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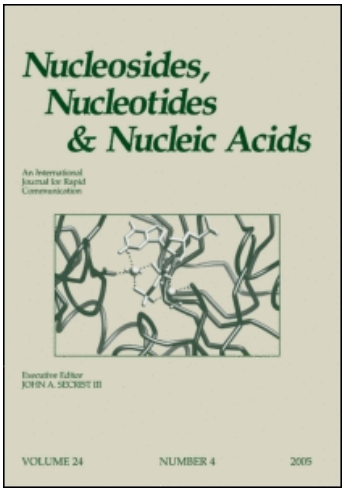

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RNA Modified Uridines VII: Chemical Synthesis and Initial Analysis of tRNA D-Loop Oligomers with Tandem Modified Uridines

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RNA MODIFIED URIDINES VII: CHEMICAL SYNTHESIS AND INITIAL ANALYSIS OF tRNA D-LOOP OLIGOMERS WITH TANDEM MODIFIED URIDINES

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Abstract: The first chemical synthesis of oligoribonucleotides with adjacent and significantly different modified uridines, the hydrophobic dihydrouridine, D, and the hydrophilic 3-[3-(S)-amino-3-carboxypropyl]-uridine, $(acp)^3U$, is reported. The trimers Dp-acp^3UpA, Up-acp^3UpA, DpUpA and UpUpA, and the dimer Dp-acp^3U were synthesized and initial structural analysis performed. The synthesis included a combination of protecting groups that is generally applicable to oligonucleotide syntheses in combination with various 2'OH protecting groups. The protecting groups did not cause racemization of the amino acid residue of $(acp)^3U$ during deprotection. The assignment of all ^{1}H NMR resonances of modified nucleoside-containing oligoribonucleotides includes heteronuclear one and two dimensional NMR.

Introduction

Dihydrouridine, D, is found so often in Loop I of the tRNA cloverleaf structure of all species of tRNA from all organisms that this domain of the molecule is referred to as the D-stem and loop.[1] In contrast, the nucleoside 3-[3-(S)-amino-3-carboxypropyl]-uridine, $(acp)^3U$, is a structural component of the dihydrouridine loops of Arg, Asn, Gly, Met, Tyr and Val and "extra arms" of Arg, Ile, Lys, Met, Met_i, Phe, Tyr and Val tRNAs.[2] It is noteworthy that the hydrophilic $(acp)^3U$ follows the relatively hydrophobic D in the sequence Dp-$(acp)^3$UpA in the D-loops of tRNAs Asn and Val

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and the very polar, positively charged N^7-methylguanosine (m^7G) in the "extra arm" sequence m^7Gp-$(acp)^3UpC$ of several tRNAs from eukaryotes and prokaryotes, respectively.[2] Pseudouridine, methylated at N1 and analogously modified at N3, $m^1(acp)^3\Psi$, has been found in the hydrolysis products of 17S and 18S rRNA.[3,4] Little is known about biological functions of uridines modified at the N3-position with α-amino acid side chains and occurring in tRNAs[5] or rRNAs.

Synthetic oligoribonucleotides are useful models for the study of structure and function relationships of the naturally occurring nucleic acids. However, relatively limited numbers of hypermodified uridines have been incorporated into synthesized oligoribonucleotides having tRNA sequences, either by the chemical[5-7] or semi-enzymatic (RNA recombinant technology) methods.[8] D has been used by several groups as a component for the synthesis of oligoribo-nucleotides[9,10] and tRNA D-loop fragments.[11] Dihydrothymidine has been used for the synthesis of DNA fragments by phosphoramidite chemistry[12] and by the solid phase phosphotriester approach.[13] Previously, we reported the synthesis of $(acp)^3U$ and $m^1(acp)^3\Psi$.[14,15] Here, we describe the first chemical synthesis of two tRNA D-loop fragments with uniquely different tandem modified uridines Dp-$(acp)^3U$, Dp-$(acp)^3UpA$ and the related sequences DpUpA, Up-$(acp)^3UpA$ and UpUpA.

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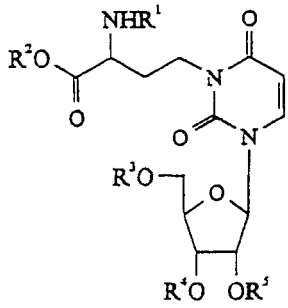
Results and Discussion

Syntheses of Oligonucleotides. The multimilligram scale syntheses of the oligoribonucleotides Dp-$(acp)^3U$ **5**, Dp-$(acp)^3UpA$ **6**, DpUpA **7**, Up-$(acp)^3UpA$ **8** and UpUpA **9** (Figures 1-3) were achieved with adaptations of the classical phosphotriester approach.[16,17] The syntheses are given in the Experimental section in some detail. Dihydrouridine, D, was obtained by catalytic hydrogenation of uridine.[18] The modified nucleoside $(acp)^3U$ **1** was chemically synthesized by condensation of 2'3'-O-isopropylidene-uridine with benzyl 2-(S)-benzyloxycarbonylamino-4-iodobutyrate. Then, the benzyloxycarbonyl- (Cbz), benzyl- (Bzl) and the isopropylidene protecting groups were removed.[14] Selective deprotection of the isopropylidene group from the product of the condensation allowed us to obtain the amino acid protected nucleoside **1a**[14] which was used as the starting compound for the synthesis of the protected nucleoside **1d**.



1

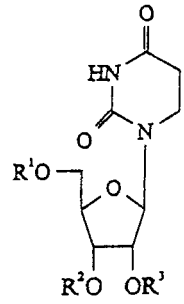
1 $R^1=R^2=R^3=R^4=R^5=H$

1a R^1=Cbz, R^2=Bzl
$R^3=R^4=R^5=H$

1b R^1=Cbz, R^2=Bzl, R^5=H
R^3R^4=Si(iPr)$_2$-O-Si(iPr)$_2$

1c R^1=Cbz, R^2=Bzl, R^5=THP
R^3R^4=Si(iPr)$_2$-O-Si(iPr)$_2$

1d R^1=Cbz, R^2=Bzl, $R^3=R^4$=H
R^5=THP



2

2 $R^1=R^2=R^3=H$

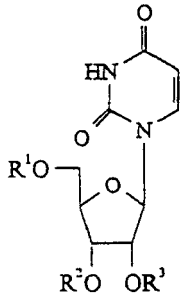
2a $R^1=R^2$=Si(iPr)$_2$-O-Si(iPr)$_2$,
R^3=H

2b R^1R^2=Si(iPr)$_2$-O-Si(iPr)$_2$

R^3=THP

2c $R^1=R^2$=H, R^3=THP

2d R^1=MMT, R^2=H, R^3=THP



3

3 $R^1=R^2=R^3=H$

3a R^1R^2=Si(iPr)$_2$-O-Si(iPr)$_2$

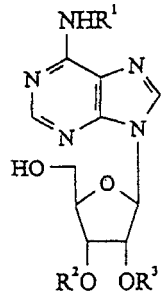
R^3=H

3b $R^1=R^2$=Si(iPr)$_2$-O-Si(iPr)$_2$

R^3=THP

3c $R^1=R^2$=H, R^3=THP

3d R^1=MMT, R^2=H, R^3=THP



4

4 $R^1=R^2=R^3=H$

4a R^1=PAC, $R^2=R^3$=H

4b R^1=PAC, R^2R^3 - methoxymethylidene

Abbreviations:
Cbz-benzyloxy-carbonyl
Bzl-benzyl
MMT-monomethoxytrityl
iPr-isopropyl
PAC-phenoxyacetyl
THP-tetrahydrofuranyl

FIG 1. Synthesis of the protected mononucleoside components. The figure illustrates the pathways for synthesis of the protected monoucleosides subsequently used in production of the oligoribonucleosides: for protection of 3-[3-(S)-amino-3-carboxypropyl]uridine, (acp)^{3}U **1**, **1** → **1a** → **1b** → **1c** → **1d**; dihydrouridine, D **2**, **2** → **2a** → **2b** → **2c** → **2d**; uridine, U **3**, **3** → **3a** → **3b** → **3c** → **3d**; and adenosine, A **4**, **4** → **4a** → **4b** → **4c** → **4d**.

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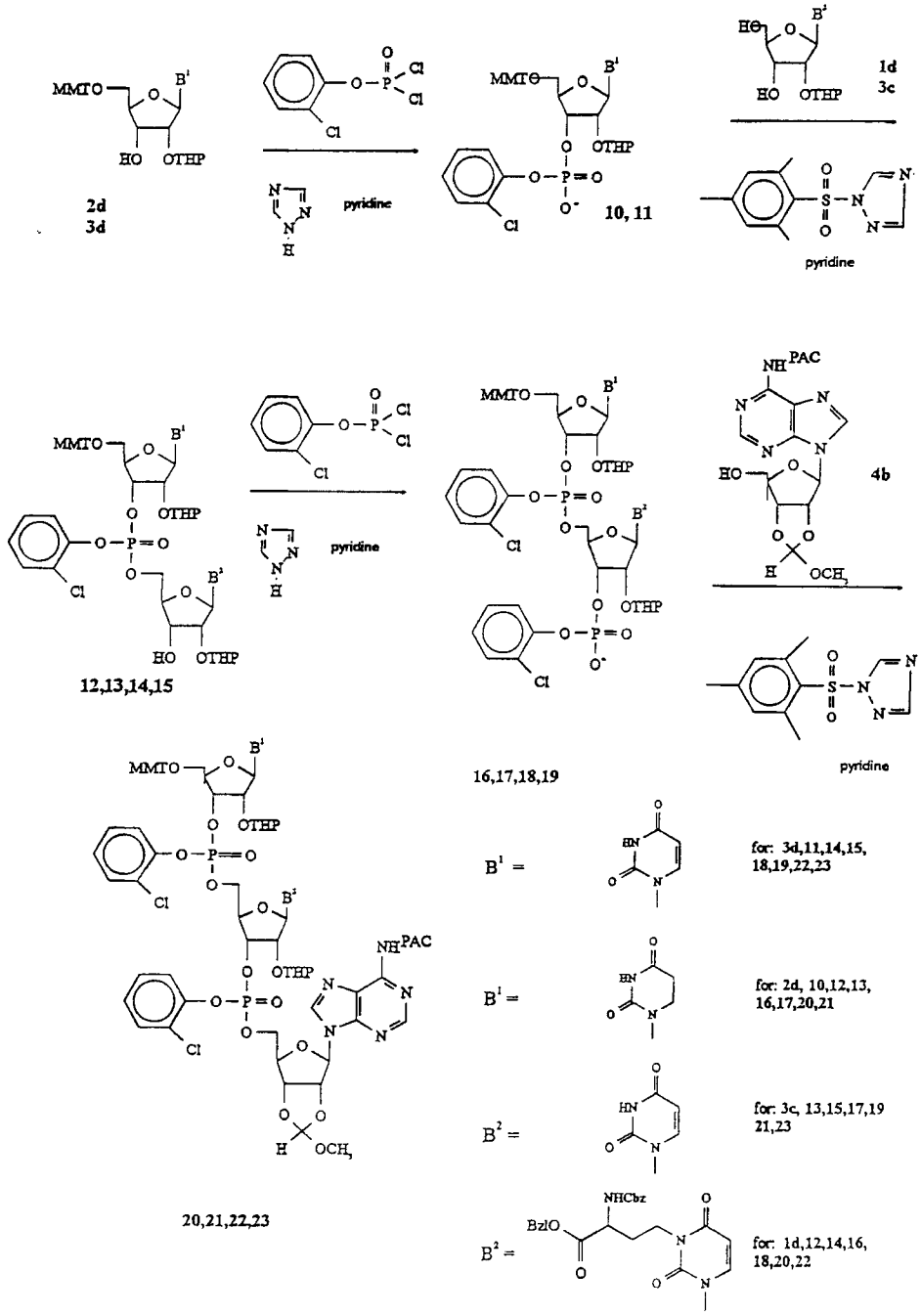

FIG 2. Synthesis of fully protected oligoribonucleotides. The diagram illustrates the synthesis of the fully protected dimer and trimers: Dp-(acp)^{3}U, **12**; Dp-(acp)^{3}UpA, **20**; DpUpA, **21**; Up-(acp)^{3}UpA, **22**; and UpUpA, **23**.

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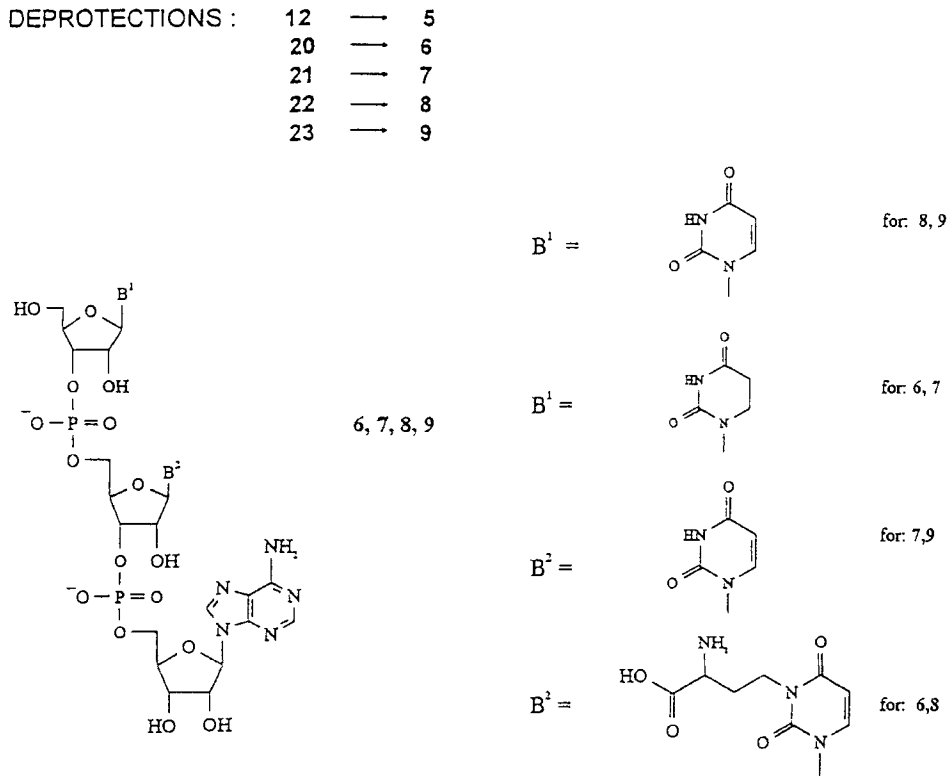

FIG 3. Deprotection of the oligoribonucleotides. The figure depicts the deprotection and molecular structures of the synthesized oligoribonucleotides: Dp-(acp)^{3}UpA, **6**; DpUpA, **7**; Up-(acp)^{3}UpA, **8**; and UpUpA, **9**.

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Protection of the monomeric starting units and their couplings are outlined in Figures 1 and 2, respectively. The 2'OH functions of the nucleosides **1a**, **2** and **3** were protected according to the procedure of Markiewicz.[19] The sub-sequent monomethoxytritylation (MMT) of the 5'OH group was accomplished according to Schaller et al.[20] The advantage of our approach is that the combination of protecting groups (Cbz and Bzl) used here could very well be generally applicable to oligonucleotide syntheses and could be combined with the various 2'OH protecting groups such as t-butyldimethylsilyl[10], 1-[(2-chloro-4-methyl)-phenyl]-4-methoxypiperydynyl (CTMP)[18] or 1-(2-chloroethoxy)-ethyl[21]. Because protection of the exocyclic amine function of adenosine was correlated with a low stability of D in alkaline media,

phenoxyacetyl protection was used for D. Such a combination of protecting groups did not cause racemization of the amino acid residue during deprotection under the conditions employed.[14,22] The protected monomeric units were produced from the 2'-O-tetrahydropyranyl- derivatives with yields ranging from 60% for adenosine to 90% for the different uridines.

All reactions of phosphorylation, coupling and deprotection (Figures 2-3) were accomplished under conditions routinely used for oligoribonucleotide synthesis.[16,17] Thus, dichloro-(2-chlorophenyl)-phosphate was used as a phosphorylating agent in the presence of the activating agent 1,2,4-triazole[23] and the products obtained in yields of 80-95%. All of the coupling reactions were accomplished in the presence of 1-(2-mesitylenesulfonyl)-1,2,4-triazole and the products were obtained in yields of 41-66%. The fully protected dimer Dp-(acp)^{3}U **12** and the trinucleotides, Dp-(acp)^{3}UpA **20**, DpUpA **21**, Up-(acp)^{3}UpA **22**, and UpUpA **23** were purified (silica gel columns) and precipitated from hexane. They were deprotected by removal of protection from the inter-nucleotide bond, the amino acid side chain of (acp)^{3}U, the acid and then the base labile protecting groups to yield Dp-(acp)^{3}U **5**, Dp-(acp)^{3}UpA **6**, DpUpA **7**, Up-(acp)^{3}UpA **8** and UpUpA **9**. The 2-chlorophenyl group was removed by action of 4-nitro-benzaldehydoxime in the presence of 1,1,3,3-tetramethylguanidine.[24] Cbz and Bzl groups were removed by hydrogenolysis in 80% acetic acid.[14] It is important that conditions for release of the Cbz and Bzl groups by hydrogenolysis do not lead to the reduction of the double bond of N3-modified uracil residue. These hydrogenolysis conditions do not cause hydrogenation of the C5-C6 double bond of cytidine[25] which, therefore, could be used together with blocked (acp)^{3}U as components of oligoribonucleotide syntheses.

Removal of the acid labile groups (MMT, methoxymethylidene and tetrahydrofuranyl, THP) was achieved under acidic conditions and additionally by action of 0.025 N HCl/H_2O and dioxane in a 1/1 (v/v) solution. Deprotection of oligomers bearing dihydrouridine in concentrated aqueous ammonia (for 2 hr)[12] produced massive degradation of both D and the oligomers with this nucleoside. In contrast, D did not undergo degradation, even under 24-hr treatment with 0.5 N methanolic ammonia and aqueous 0.01 N HCl for removal of phenoxyacetyl and acid labile tetrahydropyranyl, mono-methoxytrityl and methoxymethylidene groups. Yields of

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the deprotected dimer and trimers ranged from 65% for DpUpA to 96% for Dp-(acp)^{3}pA. The homogeneity of each product was confirmed by HPLC, LSIMS, UV and NMR analysis. Milligram quantities of hypermodified ribonucleoside-containing oligomers were produced for physical studies.

NMR Signal Assignments. The determination of nucleoside resonances within the oligomers Dp-(acp)^{3}U, Dp-(acp)^{3}UpA, DpUpA, Up-(acp)^{3}UpA and UpUpA involved the identification of three different spin systems. NMR analyses of the mononucleosides D, and (acp)^{3}U under the same conditions as the dimer and trimers helped to identify their respective signals in the oligomers and served as a useful reference. Signals were assigned by comparison of spectra, with the aid of already assigned signals for the mononucleosides[15] and with established methods for modified uridine oligonucleotides.[12,26,27] Single dimension ^{1}H-NMR spectra of (acp)^{3}U, Dp-(acp)^{3}U, and Dp-(acp)^{3}UpA are shown in Figure 4; the ^{31}P coupled and decoupled, ^{1}H spectra of Dp-(acp)^{3}U, and Dp-(acp)^{3}UpA are shown in Figure 5. The one-dimensional ^{1}H spectra of (acp)^{3}U, Dp-(acp)^{3}U and Dp-(ac)p^{3}UpA, shown in Figure 4, demonstrate the advantage of having the monomer, dimer and trimer for making signal assignments. The complete ^{1}H signal assignments for Dp-(acp)^{3}U, Dp-(acp)^{3}UpA, DpUpA, Up-(acp)^{3}UpA and UpUpA in phosphate buffered D_2O, as well as that for the mononucleosides D and acp^{3}U in phosphate buffered D_2O, are given in Table I.

The adenosine ^{1}H(2) and ^{1}H(8) resonances of Dp-(acp)^{3}UpA, DpUpA, Up-(acp)^{3}UpA and UpUpA are the farthest downfield signals. For (acp)^{3}U the two peaks furthest downfield are assigned to the C(5) and C(6) protons of (acp)^{3}U as a mononucleoside or as part of the oligomers. The deshielding and downfield shift of these protons is caused by the adjacent heteroatom, nitrogen-4, as well as the sp^2 character of the double bond. This is in contrast to the upfield resonances of the ^{1}H(5) and ^{1}H(6) of D. The ^{1}H(6) resonances of (acp)^{3}U (mononucleoside, dimer and trimer) are coupled to the ^{1}H(5) and, therefore, appear as doublets.

The COSY spectrum (Figure 6A) crosspeak from each ^{1}H(6) of acp^{3}U and of D, as mononucleoside, or within the dimer or trimer, locates the respective ^{1}H(5) signal. The remaining non-exchangeable protons of the bases are those of the amino acid side chain of (acp)^{3}U which had been assigned previously.[15] Within the dimer and the

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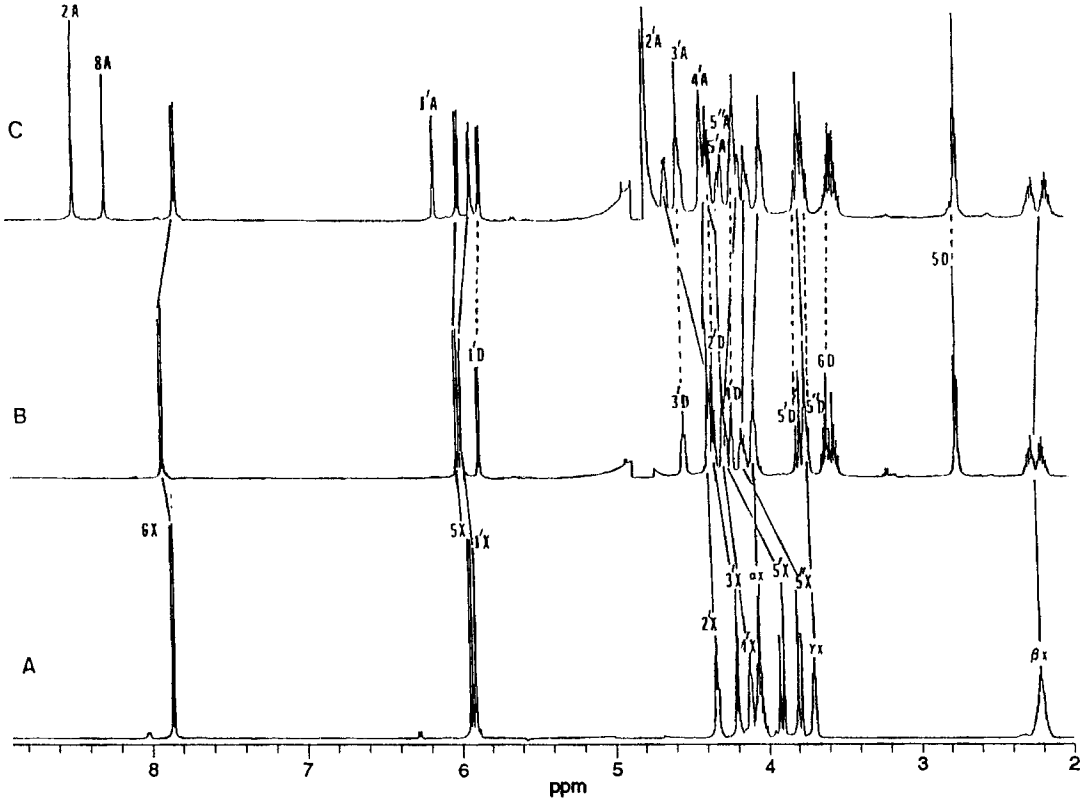

FIG 4. One dimensional ^{1}H spectra of the mononucleoside (acp)^{3}U; Dp-(acp)^{3}U; and Dp-(acp)^{3}UpA. The upfield portions of the ^{1}H spectra are shown. The resonances from (acp)^{3}Up are labelled X in spectrum A. Numbers refer to positions on the bases, whereas primed numbers refer to positions on the ribose (cf Figure 2 in reference 26). The α, β and γ refer to positions on the butyric acid side chain of (acp)^{3}U, with α referring to the carbon directly bonded to the N-3 of the uridine. Solid lines connect the (acp)^{3}U resonances in the spectrum of the monomer (A) to the corresponding resonances in the spectrum of the dimer (B), and trimer (C). The resonances from D in Dp-(acp)^{3}U are labelled with a similar protocol in spectrum B. Dashed lines connect the D resonances in the dimer (B) to that of the trimer (C). The resonances from A in Dp-(acp)^{3}UpA are labelled in spectrum C using the conventional numbering.

trimer in aqueous solution, the ^{1}H(β) protons of acp^{3}U exhibit a magnetic non-equivalence not seen in the mononucleoside except when all three are compared in DMSO.[28] We have attributed the non-equivalence of the ^{1}H(α) resonances of s^{2}mnm^{5}U in uridine dimers to hydrogen bonding of the side chain, possibly through H_2O to one of the two carbonyls.[26,29]

As seen with other nucleosides[26,30], the anomeric proton, ^{1}H(1'), is shifted downfield since it is on a carbon directly bonded to two heteroatoms. Given only the

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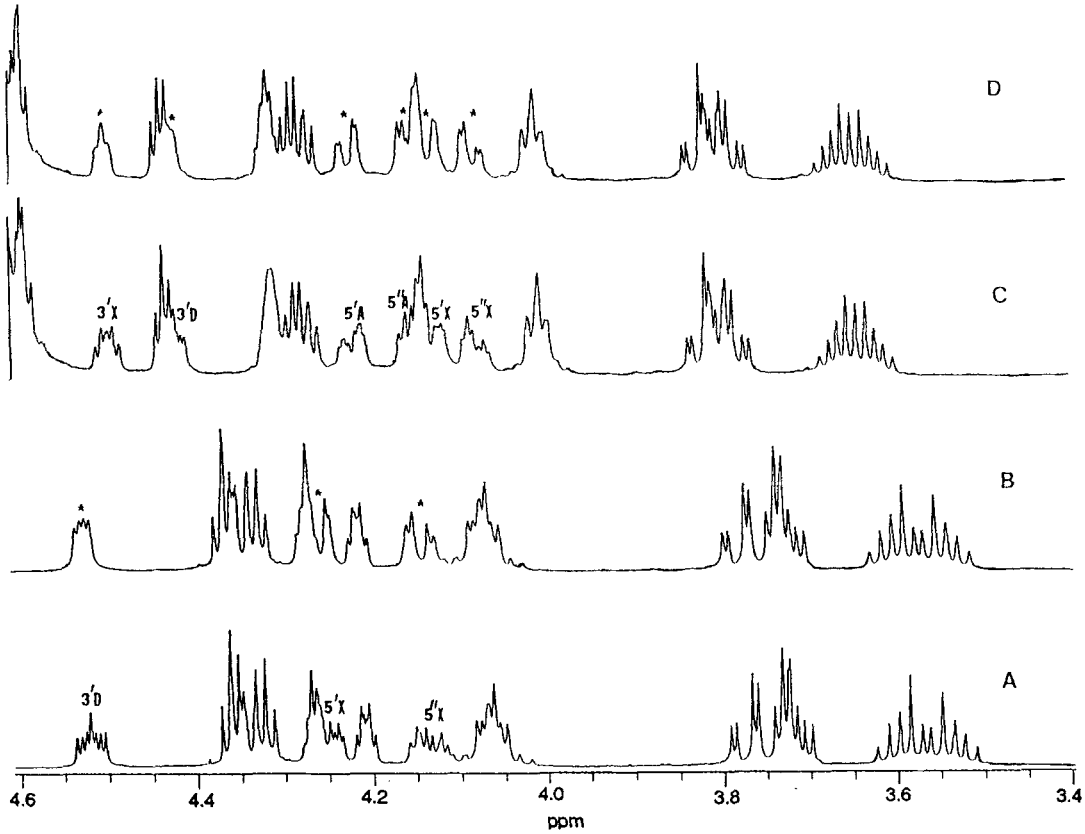

FIG 5. One dimensional ^{31}P coupled and decoupled, ^{1}H spectra of Dp-(acp)^{3}U and Dp-(acp)^{3}UpA. The middle portions of the ^{1}H spectra are shown. All four spectra were collected under identical conditions except that spectra B and D included broadband irradiation at the phosphorous frequency as described in the Experimental Section. A and B are the coupled and decoupled spectra of Dp-(acp)^{3}U, respectively; C and D are the coupled and decoupled spectra of Dp-(acp)^{3}UpA, respectively. The resonances from (acp)^{3}Up are labelled X in spectra A and C. Stars in spectra B and D specify those multiplets which collapse as a result of disrupted ^{31}P-^{1}H coupling.

vicinal coupling information, the various ribose protons of the dimer and trimer could not be assigned to a specific nucleotide subunit because of chemical shift similarities. This was overcome by the multiple bond correlations in the HOHAHA experiment (Figure 6B) and by comparison of ^{31}P coupled and uncoupled ^{1}H spectra (Figure 5) to identify the ^{1}H(2'), ^{1}H(3'), ^{1}H(4'), and ^{1}H(5',5") couplings of each nucleoside of Dp-(acp)^{3}UpA. Phosphate-bound protons were identified by using broadband decoupling at the ^{31}P frequency and comparing the resulting spectrum with the ^{31}P-coupled

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TABLE I. Proton Chemical Shifts[a]

	D	acp^{3}U	Dp----acp^{3}U		Dp-----acp^{3}Up----A			Dp----Up---A			Up-----acp^{3}Up----A			Up----Up---A		
2							8.23			8.21			8.23			8.20
5	2.75	5.93	2.73	6.00	2.71	5.95		2.7	5.86		5.77	5.92		5.72	5.78	
5[b]	2.75		2.73		2.71			2.7	5.86		5.77	5.92		5.72	5.78	
6	3.57	7.85	3.56	7.91	3.52	7.79		3.51	7.76		7.93	7.92		7.71	7.74	
6[b]	3.57		3.56		3.52			3.51	7.76		7.93	7.92		7.71	7.74	
8							8.44			8.44			8.46			8.44
α		4.05			4.07		3.98					3.98				
α[b]		4.05			4.07		3.98					3.98				
β		2.20			2.19		2.12					2.24				
β[b]		2.20			2.24		2.23					----				
γ		3.68			3.73		3.72									
1'	5.85	5.90	5.86	5.98	5.81	5.88	6.11	5.80	5.82	6.10	5.82	5.91	6.18	5.81	5.86	6.10
2'	4.29	4.33	4.36	4.37	4.31	4.33	4.72	4.30	4.29	4.72	4.41	4.34	4.70	4.38	4.29	4.69
3'	4.14	4.20	4.53	4.33	4.51	4.60	4.52	4.89	4.60	4.52	4.61	4.59	4.52	4.53	4.58	4.51
4'	4.01	4.12	4.21	4.27	4.15	4.37	4.37	4.14	4.33	4.37	4.27	4.38	4.37	4.22	4.33	4.36
5'	3.71	3.79	3.72	4.14	3.68	4.08	4.14	3.75	4.27	4.07	3.90	4.20	4.10	3.84	4.14	4.08
5''	3.79	3.91	3.78	4.26	3.68	4.12	4.23	3.79	4.28	4.04	3.81	4.25	4.18	3.79	4.24	4.14

[a] Chemical shifts in phosphate buffered D_2O relative to TMS.

[b] The 5 position protons of D are equivalent, and so are the 6 protons. The α protons of acp^{3}U are equivalent, but the β protons of acp^{3}U are equivalent only in the monomer.

spectrum (Figure 5). Proton resonances of the ribose hydroxyls are not detected since they exchange with the deuterated bulk solvent, but are detected in DMSO.[28]

Assignments were confirmed by the superior resolution of the heteronuclear (^{1}H-^{13}C) correlation experiment.[26] Carbon chemical shifts in phosphate buffer and in DMSO[28] were very similar. The two-dimensional HMQC spectral analysis of Dp-(acp)^{3}UpA takes advantage of heteronuclear scalar interactions to correlate a proton to the carbon to which it is directly bonded. Additional resolution is realized by modulation in the carbon frequency. The four methylene protons of the two ribose 5' carbons are easily identified since there are two proton resonances correlating to each carbon resonance (Figure 6). The chemical shifts of the correlated carbon resonances (Table II) also provide a useful check of the proton assignments because carbon chemical shifts more reliably reflect the electronic environment.[26,31] The ^{13}C(4') chemical shifts are always very similar, but that of the 5'-terminal nucleoside had the most upfield ^{13}C(4') signal. The phosphate-bound ^{13}C(5') and ^{13}C(3') signals are shifted downfield of the ^{13}C(3') and ^{13}C(5') resonances of the free termini. The ^{13}C(2') and ^{13}C(3') resonances of the 3' terminal nucleoside were well separated. This completes the assignment of all protons and all proton-bound carbons. To assign the five quaternary carbons (Table II), we used a multiple bond heteronuclear correlation experiment, HMBC.[26]

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Effects of Modification on Chemical Shift. With the exceptions of the phosphorous-bound positions, the ^{1}H chemical shift patterns for D, (acp)^{3}U, Dp-(acp)^{3}UpA, DpUpA, Up-(acp)^{3}UpA and UpUpA in phosphate buffered solution are quite similar. The proton chemical shifts of a nucleoside in the dimer and trimer differ from those of the monomer by less than 0.1 ppm for all positions except ^{1}H(3'), ^{1}H(4'), or ^{1}H(5') ^{1}H(5"), in which cases the phosphate group causes a downfield shift of 0.1-0.4 ppm. In addition, adenosine ^{1}H(2) and ^{1}H(8) are shifted downfield in the trimer, and the chemical shift of the β-protons of (acp)^{3}U are equivalent only in the monomer in D_2O, as noted above. The similarity of dimer and trimer to monomer in proton chemical shifts suggests a lack of stacking interaction in the oligomers. Some carbon chemical shifts for the dimer and trimer differ noticeably from those of the corresponding monomers. The C2 and C4 carbonyl carbons of D are shifted down-

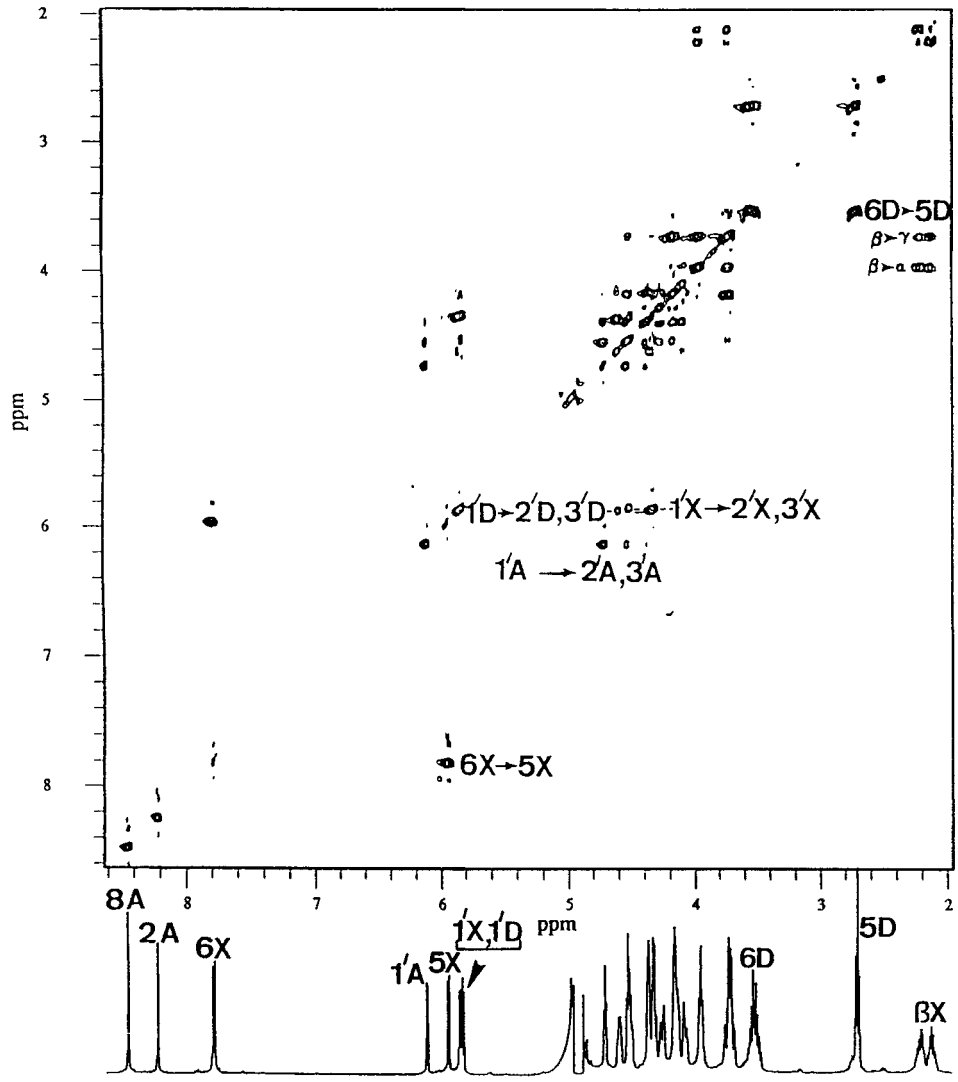

FIG 6A. Two-dimensional homonuclear and heteronuclear correlation spectra of Dp-(acp)^{3}UpA in D_2O. A. The full double quantum filtered COSY spectrum. Some of the assignments are shown on the figure.

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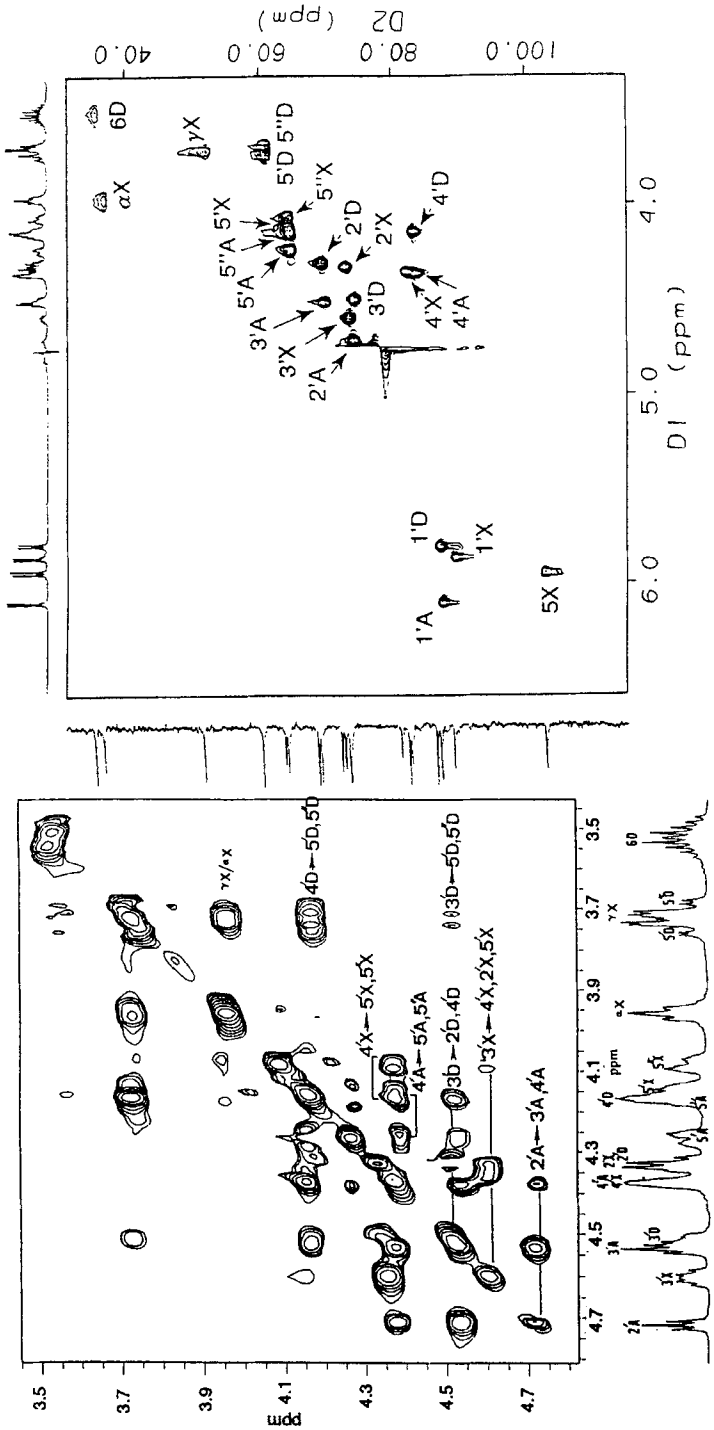

FIG 6B and 6C. B. The expanded upfield region of the HOHAHA spectrum. The one-dimensional ^{1}H spectra are labelled below the horizontal axis. Correlations for the three ribose rings are labelled. C. ^{1}H-^{13}C HMQC spectrum. The ribose proton-carbon correlations are shown. All spectra were collected and processed as described in the Experimental section. Resonances from (acp)^{3}Up are labelled X in the spectra.

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TABLE II. Carbon Chemical Shifts[a]

	D	acp^{3}U	Dp-----acp^{3}U		Dp-----acp^{3}Up--A			Dp-----Up------A			Up-----acp^{3}Up---A			Up-----Up------A		
2	153.0	151.6	154.5	151.9	154.4	151.7	152.8	154.4	151.5	152.8	151.4	151.7	152.9	151.4	151.6	152.8
4	172.2	165.0	173.7	164.1	173.7	164.7	148.8	173.6	166.0	148.9	165.9	164.6	148.9	164.0	165.4	148.8
5	28.5	101.4	30.0	101.8	30.0	102.0	118.6	29.9	102.5	118.6	102.0	101.9	118.6	102.4	102.6	118.6
6	34.8	139.7	36.2	139.7	36.1	139.3	155.5	36.1	141.1	155.5	141.3	139.2	155.5	140.3	140.8	155.4
8							139.6			139.5			139.2			139.4
α		37.3		37.4		37.5						37.5				
β		28.0		28.0		28.0						28.1				
γ		52.3		52.4		52.4						52.4				
ε		174.2		173.4		173.9						173.7				
1'	85.9	90.1	86.9	89.8	86.8	89.1	87.4	86.9	88.1	87.3	89.0	89.2	87.3	89.1	88.1	87.1
2'	68.8	73.5	69.5	73.7	69.4	73.0	74.1	69.5	72.8	74.1	72.9	73.1	74.3	72.7	72.8	74.3
3'	68.5	69.0	74.2	69.3	74.3	73.5	69.9	74.2	73.6	69.9	73.0	73.2	70.0	73.0	73.4	70.0
4'	81.8	83.8	82.7	82.7	82.9	81.9	83.3	82.8	81.8	83.3	83.4	81.9	83.4	83.4	81.6	83.2
5'	59.8	60.4	61.1	64.5	61.1	64.5	64.9	61.0	64.9	64.5	60.2	64.8	64.3	60.2	64.8	64.3

[a]The chemical shifts in phosphate buffered D_2O are relative to TMS.

field in the oligomers. Some of the differences between the ^{13}C chemical shifts of the mononucleoside and that in the oligomers [for example, $^{13}C(3')$ of D and $(acp)^3U$; $^{13}C(5')$ of $(acp)^3U$] are due to the presence of the phosphate group, whose influence may be propagated through the carbon skeleton.

Experimental

Chromatography and Analysis. TLC was accomplished with silica gel plates (Merck, DC-Fertigplattenn kieselgel 60F-254, 0.25 mm). Flash column chromatography was performed with silica gel 60 F (70-230 mesh ASTM). Paper chromatography was accomplished with Whatman 3MM paper. The following solvent systems were used: A - chloroform/methanol, 80/20; B - chloroform/methanol, 90/10; C - chloroform/methanol, 85/15; D - chloroform/methanol, 95/5; E - 2-propanol/ 25%, NH_4OH/water, 7/1/2; and F - 2-propanol/25% NH_4OH/water, 11/2/7. Purity of the nucleosides and oligomers was determined by NMR with a 300 MHz Bruker MSL-300 spectro-meter; LSIMS, an AMD-604 spectrometer and for MS at 15eV and 70eV, a GC LKB-2091 spectrometer; and by UV spectroscopy with the Specord UV-VIS spectro-photometer. Analytical HPLC was accomplished with a gradient elution (5-50% methanol in 0.02 M $NH_4H_2PO_4$; 0 and 30 min) from a Sperisorb C-18 column (25cm x 4.6 mm) at a flow rate of 1 ml/min and monitored at 250 nm.

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Synthesis of Oligoribonucleotides. Procedures for the synthesis of individual components, the oligomers in general and the unique synthesis of adjacent modified uridines in Dp-$(acp)^3U$ and Dp-$(acp)^3UpA$, were adaptations of published methods. The syntheses of Dp-$(acp)^3U$, Dp-$(acp)^3UpA$, DpUpA, Up-$(acp)^3UpA$ and UpUpA are depicted in Figures 1-3.

1. Synthesis of monomeric units.

a. 2'-O-Tetrahydropyranyl (2'OTHP) derivative of monomeric units **1a, 2** and **3**.[19,33] The reagent 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (1.89 g, 6 mmol) was added to a solution of an appropriate nucleoside in pyridine (30 ml; 5 mmol). The reaction mixture was stirred (room temperature for 3 hr), water (30 ml) added and the solution was extracted three times with chloroform (100 ml). The organic layer was dried with $MgSO_4$, concentrated and the crude product chromatographed on a <flash> silica gel column in a chloroform/ methanol (99.5/0.5,v/v) solvent system. Pure

products **1b** (R_f=0.85 (B)), **2a** (R_f=0.74 (B)) and **3a** (R_f=0.75 (B)) were obtained with 72, 86, and 85% yields, respectively. The reagent 2,3-dihydropyran (3.7 ml) was added dropwise to a solution of each 3',5'-O-(1,1,3,3-tetraisopropyldisiloxan-1,3-yl) nucleoside derivative, **1b, 2a,** or **3a** (4.3 mmol). Then, p-toluenesulfonic acid (55 mg, 0.32 mmol) was added to the reaction mixture to pH 4. After 4 hr, by which time starting materials had completely disappeared as determined by TLC with system A, ammonium hydroxide (25% aqueous solution, 0.2 ml) was added. The solvent was evaporated and the residue was co-evaporated twice with toluene and finally dissolved in toluene (10 ml). The precipitated solid of ammonium p-toluenesulfonate was removed by filtration. The filtered solution was concentrated under reduced pressure to give the crude 3'5'-protected 2'-OTHP derivative (**1c, 2b, 3b**) which was dissolved in 1 M triethylammonium fluoride in tetrahydrofurane (10 ml). The reaction mixture was kept at room temperature for 24 h. Then an aqueous solution of sodium bicarbonate (1.68 g of $NaHCO_3$ in 10 ml of water) and pyridine (20 ml) were added. Solvents were evaporated and co-evaporated with pyridine. The precipitated solid was removed by filtration. The solution was concentrated, and the crude product was co-evaporated five times with ethanol, subjected to chromatography on a <flash> silica gel column and eluted with a gradient of chloroform/methanol (99/1 to 95/5, v/v). The pure 2'OTHP protected derivatives (**1d, 2c, 3c**) were obtained with ca. 70% yields as a mixture of diastereoisomers. The chromatographic and spectral characteristics of the compounds follow.

1d: R_f=0.33 and 0.41 (B); MS (15 eV), m/z: 568 (M-85, 4.74%), 433 (32.39%), 421 (9.29%), 191 (9.14%), 190 (70.87%), 113 (13.93%), 108 (45.18%), 107 (21.76%), 91 (100%), 85 (30.73%), 77 (5.18%); ^{1}H NMR ($CDCl_3$), δ (ppm): 7.60 (d, J=8 Hz, 1H, H-6), 7.43-7.33 (m, 10H, aromatic protons), 6.08 (d, J=8 HZ, 1H, H-5), 5.98 (s, 1H, H-1'), 5.69-5.62 (m, 4H, CH_2 of benzyl and Cbz), 5.12-5.02 (m, 1H, THP acetal proton), 4.65-3.80 (m, 10H, H-2', 3', 4' 5' 5", NH, NCH_2, and $O\underline{CH_2}CH_2$), 2.21-2.10 (m, 2H, $NCH_2\underline{CH_2}$), 1.80-1.51 (m, 6H, THP alkyl ^{1}H). **2c**: R_f=0.61 (m.p. 173.5°C from methanol) and 0.77 (C). The spectral characteristics of the more polar diastereoisomer (R_f=0.61) are: MS (15 eV), m/z: 330 (M, 0.05%), 329 (M-1, 0.16%), 245 (M-85, 12.17%), 143 (19.07%), 131 (36.87%), 115 (4.92%), 114 (1.35%), 85 (100%); ^{1}H NMR (D_2O), δ(ppm): 5.96 (d, J=6.68 Hz, 1H, H-1'),

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4.88-4.72 (m, 1H, acetal proton of THP), 4.40-4.33 (m, 1H, H-2'), 4.27-4.22 (m, 1H, H-3'), 4.01 (dt, J=3.5 Hz, J=5 Hz, 1H, H-4'), 3.82-3.53 (m, 6H, H-5', 5", 6, -O$\underline{CH_2}CH_2$), 2.74 (t, J=7 Hz, 2H, H-5), 1.81-1.51 (m, 6H, alkyl protons of THP).

3c: R_f=0.66 and 0.52 (C) [16]. Less polar diastereoisomer of **3c** (R_f=0.66) was used for further reactions.

b. 5'-O-monomethoxytritylation, 5'-OMMT[20] of **2c** and **3c**. Monomethoxytrityl chloride (701 mg, 2.27 mmol) was added to a pyridine (anhydrous; 20 ml) solution of the appropriate 2'-O-tetrahydropyranyl (2'-OTHP) derivative **2**, or **3c** (1.89 mmol) and stirred for 3 days (room temperature). Progress was monitored by TLC in system D. The reaction mixture was poured into ice/water (50 ml) and the product was extracted with chloroform (100 ml). Organic layers were combined, dried with $MgSO_4$ and concentrated to give the crude product, which was purified by column chromatography in a chloroform/methanol (99.5/0.5%, v/v) solvent system. The pure 5'OMMT, 2'OTHP derivatives, **2d** or **3d**, were obtained in 90% yield and had the following characteristics.

2d: R_f=0.55 and 60 (B); MS (15 eV), m/z: 602 (M, 3.45%), 517 (M-85, 2.35%), 312 (M-274, 3.64%), 274 (57.17%), 273 (100%), 229 (6.96%), 131 (3.24%), 115 (1.29%), 114 (0.75%), 85 (43.73%); ^{1}H NMR ($CDCl_3$), δ(ppm): 7.4-7.19 (m, 14H, aromatic protons), 6.822 (d, J=9 Hz, 2H, aromatic ortho OCH_3 protons), 6.09-5.98 (two d, J=3.4Hz and J=6.9 Hz, 1H, H-1' of both diastereo-isomers), 4.08-4.02 (m, 1H, H-4'), 3.79 (s, 3H, CH_3O), 3.68-3.23 (m, 6H, H-6, 5', 5" and O$\underline{CH_2}CH_2$), 2.49-2.38 (m, 2H, H-5), 1.61-1.57 (m, 6H, alkyl protons THP).

3d: R_f=0.38 (D).[16]

c. Synthesis of 2',3'-O-methoxymethylidene-N^6-phenoxyacetyl-adenosine, **4b**.[34] Trimethyl orthoformate (13.2 ml, 30 mmol) and trimethylsilyl chloride (0.48 ml, 6 mmol) were added to the solution of N^6-phenoxyacetyladenosine, **4a** (1.2 g, 3 mmol) [35] in DMF (12 ml). The resulting solution was stirred at room temperature for 30 min. Then, the reaction was quenched by addition of pyridine (3 ml) and the solvent was removed under reduced pressure. The residue was chromatographed on a silica gel column with a chloroform/methanol (99/1) solvent system and precipitated from chloroform with hexane/ethyl ether (7/1) to give 0.8 g (60%) of analytically pure

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product **4b**. Chromatography and spectroscopy characteristics of **4b**: R_f=0.37 (D); UV (MeOH): λ_{max}=273nm (ϵ=16100), MS (70 eV) m/z: 443 (M, 9.07%), 412 (M-31, 10.06%), 309 (22.37%), 218 (23.53%), 164 (100%); ^{1}H NMR ($CDCl_3$) δ(ppm): 9.52 (brs, 1H, NH), 8.77 and 8.76 (2s, 1H, H-2), 8.10 and 8.12 (2s, 1H, H-8), 7.37-7.31 (m, 2H, aromatic), 7.08-7.03 (m, 3H, aromatic), 6.28 and 5.93 (m and d, J=4.8 Hz, 1H, H-1'), 6.05 and 5.98 (2s, 1H, O-CH-O), 5.36-5.19 (m, 2H, H-2', 3"), 4.87 (s, 2H, CH_2O), 4.57 (m, 1H, H-4'), 4.01 and 3.98 (2dd, $J_{4',5'}$=1.7 Hz, $J_{5',5'}$=12.6 Hz, 1H, H-5'), 3.85 (m, 1H, H-5"), 3.37 and 3.34 (2s, 3H, OCH_3).

2. General procedure for phosphorylation.[23]

The reagents 1,2,4-triazole (1.36 mmol, 94 mg) and dichloro-(2-chlorophenyl)-phosphate (0.68 mmol, 100 μl) were added to the monomeric or dimeric unit (0.4 mmol) that had been co-evaporated three times with anhydrous pyridine and finally dissolved in 1 ml of pyridine. After 1 hr. cold 50% aqueous pyridine (20 ml) and 0.1 N triethylammonium bicarbonate (10 ml) were added to the cooled (0°C) reaction mixture. After 15 min, the product was extracted with chloroform (50 ml). The organic layer was washed twice with 0.1 N triethyl-ammonium bicarbonate (10 ml) and once with water, dried with anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was co-evaporated twice with toluene, dissolved in chloroform (1 ml) and precipitated with hexane (100 ml). The product was used directly for the next reaction without purifica-tion. This procedure was used to transform compounds **2d**, **3d**, **12**, **13**, **14** and **15** to phosphates **10**, **11**, **16**, **17**, **18** and **19**, respectively, with 80-95% yields.

3. General Procedure for Condensation.[35]

Nucleoside (1.2 mmol) and nucleotide (1.0 mmol) were combined and coevaporated three times with anhydrous pyridine, and dissolved in pyridine (3.5 ml). The reagent 1-(2-mesitylenesulfonyl)-1,2,4-triazole (0.2 mmol, 502 mg) was added to the reaction mixture. The mixture was allowed to react for 6 days at room temperature. Aqueous pyridine (50%, 30 ml) was added to the reaction mixture. After 15 min the product was extracted with chloroform (50 ml). The organic layer was washed with 0.1 N triethylammonium bicarbonate (10 ml) and water (10 ml), dried with $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography in chloroform/methanol (99.5/0.5, v/v) solvent system to give, after

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precipitation from hexane, the pure product. This procedure of coupling **10** with **1d** gave dimer **12**, Dp-(acp)^{3}U (66% yield; R_f=0.67 and 0.73 (D)); **10** with **3c** gave the dimer **13**, DpU (63% yield; R_f=20 (D)); **11** with **1d** gave the dimer **14**, Up-(acp)^{3}U (50% yield; R_f=40 (D)); **11** with **3c** gave the dimer **15**, UpU (57% yield; R_f=0.25 (D)); **16** with **4b** gave trimer **20**, Dp-(acp)^{3}UpA (47% yield; R_f=0.50 (D)); **17** with **4b** gave trimer **21**, DpUpA (53% yield; R_f=0.38 (D)); **18** with **4b** gave trimer **22**, Up-(acp)^{3}UpA (41% yield; R_f=0.49 (D)); **19** with **4b** gave trimer **23**, UpUpA (42% yield; R_f=0.42 (D)).

4. General Procedure of Deprotections

a. Removal of 2-chlorophenyl group.[24] Dimer (0.1 mmol) or trimer (0.05 mmol) was dissolved in acetonitrile (4 ml or 2 ml, respectively) and 4-nitrobenzald-oxime (1.15 mmol, 192 mg) and 1,1,3,3-tetramethylguanidine (1 mmol, 126 μl) were added. The reaction mixture was maintained at room temperature for 3 hrs. By-product was precipitated by dropping of the reaction solution into anhydrous ethyl ether (100 ml or 50 ml, respectively). Dowex ion-exchange resin (pyridynium salt; 1 ml) was added to the aqueous solution, and stirred for 1 hr. The ion-exchanger was removed by filtration and washed three times with aqueous pyridine. The filtered solutions were combined and concentrated under reduced pressure, and the residue was evaporated twice with toluene. The product was used directly in the next step without purification.

b. Removal of benzyloxycarbonyl (Cbz) and benzyl (Bzl) groups under hydrogenation condition.[14] Crude product of the former reaction was dissolved in 80% aqueous acetic acid (4 ml) and 10% Pd/C (10 mg) was added. H_2 was passed through the stirred reaction mixture (1.5 hr). Catalyst was removed by filtration and washed twice with 80% acetic acid (2 ml). Filtered solutions were combined, lyophilized and the product used directly in the next reaction.

c. Removal of acid labile groups (MMT, THP, methoxymethylidene groups).[36] The crude product of the former reaction was dissolved in a mixture of 0.025 N HCl/water and dioxane (1/1) (2 ml) and left for 4 hr. Then, 0.1 N ammonium hydroxide (0.25 ml) was added and the reaction mixture was lyophilized.

d. Removal of base labile, phenoxyacetyl group.[35] Methanolic ammonia (0.5 N, 2 ml) was added to the crude product of the former reaction and the reaction mixture

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was stirred for 3 hrs. Then, the residue was concentrated, co-evaporated twice with methanol. The crude product of the deprotections was dissolved in water (0.5 ml) and purified by paper (3 MM) chromatography in an n-propanol/water/ammonium hydroxide (11/7/2, v/v/v) solvent system. The product was eluted from the paper with water, additionally purified by reverse-phase HPLC (10 x 250 mm Dynamax C-18 column) with a gradient elution (0.1 N TBAF and 70% acetonitrile/water; 0-50% of the latter in 60 min) and lyophilized. Deprotection of dimer **12** resulted in the dimer Dp-acp^{3}U, **5** (84% yield): R_f= 0.22 (E); R_f= 0.62 (F); HPLC R_t=3.17 min; LSIMS (thioglycerol) positive ions: 654 (M+H), 676 (M+Na); negative ions: 652 (M-H), 674 (M+Na-2H); electrophoretic mobility (1200 V, 0.02 M Na_2HPO_4, 80 min) of 0.47 (relative to 5'-pU); and UV (D_2O) λ_{max}=266 nm (ϵ=5.66x10^3). Deprotection of compound **20** produced the trimer, Dp-(acp)^{3}UpA **6** (96% yield): R_f=0.17 (E); and R_f=0.51 (F); HPLC R_t=9.77 min; LSIMS: (thioglycerol) positive ions: 983 (M+H), 1005 (M+Na), 1027 (M+2Na-H), 1049 (M+3Na-2H), 1071 (M+4Na-3H), (glycerol) negative ions: 981 (M-H), 1003 (M+Na-2H), 1025 (M+2Na-3H); electrophoretic mobility (conditions as above) of 0.56; and UV (H_2O) λ_{max}=260 nm (ϵ=1.24x10^4). Deprotection of the trimer **21** according to the procedures described resulted in the trimer **7** (65% yield): R_f=0.45 (F); HPLC R_t=9.54 min; LSIMS: (glycerol) negative ions: 979 (M-H), 1001 (M+Na-2H). Deprotection of the trimer **22** resulted in the trimer **8** (86% yield): R_f=0.48 (F); HPLC R_t=9.26 min; LSIMS: (glycerol) negative ions: 880 (M-H), 902 (M+Na-2H). Deprotection of the trimer **23** produced the trimer **9** (84% yield): R_f=0.34 (F); HPLC R_t=10.12 min; LSIMS: (glycerol) negative ions: 878 (M-H), 900 (M+Na-2H).

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NMR Spectroscopy. Lyophilized samples were dissolved at a concentration of ≈8 mM in either phosphate buffer, or 98% D_2O, phosphate buffer.[26] Spectra were collected on a GE Omega 500 at ambient temperature, unless otherwise noted. One-dimensional ^{1}H spectra were typically collected with 8K data points and enough scans to obtain a good signal-to-noise ratio.

1. ^{31}P decoupling. Phosphorus decoupled, proton spectra at 202 MHz were obtained with a dedicated reverse polarization transfer probe with detection through the decoupling coil. The spectral width was 5000 Hz over 8K data points. For the ^{31}P

decoupled spectrum, broadband phosphorous decoupling with 2WALTZ 16 modulation was applied for the duration of the experiment.

2. ^{13}C spectra. One-dimensional ^{13}C spectra were recorded with a dedicated carbon probe at 125 MHz. The spectral width was 25,000 Hz with 8K data points and broadband proton decoupling with WALTZ modulation was applied for the duration of the experiment. In order to measure ^{13}C-^{31}P coupling constants, the resolution was improved by zerofilling to 16K data points and the Fourier transformation was without apodization or with a $\sin^2$ 10 apodization function.

3. Two-dimensional homonuclear and heteronuclear correlation spectra. COSY, HOHAHA (Hartman-Hahn) and ^{1}H-^{13}C HMQC spectra were collected according to previously published methods.[15,26,30,37]

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