partners is much greater than the absolute concentration of any single homocoupling-prone substrate.

#### 4. DNA-Templated Polymerization

DNA- and RNA-templated phosphodiester formation and amine acylation reactions are iterated in nature to



**Figure 14.** Duplex-DNA-templated synthesis. a) Replication of palindromic double-stranded DNA by using both single-stranded-DNA- and double-stranded-DNA-templated phosphodiester formation.<sup>[15]</sup> b) Double-helical-DNA-templated dimerization of polyamides through sequence-specific minor-groove binding.<sup>[120]</sup>

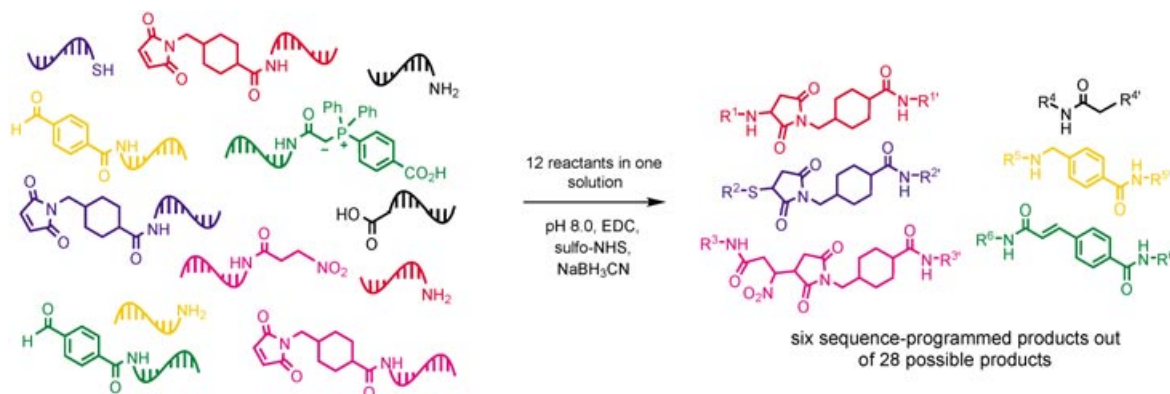
biosynthesize functional macromolecules. The efficient laboratory synthesis of sequence-defined synthetic heteropolymers of similar length to functional proteins and nucleic acids remains a daunting challenge. DNA polymerases,<sup>[128–133]</sup> RNA polymerases,<sup>[134–137]</sup> and the ribosomes<sup>[138–142]</sup> are known to tolerate modified building blocks thus enabling the incorporation of modified nucleic acid bases and amino acids into nucleic acid and protein polymers, respectively. Natural enzymes for generating biopolymers, however, typically do not accept monomers containing nonnatural backbones, although as a notable exception, Chaput and Szostak recently

reported the ability of Deep Vent(exo-) DNA polymerase to extend a DNA primer by three  $\alpha$ -L-TNA nucleotides.<sup>[143]</sup> Nucleic acid templated polymerization has therefore attracted the interest of organic chemists because it may provide access to sequence-defined synthetic heteropolymers free from constraints imposed by polymerase or ribosome acceptance.

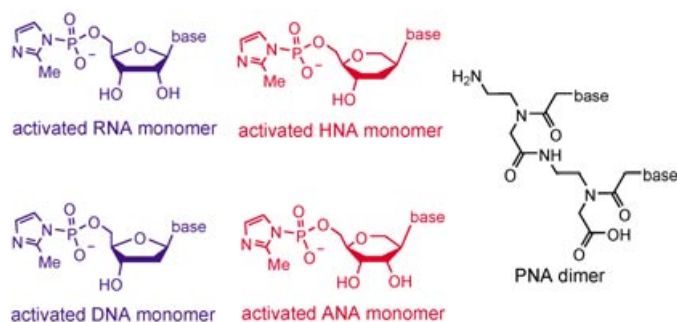
#### 4.1. DNA-Templated Polymerization of DNA and RNA

Polymerization reactions are an especially challenging form of DTS because they require many successive reactions to take place efficiently and sequence specifically without the benefit of intermediate purification. A hypothetical DNA-templated coupling reaction that generates a product that is 80% sequence-specific in 80% yield only provides 1% overall yield of a final 10-mer product of correct sequence. The simplest (in retrospect, deceptively so) target for templated polymerization is the polymerization of activated DNA or RNA monomers (Figure 16). These studies, led by the pioneering work of Orgel and co-workers,<sup>[1,85,92,144–150]</sup> demonstrated that monomers containing activated phosphate units could induce a small number of DNA-, RNA-, PNA-, HNA-, or ANA-templated phosphoesterification reactions between mono-, di-, tri-, or oligonucleotides to generate oligomeric DNA or RNA products with modest efficiency (generally < 50% yield per monomer coupling).

Acevedo and Orgel achieved the DNA-templated synthesis of an RNA 14-mer by using a DNA template and G and C 5'-phospho-2-methylimidazole monomers.<sup>[147]</sup> The full-length polymer resulting from 13 DNA-templated coupling reactions was generated in  $\leq 2\%$  overall yield. The sequence specificities of this oligomerization and other early DNA-templated polymerization reactions<sup>[1,85,92,144,146,147,149,150]</sup> were not investigated in detail, however, and templates usually consisted of poly(G), poly(C), or mixed G/C bases. Subsequent studies by Stutz and Richert suggest that the error rates of related DNA-templated phosphoimidazole mononucleotide coupling reactions are as high as 30% for forming G:C pairs, and > 50% for forming A:T pairs,<sup>[151]</sup> suggesting that these systems may not maintain sufficient sequence specificity to faithfully translate templates into sequence-defined synthetic polymers.



**Figure 15.** DTS can control multiple, otherwise incompatible reactions in a single solution. R<sup>1</sup>, R<sup>2</sup>: linker or DNA oligonucleotide.<sup>[126]</sup>



**Figure 16.** DNA and RNA monomers suitable for oligonucleotide-templated polymerization.<sup>[24,148–150]</sup>

#### 4.2. Nonnatural Polymers Generated by DNA-Templated Polymerization

The DNA-templated oligomerization of non-DNA or non-RNA monomers has also been achieved. Nucleic acid analogues that have been oligomerized by DTS include peptide nucleic acids (PNAs)<sup>[24,149]</sup> and altritol nucleic acid (ANA, the hydroxylated analogue of HNA) (Figure 16).<sup>[150]</sup> Bohler, Nielsen, and Orgel used DNA-templated amine acylation to oligomerize five PNA dimers gg on a dC<sub>10</sub> template.<sup>[24]</sup> This 1995 study represents the first report of a nucleic acid templated synthesis of an oligomer containing a nonnatural backbone. Yields of full-length PNAs in this and subsequent studies,<sup>[148]</sup> however, are modest (typically < 25% relative to limiting template), and the sequence specificities of these DNA-templated PNA oligomerization reactions are unclear since some oligomeric products are observed even when PNA dimers complementary to portions of the template are excluded, or when the template itself is excluded.<sup>[24,148]</sup> In the case of the nucleic acid templated oligomerization of ANA, Kozlov, Orgel, and co-workers observed only isomeric mixtures of very short oligomers of four or fewer ANA nucleotides from phosphorimidazole transesterification reactions containing ANA or RNA C<sub>10</sub> templates.<sup>[150]</sup> Chaput's and Szostak's findings that polymerases can catalyze the DNA-templated oligomerization of several TNA nucleotides<sup>[143]</sup> raises the possibility that natural or laboratory-evolved polymerases may eventually enable DNA-templated polymerizations.

Reactions other than phosphodiester formation and amine acylation have also been used to effect DNA-templated oligomerization and polymerization, in some cases with remarkable results. In 2000, Fujimoto, Saito, and co-workers used an efficient and reversible DNA-templated photochemical [2+2] cycloaddition (Figure 5b) to oligomerize five DNA hexamers each containing a 5'-exocyclic vinyl group and a 3' pyrimidine on a complementary 30-mer DNA template. The full-length 30-mer product containing four cyclobutane linkages was generated in high yield upon irradiation at 366 nm and could be fragmented back to the monomers quantitatively by irradiation at 302 nm.<sup>[101]</sup>

Li, Lynn, and co-workers significantly advanced the field of templated polymerization in 2002 by adapting their

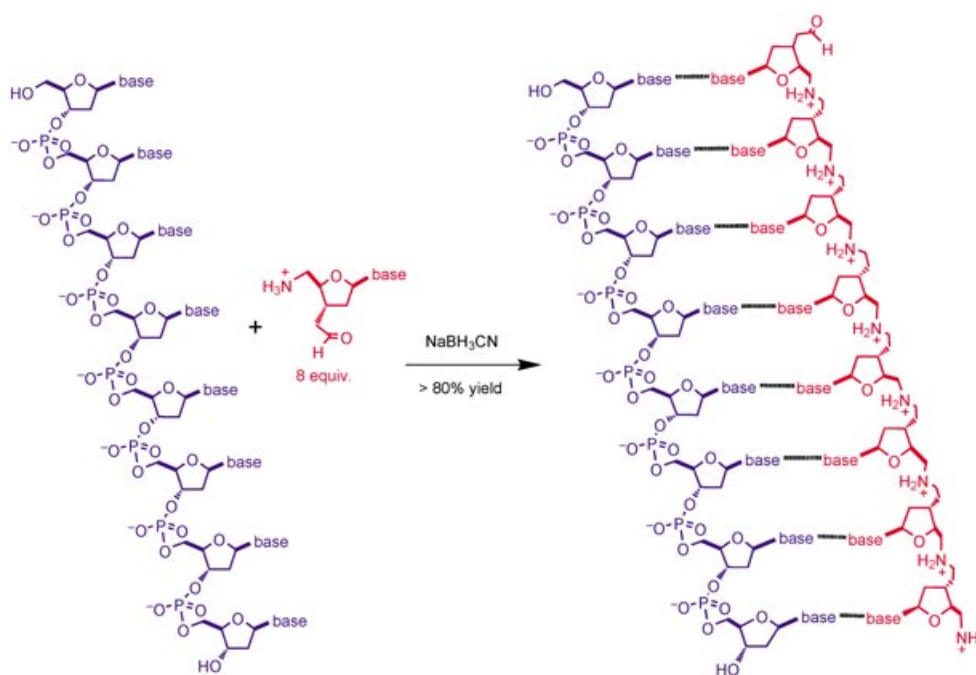
previously described DNA-templated coupling of 5'-amino and 3'-formyl DNA analogues to address DNA-templated polymerization.<sup>[31–35,38,39]</sup> In contrast with the DNA-templated DNA, RNA, PNA, and ANA oligomerization reactions described above (Figure 16) which generally proceed in low yields and with modest chain-length and sequence specificity, Li, Lynn, and co-workers found that reductive amination mediates the efficient coupling of eight 5'-amino-3'-formyl dT mononucleotides on a dA<sub>8</sub> template to generate the full-length octamer product in > 80% yield (Figure 17). Importantly, products larger than eight nucleotides were not observed, oligomerization did not proceed in the absence of template, and studies using templates containing A and T bases showed that oligomerization does not occur when monomer and template sequences cannot form base pairs.<sup>[34]</sup> These findings demonstrated that DTS can generate synthetic polymers efficiently with sequence and length specificity.

Our group studied the efficiency, regioselectivity, and sequence specificity of polymerization reactions of PNAs or formyl-PNAs by using amine acylation or reductive amination templated by 5'-amino-terminated hairpin DNA oligonucleotides.<sup>[152]</sup> Consistent with the previous observation<sup>[43,126]</sup> of the distance independence of DNA-templated amine acylation, poor regioselectivity and poor yields of full-length products were observed when the polymerization was mediated by amine acylation. In contrast, polymerization mediated by the highly distance-dependent<sup>[43,46]</sup> reductive amination reaction proceeded very efficiently (> 90% yield of full-length products) and with excellent sequence specificity and regioselectivity (Figure 18),<sup>[152]</sup> consistent with the findings of Lynn and co-workers.

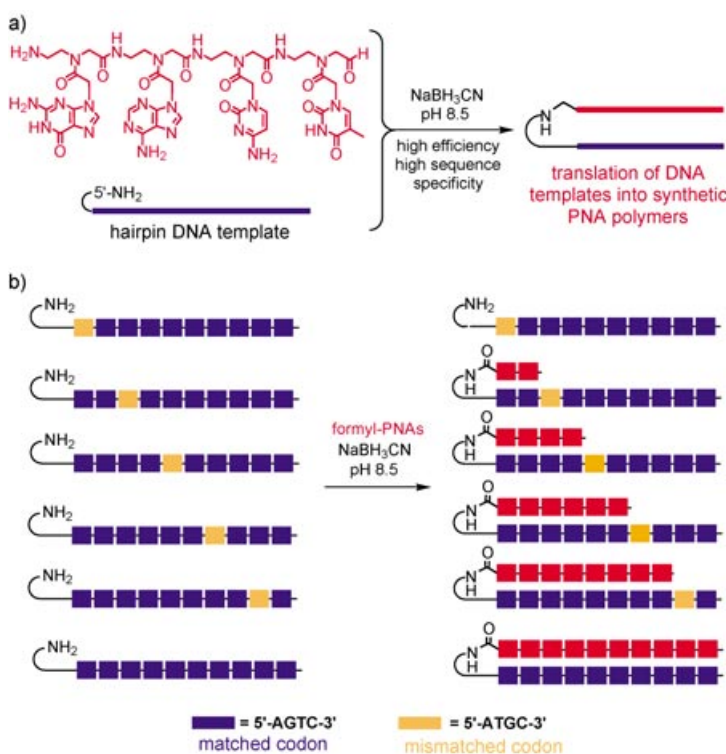
We systematically examined the sequence specificity of DNA-templated formyl-PNA polymerization reactions with templates of mixed sequences containing all four bases,<sup>[152]</sup> and found that tetrameric formyl-PNA of sequence gvvt (v = g, a, or c) were polymerized with excellent sequence specificity even in the presence of mixtures of all nine possible gvvt formyl-PNAs. In all cases, the polymerization terminated upon reaching the first template codon that did not complement any of the formyl-PNAs in solution. Integrating these findings, DNA-templated reductive amination was used to translate nine different DNA templates, each containing a 40-base coding region with approximately equal percentages of A, G, C, and T (ten consecutive four-base codons), into corresponding sequence-defined synthetic PNA heteropolymers (Figure 18).<sup>[152]</sup> Full-length heteropolymeric products were generated in good yields only when the formyl-PNAs complementing all template codons were present. These studies established that synthetic polymers of length comparable to that of natural biopolymers with binding or catalytic properties<sup>[153]</sup> can be generated efficiently and sequence specifically by nucleic acid templated synthesis.

### 5. Toward a Physical Organic Understanding of DNA-Templated Synthesis

Understanding key aspects of DNA-templated synthesis is valuable not only because it enhances the development of



**Figure 17.** DNA-templated polymerization of 5'-amino-3'-formyl-modified dT monomers.<sup>[34]</sup>



**Figure 18.** DNA-templated formyl-PNA polymerization. a) A 5'-amino-terminated DNA template (blue) directs the efficient oligomerization of modified formyl-PNAs (red) with high sequence specificity; b) mismatched codons (orange) in the templates halt the polymerization of formyl-PNAs and generate predominantly truncated products, demonstrating regioselectivity.<sup>[152]</sup>

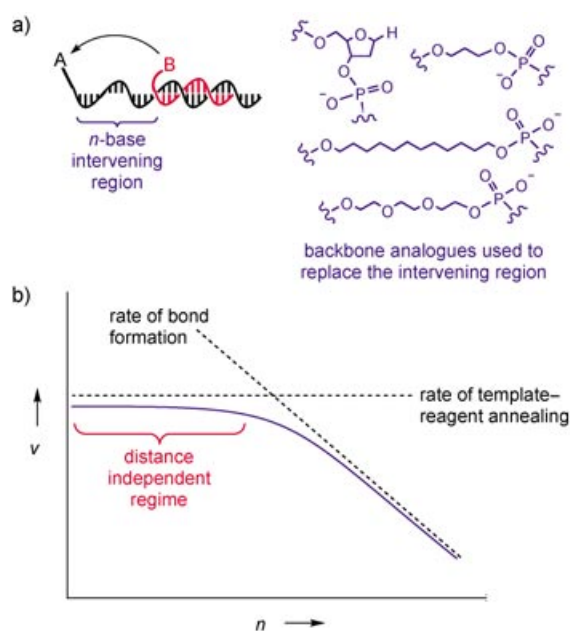
DTS but also because it reveals underlying principles that deepen our understanding of analogous biological and chemical systems. In this section we discuss three central

features of DTS for which an understanding of underlying principles is emerging.

### 5.1. Understanding Distance-Independent DNA-Templated Synthesis

One of the most unexpected and enabling properties of DTS is its ability to direct efficient reactions even when many intervening template nucleotides separate hybridized reactive groups (see Figure 9).<sup>[44]</sup> This property raises two questions: 1) why is the rate of product formation for some, but not all, DTS reactions independent of the intervening distance between the hybridized reactive groups; and 2) why is long-distance DTS efficient at all, in contrast with the notorious difficulty<sup>[105,106]</sup> of synthesizing macrocycles (which mimic the structure of long-distance DTS products)?

Our group began to address the first question by determining the role of the DNA backbone in mediating efficient long-distance DTS.<sup>[43,44]</sup> The intervening nucleotides separating the reactive groups were systematically replaced with structural analogues of similar length but lacking the aromatic base, lacking the entire ribose ring, lacking the ribose and phosphate groups, or lacking nearly all heteroatoms (Figure 19a). In all cases, efficient long-distance DTS was still observed.<sup>[44]</sup> The efficiency of long-distance DTS was significantly reduced, however, when the intervening region was rigidified by hybridization with a complementary DNA oligonucleotide. These results established that structural elements of the DNA backbone are



**Figure 19.** Understanding distance-independent DTS. a) The intervening region of a long-distance A+A' template was replaced with DNA backbone analogues. b) Conceptual model of distance-independent DTS.<sup>[44,47]</sup>

not responsible for distance independence, although flexibility in the intervening region is required.

Subsequent studies by our group<sup>[43]</sup> demonstrated that product formation for distance-independent DNA-templated reactions exhibits second-order kinetics (first order in each of the two DNA-linked reactants). This simple finding began to unravel the mystery of distance-independent DTS because it indicated that bond formation between the reactive groups in the hybridized complex (a pseudo-unimolecular process) cannot be rate-determining for these reactions. Instead, the results suggested that hybridization of the two DNA-linked reactants (a bimolecular process) is rate-determining for these reactions. Distance independence therefore can occur when the effective molarity of the hybridized reactive groups is sufficiently high that bond formation occurs faster than DNA hybridization. Increasing the number of intervening nucleotides between the reactive groups in this situation does not decrease the observed rate of product formation until bond formation rates begin to approach or fall below rates of hybridization (Figure 19b).

This simple kinetic model for distance-independent DTS explains differences in behavior among different DNA-templated reactions such as the progressive loss of distance independence in the following series of reactions: Cu<sup>I</sup>-mediated Huisgen cycloaddition (fastest rate of bond formation), amine acylation, Wittig olefination, and 1,3 dipolar nitrene cycloaddition (slowest rate of bond formation).<sup>[46]</sup> One DNA-templated reaction of particular importance (see Section 4.2), however, does not fit this model: reductive amination<sup>[34,35,43]</sup> is highly distance-dependent,<sup>[126]</sup> yet generates the product more rapidly than should be possible under the model in Figure 19b. The origins of this discrepancy are

not yet understood but could be explained if the rate of imine hydrolysis is enhanced by intervening single-stranded template bases, or if imine reduction is inhibited by intervening template nucleotides.

## 5.2. The Role of High Dilution and Aqueous Solvent

How can long-distance DTS be much more efficient than the equivalent non-templated (intermolecular) reaction considering that macrocyclizations are generally challenging synthetic reactions? There are at least two explanations. The first is the incongruity between reference states of DTS and conventional organic synthesis. Most of the DNA-templated small-molecule syntheses described above are performed at mid-nM reactant concentrations. At these concentrations, rates of nearly all intermolecular reactions including reactions between reactants linked to mismatched DNA are negligible. These intermolecular reaction pathways also include the dimerization and oligomerization of reactants and products—common sources of undesired products in traditional macrocyclization reactions even when performed under “dilute” (typically  $\mu\text{M}$ – $\text{mM}$ ) conditions. By eliminating the possibility of significant dimerization or oligomerization without impairing the formation of desired products, the extremely high dilution of DTS reactions contributes to their viability even in long-distance (pseudo-macrocyclic) format.

An additional key factor behind the efficiency of long-distance DTS compared with conventional synthetic macrocyclization reactions is the use of aqueous solvents in DTS reactions and predominantly nonaqueous solvents in the latter. Aqueous solvents can assist long-distance DTS in several ways. Water is a better solvent than nonaqueous alternatives for the wide range of reactions described above because the rate-determining transition states of these reactions (and indeed of most synthetic transformations) are generally more polar than the starting materials. For some DTS reactions, the aqueous environment enables bond formation rates to exceed the rate of DNA hybridization, resulting in distance independence. In addition, water is well-known to minimize the volume of organic reactants as a consequence of the entropic penalty incurred by ordered water molecules at the water–organic interface. This tendency is reflected in the unusually high cohesive energy density of water.<sup>[154]</sup> The tendency of aqueous solvents to contract reactant volume makes water especially well-suited for macrocyclic joining reactions including long-distance DTS. Consistent with this analysis, previous comparisons of macrocyclization efficiencies in water and organic solvents<sup>[105,106,154–158]</sup> highlight the benefits of aqueous media.

Both of the above proposed roles of aqueous solvents predict that DTS in organic solvents should be less efficient than DTS in water, and more distance-dependent. Early unpublished results by our group (Calderone and Liu) suggest that this is indeed the case. The use of long-chain tetraalkylammonium salts enable DNA-linked reactants to dissolve in organic solvents including dichloromethane, DMF, and methanol.<sup>[159,160]</sup> Remarkably, DTS can be sequence-specific in organic solvents, suggesting that base-pairing of some form

can still take place. However, DNA-templated amine acylation reactions, normally efficient and distance-independent in aqueous solvents, can be less efficient and more distance-dependent when performed in organic solvents.

While in some respects an aqueous solvent is a constraint that prevents the use of strongly basic or strongly acidic reactants, the above analysis suggests that water is also a key enabling aspect of DTS. The insolubility of organic reactants in aqueous solvents frequently precludes the use of water in conventional organic synthesis. In contrast, DNA-linked reactants for DTS, by virtue of their attached oligonucleotides and NM working concentrations, are not constrained by limited solubility in water.

### 5.3. Probing Template-Induced Effects by Using Stereoselectivity in DNA-Templated Synthesis

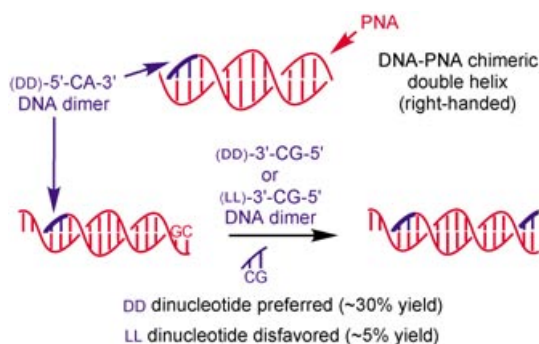
DTS is most general when the oligonucleotides modulate the effective molarities of reactants but do not perturb reaction outcomes. DNA-induced stereoselectivity during DTS is a sensitive probe of template-induced effects beyond elevating effective molarities. Moreover, stereoselective DTS serves as a model for how the chirality of an information carrier, in addition to its sequence, can be transferred to the products of a templated synthesis. In theory, stereoselective DTS could also be used to alter the distribution of stereoisomeric products arising from templated reactions to favor desired stereoisomers, although predicting and measuring the sense and magnitude of stereoselection on the minute molecular biology scale of DTS reactions are formidable challenges.

The earliest studies on stereoselectivity in nucleic acid templated synthesis were performed on systems that generated nucleic acid analogues. Joyce, Orgel, and co-workers showed in 1984 that the poly( $C_n$ )-templated oligomerization of *D*-guanosine 5'-phospho-2-methylimidazole (*D*-2-MeImpG) to generate oligo(G) was highly sensitive to inhibition by the enantiomeric monomer *L*-2-MeImpG.<sup>[161]</sup> Interestingly, *L*-2-MeImpG is efficiently coupled by templated synthesis in response to the poly( $C_n$ ) template, but the resulting product is effectively capped and cannot undergo further extension. These findings introduced the importance of minimizing inhibition from enantiomeric monomers in prebiotic models of translation. Enantiomeric cross-inhibition was also observed in PNA-templated RNA oligomerization.<sup>[162]</sup>

Bolli, Micura, and Eschenmoser demonstrated that stereoselectivity in nucleic acid templated synthesis extends beyond RNA synthesis and includes the synthesis of non-natural nucleic acid analogues.<sup>[25]</sup> For example, the *D*-pyranosyl-RNA-templated oligomerization of complementary pyranosyl-RNA tetramers proceeds diastereoselectively, favoring the coupling of *D*-tetramers over tetramers with mixed pyranose chirality.<sup>[25]</sup> In an elegant example of stereoselective DTS, Kozlov, Orgel, and Nielsen showed that as few as two *D*-DNA nucleotides when appended to an achiral PNA template could favor the enantioselective template-directed coupling of *D*-DNA dinucleotides in the  $A+B+A'B'$  archi-

tecture (Figure 20).<sup>[146]</sup> This enantioselectivity is striking because bond formation occurs far away from the inducing chiral groups, and on a different molecule.

We recently investigated stereoselectivity in the DTS of products unrelated to the nucleic acid backbone. The chirality



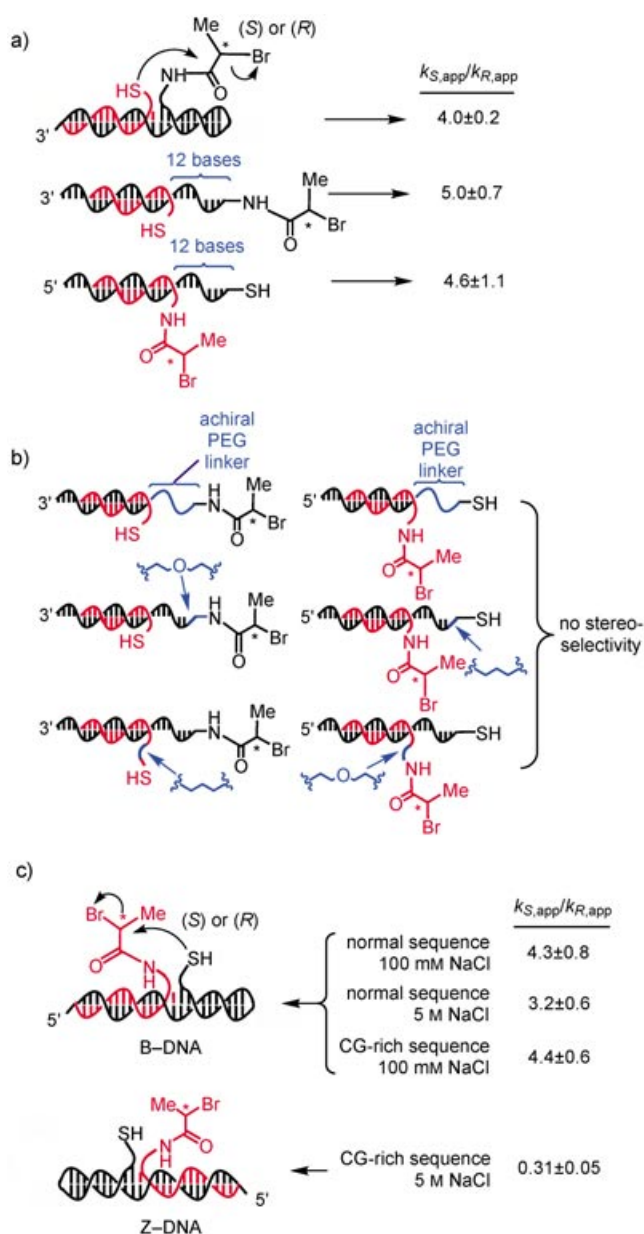
**Figure 20.** The chirality of a DNA dinucleotide (blue) terminally incorporated in a PNA template affects the stereoselectivity of a remote PNA-templated PNA–DNA coupling. As a result, the (DD)-3'-CG-5' DNA dinucleotide substrate is preferred over the (LL)-3'-CG-5' dimer.<sup>[146]</sup>

of a DNA template was observed to induce modest stereoselectivity in DNA-templated thiol substitution reactions (Figure 21 a).<sup>[48]</sup> The observed stereoselectivity was surprisingly independent of the template architecture, favoring the *S* substrate by a similar degree in either hairpin ( $A+BB'A'$ ) or long-distance  $A+A'$  architectures. Stereoselectivity was abolished, however, when flexible achiral linkers (three or more  $CH_2$  or  $O$  groups) were introduced between the reactive groups and the DNA oligonucleotides (Figure 21 b).<sup>[48]</sup> These findings indicated that even short flexible linkers can remove template influences beyond modulation of the effective molarity of reactants, suggesting that the use of such linkers is important when DTS is to be performed in its most general form.

The observed stereoselectivity in small-molecule substitution reactions was traced to the macromolecular helical conformation of the single-stranded or double-stranded template, rather than to the chirality of any particular nucleotide group.<sup>[48]</sup> This hypothesis was supported by the additional observation that stereoselectivity is inverted when the conformation of template hairpin DNA is transitioned from right-handed B-DNA to left-handed Z-DNA (Figure 21 c). These findings also demonstrate how the chirality of information carriers can be transferred through their helicity to products unrelated to the structure of the template.

## 6. Applications of DNA-Templated Synthesis

DTS connects three broadly important components of chemical and biological systems: nucleic acid sequences, synthetic products, and reactions. This connection in principle allows mixtures of any one of the above three components to



**Figure 21.** a) Stereoselective DNA-templated substitution reactions. b) Flexible achiral linkers abolish stereoselectivity during DNA-templated substitution reactions. c) Stereoselectivities are inverted when DNA undergoes a B-form (right-handed) to Z-form (left-handed) transition.<sup>[48]</sup>  $k_{app}$ : apparent reaction rate.

be searched for a desired solution while the other two components are defined. This conceptual framework suggests three types of discovery-oriented applications for DTS: 1) detection of nucleic acid sequences for the DTS of a specific product (nucleic acid sensing), 2) identification of DNA-templated synthetic products with desired properties that arise from DTS (discovery from synthetic libraries), and 3) discovery of DNA-templated reaction schemes that enable template sequences to generate products (reaction discovery). Early studies have already begun to realize the potential of DTS-based approaches for each of these emerging

applications and are presented in the following sections (see the *Note Added in Proof* regarding the application of DTS to reaction discovery).

### 6.1. Nucleic Acid Sensing

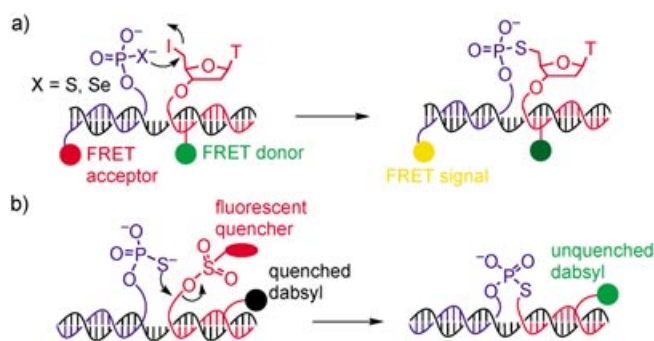
The sequence specificity of DTS enables products to form exclusively in the presence of complementary templates. When the DNA-templated reactions and the product structures are chosen to facilitate the detection of DTS events, the resulting systems can be used to detect the presence of specific nucleic acid sequences.

Ma and Taylor described one of the earliest applications of DTS for nucleic acid detection (see Figure 8a).<sup>[49]</sup> DNA templates brought together DNA-linked imidazole and DNA-linked *p*-nitrophenyl esters, inducing imidazole-catalyzed ester hydrolysis. Simple Michaelis–Menten kinetic behavior was observed with a  $k_{cat}$  of  $0.018 \text{ min}^{-1}$  when the ester-linked oligonucleotide was sufficiently short to allow dissociation from the template after hydrolysis. The authors proposed that this system might lead to the sequence-specific release of small-molecule drugs, although localizing DNA-linked reagents to target nucleic acids within living organisms is a significant challenge. This strategy might also be adapted to release a readily detected chromophore or fluorophore in response to a DNA or RNA analyte.

Mattes and Seitz used DNA-templated amine acylation to ligate two octamer PNA reagents for DNA detection.<sup>[50]</sup> The formation of coupled PNA products, and therefore the inferred presence of complementary template sequences, was confirmed by MALDI-TOF mass spectrometry. Three template sequences could be detected simultaneously and independently by choosing PNA reagent sequences and lengths such that product masses are distinguishable. Increasing the sensitivity and number of templates that can be simultaneously detected may eventually enable efficient DNA single-nucleotide polymorphism (SNP) detection by this approach.

Kool and co-workers used DNA-templated substitution reactions between 3'-phosphorothioates and 5'-electrophilic groups in two distinct approaches to nucleic acid detection.<sup>[27–30]</sup> In the first approach,<sup>[28,29]</sup> DNA- or RNA-templated  $S_N2$  reactions covalently link fluorescence resonance energy transfer (FRET) donor and acceptor fluorophores to the same oligonucleotide product (Figure 22a). The resulting proximity of the FRET donor and acceptor fluorophores generates a distinct signal. This approach was used to distinguish mixtures of complementary (matched) and mismatched RNA and DNA templates sequence specifically. In the second approach,<sup>[30]</sup> Sando and Kool used DTS to induce the loss of a fluorescent quencher from a fluorescein-linked oligonucleotide probe conjugated to an dabsyl leaving group (Figure 22b). Using these reagents, the presence of complementary rRNA within fixed cells was detected by fluorescence unquenching.<sup>[30]</sup>

DTS-based strategies for nucleic acid detection are attractive compared with existing enzyme-based approaches<sup>[51–60]</sup> because the detection signal is transduced



**Figure 22.** Nucleic acid sensing through DTS. a) A DNA- or RNA-templated substitution reaction enforces fluorophore proximity, creating a detectable FRET signal. b) RNA-templated ligation reactions can induce the unquenching of a tethered dabsyl group.<sup>[27–30]</sup>

through chemistry chosen by the researcher rather than through the narrow range of ligation and polymerization reactions that can be mediated by enzymes. Indeed, the structures generated by the small number of early examples above have already significantly expanded the diversity of signals that can arise from nucleic acid sensing. Advances in sensitivity or turnover as well as more extensive use of the inherent ability of DTS to be multiplexed are still needed, however, before DTS-based nucleic acid sensing is likely to achieve widespread and general use.

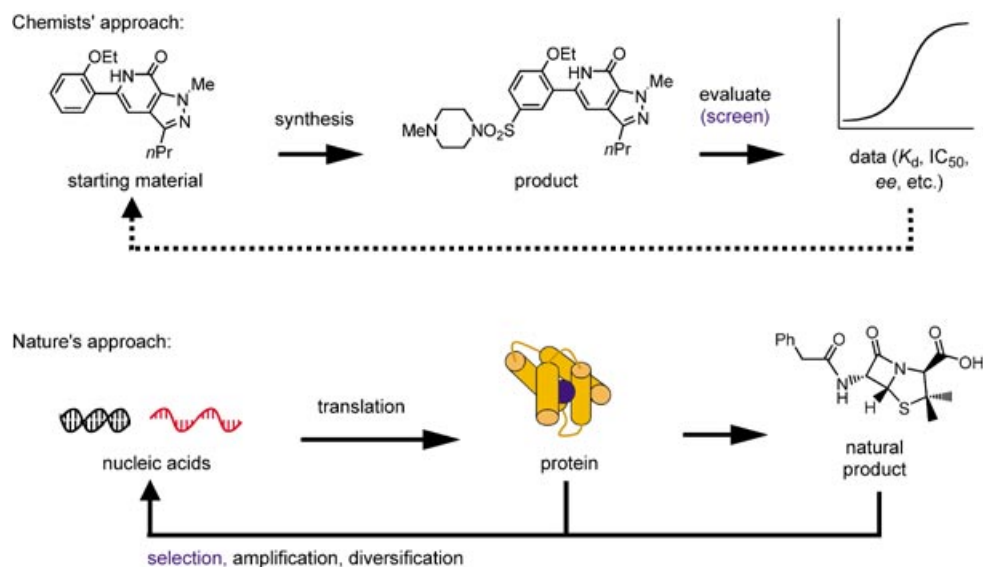
## 6.2. Synthetic Small-Molecule and Polymer Evolution

The development of synthetic small molecules and polymers with desired properties is a persistent and widespread challenge in chemistry. Chemists most frequently address this challenge by synthesizing or isolating from nature candidate structures, then evaluating (screening) the candidates for desired compounds (Figure 23). Nature's approach

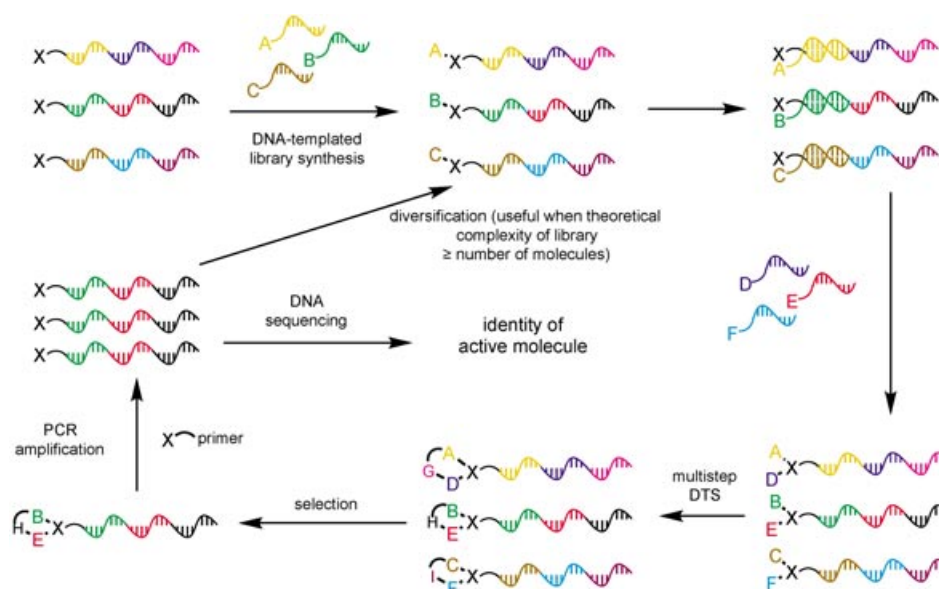
to functional biological molecules,<sup>[163–169]</sup> in contrast, involves 1) the translation of nucleic acids into proteins in a manner that preserves their association, 2) the selection of proteins (and their associated encoding nucleic acids) with favorable properties, and 3) the amplification and occasional diversification of nucleic acids encoding functional proteins that survived selection (Figure 23). Compared with the chemists' approach, nature's evolutionary approach offers advantages including unparalleled sensitivity, efficiency, and throughput without the significant infrastructure requirements associated with conventional library synthesis, spatial separation, and screening.<sup>[82,153,170–172]</sup>

Nature's evolution-based approach to discovery can only be applied to molecules that can be translated from amplifiable information carriers. The ribosomes and polymerases address this requirement for proteins, nucleic acids, and their close analogues, but cannot create general synthetic structures. Based on the properties of DTS described above, we hypothesized that DTS could be used to translate libraries of DNA templates sequence specifically into corresponding libraries of synthetic small molecules and polymers,<sup>[44]</sup> addressing the major challenge involved in the evolution of synthetic molecules.

DTS products remain covalently associated with the encoding template if architectures such as A+A' or A+BB'A' (hairpin) are used, analogous to the association between nucleic acids and their encoded proteins that is required for protein evolution. Unlike natural translation, however, DTS is not limited to structures that are compatible with biological machinery. A scheme for the evolution of synthetic small molecules proposed by our group in 2001<sup>[44]</sup> is shown in Figure 24. Multistep DTS was proposed as a means of translating a library of DNA templates into the corresponding complex synthetic small molecules. The resulting template-linked library could then be subjected to *in vitro* selections for desired properties. The templates conjugated to and encoding library members surviving selection could be



**Figure 23.** Two approaches to discovering functional molecules.



**Figure 24.** General scheme for the creation and evolution of libraries of synthetic molecules by using DNA-templated library synthesis, in vitro selection, PCR amplification, and DNA sequence diversification.<sup>[44]</sup>

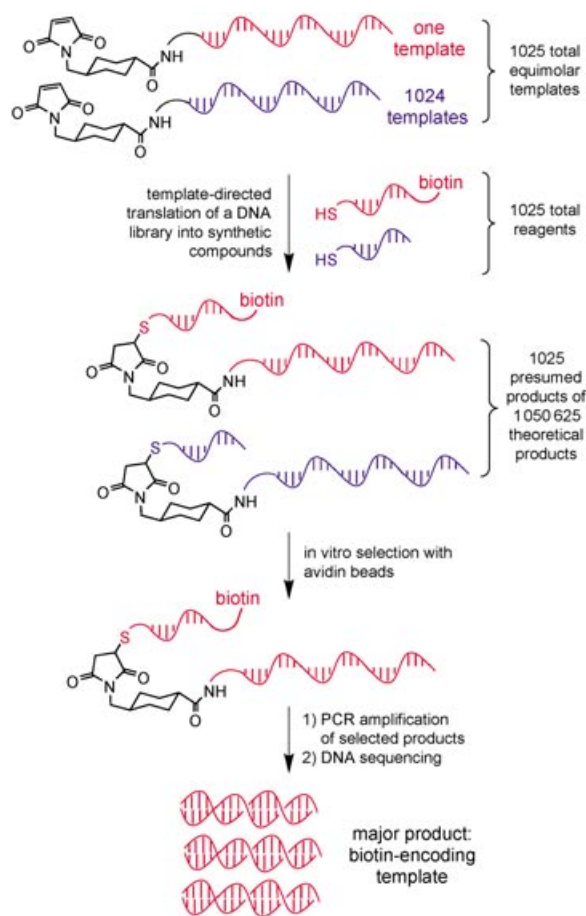
amplified by PCR and either sequenced to identify desired compounds, or diversified and subjected to additional cycles of DTS (translation), selection, and amplification.

The scheme in Figure 24 requires that DTS retains its efficiency and sequence specificity when performed in a library format, as opposed to a single-template format. To evaluate the sequence specificity of library-format DTS, we combined a library of 1025 maleimide-linked DNA templates with 1025 complementary thiol-linked reagents in a single solution (Figure 25).<sup>[44]</sup> The templates that reacted with one of the 1025 thiol reagents (the only thiol reagent that was biotinylated) were isolated by in vitro selection, amplified by PCR, and characterized by restriction digestion and DNA sequencing. The predominant template was found to be the one complementary to the biotinylated thiol reagent.<sup>[44]</sup> These results suggested that DTS can be sufficiently sequence-specific in a library format to enable templates to react with sequence-programmed reagents even in the presence of a large molar excess of mismatched, noncomplementary reagents.

The approach in Figure 24 also requires selections for DNA-linked synthetic molecules with desired properties. Our group developed highly sensitive and effective in vitro selections for DNA-linked synthetic small molecules with protein binding affinity or specificity.<sup>[82]</sup> As few as  $10^{-20}$  mol of DNA-linked protein-binding small molecules could be enriched and identified following affinity selections against six different proteins. Iteration of these selections enabled minute quantities of a DNA-linked protein ligand to be enriched starting from a mixture containing a  $10^6$ -fold excess of DNA-linked nonbinding control molecules.<sup>[82]</sup>

Our group recently integrated the generality, sequence specificity, distance independence, and multistep synthetic capability of DTS to translate a library of DNA templates into a library of corresponding complex synthetic small molecules.<sup>[81]</sup> Three successive DNA-templated reactions, each

encoded by a distinct 12-base region of a DNA template, followed by an efficient aqueous Wittig macrocyclization,



**Figure 25.** A model library-format DNA-templated synthesis, selection for protein binding, and PCR amplification.<sup>[44]</sup>

were used to generate macrocyclic fumaramides conjugated to their encoding DNA templates. A pilot library of 65 macrocyclic fumaramides was translated sequence specifically in this manner from a single solution containing 65 DNA templates. The ability of libraries of DNA-templated synthetic small molecules to be selected for properties such as protein binding affinity was established by performing an *in vitro* selection on this 65-membered macrocyclic fumaramide pilot library. Two iterated rounds of selection for carbonic anhydrase affinity<sup>[82]</sup> (without retranslation between rounds) enriched a single member of the 65-membered library. Sequence characterization of the PCR-amplified template emerging from this selection indicated that the selected macrocyclic fumaramide uniquely contained a phenyl sulfonamide group known to confer carbonic anhydrase affinity (Figure 26).<sup>[81]</sup> These results collectively indicate that library-format DTS coupled with *in vitro* selection enables the translation, selection, and amplification of DNA sequences encoding not biological macromolecules but rather synthetic small molecules.

By analogy, recent successes in translating DNA templates sequence specifically into synthetic polymers even in the presence of several monomers of different sequence (see Figure 18, Section 4)<sup>[152]</sup> suggest that it may be possible to evolve sequence-defined synthetic heteropolymers by analogous processes. Compared with the small-molecule discovery methods described above, DTS-driven synthetic-polymer discovery offers the additional attraction that the theoretical complexity of heteropolymers of even relatively modest length can easily exceed the total number of molecules present in a typical pmol-scale library ( $10^{12}$  molecules). Such an enormous sequence space can in principle be explored efficiently by iterated cycles of DTS-based translation, selection for desired binding or catalytic properties, template amplification by PCR, and template diversification by mutagenesis or recombination, representing a true evolutionary process. The possible structures of synthetic heteropolymers

evolved in this manner, however, are constrained to arise from monomers that can sequence specifically hybridize to a DNA template, or that can be cleaved from adapter molecules (analogous to natural tRNAs) that hybridize to DNA.

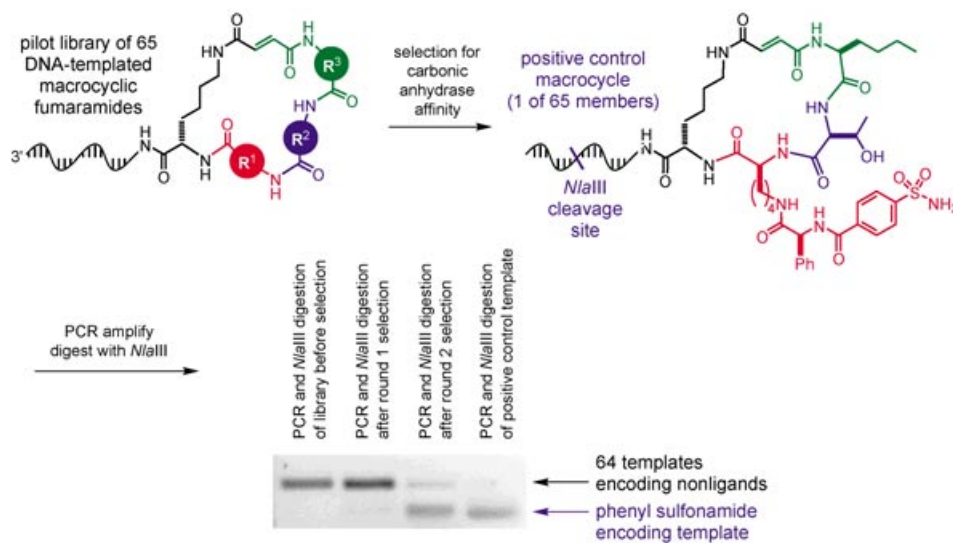
## 7. Summary and Outlook

DNA-templated synthesis has evolved dramatically over the past 40 years. DTS was first examined as a model system for prebiotic self-replication through phosphodiester formation. The recently discovered abilities of DTS to sequence specifically generate products unrelated to the phosphoribose backbone<sup>[43–48]</sup> and to mediate sequence-programmed synthesis between groups separated by long distances on DNA templates<sup>[47,102]</sup> have established DTS as a general method that enables the reactivity of synthetic molecules to be controlled by modulated effective molarities. These discoveries have also led to new developments that have rapidly expanded the synthetic capabilities of DTS, including multi-step DNA-templated small-molecule synthesis, new template architectures, synthesis templated by double-stranded DNA, efficient and sequence-specific DNA-templated polymerization, and DNA-templated library synthesis.

Controlling reactivity with DNA-programmed effective molarity rather than with conventional intermolecular reactions allows synthetic molecules to be manipulated in ways previously available only to the substrates of natural macromolecular templates. For example, otherwise incompatible reactions can take place in a single solution. Some reactions that cannot easily be performed by conventional synthetic methods, such as heterocoupling reactions between substrates that preferentially homocouple, can also take place in a DNA-templated format (see the *Note Added in Proof*). We anticipate that DTS may eventually enable ordered multistep syntheses in a single solution between reactants that would

normally generate uncontrolled mixtures of products. These unique features of effective-molarity-controlled reactivity may expand the accessibility and structural diversity of libraries of synthetic small molecules and heteropolymers beyond what is possible with current approaches.

The ability of DTS to translate amplifiable information into synthetic structures has also led to fundamentally new approaches to widespread discovery challenges that are faced by chemists. These challenges, including nucleic acid detection, synthetic small-molecule and polymer discovery, and reaction discovery, in principle can now be addressed



**Figure 26.** *In vitro* selection of a carbonic anhydrase ligand from a 65-membered library of DNA-templated macrocyclic fumaramides.<sup>[81]</sup>

with the assistance of powerful translation, selection, amplification, and diversification strategies previously available only to biological macromolecules.

Several remaining goals must still be met for the vision presented herein to be fully realized. These goals include 1) continuing to expand the scope and synthetic capabilities of DTS beyond the modest fraction of synthetic organic chemistry represented above, 2) continuing to develop and apply new modes of controlling synthetic reactivity through DTS that cannot be realized by conventional synthetic methods, 3) discovering additional reactions that occur efficiently in a DTS format and that are not known to exist or that cannot take place in a nontemplated format, and 4) discovering functional synthetic small molecules and polymers that are difficult or impossible to find by other approaches. Leslie Orgel presciently wrote in 1995 that the development of chemical systems that incorporate fundamental and powerful features of biological molecules “will require a combination of the techniques of organic chemistry ... and the methods of molecular biology.”<sup>[1]</sup> Less than a decade later, DNA-templated synthesis has transformed this prediction into a fertile frontier for organic chemistry.

**Note Added in Proof** (August 16, 2004): The third proposed application of DTS listed in section 6, reaction discovery, has now been realized and is reported in “Reaction Discovery Enabled by DNA-Templated Synthesis and In Vitro Selection”: M. W. Kanan, M. M. Rozenman, K. Sakurai, T. M. Snyder, D. R. Liu, *Nature* **2004**, in press.

## 8. Abbreviations

ANA	altritol nucleic acid
CDI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
DMT-MM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
Dabsyl	5-dimethylaminonaphthalene-1-sulfonyl
DTS	DNA-templated synthesis
EDC	3-(3-dimethylaminopropyl)-1-ethylcarbodiimide
FRET	fluorescence resonance energy transfer
HNA	hexitol nucleic acid
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
PCR	polymerase chain reaction
Sulfo-NHS	<i>N</i> -hydroxysulfosuccinimide sodium salt
TNA	threose nucleic acid

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