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Efficient and Simple Solid-Phase Synthesis of Short Cyclic Oligodeoxynucleotides Bearing a Phosphorothioate Linkage**

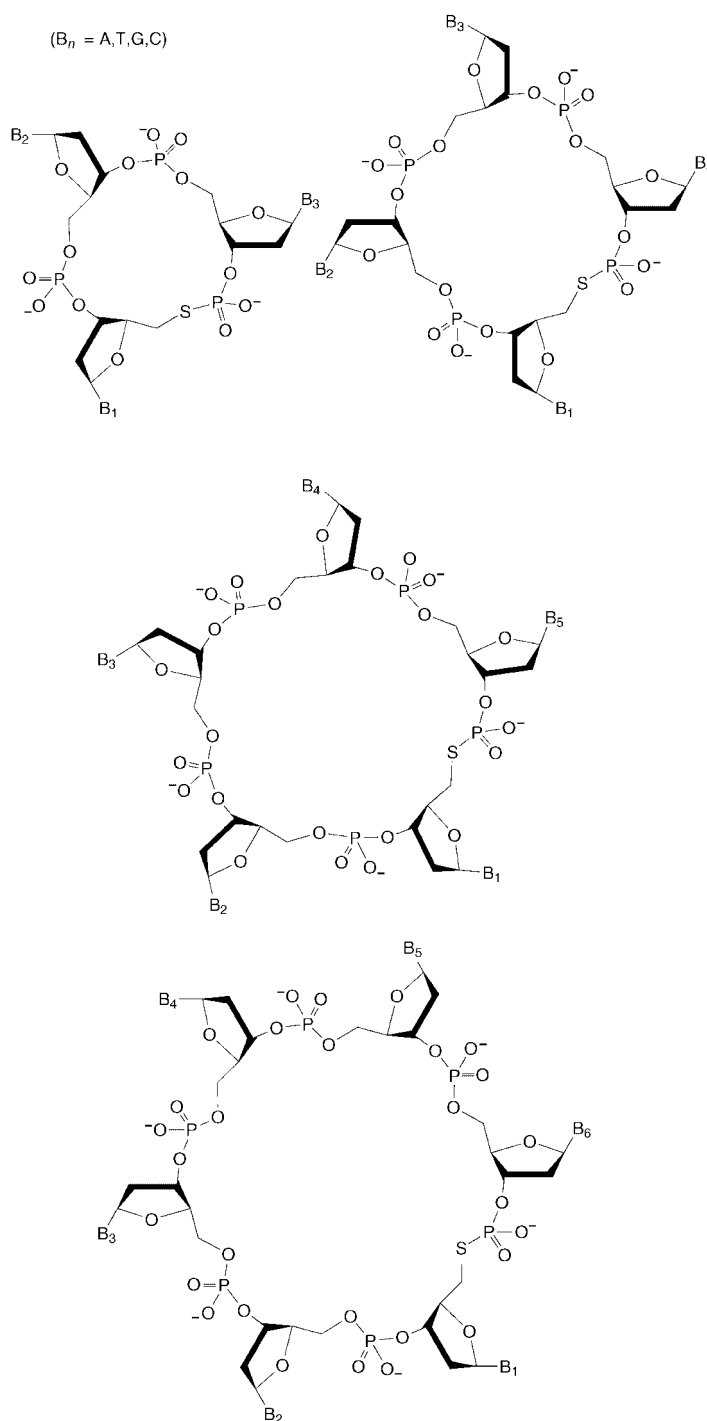
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There has been an increasing interest of late in the unusual chemical and biological properties of synthetic cyclic DNAs and RNAs.^[1] Such molecules are distinct from standard linear oligonucleotides in several respects: they are often unusually good substrates for polymerase enzymes, they are remarkably stable in biological media, and they show unusual recognition abilities with other nucleic acids.^[2] Such properties have been reported for a wide range of such molecules, from larger cyclic sequences (e.g., > 100 nt in size),^[3] to intermediate-sized compounds (e.g., 18–72 nt in size),^[4] and even to the smallest ones (2–10 nt in size).^[5] As for this latter class of molecules, cyclic dinucleotides have been suggested as promising biological agents. For instance, *c*(GG) is an activator of cellulose synthase in *Acetobacter xilinum*,^[5c] and *c*(UU) and *c*(AU) are inhibitors of DNA-dependent RNA polymerase of *E. coli*.^[5d] The hypothesized application of short cyclic oligonucleotides in elucidating enzyme mechanisms and as lead structures for development of new drugs,^[6] calls for the definition of an easy and efficient production of such compounds.

So far, several methods have been proposed for the synthesis of short cyclic oligonucleotides, in solution using the phosphotriester^[7–9] or H-phosphonate^[10,11] method, or on polymeric support.^[12–14] Unfortunately, these procedures have two main drawbacks that limit their use: they are not compatible with the more common phosphoramidite chemistry, and they require additional protection and deprotection steps. Moreover, the yield for cyclization never exceeded 50 % using those approaches. De Napoli et al. wisely aimed to circumvent these problems by the use of a glass (CPG)

support for a general synthesis of cyclic oligonucleotides. However, cyclization yields were low (20 %) even in the case of very short oligomers (2–4 residues).^[15] The useful solid support developed by Pedroso et al.^[12] offers a moderate to good 50 % yield for the smallest cycles; however, only the T-support is commercially available.

In this study, we describe the first solid-phase synthesis of cyclic oligonucleotides using the standard β -cyanoethylphosphoramidite method. Oligodinucleotides **1** and larger cyclic oligomers (Scheme 1) bearing one 5'-bridging phosphorothioate linkage are obtained in good to excellent yields. The



Scheme 1. Cyclic oligonucleotides (trimer through hexamer).

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cyclization occurs spontaneously during deprotection of the oligonucleotide, and all four nucleobases can be used without restrictions.

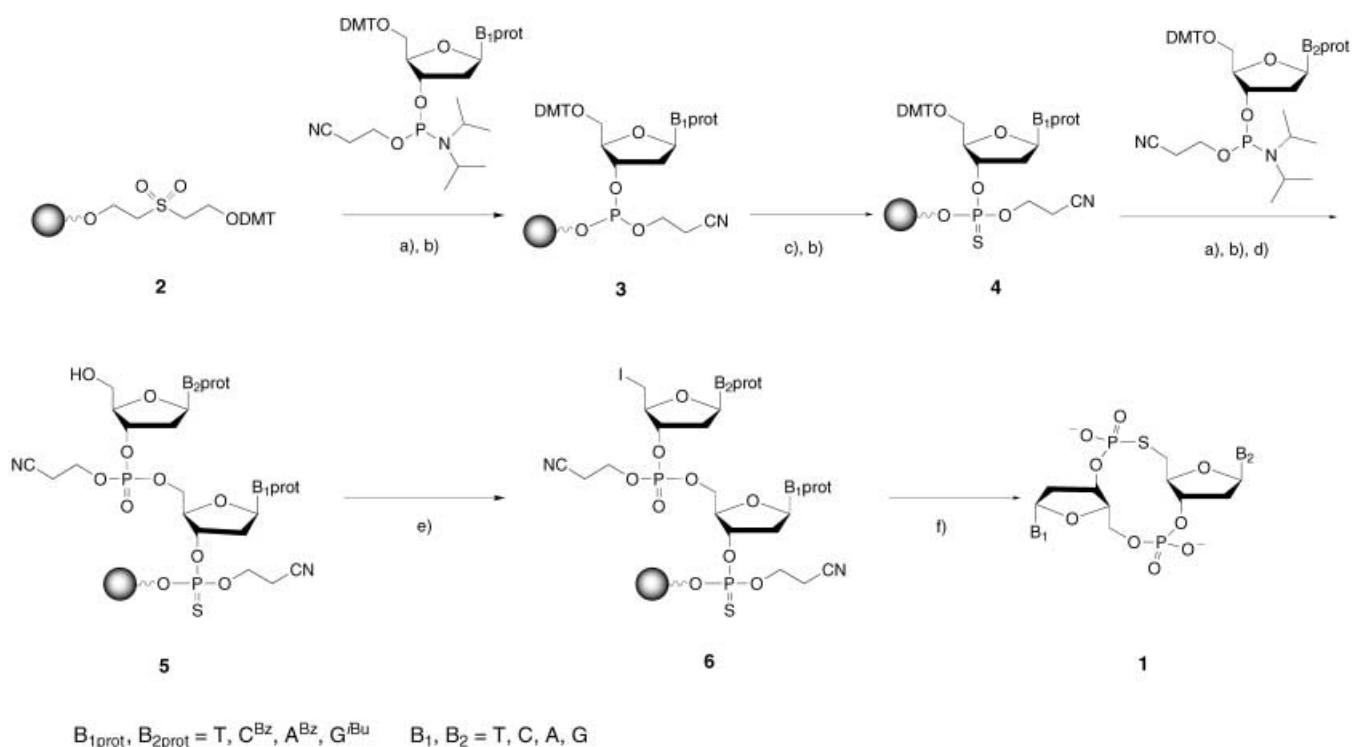
We have previously studied the nucleophilic displacement of 5'-iodo-nucleoside groups.^[16] This has proven to be a useful approach to nonenzymatic ligation of DNAs, involving the reaction of phosphothioates at the 3'-end of one strand with an iodide as leaving group on thymidine on another strand. Very recently, we described a rapid, inexpensive automated method to generate 5'-iodo-oligonucleotides directly on the DNA synthesis column in high yield using $(\text{PhO})_3\text{PCH}_2\text{I}$ as an iodination reagent.^[17] On the basis of these methodologies, we envisioned the formation of cyclic oligodinucleotides, with a sulfur atom replacing one of the bridging phosphodiester oxygen atoms. Of course it remains to be seen how this single sulfur-for-oxygen replacement affects biological activities; however, an earlier study revealed a number of enzymes that were unaffected by this difference in larger oligonucleotides,^[16b] and recent progress in medicinal development of phosphorothioate-containing oligonucleotides also suggests the benign nature of this replacement.^[18]

Starting with solid support **2**, the scheme for synthesis of cyclic oligodinucleotides was initiated on an automated DNA synthesizer on a 1 μmol scale, using two cycles of the standard DNA synthesis protocol that employed phosphoramidite chemistry (Scheme 2). The first nucleotide unit was added yielding the phosphite triester **3**. Oxidative sulfurization of **3** with the commercial reagent 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent) led to the thiophosphotriester **4**.^[19] The second cycle was a standard DNA coupling cycle, and the terminal 5-dimethoxytrityl group was removed.

Iodination of the 5'-end of **5** was performed in one step, either on the synthesizer using the automated "iodo-cycle" or by following the manual procedure (see Experimental Section),^[17] leading to 5'-iodinated dinucleotides **6**. Deprotection of bases, release from the CPG support, and cyclization by $\text{S}_\text{N}2$ displacement, were all conducted in a single step by treatment with concentrated ammonium hydroxide for 24 h at room temperature. Overall, then, this procedure is virtually identical to the automated synthesis of a standard dinucleotide, but with a single additional step (the iodination step).

To test the generality of the method, a set of eleven cyclic oligodinucleotides **1** bearing a phosphorothioate linkage was prepared by using this approach. These dimers were characterized by ^1H and ^{31}P NMR spectroscopy and by electrospray mass spectrometry (see Table 1). They were purified by preparative reverse-phase HPLC and quantitated by UV absorbance.^[10] Phosphorus NMR analysis clearly revealed two signals, indicating the presence of one phosphorothioester and one phosphodiester bond. In particular the P(S) resonance signals observed ($\delta \approx 20$ ppm) clearly demonstrate the presence of a 5'-bridging phosphorothioate linkage, whereas unbridged P(S) linkages usually resonate at lower field ($\delta \sim 50$ ppm).^[19] In the ^1H NMR spectra, the signals corresponding to the different protons of each mononucleotide were well separated in the low-field regions, whereas some overlap occurred in the 4'-, 5'-, and 2'-sugar proton regions.

On the basis of a 1 μmol CPG column, the yields of the set of eleven cyclic dinucleotides ranged between 40 and 80 %, demonstrating the high efficiency of the method. The reaction time allowed for reaching these yields was routinely 24 h, although yields were approaching these values after only 16 h



Scheme 2. Synthesis of cyclic oligodinucleotides. a) coupling; b) capping; c) sulfurization: 3*H*-1,2-benzodithiol-3-one-1,1-dioxide, CH_3CN ; d) oxidation: I_2 , 0.02 M (H_2O , pyridine, THF); e) iodination: $(\text{PhO})_3\text{PCH}_2\text{I}$, DMF; f) cleavage: 28 % aq. NH_4OH , RT, 24 h.

Table 1. Analytical data for cyclic compounds.^[a] All contain one sulfur atom (replacing 5'-oxygen atom) immediately 5' to the first nucleotide listed.

Entry	Compound	³¹ P NMR [ppm] ^[b]		H-8	H-2	¹ H NMR [ppm]		H-1'	H-3'	[M + H] ⁺ ^[c]	RT [min] ^[d]	Yield [%]
		PS	PO			H-6	H-5					
1	c(TT)	20.24	−0.23			7.68 7.52		6.32 6.24	5.03	623.1	10.21	71
2	c(TC)	20.15	−0.20			7.93 7.79	6.07	6.29 6.21	5.00 4.91	608.1	9.07	78
3	c(TA)	20.22	−0.17	8.41	8.22	7.80		6.48 6.30	5.13 5.02	632.1	10.57	75
4	c(TG)	20.22	−0.14	8.04		7.50		6.16 5.03	5.15 5.03	648.1	9.65	77
5	c(GG)	20.39	−0.14	8.03 8.00				6.31	5.17	673.1	9.02	60
6	c(GA)	20.46	−0.17	8.41 8.01	8.24			6.50 6.31	5.23	657.1	9.88	55
7	c(CG)	20.31	−0.13	8.01		7.70	6.04	6.30	5.15 4.98	633.2	8.92	45
8	c(CC)	20.25	−0.21			8.25 7.93	5.88 5.79	6.14	4.98	593.1	8.12	65
9	c(CA)	20.36	−0.16	8.42	8.23	7.77	6.04	6.50 6.30	5.13 4.99	617.1	9.26	52
10	c(AA)	20.29	−0.18	8.42 8.40	8.24 8.22			6.50	5.02	641.1	10.65	36
11	c(AT)	20.28	−0.25			nd				632.1	10.64	50
12	c(T ₃)	19.58	−0.78 −1.11			7.76 7.75 7.59		6.39 6.36 6.27	4.88 4.83	927.1	13.31	70

[a] The NMR spectra were recorded in D₂O at 500 MHz. [b] ³¹P chemical shifts were measured with respect to 85 % H₃PO₄ as external standard. [c] Electrospray MS. [d] Retention time (RT) by analytical reverse-phase HPLC using a gradient of 0 to 20 % of acetonitrile over 20 min, at a flow rate of 1 mL min^{−1}. nd: not determined.

(by HPLC). A common feature of the HPLC profiles of all the crude cyclization/deprotection reactions is a single major peak representing the cyclic product (typically more than 70 % of the total integrated area) accompanied by only small impurities. Most of the sequences behaved similarly. However, particularly striking is the difference in yield between the two syntheses of c(TA) (Table 1, entry 3) and c(AT) (Table 1, entry 11): much lower yield was obtained from 5'-terminal iodo-dA. A possible explanation for this is the formation of a cyclonucleotide by N3 intramolecular attack either on the 5'-O-phosphonium Moffatt intermediate or on the subsequent 5'-iodo derivative.^[20]

Work is underway to apply a similar strategy to the preparation of short cyclic oligoribonucleotides. Such compounds, because of their greater reactivity, are commonly much more difficult to prepare than the corresponding 2'-deoxy analogues.^[13] Micura et al. recently developed a method for the synthesis of small cyclic RNAs by the use of 2'-O-triisopropylsilyloxymethyl (TOM)-protected amidites to circumvent the difficulties and limitations generally encountered.^[21] Preliminary results obtained in our laboratory show that 2'-O-methoxyribonucleotides are well iodinated on the 5'-end allowing the high-yield formation of mixed cyclic oligonucleotides (e.g. c(TU_(OMe)) (data not shown). However the synthesis of cyclic oligodi-(2'-Ome)-ribonucleotides appears more difficult to achieve.

Finally, the synthesis of some larger cyclic oligodeoxynucleotides was undertaken to further evaluate the utility of the method. Although the formation of trimers appears to be as good as dimers (Table 1, entry 12), the cyclization yields decrease significantly, not surprisingly, as the size of the

oligomers increases (after 5 days at 50 °C, c(T₄) 50 %, c(T₅) 25 %, and c(T₆) 15 %). We noted that with the larger macrocycles these products appear with concomitant formation of an unexpected, apparently dethiophosphorylated by-product as indicated by mass spectrometry (data not shown), which apparently competes against formation of the desired cyclic product.^[22] Although the yield drops significantly as cyclic pentamers and hexamers are prepared, it is worth noting that these yields are still competitive with those obtained by previous approaches.

In conclusion, we present a very efficient procedure for the synthesis of short cyclic oligonucleotides using a standard commercial CPG support. Its suitability with standard DNA synthesizers and phosphoramidite chemistry, as well as the ease of execution, make the method quite attractive for general use. The higher final yields and shorter reaction times in comparison with previously described methods also speak for the utility of the approach. Elaboration of a library of cyclic molecules using modified nucleotides, as well as evaluation of such compounds against specific biological targets will be presented elsewhere.

Experimental Section

All cyclic oligonucleotides were prepared on a DNA synthesizer using standard phosphoramidite chemistry and standard synthesis cycles. After coupling the second nucleotide (DMT-off), columns were removed and 1 mL syringes were attached to both ends of the columns. (PhO)₃PCH₃I (1 mL, 0.5 M in anhydrous DMF) was then passed from one syringe, through the column, to the other syringe for 5 min before the sample was put on a shaker at room temperature for 25 min. The iodination reagent was then removed and the column washed with CH₂Cl₂ (10 mL), CH₃CN (10 mL), and CH₂Cl₂ (10 mL). Cleavage and cyclization of the nucleotides was



achieved by soaking the beads in concentrated ammonia at room temperature for 24 h. Cyclic compounds were purified by preparative reverse-phase HPLC using a gradient of 0–20% of acetonitrile over 20 min at a flow rate of 3 mL min⁻¹. Analytical data are given in Table 1.

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Kinetics of a Reversible Covalent-Bond-Forming Reaction Observed at the Single-Molecule Level**

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The examination of single molecules can reveal detail that is obscured in observations of ensembles. A great deal of recent work at the single-molecule level has focussed on biological problems;^[1–6] there has been less emphasis on chemistry, especially the making and breaking of covalent bonds. Seminal experiments have resulted in the observation of individual reaction steps catalyzed by enzymes^[7] and ribozymes.^[8] But, this work has employed optical techniques that are restricted to the use of fluorescent substrates or the investigation of nanoscale movements.^[9] We have previously used electrical recording, which does not suffer from these limitations, to visualize individual noncovalent interactions occurring in the lumen of a protein pore.^[10] Others have used electrical recording to study individual unidirectional covalent-bond-forming reactions^[11] and a reversible thermal isomerization.^[12] Herein, we demonstrate that reversible covalent-bond-forming chemistry can be observed by electrical detection. Reversible covalent-bond-forming chemistry at the single-molecule level has not been observed previously by any means.

To demonstrate our approach, we examined the reaction of organoarsenic(III) compounds with thiols, a reaction of considerable importance in the toxicology of environmental contaminants, chemotherapeutic agents, and chemical weapons.^[13–21] The reactions of organoarsenic(III) compounds with dithiols to form stable cyclic 1,3-dithia-2-arsolanes have been thoroughly investigated (Figure 1a, upper).^[22–25] Such reactions are essentially irreversible with formation constants of the order of $K_f = 10^7 \text{ M}^{-1}$.^[26,27] But, we reasoned that reactions with monothiols would be reversible (Figure 1a, lower); indeed, unstable adducts of organoarsenic(III) compounds with monothiols are known, although their chemistry has been hardly studied.^[28,29]

In our experiments, organoarsenic compounds were present in solution, while thiols were incorporated onto the luminal face of the α -hemolysin protein pore by site-directed muta-

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