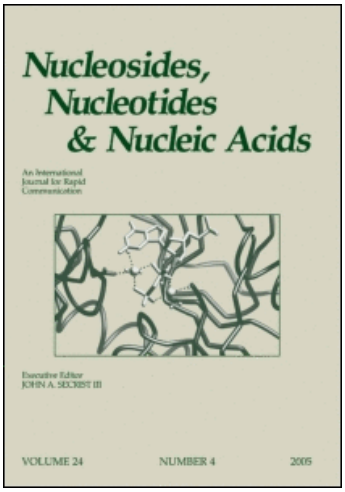

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Synthesis and Physical Characterization of Bis 3'→5' Cyclic Dinucleotides (NpNp): RNA Polymerase Inhibitors

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SYNTHESIS AND PHYSICAL CHARACTERIZATION OF BIS 3'→5' CYCLIC DINUCLEOTIDES (⌐NpNp¬): RNA POLYMERASE INHIBITORS‡

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Abstract

A defined chemical synthesis of the cyclic dinucleotides, ⌐ApAp¬, ⌐ApUp¬, and ⌐UpUp¬ has been devised, based on modern phosphotriester methods. These cyclic dinucleotides have been shown to inhibit RNA polymerase. The ^{1}H NMR spectra of the protected dimers show a preference for the ^{2}E form, while the spectra of the unprotected dimers show the ^{3}E form to be favored. The circular dichroism spectra show a negative long wavelength transition; however, ⌐ApUp¬ does not show the short wavelength maximum present in the spectra of ⌐ApAp¬ and ⌐UpUp¬.

Introduction

Cyclic ribodinucleotides have been shown by Hsu and Dennis[1] to act as potent linear competitive inhibitors for the DNA dependent RNA polymerase of E. coli, specifically during the initiation phase of the enzymatic reaction. This inhibition was predicted to occur due to the simultaneous binding at both active sites on the enzyme according to a model for rotational translocation proposed by Dennis and Sylvester[2].

Previous routes to cyclic dinucleotides have relied on isolation of the products from the complex mixtures obtained from homopolymerization

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reactions (Lapidot and Khorana,[3]; Schaller and Khorana[4]; Coutsogeorgopoulos and Khorana[5]; and Ohtsuka et al.,[6]. This is an inefficient procedure that is necessarily limited to homocyclic molecules. We have devised a specific phosphotriester procedure for the synthesis of both homo- and heterocyclic dinucleotides, employing high dilution cyclization of the appropriately protected ribonucleotides (Scheme I). The synthesis and characterization of ⌐UpUp¬, ⌐ApUP¬ and ⌐ApAp¬ are presented.

Our conclusions from NMR studies of these cyclic compounds are that the pentose ring in solution is rigid; existing in the ^{2}E conformation for the fully protected cyclic dinucleotides, and the ^{3}E conformation in the case of the unprotected cyclic dinucleotides.

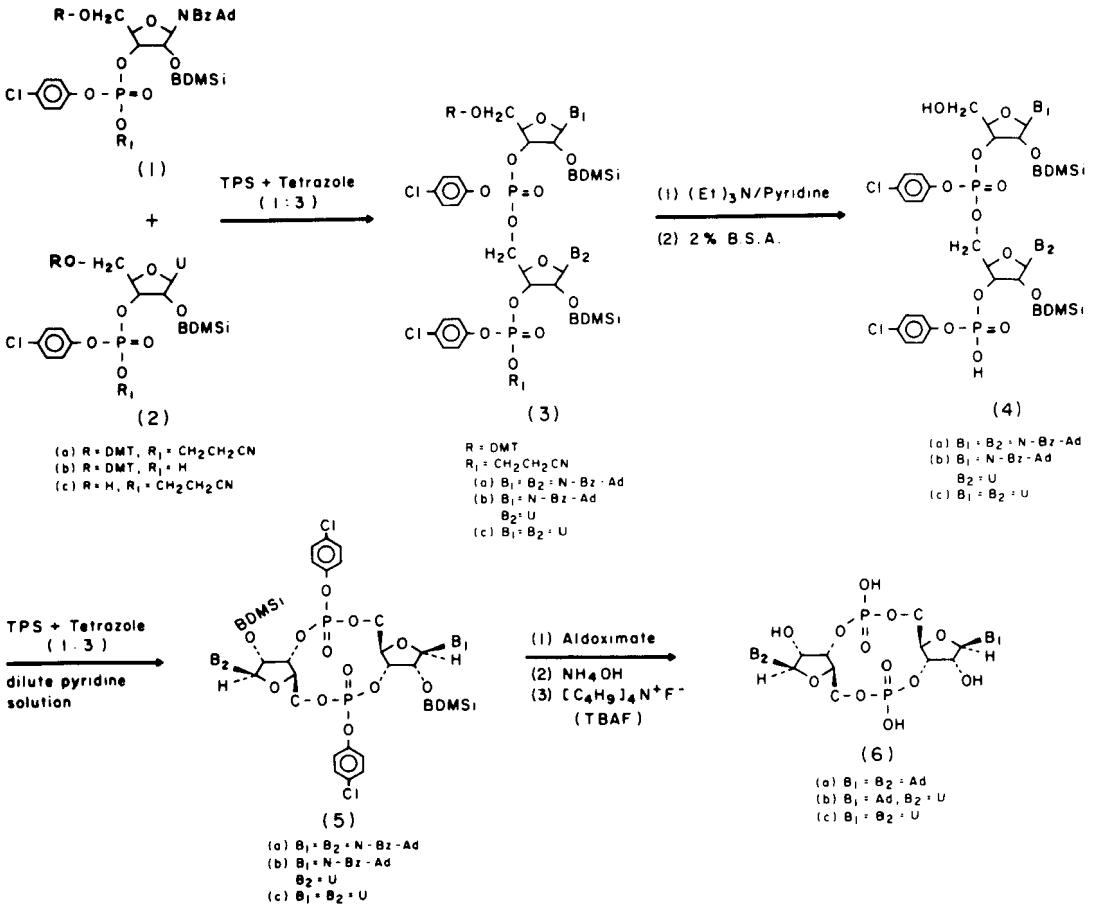
MATERIALS AND METHODS

Mallinckrodt CC-7 silica gel was used for column chromatography. Preparative silica gel GF plates (20 x 20 cm, 1500μ) were purchased from Analtech. The plates were eluted once with acetone prior to sample loading. Separations were typically carried out on 100-200 mg of material.

Analytical cellulose TLC was carried out on EM pre-coated cellulose F-254 aluminum sheets. Solvent systems were: solvent 1, n-propanol:conc. ammonium hydroxide:water = 55:10:35; solvent 2, isobutyric acid:conc. ammonium hydroxide:water = 66:1:33 (IBAW).

High pressure liquid chromatography (HPLC) was performed on a Varian model 5000 liquid chromatograph with a Varian-Chrom UV-Vis detector. A MCH-10 C18 reverse phase column (0.4 x 30 cm) with a micropak guard column was used. The flow rate was 2 ml/min. The operating temperature was 30°C. The purity of all synthesized compounds (>95%) was assessed by HPLC analysis.

RNase M was kindly supplied by Dr. Masachika Irie, Hoshi College of Pharmacy. Samples of 3-4 OD_{260} of cyclic dinucleotides were treated with 30 μg RNase M in 0.1 M ammonium acetate (pH 5.0) in a total volume of 115 μl. The reaction was allowed to proceed 5 hrs. at 37°C. A solution of the enzyme alone in the buffer and another solution of the cyclic dimer alone in the buffer were used as the blank and reference for cellulose TLC analysis and UV absorption at 260 nm. A 20 μl sample of each was taken for UV and a 10 μl sample of each was taken for TLC.



(1)

(2)

(a) R = DMT, R_1 = CH_2CH_2CN
(b) R = DMT, R_1 = H
(c) R = H, R_1 = CH_2CH_2CN

(3)

R = DMT
R_1 = CH_2CH_2CN
(a) B_1 = B_2 = N-Bz-Ad
(b) B_1 = N-Bz-Ad, B_2 = U
(c) B_1 = B_2 = U

(4)

(a) B_1 = B_2 = N-Bz-Ad
(b) B_1 = N-Bz-Ad, B_2 = U
(c) B_1 = B_2 = U

(5)

(a) B_1 = B_2 = N-Bz-Ad
(b) B_1 = N-Bz-Ad, B_2 = U
(c) B_1 = B_2 = U

(6)

(a) B_1 = B_2 = Ad
(b) B_1 = Ad, B_2 = U
(c) B_1 = B_2 = U

^{1}H-NMR spectra were recorded using a Bruker WM-250 (250.13 MHz) spectrometer in a Fourier transform mode. Tetramethylsilane was used as the internal reference. Sample solutions were 5-100 mM in $CDCl_3$ or deuterium oxide contained in a 5mm tube. The accuracy of the chemical shifts was ±0.01 ppm. ^{31}P-NMR spectra were measured at 101.27 MHz. Orthophosphoric acid was used as the external standard. Sample solutions were 1-10 mM contained in a 10 mm tube. The accuracy of the chemical shifts was ±0.1 ppm.

The assignments of each proton of the fully blocked and the fully deblocked cyclic dinucleotides were obtained by comparison with the literature data, examination of the peak splitting pattern and, most definitively, by decoupling. The coupling constants were obtained by

analysis of the fully coupled ^{1}H spectra, the selectively spin decoupled ^{1}H spectra and the ^{1}H-coupled ^{31}P spectra as well as some computer simulation using an ITRCAL program.

The starting materials, 1a, 1b and 2a, 2b, were synthesized from the corresponding nucleosides according to published procedures (Ogilvie et al.[7, 8]).

The Fully Protected Linear Dinucleotides (3). To a mixture of 0.15-0.3 mmole of (1b-2b) and 0.6-1.4 equivalents of (1c-2c) rendered anhydrous by repeated evaporation of pyridine was added a solution of 3 eq. of 2,4,6-triisopropyl-benzenesulfonylchloride (TPSCl) and 9 eq. of tetrazole in anhydrous pyridine. The total volume was 1-2 mL. The reaction was allowed to proceed for 2 hrs. under nitrogen at room temperature. Water was added and the product was extracted into chloroform. The layers were separated and the chloroform layer was washed with 0.1 M sodium bicarbonate (2X), with water, and was dried over calcium sulfate and concentrated. The remaining pyridine was removed by evaporation with toluene. The residue was dissolved in 1 mL chloroform and applied either to a silica gel column (3 x 20 cm) or to a preparative plate, using 2.5% ethanol/chloroform as the eluant. The appropriate fractions were evaporated to a dry foam and dried above phosphorus pentoxide in vacuo.

Decyanoethylation and Detritylation of the Fully Protected Dimer (4) The fully blocked dinucleotide (3, 50-100 μmole) was dissolved in 1-2 mL of double distilled triethylamine and an equal volume of anhydrous pyridine. The solution was kept at room temperature for 2 hrs and was then evaporated with additions of pyridine. The residual concentrated solution was added dropwise to 100 mL of a mixture of diethyl ether and petroleum ether (1:2) with stirring. The white precipitate was collected by centrifugation and dried over phosphorus pentoxide in vacuo. The dried product was then treated with 3 mL of 2% benzenesulfonic acid in a mixture of chloroform and methanol (7:3) in an ice bath. After 20 min., the solution was washed with 0.1 M sodium bicarbonate (2 x 5 mL) and water. Each aqueous phase was back extracted with chloroform. The combined chloroform solution was evaporated with additions of pyridine to a concentrated solution which was then added dropwise to 60 mL of a stirred mixture of ether and pet ether (1:1). The precipitated product (4) was collected by centrifugation.

Intramolecular Condensation for the Synthesis of the Fully Protected Cyclic Dinucleotides (5). To a dried pyridine solution of the partially deblocked dimer (4, 40-50 μmole) was added an anhydrous pyridine solution of 6 equivalents of TPSCl and 18 eq. of tetrazole. The final concentration of the dinucleotide in pyridine was 5mM. After 5-10 hrs., most of the starting material was converted to the highly nonpolar cyclic product as judged by TLC. The reaction was stopped by the addition of water and additional 50% aqueous pyridine was added. The cyclic product was then extracted with chloroform (3 x 20 mL), which was then washed with 0.1 M sodium bicarbonate and water. The aqueous wash was further extracted with chloroform. The combined chloroform extract was concentrated and the residue applied to a preparative silica gel plate, which was then developed in ethanol/chloroform 1-3 times until a good separation was obtained.

Complete Deblocking to Obtain the Fully Unprotected Cyclic Dinucleotides (6). Method A: To 25 mg of the fully protected cyclic dimer (5) was added 1 mL of a solution of 0.3 mmole of tetramethylguanidine (TMG) and 0.3 mmole of 2-nitrobenzaldoxime (NBO) in dioxane and water (1:1) according to Reese et al.[9]. After 3 hrs., an additional 0.15 mmole of TMG was added and the reaction mixture was kept at room temperature overnight. This mixture was treated with 10 mL of concentrated aqueous ammonia overnight and then heated at 50°C for 3 hrs. Preparative silica gel TLC (5% ethanol/chloroform) was used to remove non-nucleotidic material. The separated product was then treated with 1.0 mL of a pyridine solution of tetra-n-butylammonium fluoride (TBAF, 0.2 mmole and HF (0.1 mmoles; Jones et al.[10]). The extent of reaction was monitored by silica gel TLC in 20% methanol/ethylacetate and cellulose TLC in solvent 1. After 3 hrs., about 15 mL of a suspension of pyridinium Dowex 50-X8 was added and the mixture was stirred for 1 hr. The resin was filtered off and the filtrate was concentrated under reduced pressure. The fully deblocked product was separated by HPLC using a linear gradient of 4-35% acetonitrile in 0.1 M triethylammonium bicarbonate (TEAB). The collected eluate was concentrated to a small volume and lyophilized.

Method B: To 27 mg of 5b was added 10 mL of concentrated aqueous ammonia and 0.5 mL of pyridine. After 3 days, the mixture was

evaporated with additions of pyridine to a volume of about 1 mL. The mixture was then treated with TBAF/HF and purified by HPLC as above.

Results and Discussion

SYNTHESIS The fully protected adenosine and uridine phosphotriester derivatives 1a and 2a were prepared according to the literature (Ogilvie et al.[7,8]). The synthesis of the linear linear dinucleotides was then carried out by: 1. Treatment of 1a/2a with triethylamine to give the 3'phosphodiester derivative 1b/2b. 2. Treatment of 1a/2a with 2% benzenesulfonic acid to give the 5' hydroxyl derivative 1c/2c. 3.Condensation of (1b + 1c), (1b + 2c), (2b + 2c) using triisopropylbenzenesufonyl chloride (TPS-Cl) and tetrazole (Seth and Jay[11].) to give, respectively, 3a-c, in an average yield of 63% after purification by chromatography on silica.

The fully protected linear dinucleotides 3a-c were analogously 5' and 3' deprotected to give 4a-c in isolated yields of 75-82%. Cyclization of 4a-c to 5a-c was then effected using TPS-Cl/tetrazole as above but at high dilution (5 mM vs. 400 mM) to avoid oligomerization. After purification by preparative silica gel TLC, 5a-c were obtained in yields of 60%, 68% and 54%, respectively, as mixtures of diastereomers. These mixtures were separated by further preparative TLC.

Deprotection of 5a-c by standard procedures followed by purification by reversed phase HPLC gave 6a-c in 50-66% yield. Each was homogeneous by HPLC and by TLC on cellulose. Ohtsuka et al.[6] reported that ⌐ApAp¬ (6a) is hydrolyzed by the enzyme RNase M to yield adenosine-3'-phosphate as the sole product. Treatment of compound 6b ⌐ApUp¬ with RNase M produced two products adenosine-3'-phosphate and uridine-3'-phosphate in equal molar quantities by quantitative analysis on cellulose TLC using solvent 2.

NMR Conformational Analysis

The major diastereomers of 5a-c were studied by both ^{31}P and ^{1}H NMR. The homocyclic dimers 5a and 5c showed only a single ^{31}P resonance, and the heterocyclic dimer 5b showed only two. In addition, the ^{1}H NMR of 5a and 5c showed only one set of proton resonances. Thus both ^{31}P and ^{1}H NMR indicate identical conformations for the two base-ribose-phosphates. Moreover, the $J_{1',2'}$ values of 7.3 Hz, and $J_{3',4'}$ values near zero (5a and 5c, Table 1) define the sugar ring

Table 1

Coupling Constants (Hz) in Fully Protected Cyclic Dinucleotides

Compounds		$J_{5,N3}$	$J_{5,6}$	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{4'5''}$	$J_{5'5''}$	$J_{5'p}$	$J_{5''p}$	$J_{3'p}$
[ApAp][a]		-	-	7.3	4.7	0.5 ±[b] 0.5	10.5	3.6	-10.5	10.8	10.4	9.0
[UpUp]		2.0	8.2	7.3	5.3	0.5 ±[b] 0.5	9.2	3.0	-9.5	9.0	9.0	8.5
[ApUp]	-pAp-	-	-	4.7[c]	-	~0	-	-	-	-	-	-
	-pUp-	~0	7.7	5.6[c]	-	~0	-	-	-	-	-	~8.0

a. Protecting groups are N-benzoyl, 2'-t-butyldimethylsilyl, P-(p-chlorophenyl).
b. Analysis from computer line-shape simulation.
c. Smaller than the real value due to the second order effect.
- Non-determined due to great overlap of protons.

conformation as C(2')-endo (^{2}E), while the $J_{4',5'}$ and $J_{4',5''}$ values indicate that the C(4')-C(5') bond has the tg conformation.

The coupling values for $J_{5'p}$ and $J_{5''p}$ in both 5a and 5c are about 10 Hz. This value is not characteristic of either the typical trans (~21 Hz) or the typical gauche (~3 Hz) relationship of the O(5')-C(5') bond. It is unlikely that the bond exists as a mixture of conformers g'g', t'g' and g't' using the method of rotamer analysis (Davies[12]; Cheng and Sarma[13]; Davies and Danyluk[14]; Lee et al.[15]) because (i) all the other backbones, i.e. the sugar ring, the C(4')-C(5') bond (described above) and the C(3')-O(3') bond (described later), exist in an unique conformation, and (ii) the g'g' conformation was found to be exclusively preferred in all the previously studied molecules. It is thus assumed that the O(5')-C(5') bond conformation is g'g' and that the larger than normal value observed is due to the electronegative effect of the p-chlorophenyl group on the internucleotidic phosphodiester linkage. This assumption was supported after the protecting groups were removed.

The vicinal H-P coupling values, $J_{3'p}$, for all three of the protected cyclic dimers were essentially the reported literature value of 8-9 Hz indicating a dihedral angle of 36° that is consistent for that seen with linear molecules.

The Fully Unprotected Cyclic Dimers. The spectra of the fully unprotected cyclic dimers are not as well resolved as those of the fully protected dimers. The coupling constants obtained are listed in Table 2. The remarkable difference between the protected and the unprotected compounds is the sugar ring puckering which changes from ^{2}E in the former to ^{3}E in the latter. The ^{1}H nmr spectra of all three dimers showed a single peak for each C(1') proton. The splitting pattern of

Table 2

Coupling Constants (HZ) in Fully Unprotected Cyclic Dinucleotides

Compounds	$J_{5,6}$	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{3'p}$	$J_{5''p}$	$J_{5'5''}$
⌐ApAp¬	-	0	~5.0	~9.0	~9.0	-	-
⌐UpUp¬	8.17	0	4.73	~9.0	~9.0	~3.0	12.0
⌐ApUp¬ pAp	-	0	4.3	-	-	-	12.0
pUp	8.0	0	4.5	~8.5	~8.5	-	12.0

the C(3') proton, which is a double doublet in the protected compounds, changes to a triple doublet in the unprotected compounds (Fig. 1) due to the 3',4' coupling ($J_{3',4'}$ = 9.0 Hz). The coupling constants $J_{1'2'}$ and $J_{3'4'}$ indicate that the conformation of the sugar ring of bis-(3'→5')-cyclic dinucleotides are C(3')-endo (^3E).

The $J_{3'p}$ value remained the same as that in the protected compounds. The unique conformation of the C(3')-O(3') bond, g^+ (θ=36°), is thus maintained.

The $J_{5''p}$ was obtained, using the decoupling method, as ~3.0 Hz. This value is the typical J_{HP} in a gauche relationship. A g'g' conformation, as in the protected compounds, is thus assigned to the O(5')-C(5') bond. Furthermore, these values support our assumption that that the unusually large value of 10.0 Hz observed in the protected cyclic dimers for $J_{5'p}$ and $J_{5''p}$ in g'g' conformation is due to the substituent on the internucleotidic phosphodiester linkage.

Some differences in the chemical shifts of the relative base and ribose protons in the three cyclic dimers (Table 3) were observed. The A-H8, A-H2 and A-H1' in ⌜ApUp⌝ all shift upfield by ~0.25 ppm from those in ⌜ApAp⌝. The U-H6, U-H5 and U-H1' in ⌜ApUp⌝ shift upfield by 0.28,

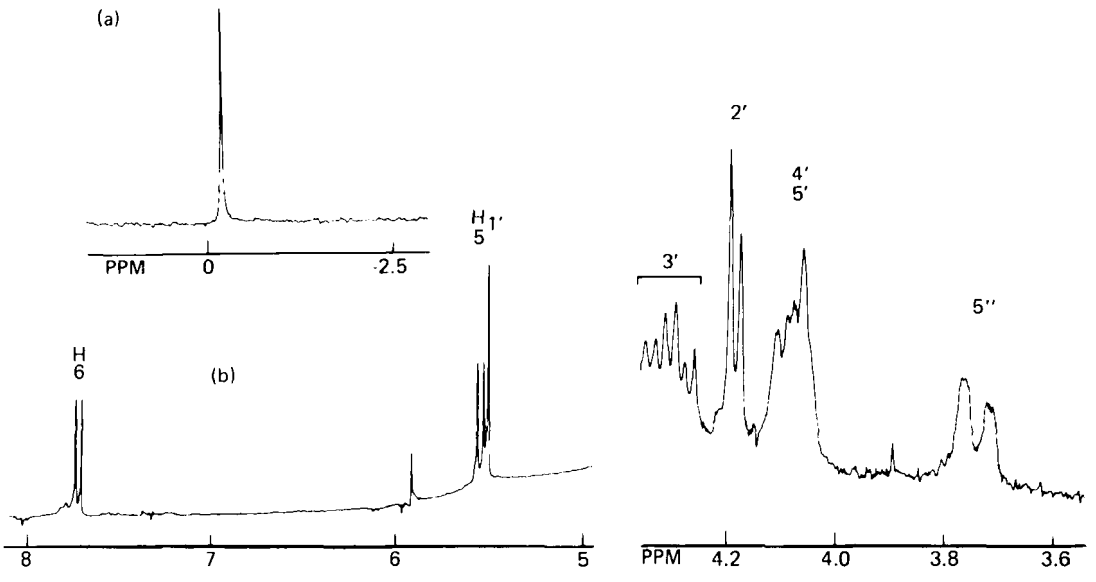


Figure 1. (a) The ^{31}P NMR spectra of ⌜UpUp⌝ (6c) in D_2O.
(b) The ^1H NMR spectra of ⌜UpUp⌝ (6c) in D_2O.

Table 3

Summary of ^{1}H-NMR Chemical Shifts (ppm) of The Cyclic Compounds

Compound / protons		1'	2'	3'	4'	5'	5"	2	8	5	6
5a'		5.87	5.67	5.46	4.56	4.93	4.19				
5b'	(A)	5.9	5.46	5.46	--	--	4.20				
	(U)	5.6	--	5.21	--	--	4.20			5.68	
5c'		5.81	4.80	5.07	4.27	4.66	4.16			5.69	
6a[2]		5.97	4.83	4.28				7.93	8.20		
6b[2]	20°C (A)	5.68	--	--	--	--	--	7.69	7.95		
	(U)	5.27	--	--	--	--	--			5.01	7.44
6b[2]	57°C (A)	5.89	4.54	--	--	--	--	7.95	8.14		
	(U)	5.53	4.57	4.60	--	--	--			5.46	7.68
6c[2]		5.51	4.19	4.30	--	--	--			5.55	7.72

1. Dissolving solvent is d-chloroform.

2. Dissolving solvent is D_2O.

-- Non-resolved due to extensive overlap of adjacent protons.

0.54 and 0.24 ppm, respectively from those in ⌐UpUp¬. Base-stacking effects and changes in the glycosidic torsion angle may be the major contributors to the observed shifts (Lee et al.[15]) since the circular di(ribose-phosphate) backbones of the three cyclic dimers are the same. A quantitative sorting out of the individual contributors is not possible at this moment.

Circular dichroism spectra of the three cyclic dinucleotides in aqueous and organic solvents were measured. Cantor et al.[16] and Ohtsuka et al.[6], have noticed a remarkable difference between the linear and the cyclic dithymidylic acids and diadenylic acids, respectively. The differences reside in the opposite signs of the Cotton effect at both

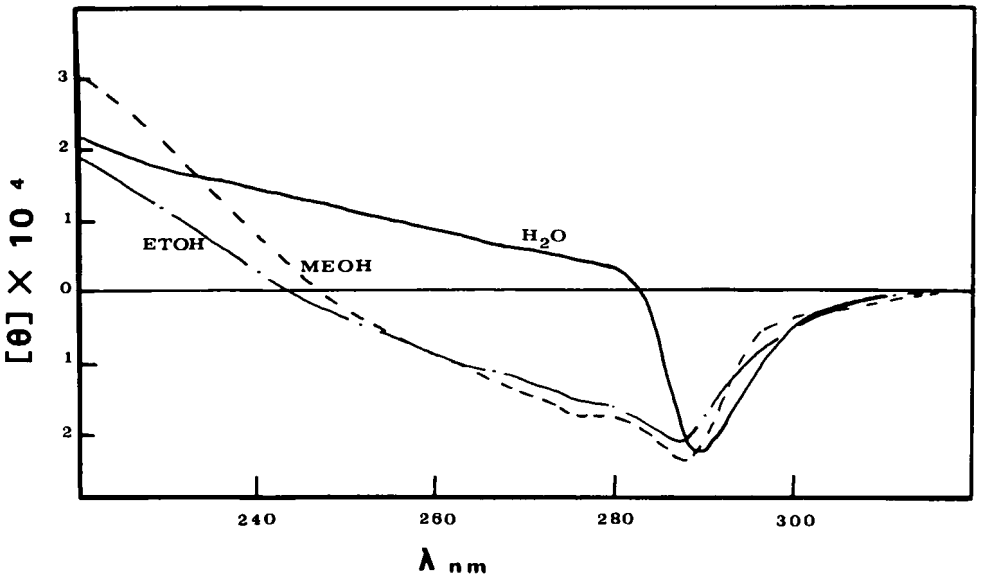


Fig. 2 The CD spectra of ⌜ApUp⌝ (6b) in different solvents

long and short wavelengths in aqueous solution. It was reported that almost all of the linear dinucleotides have positive Cotton effects at long wavelength (the π-π* transition, Cantor and Warshaw[17]; Warshaw and Cantor[18]). Both d⌜TpTp⌝ and ⌜ApAp⌝ have a negative long wavelength band, which seems to be characteristic of the cyclic dinucleotides. All three cyclic dinucleotides examined showed negative π-π* transition. The CD spectra of ⌜ApUp⌝ in different solvents are shown in Fig. 2. Unlike the homocyclic dimers, ⌜ApUp⌝ does not have a short wavelength maximum. The well defined negative Cotton effect at long wavelength is disrupted by the alcoholic solvents. The spectrum of ⌜ApAp⌝ is also greatly perturbed by ethanol. It was reported that the ordered stacking of d⌜TpTp⌝ is greatly disrupted in methanol (Cantor and Warshaw[17]; Warshaw and Cantor[18]).

Concluding Remarks

Three bis-(3'→5')-cyclic dinucleotides were synthesized using a phosphotriester procedure and the backbone conformations were determined

by NMR. Both the fully protected and unprotected cyclic dimers are rigid molecules. The conformation of the fully protected cyclic dimers are (^{2}E/tg/g'g'/g+(θ=36°). The sugar ring conformation changes to ^{3}E exclusively in the unprotected cyclic dimers, while all other backbone conformations remain the same. The cyclic di(ribose-phosphate) backbone is rigid and its conformation is independent of the base sequence. The coupling values found with these rigid molecules are consistent with the idea that a unique coupling constant characterizes a unique dihedral angle.

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