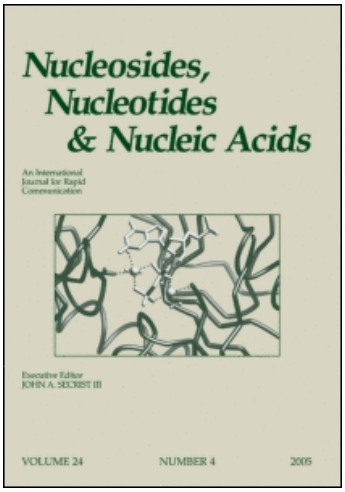

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CHEMICAL SYNTHESIS OF FULLY AND PARTIALLY XYLOADENOSINE-SUBSTITUTED 2',5'-OLIGOADENYLATES DESIGNED AS NEW POTENTIAL ANTIVIRAL AND ANTITUMOR AGENTS

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ABSTRACT.

Seven 2',5'-oligoadenylate analogues containing 9-β-D-xylofuranosyladenine (xyloaadenosine, XyloA) at the 2'-end or at other positions were synthesized by the phosphotriester method. These new analogues, from dimer to tetramers, exhibit antiproliferative activity which is probably due to their degradation to xyloadenosine units.

INTRODUCTION.

The 5'-O-triphosphorylated oligoriboadenylates containing the unusual 2',5'-phosphodiester linkage (abbreviated as 2-5A) are biologically important substances involved in the antiviral and antiproliferative action of interferon. Synthesized in cells from ATP by a synthetase which is induced by interferon and activated by double-stranded RNA, 2-5A activates a latent endoribonuclease which cleaves viral and cellular single-stranded RNA, resulting in inhibition of protein synthesis (for recent reviews, see Ref. 1).

The involvement of 2-5A as a mediator of interferon, as well as its possible role in the control of cell proliferation and differentiation, raised the hope that these oligonucleotides or their derivatives might be useful chemotherapeutic agents in the treatment of viral and neoplastic diseases.[2] Despite their demonstrated *in vitro* activity, the *in vivo*

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utilization of 2-5A is severely limited by two factors : (1) cellular impermeability and, (2) enzymatic degradation. Due to their highly ionic character these 5'-O-triphosphorylated molecules are unable to penetrate intact cells effectively. Artificial *in vitro* methods of introduction, such as calcium phosphate coprecipitation, [3] permeabilization in hypertonic media, [4] lysolecithin treatment [5] and microinjection, [6] have been successfully employed, but these routes are inadequate for realistic assessment *in vivo* chemotherapeutic potential. Rapid degradation of intracellular 2-5A to 5'-AMP and ATP by 2'-phosphodiesterase and 2',3'-exoribonuclease accounts for their observed transient biological effects. [1,2] To circumvent these difficulties attempts have been made to improve both cellular permeability and metabolic stability. Since three AMP residues in 2-5A are required for maximal activity, [4] most attempts have dealt with trimeric entities.

Removal of the 5'-O-triphosphate group may enhance cellular permeability. Accordingly, various syntheses of the 5'-nonphosphorylated molecules, referred to as 2-5A cores, have been published. They have involved either unambiguous direct synthesis [8-22] or alkaline phosphatase treatment of chemically [23-29] or enzymatically [29] synthesized 2-5A. The cores, which occur naturally in mammalian tissues, as well as in yeast and bacteria, can be significantly increased by interferon treatment of virally infected cells. [30-33] Added exogenously to intact cells, the 2-5A cores exhibit a variety of effects, [34-55] among which the most interesting are antimitogenic [34-40] and antiviral [43-47] activities. Since these cores are inactive as inhibitors of protein synthesis in cell-free extracts, and neither activate nor bind to the 2-5A-dependent endoribonuclease, [56,57] it was originally believed that they exert their action only after intracellular 5'-phosphorylation to 2-5A and subsequent activation of the endoribonuclease. [36,59] However more recent investigations have shown that the biological effects of the cores are not the consequence of activation of this enzyme. [39,40,49,50,59,60] Rather, the observed activities might often result from degradation of the cores to monomeric units. This supposition was further supported by the demonstration that the antiproliferative action of the natural trimeric core is related to its degradation by serum phosphodiesterases.[61]

The problem of enzymatic degradation has been approached by introducing modifications on the cores or the 2-5A, either in the ribose or adenine moieties, or at the terminal or internucleotidic phosphates. These modification have been carried out at the 2'-end or in all units.

[12,14,19,27,37,40,59,62-95] Some of these analogues have been found to be more potent than their parent 2-5A or cores.

In a preliminary communication we have reported the synthesis of the 2',5'-linked trimeric core of 9-β-D-xylofuranosyladenine (Fig. 1, 1b).[96] This compound, for which the conformational analysis has been described,[97] has proven resistant to hydrolysis by cell-free extract[39] and by homogeneous poly(A)-specific 2',3'-exoribonuclease.[98] Furthermore it is a potent inhibitor of DNA synthesis[39] and herpes virus growth.[99] Additionally it has been shown that the activity of this compound does not involve activation of the 2-5A-dependent endoribonuclease, and that its mechanism of action involves its degradation to monomeric units.[100, 101]

The present paper describes the detailed chemical synthesis and characterization of 1b , as well as six new 2-5A core derivatives containing xyloadenosine either at the 2'-end or at other positions (Fig. 1).

RESULTS AND DISCUSSION.

The strategy followed for the chemical synthesis of the desired oligonucleotides 1a-1g was based on the modified solution-phase phosphotriester method.[102] First the properly protected monomeric building blocks in the xylose series (4 and 5, Scheme 1) and the ribose series (14, 15, Scheme 2) were prepared and then condensed to give the fully protected 2'-5'-linked dimers (6, Scheme 1 ; 16, Scheme 2). Sequential selective removal of the 5'-OH or 2'-phosphate protecting groups allowed further condensations to the fully protected oligomers (8, 10, 21-24, Scheme 1-3). These latter were then deblocked to afford the unprotected dimer 1a, trimers 1b, 1d, 1e and tetramers 1c, 1f, 1g which were fully characterized.

Protecting groups and preparation of monomers.

A successful scheme for the synthesis of 2',5'-oligonucleotides requires a judicious choice of selective protecting groups. Of particular importance is the need to differentiate between the two vicinal hydroxyl functions (2',3') of the ribose and xylose moieties.

In our strategy, the 5'-hydroxyl functions of the nucleosides were temporarily protected with monomethoxytrityl groups (mMTr) and the other positions (3'-OH and exocyclic NH_2) were blocked by benzoylation (Bz). Phosphorus protection was accomplished with the 2-chlorophenyl group in

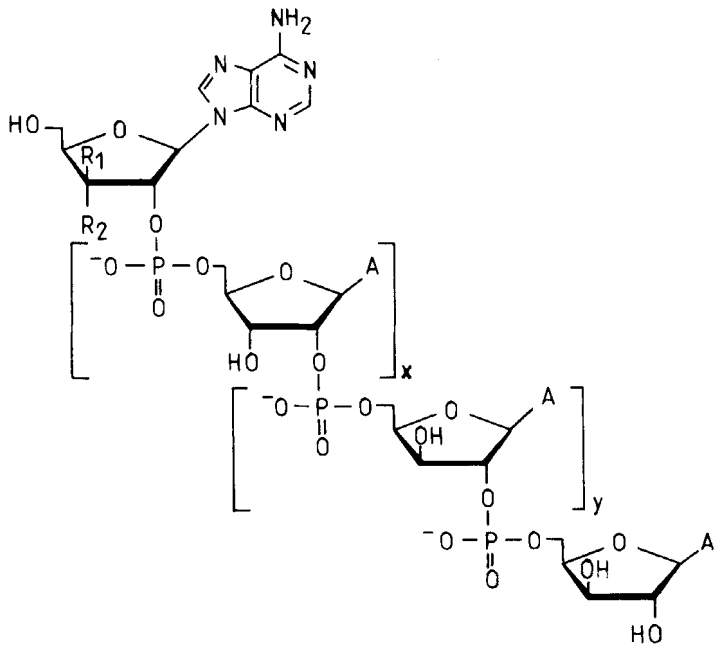
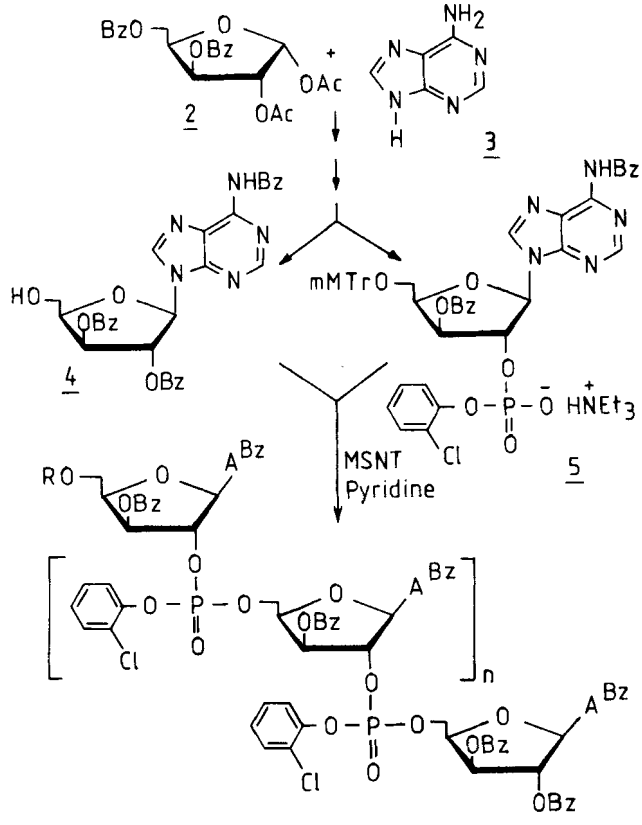


Fig.1.

1a, R_1 = OH, R_2 = H, x = y = 0 : xyloadenylyl-(2'→5')-xyloadenosine (XyloA2'p XyloA) ; 1b , R_1 = OH, R_2 = H, x = 0, y = 1 : xyloadenylyl-(2'→5')-xyloadenylyl-(2'→5')-xyloadenosine ($(XyloA2'p)_2XyloA$) ; 1c , R_1 = OH, R_2 = H, x = 0, y = 2 : xyloadenylyl-(2'→5')-xyloadenylyl-(2'→5')-xyloadenylyl-(2'→5')-xyloadenosine ($(XyloA2'p)_3XyloA$) ; 1d, R_1 = H, R_2 = OH, x = 0, y = 1 : adenylyl-(2'→5')-xyloadenylyl-(2'→5')-xyloadenosine (A2'p XyloA2'p XyloA) ; 1e, R_1 = H, R_2 = OH, x = 1, y = 0 : adenylyl-(2'→5')-adenylyl-(2'→5')-xyloadenosine ($(A2'pA)_2XyloA$) ; 1f, R_1 = H, R_2 = OH, x = 1, y = 1 : adenylyl-(2'→5')-adenylyl-(2'→5')-xyloadenylyl-(2'→5')-xyloadenosine ($(A2'pA)_2XyloA2'pXyloA$) ; 1g, R_1 = H, R_2 = OH, x = 2, y = 0 : adenylyl-(2'→5')-adenylyl-(2'→5')-adenylyl-(2'→5')-xyloadenosine ($(A2'pA)_3$ XyloA)

internucleotidic linkages as well as the 2-cyanoethyl group in ribonucleotide units containing 2'-phosphotriester substitution.

In the xylose series, the terminal unit 4 and the monomer 5 (Scheme 1) were prepared as previously described.[103] For the ribose series, the key intermediate 12 [82,104] (Scheme 2) was synthesized from adenosine by the method of Engels.[104] Phosphorylation of 12 with 2-chlorophenyl phosphoryl ditriazolide in pyridine [105] occurred readily without any detectable migration. Subsequent addition of 2-cyanoethanol gave the fully protected ribomononucleotide 13, isolated by short silica gel column chroma-



PTS/$CHCl_3$, MeOH
5, MSNT/pyridine
PTS/$CHCl_3$, MeOH
5, MSNT/pyridine

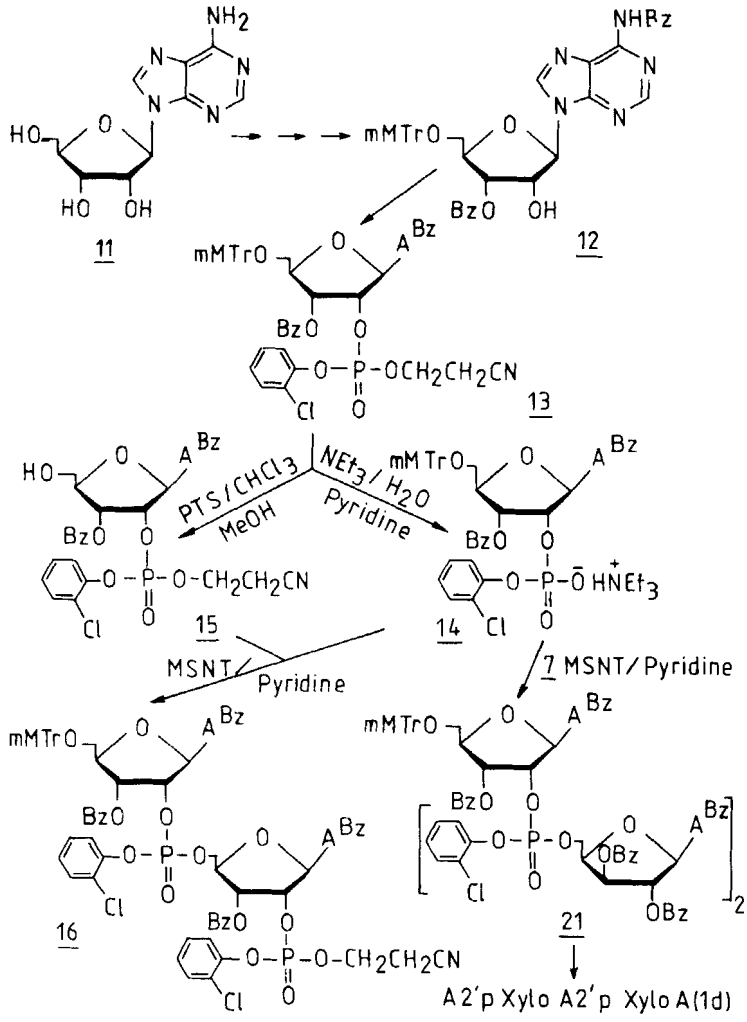
6 R = mMTr , n = 0
7 R = H , n = 0 → XyloA2'pXyloA (1a)
8 R = mMTr , n = 1 → $(XyloA2'p)_2XyloA$ (1b)
9 R = H , n = 1
10 R = mMTr , n = 2 → $(XyloA2'p)_3XyloA$ (1c)

Scheme 1

tography in 73 % yield. Compound 13 was selectively decyanoethylated by treatment with triethylamine-pyridine [106] to give the phosphodiester 14; it was also detritylated with *p*-toluenesulfonic acid (PTS) in chloroform/methanol [107] to produce the free 5'-OH 3'-phosphotriester 15. Both of these monomers (14 and 15) were easily isolated after column chromatography in 91 and 81 % yields respectively (Table 1).

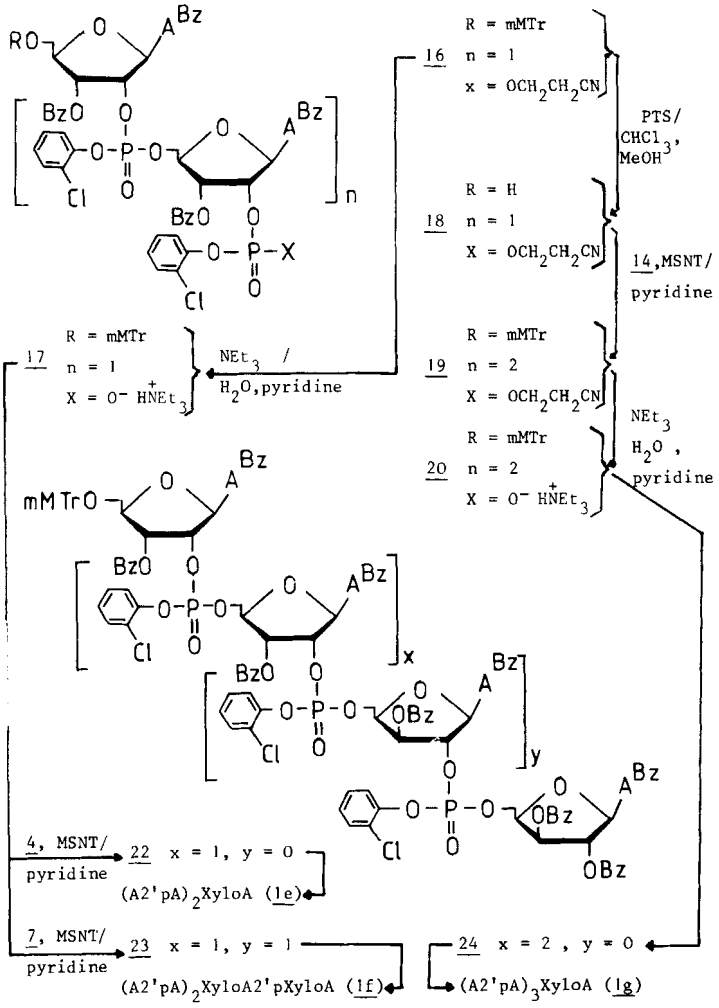
Preparation of the fully protected oligonucleotides.

Our general strategy for the synthesis of 2',5'-oligomers required building of oligonucleotide chains from the 2'- to the 5'- position.



Scheme 2

Thus the method involved reaction between a 2'-phosphodiester intermediate and the 5'-OH function of a nucleoside or oligonucleotide component, in the presence of an activating agent. Owing to its stability and efficiency,1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) [108] was chosen as the activating agent. Solutions of the triethylammonium salts of the 2'-phosphodiester derivatives (5, 14, 17 or 20) and the free 5'-OH compounds (4, 7, 9, 15 or 18) treated with 2.5 molar equivalents of MSNT in anhydrous pyridine at room temperature gave the fully-protected oligomers (6, 8, 10, 16, 19, 21-24). The products, obtained as



Scheme 3.

mixtures of diastereoisomers due to the chirality of the phosphorus triesters, were isolated in 58-84 % yields by silica gel short column chromatography (Table 2).

Detritylated 7, 9, 18 or decyanoethylated 17, 20 were obtained from the fully-protected oligomers 6, 8, 16 and 16, 19 using *p*-toluenesulfonic acid or triethylamine respectively, as described above for 13. Relevant data for the synthesis and properties of these compounds are given in Table 1.

TABLE 1. Relevant data for the synthesis and properties of decyanoethylated compounds 14, 17, 20 and detritylated compounds 7, 9, 15, 18.

Starting Compound		*Product*						
N°	mmole	N°	eluent[a]	Yield[b] mmole	Yield[b] %	TLC R_f values[d] A / B / C	^{31}P NMR[f] (solvent)
13	2.52	14	CH_3OH in (CH_2Cl_2 - 1% NEt_3) from 0 to 3 %	2.31	91	0.18	- 6.97 (CH_2Cl_2)
16	0.44	17	CH_3OH in ($CHCl_3$ - 1% NEt_3) from 0 to 3 %	0.40	91	≃ 0.15[e]	- 6.91 - 7.03 - 7.66 - 8.06 ($CDCl_3$)
19	0.29	20	CH_3OH in ($CHCl_3$ - 1% NEt_3) from 0 to 4 %	0.24	83	≃ 0.17[e]	- 6.74 - 6.91 - 7.14 - 7.49 - 7.71 - 7.83 - 7.94 - 8.06 - 8.17 - 8.28 ($CDCl_3$)
6	≃3.6	7	CH_3OH in $CHCl_3$ from 0 to 3%	2.7[c]	74[c]	0.17[e] 0.24[e]	- 8.06 - 8.23 ($CHCl_3$)
8	0.21	9	CH_3OH in $CHCl_3$ from 0 to 2%	0.13	62	≃ 0.3[e]	ND[g]
13	2.65	15	CH_3OH in CH_2Cl_2 from 0 to 3 %	2.15	81	0.29[e] 0.36[e]	- 8.51 - 8.86 (CH_2Cl_2)
16	0.92	18	CH_3OH in CH_2Cl_2 from 0 to 2 %	0.60	65	≃ 0.27[e]	- 7.43 - 8.06 - 8.40 - 8.63 - 8.74 ($CDCl_3$)

[a] For column chromatography purification. [b] After column chromatography purification. [c] Based on 4. [d] On silica gel plates in systems : A(chloroform/methanol 94:4, v/v) ; B(dichloromethan/methanol 90:10, v/v) ; C(ethylacetate). [e] TLC homogeneous, elongated or multiple spots due to diastereoisomers on phosphorus. [f] For a compound with n phosphorus atoms of which m are asymmetric, there are theoretically n x 2^m singlets ; but some signals may overlap. [g] Not determinated.

Final deblocking procedures and characterization of the unprotected 2',5'-oligoadenylates 1a-1g.

Sequential removal of the protecting groups was crucial for the success of our synthesis. In general, the strategy involved initial transformation of the phosphotriesters into the phosphodiesters, followed by basic treatment to remove the acyl groups, and finally removal of the acid-labile groups.

In the homogeneous xylose series, for the deprotection of oligomers 7, 8 and 10, cleavage of the 2-chlorophenyl groups from phosphorus was accomplished with N^1 , N^1 , N^3 , N^3-tetramethylguanidinium *syn*-4-nitrobenzaldoximate [108] in aqueous dioxane. Removal of N- as well as O - benzoyl with aqueous ammonia was followed by treatment with 80 % acetic acid to remove the 5'-O-monomethoxytrityl groups. The mixtures obtained after deprotection were applied to a DEAE-Sephadex A-25 column and eluted with a linear gradient of triethylammonium bicarbonate to give the pure deprotected 1a-1c in good yields.

In the mixed ribose-xylose series, HPLC and analysis of the ribonuclease T_2 digestion [29] of the deprotected products obtained from 21-24 by the method described above, showed extensive internucleotidic cleavage and isomerisation. These side-reactions may have occurred during the removal of the aryl groups from phosphorus. Concomitant removal of the 3'-O-benzoyl group by 4-nitrobenzaldoximate might indeed lead to 2' → 3' isomerisation as well as cleavage. Alternatively, these side-reactions may also have occurred during the subsequent basic then acidic steps.[116] Milder reaction conditions were therefore sought to minimize decomposition. Greatly reduced internucleotidic cleavage and isomerisation resulted when the phosphotriesters were deprotected with tetrabutylammonium fluoride in tetrahydrofuran-pyridine-water [109] at room temperature, and the benzoyl groups were removed with *n*-butylamine-methanol-dioxane. [110] Brief treatment (1-2h) with 80 % acetic acid effected the removal of the monomethoxytrityl groups. Under these conditions, after DEAE-Sephadex chromatography, TLC and HPLC analysis showed oligomers 1d-1g to be the main products, but detectable amounts of other isomers and possibly side-products were present. Pure oligomers 1d-1g were isolated after ribonuclease T_2 hydrolysis [29] of the unwanted 3' → 5' linkages, followed by alkaline phosphatase treatment [29] and DEAE-Sephadex chromatography.

Relevant data for the unprotected oligonucleotides 1a-1g are given in Table 3. These compounds were found to be pure by TLC and HPLC (Table 3). Their 2' → 5' internucleotidic phosphodiester linkages were corroborated

TABLE 2. Relevant data for the synthesis and properties of fully protected oligonucleotides 6,8,10,16,19,21-24

Starting 2'-Phosphodiester		*Product*							
N°	mmole	N°	mmole	N°	Eluent[a]	Yield[b] mmole	Yield[b] %	TLC R_f values[d,e]	^{31}P NMR[f] (solvent)
5	4.28	4	3.85	6	—	≃ quantitatif[c]		0.66 0.57	ND[g]
5	2.02	7	1.82	8	CH_3OH in $CHCl_3$ from 0 to 2 %	1.36	75	0.42 0.37 0.33 0.28	- 7.60 - 8.06 - 8.17 - 8.51 ($CHCl_3$)
5	0.14	9	0.12	10	CH_3OH in $CHCl_3$ from 0 to 2%	0.07	58	≃ 0.35	ND[g]
14	2.31	15	2.08	16	CH_3OH in CH_2Cl_2 from 0 to 2%	1.60	77	≃ 0.34	- 7.77 - 8.11 - 8.34 - 8.57 - 8.68 ($CDCl_3$)
14	0.47	18	0.43	19	CH_3OH in $CHCl_3$ from 0 to 2%	0.31	72	≃ 0.44	- 6.91 - 8.57 - 7.71 - 8.63 - 8.06 - 8.23 - 8.40 ($CDCl_3$)
14	0.48	7	0.43	21	CH_3OH in CH_2Cl_2 from 0 to 3%	0.36	84	≃ 0.30	- 7.77 - 8.17 - 8.28 - 8.57 ($CDCl_3$)
17	0.21	4	0.19	22	CH_3OH in CH_2Cl_2 from 0 to 3%	0.16	84	≃ 0.25	- 7.60 - 7.77 - 7.94 - 8.06 - 8.17 - 8.34 ($CDCl_3$)
17	0.15	7	0.13	23	CH_3OH in CH_2Cl_2 from 0 to 3%	0.10	77	≃ 0.31	- 7.77 - 8.06 - 8.17 - 8.28 - 8.40 - 8.63 ($CDCl_3$)
20	0.20	4	0.18	24	Aceton in ethylacetate from 0 to 20 %	0.13	72	≃ 0.39	- 7.66 - 7.77 - 7.94 - 8.11 - 8.23 - 8.34 ($CDCl_3$)

[a] For column chromatography purification. [b] After column chromatography purification and based on the 5'-hydroxy component.
[c] [d] On silica gel plates with chloroform/methanol 96:4, v/v. [e,f,g] See Table 1.

TABLE 3. Relevant data for the synthesis and properties of unprotected oligonucleotides 1a-1g.

Starting Compound		*Product*								
N°	mmole	N°	DEAE-Sephadex[a] chromatography		Yield[b]		TLC[d] R_f values		HPLC[e] Retention time(min)	
			Gradient (M/L)	Concn. of elution (M/L)	mmole	%	A	B	C	D
7	0.20	1a[c]	$2.0\ 10^{-3}$ - 0.2	0.12	0.16[c]	79[c]	0.55	0.43	10.45	ND[f]
8	1.00	1b	$2.0\ 10^{-3}$ - 0.5	0.29	0.82	82	0.28	0.38	12.18	16.20
10	0.064	1c	$2.0\ 10^{-3}$ - 0.4	0.36	0.048	75	0.24	0.33	13.77	10.09
21	0.186	1d	$2.0\ 10^{-3}$ - 0.35	0.29	0.061	33	0.28	0.38	ND[f]	20.00
22	0.118	1e	$2.0\ 10^{-3}$ - 0.35	0.30	0.033	28	0.28	0.38	ND[f]	17.88
23	0.072	1f	$2.0\ 10^{-3}$ - 0.4	0.35	0.021	29	0.24	0.38	ND[f]	10.09
24	0.120	1g	$2.0\ 10^{-3}$ - 0.4	0.34	0.014	12	0.24	0.33	ND[f]	11.05

[a] For elution with triethylammonium hydrogenocarbonate. [b] After DEAE-Sephadex column chromatography and lyophilization. [c] This compound was contaminated by an unknown salt of triethylammonium (^{1}H NMR) ; its purity (70%) and its yield (0.16 mmoles, 79%) were determined by spectrophotometrical analysis of a weighed aliquot after enzymatic degradation. [d] On silica gel plates in systems : A(aq. M NH_4 OAC/EtOH 2:8,v/v) ; B(isopropanol/aq. NH_3 20%/H_2O 7:2:1, v/v/v). [e] See experimental part.

TABLE 4. Enzymatic (snake venom phosphodiesterase) digestion and UV absorption data for 1a-1g.

Compound	UV [a]			Enzymatic DIGESTION [a] Molar ratios of digestion products [b]							
				XyloA		pXyloA		A		pA	
	λ_{max} (nm)	ε_{259}	Hyperchromicity (%)	Calcd.	found [c]	Calcd.	found [c]	Calcd.	found [c]	Calcd.	found [c]
1a	259	25,500	21.1	1	1.1	1	0.9	0	0	0	0
1b	259	37,800	22.3	1	1.2	2	1.8	0	0	0	0
1c	259	49,600	24.3	1	1.2	3	2.8	0	0	0	0
1d	259	40,500	14.2	0	0.1	2	1.9	1	1	0	0
1e	259	38,800	19.3	0	0.1	1	0.9	1	1.1	1	0.9
1f	259	50,700	21.6	0	0.2	2	1.8	1	1.1	1	0.9
1g	259	48,500	27.1	0	0.1	1	0.9	1	1.2	2	1.8

[a] For details see experimental part. [b] Molar ratios of the products were calculated by HPLC.
[c] Deviations from the theoretical values stemmed from some phosphomonoesterase activity in the enzyme preparation.

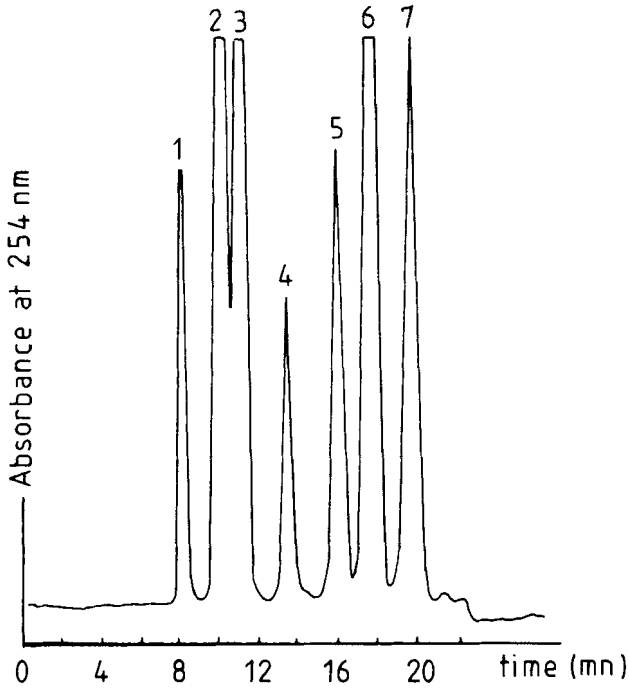


FIG.2. Elution profile of a test mixture of trimeric and tetrameric 2',5'-oligoadenylates, as detected at 254 nm. Separation conditions (D) are described under Experimental. Retention times in minutes are as follows : (1) A2'p5'A2'p5'A2'p5'A, 8.13 ; (2) XyloA2'p5'XyloA2'p5'XyloA 2'p5'XyloA + A2'p5'A2'p5'XyloA2'p5'XyloA, 10.09 ; (3) A2'p5'A2'p5'A2'p 5'XyloA, 11.05 ; (4) A2'p5'A2'p5'A, 13.65 ; (5) XyloA2'p5'XyloA2'p5' XyloA, 16.20 ; (6) A2'p5'A2'p5'XyloA, 17.88 ; (7) A2'p5'XyloA2'p5'XyloA, 20.00.

by their resistance to digestion with ribonuclease T_2 , and the assigned structures were further confirmed by their complete hydrolysis to the expected products in satisfactory ratios with venom phosphodiesterase. The enzymatic digestion and UV absorption data are tabulated in Table 4.

Analysis of trimeric and tetrameric 2',5'-oligoadenylates in the homogeneous xylose, ribose and mixed ribose-xylose series.

The chemically synthesized 2',5'-oligoadenylates Ib-Ig were analyzed together with enzymatically synthesized A2'p5'A2'p5'A^{III} and A2'p5'A2'p 5'A2'p5'A^{III} by reverse phase HPLC. Based on the method of P.J. Cayley, R.E. Brown and I.M. Kerr for the separation of the cores of natural

2-5A,[112] we have found that our new 2',5'-oligoadenylates are well resolved using a Radialpak C_{18} column 5μ and isocratic elution with 10 % methanol in 4 mM potassium phosphate buffer, pH 6.5 (conditions D in Experimental).

Fig. 2 depicts a representative chromatogram of a recombined mixture of 1b-1g and trimeric and tetrameric natural 2-5A cores.

CONCLUSION.

In the present study well-defined fully and partially xyloadenosine-substituted 2',5'-oligoadenylates were efficiently obtained by chemical synthesis. Since these compounds are analogues of the natural 2-5A cores, their antiviral and antitumor properties are of considerable interest. Preliminary data concerning their biological activities have shown all the compounds 1a-1g to be effective antimitogenic and antiproliferative agents.[113] However, as with the natural 2-5A cores [39,40,49,50,59-61] and other active analogues, [61,100,101,114] it appears that their mode of action does not involve intracellular rephosphorylation and activation of the latent endoribonuclease. Rather, it is likely that our new analogues owe their biological activity to degradation to their monomer units. Thus, they act as prodrugs.

EXPERIMENTAL.

General Procedures. Evaporation of solvents was done with a rotary evaporator under reduced pressure (water aspirator). Ultraviolet spectra (UV) were recorded on a Cary 118C spectrophotometer. Proton nuclear magnetic resonance was determined at ambient temperature on a Varian EM 390 spectrometer. Chemical shifts are expressed in parts per million downfield from internal tetramethylsilane. ^{31}P NMR spectra were recorded on a Bruker WP 80 DS instrument at 32.37 MHz. ^{31}P NMR chemical shifts (parts per million) were reported relative to external H_3PO_4. Thin-layer chromatography (TLC) was performed in an ascending system on precoated aluminium sheets of silica gel $60F_{254}$ (Merck, n°. 5554), visualization of products being a accomplished by UV absorbance followed by charring with 10 % ethanolic sulfuric acid and heating. For the protected compounds, short column chromatography was performed with silica gel 60H (Merck n° 7736) under weak nitrogen pressure (≃ 4 psi). Unprotected oligonucleotides were purified by chromatography on DEAE-Sephadex A-25 (Pharmacia) using a linear gradient of triethylammonium hydrogenocarbonate (pH 7.5). High-pressure liquid chromatographic (HPLC) studies were

carried out on a Waters Associates unit equipped with : an U_6K injector, 6000A and M-45 pumps, a M-720 solvent programmer, a 440 UV detector operating at 254 nm, a R-401 differential refractometer and a M-730 microprocessor-controlled data system. Distilled water was further purified using a Milli-Q (Millipore) system. Organic solvents were HPLC grade from Fisons. All eluents were degassed with an ultrasonic bath prior to use. Conditions for analytical HPLC (Fig. 2 and Table 3) were : C (Radialpak C_{18} column 10 µ, 8 mm x 10 cm, in Z-module ; solvent A : 2 % acetonitrile in 0.1M ammonium acetate buffer, pH 5.9 ; solvent B : 12 % acetonitrile in the same buffer ; gradient 0-50 % B in 15 min, flow rate : 3.0 mL/min); D (Radialpak C_{18} column 5 µ, 8 mm x 10 cm, in RCM 100 ; solvent : 10 % methanol in 4mM potassium phosphate buffer, pH 6.5 ; isocratic conditions, flow rate 0.5 mL/min). Snake venom phosphodiesterase (from Crotalus Durissus, EC 3.1.4.1) and calf spleen phosphodiesterase (EC 3.1.16.1) were from Boehringer Mannheim ; ribonuclease T_2 (EC 3.1.27.1) and bacterial alkaline phosphatase (type 111-R, EC 3.1.3.1) were from Sigma. Enzymic digestions were made under similar conditions as previously described.[29,115]

N^6,3'-O-dibenzoyl-5'-O-(4-methoxytrityl)adenosine-2'-O-[(2-chlorophenyl)(2-cyanoethyl)phosphate] (13)

A solution of 1,2,4-triazole (2.74 g, 40.3 mmole) and 2-chlorophenyl phosphorodichloridate (1.83 g, 7.45 mmole) in anhydrous pyridine (10.1 mL) was stirred for 35 min at room temperature. This solution was added to N^6,3'-O-dibenzoyl-5'-O-(4-methoxytrityl)adenosine (12)[104] (3.0 g, 4.03 mmole) which had been previously dried by three co-evaporations with pyridine . The reaction mixture was stirred for 15 min and a solution of 3-hydroxypropionitrile (1.15 g, 16.18 mmole) and 1-methylimidazole (1.0 g, 12.18 mmole) in pyridine (2.4 mL) was then added. After the reaction was complete (3 hours) the mixture was diluted with water (75 mL) and extracted with dichloromethane (3 x 75 mL). The combined extracts were washed with 2 % aqueous sodium bicarbonate (75 mL) and water (3 x 75 mL), dried over sodium sulfate and evaporated. The product was chromatographed on a silica gel column using as eluent a stepwise gradient of methanol (0-1.5 %) in dichloromethane. The fractions containing the pure compound were pooled, evaporated, and lyophilized from dioxane to give 13 : 2.9 g (73 %) ; ^{1}H NMR ($CDCl_3$) δ 2.43 (t, 2H, $-CH_2CN$), 3.4-3.6 (m, 2H, $-OCH_2-$), 3.66 (s, 3H, $-OCH_3$), 3.9-4.2 (m, 2H, H-5',5''), 4.3-4.5 (m, 1H, H-4'), 5.8-6.5 (m, 3H, H-1', 2' and 3'), 6.6-8.2 (m,29H, aromatic H + H-2 or H-8), 8.50 and 8.55 (2s, 1H, H-2 or H-8), 9.5 (br s, 1H, NH-6).

^{31}P NMR ($CDCl_3$)δ -8.17 ; - 8.28 ; UV (EtOH) λ_{max}, nm (ε) : 231 (43,200), 278 (25,000) ; λ_{min}, nm (ε) : 255 (16,600).

Anal. Calcd for C_{53} H_{44} N_6 O_{10} P Cl. H_2O : C, 63.06 ; H, 4.59 ; N, 8.33 ; P, 3.07. Found C, 63.15 ; H, 4.36 ; N, 8.34 ; P, 2.97.

Selective removal of β-cyanoethyl group from the fully protected ribomononucleotide 13 and ribooligonucleotides 16 and 19.

Compounds 13, 16 and 19 were dissolved in a solution of pyridine (35.5 mL per mmole of substrate), water (5.5 mL/mmole) and triethylamine (5.5 mL/mmole) were added and the reaction mixture was stirred at room temperature for 30 min. The solvent was then removed *in vacuo* and the foamy residues reevaporated with toluene solvent three times. The residues were chromatographed on a silica gel column to give the aryl phosphodiester compounds 14, 17 and 20. Chromatographic properties, yields and NMR data are given in Table 1 for the individual compounds.

Selective removal of 4-methoxytrityl group from the fully protected ribomononucleotide 13 , ribodinucleotide 16 and xylooligonucleotides 6 and 8.

Compounds 13, 16, 6 and 8 were dissolved in a solution of 2 % *p*-toluenesulfonic acid in chloroform-methanol (7:3, *ca.* 15 mL per mmole of substrate) and the reaction mixture was stirred at room temperature for 1h-3h. After the trityl cleavage was complete, the reaction mixture was taken up in chloroform (*ca.* 70 mL/mmole) and saturated aqueous sodium bicarbonate solution (*ca.* 70 mL/mmole). The aqueous layer was extracted twice with chloroform (50 mL/mmole). The combined organic layers were washed with water, dried over sodium sulfate, filtered and evaporated to dryness. Chromatography of the residues on a silica gel column gave the pure detritylated compounds 15, 18, 7 and 9, which are individually described in Table 1.

General procedure for the preparation of fully protected oligonucleotides 6, 8, 10, 16, 19, 21-24.

Protected 2'-phosphodiester derivatives 5, 14, 17, 20 and 5'-hydroxyl components 4, 7, 9, 15, 18 (0.9 mmole per mmole of phosphodiester) were first co-evaporated three times with anhydrous pyridine then dissolved in pyridine (5 mL/mmole), and 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (0.73 g, 2.5 mmole/mmole) was added to the stirred solution at room temperature. After 20-60 min. saturated aqueous sodium bicarbonate (1 mL/mmole) was added and the stirring continued for 15 min. The reaction mixtures were poured into saturated aqueous sodium bicarbonate (90 mL/mmole)

and extracted with chloroform (5 x 30 mL/mmole). The combined chloroform extracts were dried over sodium sulfate, filtered and evaporated to dryness. The residues were reevaporated three times in toluene solvent then chromatographed on silica gel to give the fully protected oligonucleotides 6, 8, 10, 16, 19, 21-24, which are individually described in Table 2.

Deblocking of fully protected xylooligonucleotides 6, 8 and 10.

A solution of the oligonucleotides 6, 8 and 10, *syn*-4-nitrobenzaldoxime (35 mmole per mmole of xylooligonucleotide) and N^1, N^1, N^3, N^3-tetramethylguanidine (35 mmole/mmole) in dioxane-water (1:1, 70 mL/mmole) was stirred at room temperature. Tetramethylguanidine (0.6 mmole/mmole) was added after 5 h and the deblocking was allowed to proceed for a total of 20 h. The products were then concentrated under reduced pressure and the resulting gums were redissolved in aqueous ammonia (*d* 0.92, 350 mL/mmole) in a pressure bottle. After 20 h at 40° the solvents were removed *in vacuo*. The residues were dissolved in 80 % aqueous acetic acid (400 mL/mmole) and stirred at room temperature for 5 h. The reaction mixture was diluted with water (*ca.* 300 mL/mmole) and extracted first with chloroform (8 x 300 mL/mmole) then with ether (4 x 300 mL/mmole). The aqueous layers were evaporated to dryness and the residues co-evaporated three times with water. The products were chromatographed on a DEAE-Sephadex A-25 column using triethylammonium bicarbonate buffer (pH 7.5, linear gradient increasing from 0.002 M) as eluent. The appropriate fractions which were found to be pure when analyzed by TLC and HPLC were combined, evaporated and reevaporated in water, then lyophilized to give the desired fully unblocked xylooligonucleotides 1a-1c, which are individually described in Table 3.

Deblocking of fully protected mixed ribo-xylooligonucleotides 21-24.

The protected derivatives 21-24 were dissolved in 0.05 M tetrabutylammonium fluoride in a mixture of tetrahydrofuran-pyridine-water (2 equiv. per phosphotriester moiety, 8:1:1 v/v/v). After stirring overnight at room temperature, the solutions were evaporated *in vacuo* and the residues partioned between chloroform (*ca.* 300 mL/mmole) and water (*ca.* 300 mL/mmole). The aqueous layers were first extracted with chloroform (3 x *ca.* 100 mL/mmole) then with ethyl acetate (*ca.* 100 mL/mmole). The combined organic layers were dried over sodium sulfate, filtered and evaporated to dryness. The resulting residues were dissolved in a mixture of *n*-butyl-

amine-methanol-dioxane (2:1:2 v/v/v, 130 mL/mmole) and stirred in a pressure bottle at 33° for 20 h. The reaction mixtures were evaporated *in vacuo* and reevaporated to dryness in water. The residues were then treated and chromatographed as described above for deblocking of the fully protected xylooligonucleotides but the time of acetic acid treatment was reduced to 1-2 h. TLC and HPLC analysis of the main peaks obtained by DEAE-Sephadex A-25 chromatography showed detectable amounts of other isomers and possibly side-products. These fractions were collected, evaporated to dryness, coevaporated with water and lyophilized before enzymatic treatment.

The lyophilized fractions were dissolved in 0.1M ammonium acetate buffer (pH adjusted to 4.5 with acetic acid, 66 µL/mg) and incubated overnight at 37° with ribonuclease T_2 (1.5 µL/mg of a solution containing 1000 U/mL H_2O). The pH was adjusted to 8.9 with 0.5M Tris-HCl buffer (*ca.* 30 µL/100 µL of incubation medium) and a suspension of alkaline phosphatase from *Escherichia Coli* (5 µL/mg) was added. After 3 h at 37°, the enzymes were inactivated by heating for 3 min. at 90°. The reaction mixtures were centrifuged and the supernatants evaporated *in vacuo* and reevaporated to dryness in water. The residues were then chromatographed on a DEAE-Sephadex A-25 column as described above. The appropriate fractions which were found to be pure by TLC and HPLC were combined, evaporated and reevaporated in water, then lyophilized to give the desired fully deblocked mixed ribo-xylooligonucleotides 1d-1g, which are individually described in Table 3.

Enzymatic hydrolysis studies of 1a-1g.

An accurate quantity of each unprotected 2',5'-oligonucleotide 1a-1g (1.2 - 1.4 mg) was dissolved in water (10 mL). With ribonuclease T_2 : 1 mL of each stock solution was first lyophilized then incubated with ribonuclease T_2 as described above for the deblocking of the fully protected mixed ribo-xylooligonucleotides. TLC and HPLC analysis showed no hydrolysis of any of the oligonucleotides 1a-1g. With calf spleen phosphodiesterase: to 0.8 mL of each stock solution were added 0.15 mL of a cocktail (6 mL of 1M KH_2PO_4 , pH adjusted to 6.1 with 1M NaOH + 5 mL of 0.1M EDTA + 6 mL of Tween 80 + 23 mL of H_2O) and 20 µL of calf spleen phosphodiesterase. The resulting solutions were maintained at 37° for 16 h. TLC and HPLC analysis showed no hydrolysis of the oligonucleotides. With snake venom phosphodiesterase : to 8 mL of each stock solution were added 0.56 mL of $MgCl_2$ (0.1M), 0.56 mL of Tris-HCl (1M, pH adjusted to 8.9 with HCl), and

the volume was adjusted to 10 mL with H_2O. Four mL of the resulting solutions were incubated with snake venom phosphodiesterase (20 µL) at 37° for 16 h. The enzyme was inactivated by heating for 3 min at 90° and the enzymatic digests were analyzed qualitatively by TLC and quantitatively by HPLC. Complete conversion of oligomers 1a-1g into the expected products was observed. Data are presented in Table 4.

Determination of hyperchromicity and yield for each unprotected 2',5'-oligonucleotide 1a-1g.

Hyperchromicity, defined as $\frac{A_D - A_I}{A_I} \times 100$, was determined for each oligonucleotide by measurement of the absorbance at 260 nm before (A_T) and after (A_D) digestion with snake venom phosphodiesterase, as described above. The concentration of each oligonucleotide was spectrophotometrically determined after snake venom phosphodiesterase digestion using an extinction coefficient of 15,400 at 260 nm for adenosine, xyloadenosine and their 5'-O-monophosphorylated derivatives. The calculated concentrations were used to determinate the yields (Table 3) and the extinction coefficients (Table 4) of the pure oligonucleotides 1a-1g in their triethylammonium form.

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