Oligonucleotide Complexes

Use of Dynamic Combinatorial Chemistry for the Identification of Covalently Appended Residues that Stabilize Oligonucleotide Complexes**

*Anthony Bugaut, Jean-Jacques Toulmé, and Bernard Rayner**

Selective molecular recognition between synthetic oligonucleotide ligands and nucleic acid targets plays an important role in molecular biology, biotechnology, and molecular medicine. Whereas natural DNA or RNA oligonucleotides, either rationally designed or identified through in vitro selection, usually generate lead compounds, the improvement of their properties (affinity, nuclease resistance, membrane permeability) requires introduction of chemical modifications. Nevertheless, the design of a chemical modification is not a trivial task, particularly for binders to structured RNA targets.[1] Lead optimization may benefit from combinatorial methods that allow the generation and screening of large populations of modified oligonucleotides.[2] Herein, we report a novel methodology, which employs dynamic combinatorial chemistry (DCC), for the identification of covalently appended small molecules that stabilize nucleic acid complexes.

DCC is an emerging field which offers an alternative approach to traditional combinatorial chemistry (CC).[3] Whereas CC involves the use of irreversible reactions to generate a static library of related compounds, DCC involves the use of reversible reactions to generate an equilibrating mixture of molecules, that is, a dynamic combinatorial library (DCL). The composition of a DCL is able to respond to molecular-recognition events resulting from the addition of a target of interest. The preferential binding of one member of the DCL to the target induces a shift in the equilibrium towards the formation of that particular compound. Thus, whereas in CC library synthesis and screening are two separate processes that are performed sequentially, DCC offers in situ screening of the combinatorial library simply by comparing its composition in the absence or presence of the target.

For our purpose, an oligonucleotide ligand bearing a reactive amino group will be allowed to react reversibly with a set of aldehydes in an aqueous media. According to the DCC

[*] A. Bugaut, Dr. J.-J. Toulmé, Prof. B. Rayner
INSERM U386
Université Victor Segalen Bordeaux 2
146, rue Léo Saignat, 33076 Bordeaux Cedex (France)
Fax: (+33) 5-5757-1014
E-mail: bernard.rayner@bordeaux.inserm.fr
Dr. J.-J. Toulmé
Institut Européen de Chimie et Biologie (IECB)
2, rue Robert Escarpit, 33607 Pessac Cedex (France)

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principle, the resulting mixture of imines at equilibrium will be responsive to its environment.[4] Addition of a nucleic acid target that develops interactions with imine products should promote the formation of the strongest binders. The reactive amino group will be introduced in the oligonucleotide as a 2′-amino-2′-deoxynucleotide. The pK_a value, determined to be 6.2, and the higher nucleophilicity of this primary amine compared to those present in nucleobases will allow the reaction to take place at near physiological conditions and specifically at this position.[5] Subsequently, the mixture of interconverting imine species can be selectively reduced by sodium cyanoborohydride ($NaBH_3CN$) in the form of chemically stable secondary amines, which will facilitate isolation and analysis of the final mixture.[6]

We first used a model system for an initial proof-of-principle study. This system was formed by a self-complementary hexadeoxyribonucleotide **1** bearing a 2′-amino-2′-deoxyuridine (U*) at its 3′-terminus (Scheme 1).[7] We assumed that hybridization between two self-complementary strands would drive the reversible reaction with a set of aldehydes towards the preferential formation of the conjugated oligonucleotide corresponding to the most stable duplex. Self-complementary oligonucleotide **1** was allowed to react with a set of three aldehydes **2a–c** in the presence of $NaBH_3CN$ (Scheme 1).[8] Aliquots of the reaction mixture were periodically withdrawn and analyzed by reversed-phase HPLC.[9] Proportions (as estimated from relative peak areas) of resulting conjugates **3a–c** were compared to those of the corresponding conjugates obtained in a control experiment involving reaction of non-self-complementary hexadeoxyribonucleotide, 5′-TTTCGU*, with the same set of aldehydes under the same conditions.[10] A plot, as a function of time, of the percentage amplification in the proportion of each conjugated self-complementary product over the corresponding non-self-complementary one is shown in Figure 1.[11]

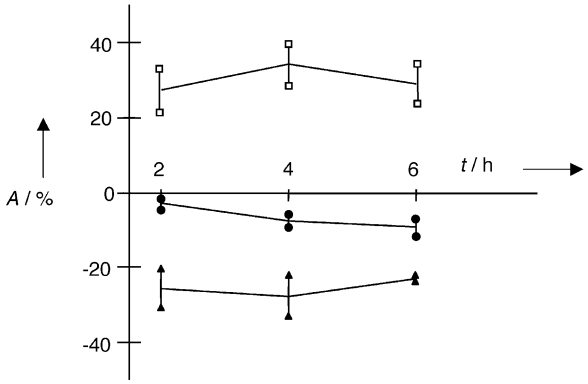
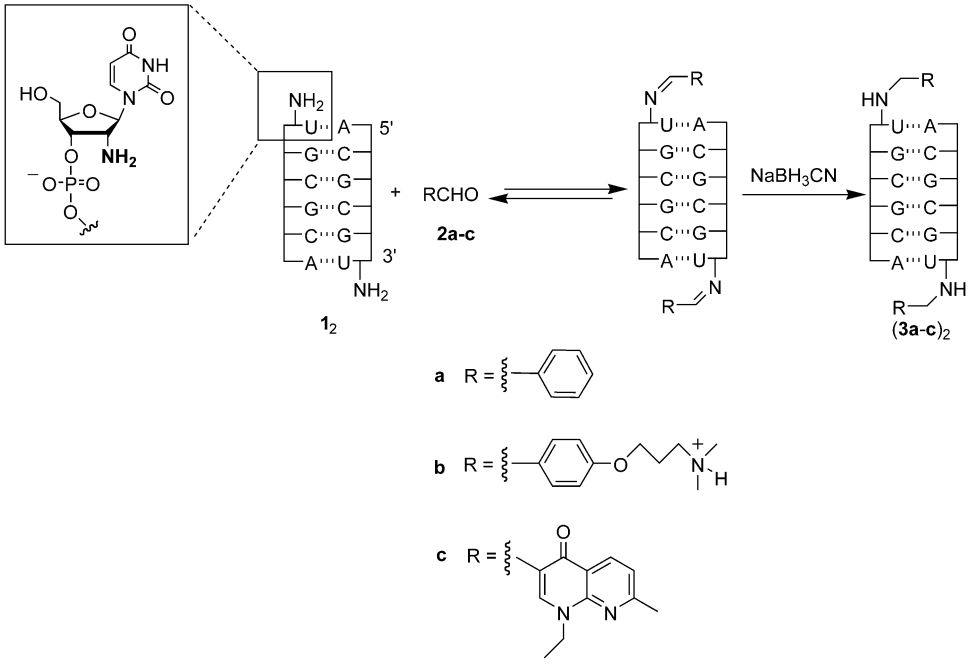


Figure 1. Time course for the DCC experiment showing the relative amplification (*A*) of **3a** (▲), **3b** (●), and **3c** (□) over the corresponding controls. **1** (0.25 mM) or 5′-TTTCGU* (0.25 mM) was incubated with **2a** (7 mM), **2b** (9 mM), and **2c** (0.8 mM) at room temperature (ca. 23 °C) in 20 mM phosphate buffer (pH 6) containing 100 mM NaCl and 5 mM $NaBH_3CN$. The reactions were complete after 6 h. Data shown correspond to duplicate experiments.

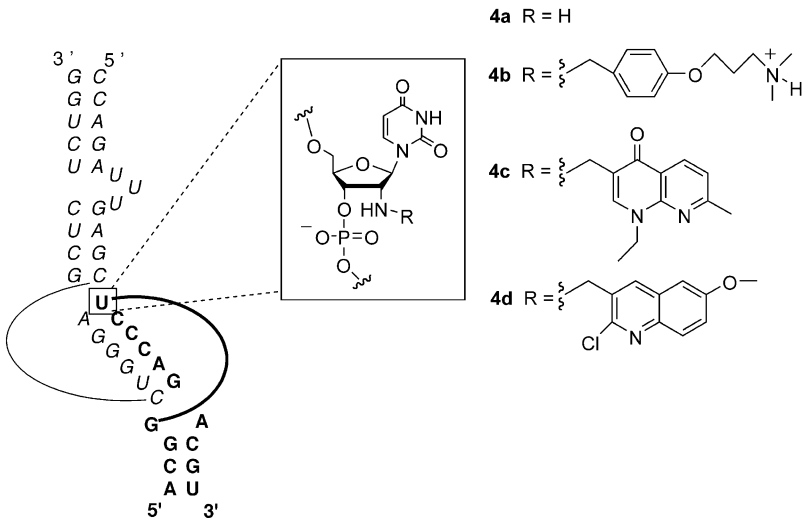
Apparent invariance of the percentage amplifications during the time-course experiment is consistent with a rapid formation of interconverting imines followed by their rate-limiting reduction. When reductive amination was performed on the reaction mixture with the self-complementary oligo-



Scheme 1. Selection by DCC of 3′-appended residues that stabilize a DNA duplex. Self-complementary oligonucleotide **1** bearing a 2′-amino-2′-deoxyuridine at its 3′-terminus is allowed to reversibly react with a set of aldehydes (benzaldehyde (**2a**), 4-[3-(dimethylamino)propoxy]benzaldehyde hydrochloride (**2b**), and nalidixic aldehyde (**2c**)) in an aqueous medium. This reaction provides a dynamic mixture of conjugated duplexes whose proportions are dictated by their relative thermodynamic stabilities. Subsequently, imines are reduced by $NaBH_3CN$ to chemically stable secondary amines, thus allowing HPLC analysis of the composition of the mixture.

nucleotide, a significant enrichment in the nalidixic conjugated product **3c** was obtained relative to the reaction involving the non-self-complementary oligonucleotide. This occurred at the expense of the other products **3a** and **3b**.

Next, to confirm that the relative amplification values are related to the thermodynamic stabilities of the three conjugated duplexes, each self-complementary product was subjected to UV-monitored melting experiments. The results (Table 1) show a direct correlation between the melting-temperature (T_m) values obtained and the ranking order of amplification effects observed in the DCC experiment. However, the dynamic amplification occurs at equilibrium and reflects the stabilizing effects of the imino-connected residues, whereas the thermodynamic stabilities were measured with reduced



Scheme 2. Structures of appended R groups (**b**–**d**) that were screened by DCC for stabilizing a kissing complex formed between the MiniTAR (italic letters) target and the aptamer (4a, bold letters) incorporating a 2′-amino-2′-deoxyuridine.

Table 1: Melting temperatures (T_m) for duplexes[a] and relative amplifications observed in the course of the DCC experiment.

Duplexes	T_m [°C]	ΔT_m [°C]	Amplification[b]
$\mathbf{1}_2$	30.0		
$(\mathbf{3a})_2$	30.3	+0.3	−28%
	(31.3)[c]	(+1.3)[c]	
$(\mathbf{3b})_2$	34.2	+4.2	−8%
$(\mathbf{3c})_2$	40.3	+10.3	+34%
	(47.3)[c]	(+17.3)[c]	

[a] UV-monitored (260 nm) melting experiments were performed with 0.25 optical density units of each oligomer in 20 mM phosphate buffer (0.2 mL; pH 6) containing 100 mM NaCl. T_m values are given with a maximum standard deviation of ±0.5 °C. [b] Values are those obtained after a four-hour reaction. [c] T_m values of the amido-linked derivatives.

conjugated duplexes. It may be argued that the amino linker present in the reduced compounds **3a**–**c** is more flexible than the imino link operating during selection. Consequently, we synthesized more rigid amido-linked analogues of products **3a** and **3c**.[9] These derivatives can be viewed as kinetically stable analogues of the transient imine compounds. We then performed UV-monitored melting experiments with these derivatives (Table 1). The results are consistent with those obtained with the amino-linked products: the amido-linked benzyl residue does not significantly improve the thermodynamic stability of the duplex, whereas the nalidixic residue increases the T_m value of duplex by 17.3 °C.[12]

Studies with this regular double-stranded model allowed us to successfully demonstrate that DCC can be used to identify covalently appended residues that increase the thermodynamic stability of a duplex. We next used this approach in the context of a tertiary-structured RNA complex formed by a loop–loop interaction between an RNA hairpin aptamer, identified by systematic evolution of ligands by exponential enrichment (SELEX) in our laboratory, and its target, the TAR RNA hairpin element of HIV-1.[13] A loop–loop complex, also known as a kissing complex, was attractive because it combines the fundamental base-pairing complementarity of nucleic acids with the added specificity of a constrained three-dimensional structure. For our experiment, we used a 14-nucleotide version of the aptamer (**4a**, Scheme 2) in which the ribonucleotide U in the loop was replaced by a U*, a set of three aldehydes (**2b**, **2c**, and 2-chloro-6-methoxy-3-quinolinecarboxaldehyde, **2d**), and a 27-nucleotide form of TAR (MiniTAR). Two reaction mixtures containing **2b**–**d** and the aptamer ligand **4a** were prepared, one in the absence and one in the presence of MiniTAR (one molar equivalent relative to **4a**).[8] Reaction mixtures were incubated at 20 °C in an aqueous buffer at pH 6 for 16 h and then analyzed by reversed-phase HPLC.[9] We observed that the presence of MiniTAR promotes the formation of **4c** (+20%) at the expense of the other products (Figure 2).[10] This amplification was correlated with a 2.6 °C increase in the T_m value of the kissing complex **4c**/MiniTAR relative to that of **4a**/MiniTAR, while a slight destabilization was found with the conjugates **4b** and **4d**.[14] To verify that the amplification effect was a consequence of loop–loop interaction and was not caused by any nonspecific effect, a mutated target, MiniTAR 3A, was also used as a control. This mutated target, in which the three central G residues in the loop are replaced by three A residues, prevents the formation of the kissing complex. As shown in Figure 2, when the reaction was carried out in presence of MiniTAR 3A, no significant change in the product distribution was observed, which demonstrates that the variations observed were caused by formation of a kissing complex between the conjugated ligands and MiniTAR.

The question arises as to why the nalidixic group was selected for stabilizing both complexes. Although the use of small libraries increases the probability of selecting the same aldehyde, the mode of interaction of the nalidixic group in stabilizing a DNA duplex or a RNA kissing complex is probably different. Structural evidence was provided that oxolonic acid (a quinolone related to nalidixic acid) disrupts the terminal T:A base-pair and stacks on the contiguous G:C

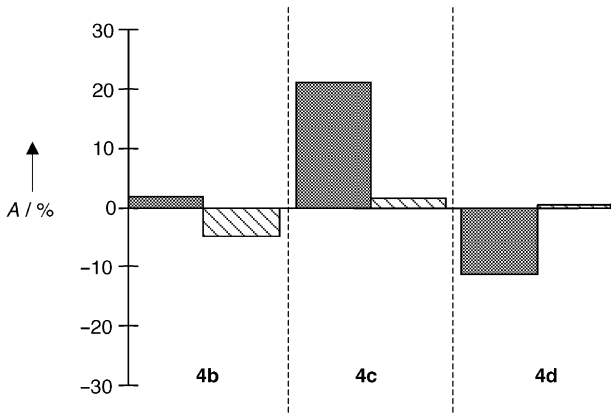


Figure 2. Relative amplifications of conjugated aptamers **4b–d**, after 16 h of incubation in the presence of 0.1 mM MiniTAR (gray) or 0.1 mM MiniTAR 3A (lined), over corresponding controls that were incubated in the absence of target. **4** (0.1 mM) was incubated at 20 °C with **2b** (1.2 mM), **2c** (0.25 mM), and **2d** (0.35 mM) in 20 mM sodium cacodylate (pH 6) containing 140 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, 5 % dimethylsulfoxide, and 5 mM $NaBH_3CN$. Results are averages of duplicate experiments.

base-pair when appended on termini of DNA duplexes.[15] Such a stabilizing but base-disrupting effect appears unlikely within the highly constrained structure of a kissing complex.[16]

In conclusion, we have described the first successful use of DCC for the rapid identification of a non nucleic acid residue appended to an oligonucleotide ligand that stabilizes the complex formed with its nucleic acid target. This was achieved both with a DNA duplex and with a tertiary-structured RNA–RNA complex. Therefore, DCC represents a powerful methodology for optimization of nucleic acid ligands. Additive effects of several appended groups would lead to a high-affinity and nuclease-resistant nucleic acid ligand. We are currently investigating the possibility of increasing the size of the set of aldehydes as well as the number of 2′-amino-2′-deoxynucleotides present in the ligand.

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Keywords: dynamic combinatorial chemistry · molecular recognition · nucleic acids · oligonucleotides · RNA structures

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[8] Initial concentrations of aldehydes were adjusted to compensate for their differences in reactivity with 2′-amino-2′-deoxyuridine and to provide comparable proportions of conjugated products. This allows a more accurate measurement of the percentage amplification (see the Supporting Information).

[9] Experimental details are given in the Supporting Information.

[10] Products were synthesized separately to validate their assignments on the chromatograms and were characterized by MALDI-TOF mass spectrometry (see the Supporting Information).

[11] Percentage amplifications (A) were calculated from the HPLC peak area as follows: $A = (P_{sc} - P_{nsc})/P_{nsc}$. For a given aldehyde, P_{sc} represents the proportion of the conjugate formed with the self-complementary oligonucleotide over the total conjugated products **3a–c** and P_{nsc} represents the proportion of the corresponding non-self-complementary product formed in the control experiment.

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[14] Melting experiments, monitored at 260 nm, were performed with 1 μM aptamer and 1 μM MiniTAR in 20 mM sodium cacodylate (pH 6) containing 140 mM KCl, 20 mM NaCl, and 3 mM $MgCl_2$.

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