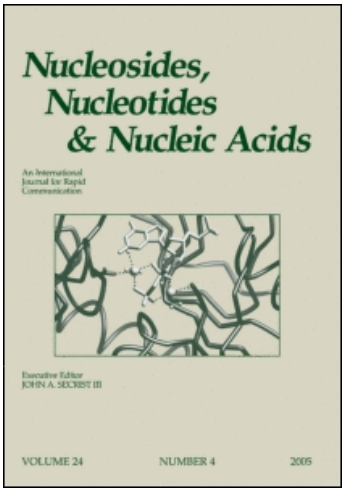

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SYNTHESIS OF CYCLIC DINUCLEOTIDES BY AN H-PHOSPHONATE METHOD IN SOLUTION

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SYNTHESIS OF CYCLIC DINUCLEOTIDES BY AN H-PHOSPHONATE METHOD IN SOLUTION

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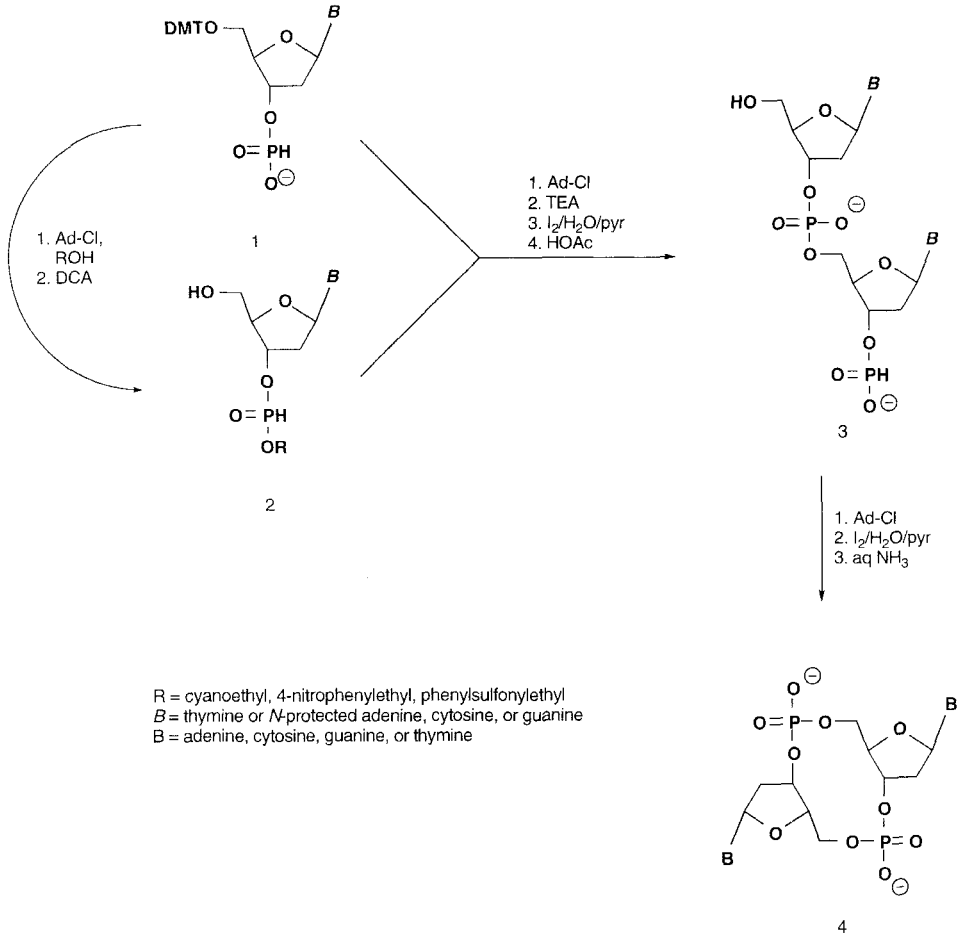
Abstract: We report synthesis of each of the ten cyclic 2'-deoxyribodinucleotides by a solution-phase H-phosphonate method. The cyclic dimers have been characterized by ^{31}P NMR, MS, UV, and enzymatic degradation.

Cyclic dinucleotides were first isolated from chemical polymerization reactions during early explorations of diester oligonucleotide synthesis.[1] They later were shown to have activity as RNA polymerase inhibitors[2] and in one case as an activator of cellulose synthase.[3] As a result, there have now been a number of reports of synthesis of cyclic dinucleotides in both the ribo and 2'-deoxyribo series. Our original work,[4] and much of the later work, relied on the phosphotriester method.[5-9] The phosphoramidite method has been used for larger cyclic oligonucleotides[10] and the H-phosphonate method has been used for cyclic dinucleotide phosphorothioates.[11] We now use the H-phosphonate method for solid-phase oligonucleotide synthesis, and chose to explore a solution-phase version for preparation of cyclic dinucleotides. We report below the synthesis and characterization of the complete set of ten cyclic 2'-deoxyribodinucleotides.

The principle disadvantage with solution-phase H-phosphonate synthesis is the lability of H-phosphonate diesters. A scheme that is applicable to synthesis of molecules with phosphorothioate linkages is to convert the H-phosphonate diesters to some form of phosphorothioate after each coupling.[10,12] In the case of our unmodified cyclic dinucleotides, we chose to explore oxidation of the linear H-phosphonate diester to the phosphate diester, as shown in Scheme 1. Since H-phosphonate monoesters are not readily oxidized, the 3' H-phosphonate monoester group is not oxidized and can be used for the cyclization. In this approach, one of the two protected 3' H-phosphonate



monoesters (e.g. **2** in Scheme 1) is coupled to an alcohol that is subject to cleavage by β-elimination. We found that the phenylsulfonylethyl derivative had the best properties for this purpose. Because of the lability of the resulting H-phosphonate diester, no extraction or chromatography is possible. But because the H-phosphonate coupling reaction is both fast and clean the product could be isolated by precipitation after a 30 sec coupling reaction. Detritylation followed by precipitation then gives the 5'-hydroxyl component for coupling with **1** to form the linear dimer. Again, the coupling is stopped after 30 sec by precipitation of the dimer, using stoichiometric quantities of each monomer, and the phenylsulfonylethyl group is cleaved using triethylamine. After another precipitation to remove the triethylamine, the internucleotide H-phosphonate diester linkage is oxidized

Tables 1 and 2. ^{31}P Chemical shifts of the linear and cyclic dimers.‡

1	ppm	pD
l-AA	5.99†, -0.50	7.4
l-AC	5.99†, -0.19	7.3
l-AG	5.44†, -0.92	6.5
l-AT	5.50†, -0.83	6.1
l-CC	5.39†, -0.54	7.3
l-CG	5.45†, -0.61	6.6
l-CT	4.99†, -1.15	7.4
l-GG	5.44†, -0.85	7.4
l-GT	5.50†, -0.82	6.1
l-TT	5.92†, -0.27	7.5

2	ppm	pD
c-AA	-0.73	7.6
c-AC	-0.74, -.083	7.6
c-AG	-0.55, -0.58	8.0
c-AT	-0.61, -0.69	7.4
c-CC	-0.68	7.8
c-CG	-0.66, -0.81	7.6
c-CT	-0.19, -0.21	7.4
c-GG	-0.99	7.4
c-GT	-0.90, -1.03	7.4
c-TT	-0.88	8.3

†d of d, ^{1}J = 641-643 Hz, ^{3}J = 8 Hz ‡D_2O, 28 mmol phosphate, 85% H_3PO_4 ext ref.

with iodine and aqueous pyridine and detritylated with acetic acid to give **3**. This linear dimer is a stable compound that is purified by preparative reversed-phase HPLC. The presence in **3** of one phosphate diester and one H-phosphonate monoester group was shown by ^{31}P NMR. The cyclization then was effected by treatment with adamantoyl chloride for 30 sec followed by precipitation into petroleum ether. The residue then was dissolved in a solution of iodine in aqueous pyridine for the final oxidation. Deprotection with aqueous ammonia followed by purification by reversed-phase HPLC completed the synthesis. The linear dimers **3** (after amino deprotection) and the cyclic dimers **4** were characterized by ^{1}H and ^{31}P NMR, MS, UV, and enzymatic degradation.

Tables 1 and 2 list the ^{31}P chemical shifts of the linear and cyclic dimers. The linear dimers each show a resonance corresponding to the internucleotide phosphate diester at -0.2 to -1.2 ppm and a resonance corresponding to the 3'-H-phosphonate at 5 to 6 ppm. The latter shows up as a doublet of doublets due to the large (642 Hz) P-H coupling and the much smaller 8 Hz coupling to the 3' proton. The ^{31}P spectra of the cyclic dimers are simpler. The homo dimers show only a single phosphate diester resonance, while the hetero dimers have two phosphate diester resonances.

Table 3 lists the masses of the species observed by laser desorption MS. These results show that, while the cyclization reaction could in principle produce higher oligomers, both linear and cyclic, the only species isolated was the cyclic dimer.

We also monitored treatment of both the linear and cyclic dimers with nuclease P1 and with spleen phosphodiesterase. The enzyme reactions were monitored by HPLC and the results are tabulated in Table 4. We found that the 3'-H-phosphomonester moiety was cleaved by P1 and, as had been reported previously by Reese,[7] the cyclic dimers are not cleaved by spleen phosphodiesterase, but are cleaved by nuclease P1. Reese has also reported that the rate of cleavage of cyclic dimers was slower than that of cyclic trimers or hexamers. We found that the cyclic dimers are much more slowly cleaved than the linear dimers, particularly as the pH is increased from 5.3 to 7. Table 5 shows the approximate times for complete digestion of linear and cyclic d[GT] as a function of pH. At pH 7, cleavage of the cyclic dimer is not complete at 48 h, while the linear dimer is completely cleaved within 2 h.

Table 3. Mass spectral data on the cyclic dimers.

	Mol Wt	Ions observed
c-AA	626.2	626.2 $(M)^+$; 642.2 $(M-2H+NH_4)^+$
c-AC	602.2	601.4 $(M-H)^+$; 623.3 $(M-H+Na)^+$
c-AG	642.2	642.0 $(M)^+$; 664.2 $(M-H+Na)^+$
c-AT	617.2	618.1 $(M+H)^+$; 639.6 $(M-H+Na)^+$
c-CC	578.1	578.4 $(M)^+$; 601.5 $(M+Na)^+$
c-CG	618.1	617.3 $(M-H)^+$
c-CT	593.1	593.1 (M^+)
c-GG	658.2	658.1 $(M)^+$; 680.7 $(M-H+Na)^+$
c-GT	633.2	632.4 $(M-H)^+$
c-TT	608.1	608.4 $(M)^+$; 630.8 $(M-H+Na)^+$

We used the resistance of the cyclic dimers to digestion at pH 7 to determine their extinction coefficients. To do this, identical volume samples of each cyclic dimer were lyophilized and dissolved in either water or pH 7 phosphate buffer to which nuclease P1 was added. After the former samples were digested, they were diluted with pH 7 phosphate to

Table 4. The reaction of cyclic and linear dimers with nuclease P1 and with Spleen phosphodiesterase.

Substrate	nuclease P1	spleen phosphodiesterase
$Tp_{(H)}$	T	-
c-AA	pA	-
c-AC	pA + pC	-
c-AG	pA + pG	-
c-AT	pA + pT	-
c-CC	pC	-
c-CG	pC + pG	-
c-CT	pC + pT	-
c-GG	pG	-
c-GT	pG + pT	-
c-TT	pT	-
l-$AAp_{(H)}$	A + pA	Ap + $Ap_{(H)}$
l-$ACp_{(H)}$	A + pC	Ap + $Cp_{(H)}$
l-$AGp_{(H)}$	A + pG	Ap + $Gp_{(H)}$
l-$ATp_{(H)}$	A + pT	Ap + $Tp_{(H)}$
l-$CCp_{(H)}$	C + pC	Cp + $Cp_{(H)}$
l-$CGp_{(H)}$	C + pG	Cp + $Gp_{(H)}$
l-$CTp_{(H)}$	C + pT	Cp + $Tp_{(H)}$
l-$GGp_{(H)}$	G + pG	Gp + $Gp_{(H)}$
l-$GTp_{(H)}$	G + pT	Gp + $Tp_{(H)}$
l-$TTp_{(H)}$	T + pT	Tp + $Tp_{(H)}$

Table 5. Approximate times for digestion with nuclease P1 as a function of pH.†

	5.3	5.6	6.0	6.3	7.0
l-d[GT]	20 min	40 min	60 min	80 min	120 min
c-d[GT]	2 h	24 h	36 h	48 h	> 48 h

†0.2 M acetate buffer at room temperature.

the same volume as the latter. Thus both the digested and undigested samples (confirmed by HPLC) had identical composition, differing only in the order of addition. The measured absorbences were all within experimental error, demonstrating that the cyclic dimer extinction is adequately represented as the sum of the monomer extinctions, as would be expected. These data are shown in Tables 6 and 7.

Experimental

General Methods. NMR spectra were recorded on a Varian XL-400 spectrometer. UV spectra were recorded on an AVIV C14. MALDITOF mass spectra were recorded on Biopro EZ1000. Preparative reversed-phase HPLC used a Waters PrepPak with two 40 x 100 mm cartridges.

General procedure for synthesis of linear dimers (3). To 0.65 mmol of a protected 3'-H-phosphonate (**1**) in 50 mL of pyridine was added 0.3 mL (2.0 mmol) of phenylsulfonylethanol. The solution was concentrated to a final volume of about 30 mL, and 0.5 g (2.5 mmol) of adamantoyl chloride was added with stirring. After 30 sec, 30 mL of ethyl acetate was added and the solution immediately poured into a flask containing 300 mL of petroleum ether. The mixture was cooled to 0 °C, the supernatant discarded, and the oily residue was dissolved in 30 mL of methylene chloride and again precipitated by addition to 300 mL of petroleum ether. To the oily residue dissolved in 30 mL of methylene chloride was added 10 mL of 5 % dicloroacetic acid in methylene chloride. After 2 min 400 mL of anhydrous diethyl ether was added to precipitate **2**. The residue of **2** was dissolved in methylene chloride and precipitated a second time by addition of diethyl ether to give **2** as a solid. This was used directly without further purification. To the precipitate of **2** dissolved in 30 mL of pyridine was added 0.65 mmol of **1** and 0.5 g (2.5 mmol) of adamantoyl chloride. After 30 sec 30 mL of ethyl acetate was added and the solution was immediately poured into 300 mL of petroleum ether. The supernatant was discarded and the precipitate was dissolved in a mixture of 75 mL of pyridine and 25 mL of triethylamine. After 20 min 400 mL of diethyl ether was added. The precipitate was dissolved in 60 mL of 2 % aqueous pyridine containing 1.0 g of

Table 6. Spectroscopic comparison of cyclic dimers and their enzymatic cleavage products.

	λ_{max}	A_{max}	A_{240}	A_{250}	A_{260}	A_{270}	A_{280}
c-d[AA]	257	0.74		0.65	0.73	0.46	
c-d[AA]†	257	0.77		0.67	0.77	0.47	
c-d[AC]	261	0.68	0.43	0.58	0.68	0.54	0.30
c-d[AC]†	261	0.65	0.42	0.55	0.65	0.52	0.29
c-d[AG]	255	0.61	0.40	0.58	0.57	0.42	
c-d[AG]†	255	0.58	0.37	0.55	0.54	0.40	
c-d[AT]	260	0.59		0.49	0.59	0.46	0.25
c-d[AT]†	260	0.60		0.49	0.60	0.48	0.25
c-d[CC]	268	0.69		0.50	0.61	0.68	0.50
c-d[CC]†	268	0.69		0.50	0.61	0.69	0.50
c-d[CG]	254	0.52	0.45	0.51	0.49	0.49	0.38
c-d[CG]†	254	0.54	0.48	0.53	0.51	0.50	0.40
c-d[CT]	267	0.59		0.43	0.55	0.57	0.41
c-d[CT]†	267	0.59		0.43	0.55	0.58	0.41
c-d[GG]	250	0.71	0.57	0.71	0.60	0.50	0.40
c-d[GG]†	250	0.75	0.60	0.75	0.63	0.55	0.45
c-d[GT]	255	0.68	0.48	0.66	0.66	0.61	0.45
c-d[GT]†	255	0.68	0.49	0.66	0.66	0.61	0.45
c-d[TT]	265	0.62	0.28	0.44	0.59	0.60	0.40
c-d[TT]†	265	0.63	0.29	0.44	0.60	0.060	0.41

†A sample of the cyclic dinucleotide after degradation with nuclease P1.

Table 7. Yields and extinction coefficients for the cyclic dimers.

	µmol	overall %	ε x $10^{-3}M^{-1}$		µmol	overall %	ε x $10^{-3}M^{-1}$
c-AA	56	13	30.6	c-CG	75	17	19.2
c-AC	73	17	22.7	c-CT	97	20	16.7
c-AG	87	15	27.1	c-GG	51	12	23.6
c-AT	160	12	24.6	c-GT	57	12	21.1
c-CC	60	14	14.8	c-TT	483	34	18.6

iodine. After 15 min the solution was concentrated to near dryness, 150 mL of 20 % acetic acid was added and the solution was extracted once with diethyl ether. After standing for 5 h, the solution was extracted with 2 50 mL portions of diethyl ether and any remaining acid neutralized by addition of portions of sodium bicarbonate until gas evolution ceased. The linear dimer (**3**) was purified by preparative reversed phase HPLC using a gradient of 0 to 25 % acetonitrile: 0.1 M ammonium bicarbonate (pH 6.9) at a flow rate of 24 mL/min. The combined fractions of pure **3** were lyophilized to dryness.

General procedure for preparation of the cyclic dimers (4). To a solution of the linear dimer **3**, dried by concentration of a pyridine solution (3 times) and dissolved in 40 mL of pyridine was added 0.5 g (2.5 mmol) of adamantoyl chloride. After 30 sec, 40 mL of ethyl acetate was added and the mixture was poured into 300 mL of petroleum ether. The precipitated solid was dissolved in 100 mL of 2 % aqueous pyridine containing 1.0 g of iodine. After 15 min, the solution was concentrated to near dryness, dissolved in 150 mL of water and extracted with three 50 mL portions of diethyl ether. The aqueous layer was concentrated to near dryness and dissolved in 30 mL of concentrated aqueous ammonia. After 2 days, the solution was concentrated on a Speed-Vac to remove most of the ammonia and lyophilized to dryness. The residue was purified by preparative reversed-phase HPLC using a gradient of 0 to 25 % acetonitrile: 0.1 M ammonium bicarbonate (pH 6.9) at a flow rate of 24 mL/min. The combined fractions of pure **4** were lyophilized to dryness. The overall yield for preparation of each cyclic dimer is listed in Table 7.

General procedure for deprotected linear dimers. The protected linear dimers **3** were treated with 15 mL of concentrated aqueous ammonia for 2 days, and purified as described above for **4.**

Nuclease P1 digestion. To a 1.0 OD sample of dimer was added 0.5 mL of 0.2 M acetate buffer (pH 5.3) and 10 μL of a solution of nuclease P1 prepared from a 100 μL solution of lyophilized P1 powder (40 units) in deionized water that was further diluted by addition of 0.4 mL of 0.2 M acetate buffer (pH 5.3) and 50 μL of 0.2 M $ZnSO_4$. The digestion was monitored by reversed-phase HPLC using a gradient of 2 to 20 % acetonitrile: 0.1 M triethylammonium acetate.

Spleen phosphodiesterase digestion. To a 1.0 OD sample of dimer was added 0.5 mL of 0.2 M acetate buffer (pH 6.3) and 10 μL of a solution of spleen phosphodiesterase prepared by dissolving 25 units of lyophilized spleen

phosphodiesterase in 0.4 mL of deionized water. The digestion was monitored by reversed-phase HPLC using a gradient of 2 to 20 % acetonitrile: 0.1 M triethylammonium acetate.

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