Chemical Synthesis of pppA2′p5′A2′p5′A, an Interferon-Induced Inhibitor of Protein Synthesis, and Some Functional Analogues

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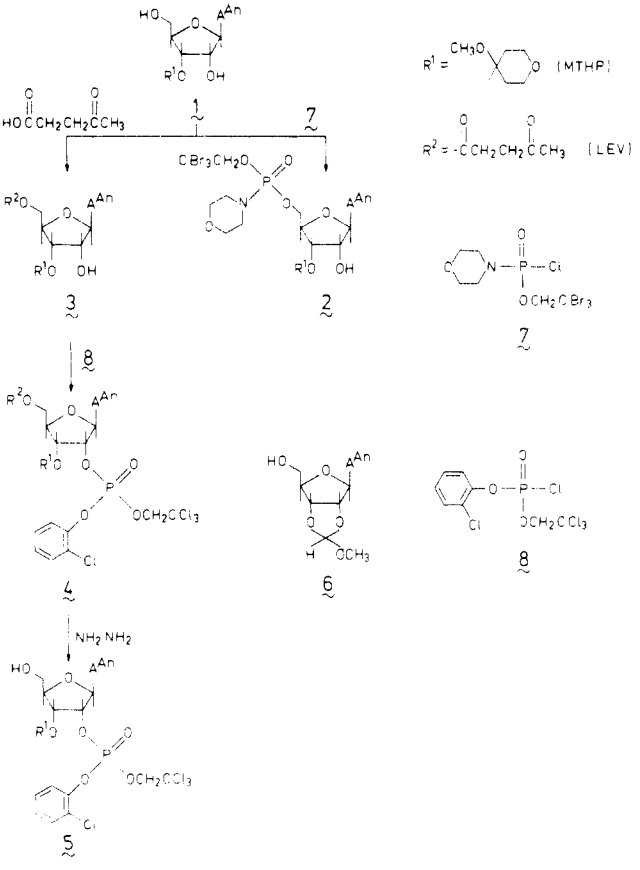
A convenient chemical synthesis via phospho triester methods of **2-5A** and some modified derivatives will be presented. The synthesis of a modified triphosphate derivative bearing a methylene function between the β- and γ-phosphorus atoms (i.e., compound **27d**) is reported. Another alteration involves the replacement of a 3′-hydroxyl group by a 3′-*O*-methyl function in the 3′(2′)-terminal adenosine (e.g., compounds **28**) or in all of the adenosine moieties (e.g., compounds **29**). For the introduction of the various 5′-phosphate groups, the monofunctional reagent 2,2,2-tribromoethyl phosphoromorpholinochloridate (i.e., compound **7**) is used. The internucleotide linkages were attained (see Scheme III) by means of 2-chlorophenyl phosphorobis(1,2,4-triazolidate) (i.e., compound **24**) or in special cases (see Scheme IV) by using 2,2,2-trichloroethyl 2-chlorophenyl phosphorochloridate (i.e., compound **8**).

In recent years a very effective inhibitor of protein synthesis has been found in eukaryotic cells and characterized as pppA2′p5′A2′p5′A (**2-5A**).[1] This 5′-triphosphate (ppp) of an adenylic acid trimer, which contains 2′–5′ internucleotide phospho diester linkages (2′p5′) instead of the common 3′–5′ linkages, as well as a series of higher homologues is synthesized by a specific synthetase in a variety of cell systems.[2-5] It has been shown[6-8] that the inhibition of protein synthesis by **2-5A** is mediated by an activatable endonuclease which degrades mRNA.

On the other hand it has been reported[9] that the biological activity of pppA2′p5′A2′p5′A is rapidly lost in cell-free systems. This inactivation has been attributed to hydrolysis of terminal phosphates[9] and to cleavage of the 2′–5′ internucleotide bonds by a specific exonuclease.[10]

Various chemical syntheses of **2-5A**[11-14] as well as its nonphosphorylated "core" (A2′p5′A2′p5′A)[15-18] have been published recently. As an extension of our previous work,[11] we now present the synthesis of some analogues of **2-5A** which were expected to be resistant to the before-mentioned degradation of **2-5A**. To achieve our goal, we adopted two distinct types of modifications. The first modification was aimed at suppressing hydrolysis of the 5′-triphosphate into a diphosphate. For this purpose a methylene function between the β- and γ-phosphorus atoms of the 5′-triphosphate group was introduced (compound **27d** in Scheme V). The thus obtained methylenephosphonate function is known to be stable in biological systems. The second alteration involved the replacement

Scheme I



(1) I. M. Kerr and R. E. Brown, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 256 (1978).
(2) A. G. Hovanessian and I. M. Kerr, *Eur. J. Biochem.*, **84**, 149 (1978).
(3) C. Baglioni, *Cell*, **17**, 255 (1979).
(4) G. R. Stark, W. J. Dower, R. T. Schimke, R. E. Brown, and I. M. Kerr, *Nature (London)*, **278**, 471 (1979).
(5) J. P. Dougherty, H. Samanta, P. J. Farrell, and P., Lengyel, *J. Biol. Chem.*, **255**, 3813 (1980).
(6) C. Baglioni, M. A. Minks, and P. A. Maroney, *Nature (London)*, **273**, 684 (1978).
(7) M. J. Clemens and B. R. G. Williams, *Cell*, **13**, 565 (1978).
(8) B. R. G. Williams, R. R. Golgher, and I. M. Kerr, *FEBS Lett.*, **105**, 47 (1979).
(9) B. R. G. Williams, I. M. Kerr, C. S. Gilbert, C. N. White, and L. A. Ball, *Eur. J. Biochem.* **92**, 455 (1978).
(10) A. Schmidt, Y. Chernajovsky, L. Shulman, P. Federman, H. Berissi, and M. Revel, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4788 (1979).
(11) J. A. J. den Hartog, J. Doornbos, R. Crea, and J H. van Boom, *Recl. Trav. Chim. Pays-Bas*, **98**, 469 (1979).
(12) S. S. Jones and C. B. Reese, *J. Am. Chem. Soc.*, **101**, 7399 (1979).
(13) M. Ikehara, K. Oshie, and E. Ohtsuka, *Tetrahedron Lett.*, 3677 (1979).
(14) H. Sawai, T. Shibata, and M. Ohno, *Tetrahedron Lett.*, 4573 (1979).
(15) A. F. Markham, R. A. Porter, M. J. Gait, R. C. Sheppard, and I. M. Kerr, *Nucleic Acids Res.*, **6**, 2569 (1979).
(16) K. K. Ogilvie and N. Y. Theriault, *Tetrahedron Lett.*, 2111 (1979).
(17) J. Engels and U. Krahmer, *Angew. Chem.*, **91**, 1007 (1979).
(18) R. Charubala and W. Pfleiderer, *Tetrahedron Lett.*, 1933 (1980).

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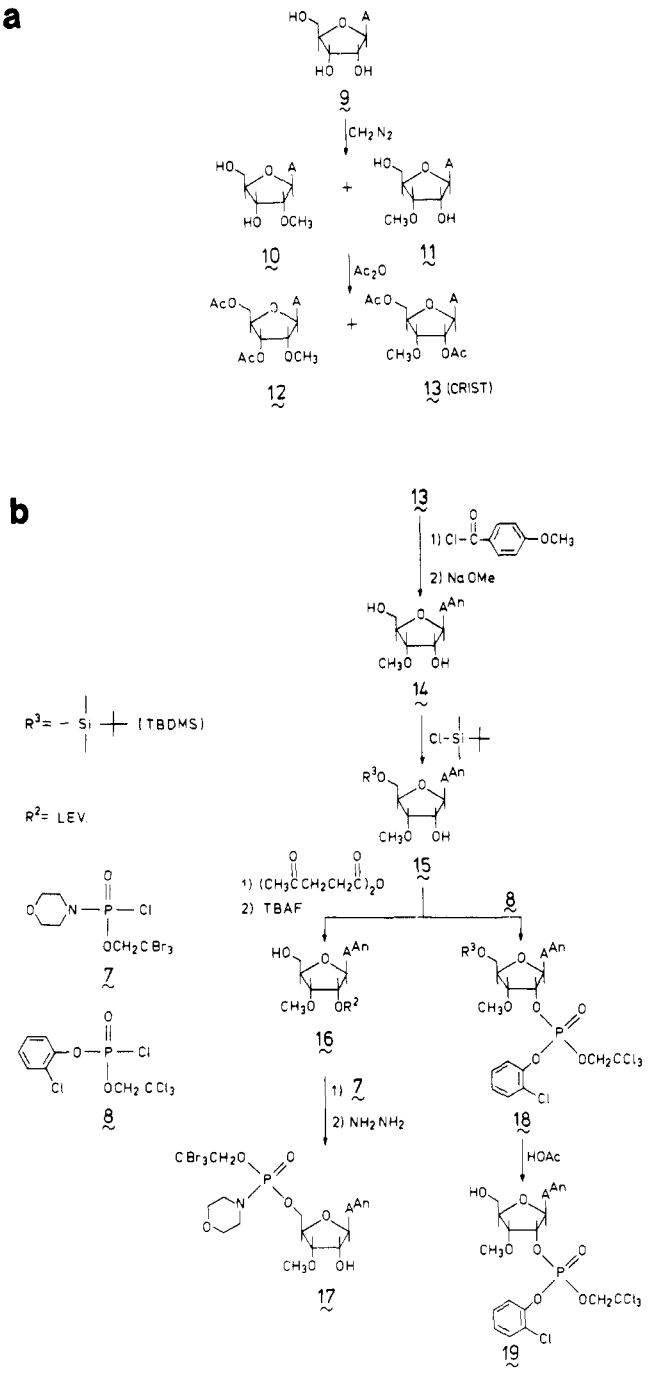
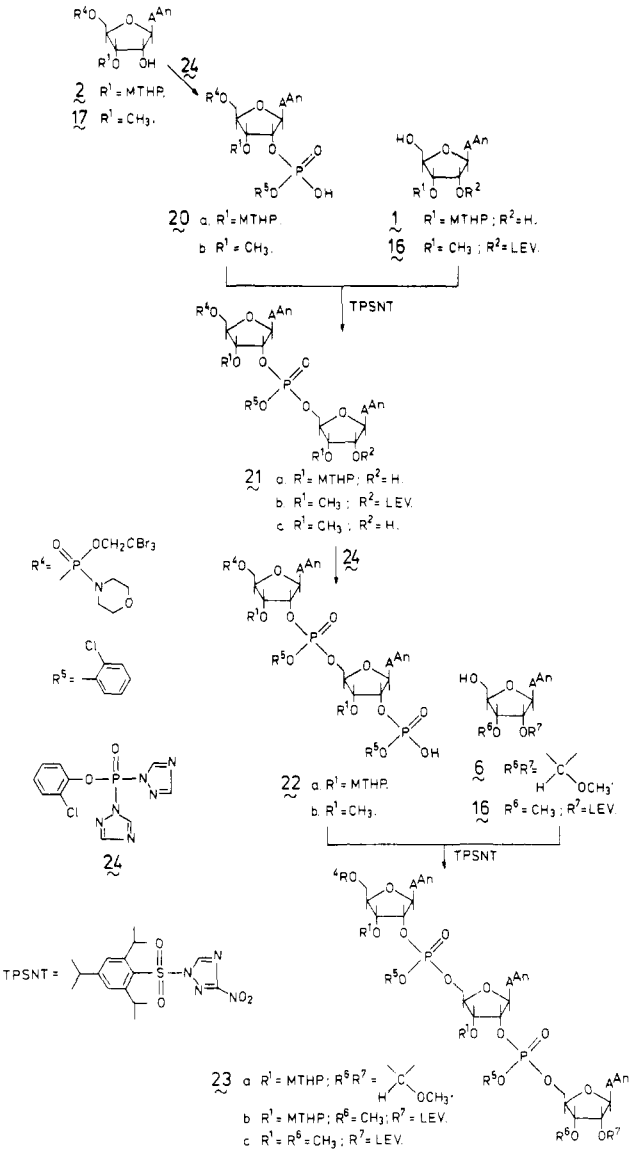
Scheme II



Scheme III



of the 3′-hydroxyl group in one (compounds **28** and **31**) or all of the ribose residues (compounds **29** and **32**) by *O*-methyl functions. The introduction of *O*-methyl groups allowed a study of the effect of this small ribose modification on the nucleolytic degradation of **2-5A**.

It has been shown[19,20] that besides pppA2′p5′A2′p5′A (i.e., compound **27c** in Scheme V), di- and monophosphorylated as well as nonphosphorylated derivatives (i.e., compounds **27b**, **27a**, and **30**, respectively) are effective inhibitors of protein synthesis in various cell systems. In order to enable an evaluation of the phosphate requirement of **2-5A** analogues, we prepared derivatives with a variable number of 5′-terminal phosphates (i.e., compounds **28a–c** and **29a–c**) or without 5′-phosphate functions ("core" derivatives, i.e., compounds **31** and **32**).

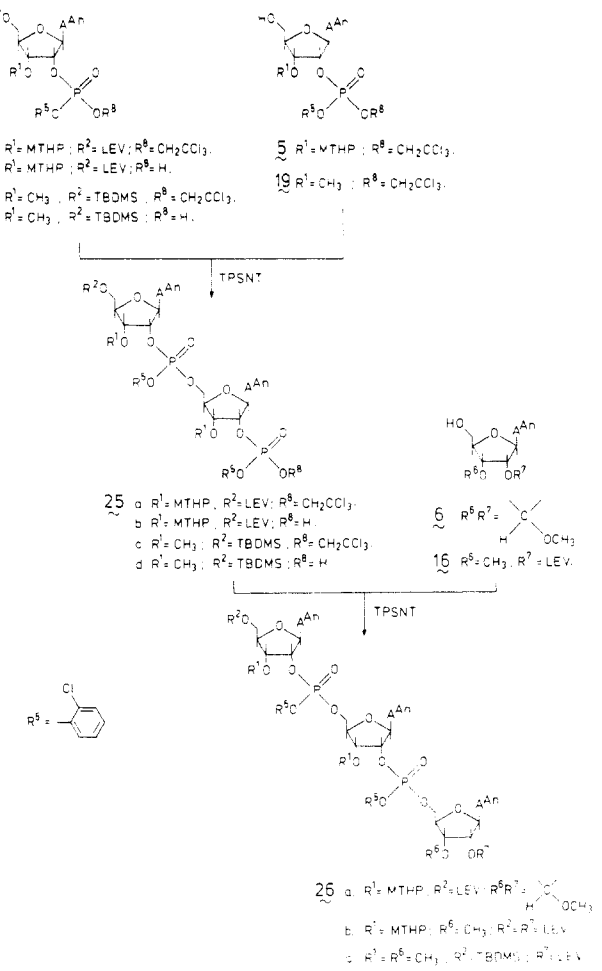
Results and Discussion

The strategy we followed for the synthesis of **2-5A** derivatives (i.e., compounds **27–32** in Schemes V and VI) consisted of the following steps: (a) preparation of properly protected derivatives of adenosine and 3′-*O*-methyladenosine (Schemes I and II), (b) synthesis of fully protected 2′–5′-linked trimers (Schemes III and IV), and finally (c) conversion of fully protected trimers into completely deblocked **2-5A** derivatives having a 5′-mono-, di-, or triphosphate or modified triphosphate function (i.e., compounds **27–29** in Scheme V) or a free 5′-hydroxyl group ("core" derivatives, i.e., compounds **30–32** in Scheme VI).

Two distinct approaches were used for the assemblage of fully protected 2′–5′-linked trimers bearing a masked 5′-phosphate function (i.e., compounds **23a–c**) and trimers with a protected 5′-hydroxyl group (i.e., compounds **26a–c**). In the synthesis of the latter type of trimer (i.e., **26**), the monofunctional reagent 2,2,2-trichloroethyl 2-chlorophenyl phosphorochloridate (**8**)[21] was applied for the introduction

(19) E. M. Martin, N. J. M. Birdsall, R. E. Brown, and I. M. Kerr, *Eur. J. Biochem.*, **95**, 295 (1979).
(20) B. R. G. Williams and I. M. Kerr, *Nature (London)*, **276**, 88 (1978).
(21) J. H. van Boom, P. M. J. Burgers, and P. H. van Deursen, *Tetrahedron Lett.*, 869 (1976).

Scheme IV



of 2′-5′ internucleotide bonds. Thus the fully protected adenosine and 3′-*O*-methyladenosine derivatives were phosphorylated on the 2′-hydroxyl group with reagent **8**. The 2′-phosphorylated products (i.e., compounds **4** and **5** in Scheme I and compounds **18** and **19** in Scheme IIb) could be used in building the non-5′-phosphorylated trimers (i.e., **26a–c**). The other type of trimer (i.e., **23**) contains a 5′-phosphate function protected with 2,2,2-tribromoethyl and morpholino protecting groups. This combination of protecting groups has been proven[22] to be suitable for the introduction of mono-, di-, and triphosphates. However, the presence of the 2,2,2-tribromoethyl group in the 5′-phosphorylated starting products **2** and **17** eliminates application of 2,2,2-trichloroethyl 2-chlorophenyl phosphorochloridate (**8**) for the formation of 2′–5′ internucletide bonds. The reason for this is that identical conditions have to be used for the removal of the tribromoethyl and trichloroethyl protecting groups. We therefore followed another approach which was based on the use of the effectively monofunctional phosphorylating agent 2-chlorophenyl phosphorobis(1,2,4-triazolidate)[23,24] (i.e., compound **24** in Scheme III) to assemble the fully protected 5′-phosphorylated trimers (i.e., **23a–c**).

Protection of Adenosine and 3′-*O*-Methyladenosine. In Scheme I the synthesis of nonmodified adenosine derivatives necessary for the construction of the **2-5A** trimers **23a,b** and **26a,b** is illustrated. All nonterminal derivatives (i.e., compounds **1**, **2**, **4** and **5**) contain a 3′-*O*-(4-methoxytetrahydropyran-4-yl) (3′-*O*-MTHP) protecting group. Treatment of key intermediate 3′-*O*-(4-methoxytetrahydropyran-4-yl)-6-*N*-*p*-anisoyladenosine (**1**)[25] with the monofunctional phosphorylating agent **7**[22] afforded the 5′-phosphorylated nucleoside **2** in good yield.

In order to phosphorylate **1** with reagent **8** on the 2′-hydroxyl group, we temporarily protected the primary 5′-hydroxyl group with a levulinyl function. The latter can be removed[26] under very mild conditions and selectively in the presence of all other protecting groups. Acylation of **1** with levulinic acid and dicyclohexylcarbodiimide[26] gave unfavorable mixtures of 5′- and 2′-levulinated and 2′,5′-dilevulinated adenosine derivatives. However, by use of levulinic acid and 1-methyl-2-chloropyridinium iodide,[27] compound **3** was obtained in relatively good yield. Starting from this intermediate, one can obtain two phosphorylated building blocks: one fully protected derivative (i.e., **4**) and the other with a free 5′-hydroxyl function (i.e., compound **5**). Thus treatment of **3** with the monofunctional phosphorylating agent **8**[21] afforded the phosphorylated derivative **4**, which after short hydrazinolysis[26] gave the 5′-hydroxyl derivative **5**. The terminal derivative 2′,3′-*O*-(methoxymethylene)-6-*N*-*p*-anisoyladenosine (**6**) was prepared as published.[26]

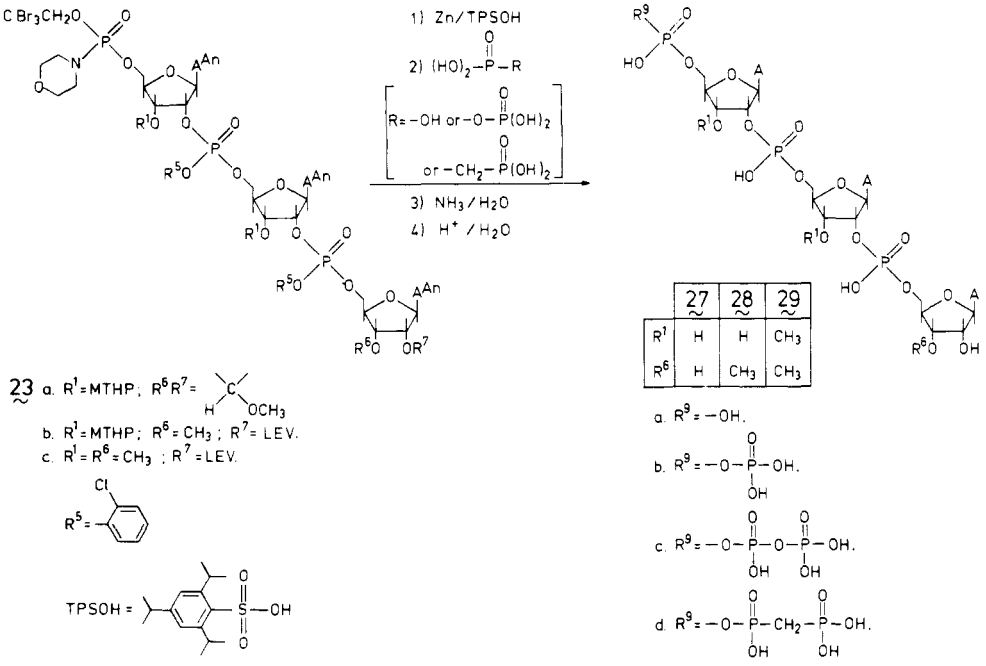
The building blocks necessary for the construction of **2-5A** analogues containing 3′-*O*-methyl groups (i.e., compounds **23b,c** and **26b,c**) were prepared by starting from 2′,5′-*O*-diacetyl-3′-*O*-methyladenosine (i.e., compound **13** in Scheme IIa). The latter derivative was obtained as a crystalline compound by the following simple procedure. Treatment of adenosine (**9**) with diazomethane, in the presence of $SnCl_2$,[28] gave a mixture containing 2′-*O*-methyladenosine (**10**) and the 3′-*O*-methyl derivative **11**. Acetylation of the latter derivatives with acetic anhydride afforded a mixture of the diacetates **12** and **13** which, after crystallization from ethanol, gave the pure isomer **13**. Diacetylated compound **13** could easily be converted into the 3′-*O*-methyl-6-*N*-*p*-anisoyl derivative **14** by reaction with anisoyl chloride followed by basic hydrolysis of the acetyl groups (see Scheme IIb).

Conversion of the obtained 3′-*O*-methyladenosine derivative **14** into the phosphorylated building blocks **17-19** could not be achieved according to the methods applied for the corresponding 3′-*O*-MTHP derivative **1** (Scheme I). No selectivity was observed in the direct phosphorylation or levulination of the 5′-hydroxyl group of **14**. To overcome this problem, we used *tert*-butyldimethylsilyl chloride (TBDMSCl)[29,30] to achieve selective protection of the 5′-hydroxyl group of **14**. Thus treatment of **14** with TBDMSCl in pyridine afforded the crystalline 5′-*O*-TBDMS derivative **15** in high yield.

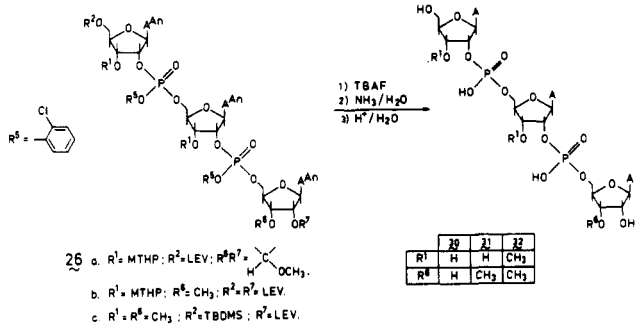
The introduction of the required 5′-phosphate function was effected via the following procedure. The 2′-hydroxyl group of **15** was temporarily blocked by reaction of **15** with levulinic anhydride[31] in the presence of 4-(dimethyl-

(22) J. H. van Boom, R. Crea, W. C. Luyten, and A. B. Vink, *Tetrahedron. Lett.*, 2779 (1975).
(23) N. Katagiri, K. Itakura, and S. A. Narang, *J. Am. Chem. Soc.*, **97**, 7332 (1975).
(24) K. L. Agarwal and F. Riftina, *Nucleic Acids Res.*, **5**, 2809 (1978).
(25) J. A. J. den Hartog and J. H. van Boom, *Synthesis*, 599 (1979).
(26) J. H. van Boom and P. M. J. Burgers, *Recl. Trav. Chim. Pays-Bas*, **97**, 73 (1978).
(27) K. Saigo, M. Usui, K. Kikuchi, E. Shimada, and T. Mukaiyama, *Bull. Chem. Soc. Jpn.*, **50**, 1863 (1977).
(28) M. J. Robins, S. R. Naik, and A. S. K. Lee, *J. Org. Chem.*, **39**, 1891 (1974).
(29) E. J. Corey and A. Venkateswarlu, *J. Am. Chem. Soc.*, **94**, 6190 (1970).
(30) K. K. Ogilvie, S. L. Beaucage, A. L. Schifman, N. Y. Theriault, and K. L. Sadana, *Can. J. Chem.*, **56**, 2768 (1978).
(31) A. Hassner, G. Strand, M. Rubinstein, and A. Patchornik, *J. Am. Chem. Soc.*, **97**, 1614 (1975).

Scheme V



Scheme VI



amino)pyridine[32] and triethylamine. After removal of the 5′-silyl function with tetra-*n*-butylammonium fluoride (TBAF),[29] the free 5′-hydroxyl group of compound **16** thus obtained was phosphorylated with the monofunctional reagent **7**. Hydrazinolysis of the fully protected intermediate gave the properly protected derivative **17**.

Starting from the 5′-silylated derivative **15**, we synthesized the two required 2′-phosphorylated building blocks, i.e., the fully protected compound **18** as well as a derivative having a free 5′-hydroxyl group (**19**). Thus reaction of **15** with the monofunctional phosphorylating agent **8** afforded compound **18**, which was finally converted into the 5′-hydroxyl derivative **19** by acidic hydrolysis.

Synthesis of Fully Protected 2′–5′-Linked Trinucleotides. The assemblage of the fully protected trimers **23a–c**, containing a masked 5′-phosphate function (R^4), is depicted in Scheme III. A common feature in the construction of trimers **23a–c** is the use of phosphorylating agent **24**[23] for the introduction of the 2′–5′ internucleotide phospho triester function. Thus the 5′-phosphorylated derivative **2** (R^1 = MTHP) was treated with **24**, which acts as a monofunctional phosphorylating agent,[24] to give the 2′-phospho diester derivative **20a**. Condensation of **20a**, in the presence of the very efficient activating agent 1-(2,4,6-triisopropylbenzenesulfonyl)-3-nitro-1,2,4-triazole (TPSNT)[33] with the adenosine derivative **1** (R^1 = MTHP) afforded dimer **21a** (R^1 = MTHP; R^2 = H). At this stage of the synthesis it is worthwhile to mention that the presence of a free secondary hydroxyl function in **1**, which may participate in the condensation reaction, gave rise to only a negligible quantity (ca. 0.1%) of an undesired 2′–2′-linked dimer. Purification of the crude condensation product by short-column chromatography[34] gave homogeneous **21a**. Subsequent phosphorylation of **21a** with **24** gave the 2′-phospho diester derivative **22a**, which was finally condensed, in the presence of TPSNT, with the 2′,3′-*O*-(methoxymethylene) derivative **6** to give the fully protected trimer **23a** (R^1 = MTHP; R^6, R^7 = methoxymethylene). On the other hand, condensation of the 2′-phosphorylated dimer **22a** obtained above with the 3′-*O*-methyl derivative **16** afforded trimer **23b** (R^1 = MTHP; R^6 = CH_3). Finally, the fully protected trimer **23c** (R^1 = R^6 = CH_3) was synthesized. Coupling of compounds **17** (R^1 = CH_3) and **16** (R^1 = CH_3), with **24** as the phosphorylating agent and TPSNT as the condensing agent, afforded the fully protected dimer **21b** (R^1 = CH_3; R^2 = LEV). In contrast to the 3′-*O*-MTHP derivative of adenosine, **1**, the analogous 3′-*O*-methyl derivative **14**, when used in a condensation reaction, gave rise to a higher amount (ca. 1%) of undesired 2′–2′-linked product. Therfore, instead of **14**, the 2′-*O*-levulinyl derivative **16** was applied. After removal of the levulinyl group from **21b**, dimer **21c** (R^1 = CH_3; R^2 = H) was coupled once more with **16** (R^6 = CH_3) to afford trimer **23c** (R^1 = R^6 = CH_3).

The assemblage of fully protected **2-5A** derivatives lacking the masked 5′-phosphate function (i.e., compounds **26a–c**) is outlined in Scheme IV. In this case the 2′–5′ internucleotide phospho triester linkages were introduced by a two-step procedure starting from 2′-phospho triester derivatives (e.g., compounds **4a** and **18a**). Thus, in the first step, the fully protected nucleotide **4a** (R^8 = CH_2CCl_3) was converted by treatment with activated zinc[35] in the presence of 2,4,6-triisopropylbenzenesulfonic acid (TPSOH)[35] into the phospho diester derivative **4b** (R^8 = H). In the second step, condensation of the latter compound with **5**,

(32) W. Steglich and G. Höffle, *Angew. Chem.*, **81**, 1001 (1969).
(33) J. F. M. de Rooij, G. Wille-Hazeleger, P. H. van Deursen, J. Serdijn, and J. H. van Boom, *Recl. Trav. Chim. Pays-Bas*, **98**, 537 (1979).
(34) B. J. Hunt and W. Rigby, *Chem. Ind. (London)*, 1868 (1967).
(35) J. H. van Boom, P. M. J. Burgers, G. van der Marel, C. H. M. Verdegaal, and G. Wille, *Nucleic Acids Res.*, **4**, 1047 (1977).

Table I. Data on the Synthesis of the Fully Protected Oligonucleotides 21, 23, 25, and 26

1st component		2nd component		product		R_f value[b]	
no.	amt, mmol	no.	amt, mmol	no.	yield,[a] %	A	B
2	1.50	1	1.20	21a	76	0.39,[c] 0.43	0.61
17	0.90	16	0.72	21b	70	0.56	0.70
				21c[d]	86	0.38	0.60
21a	0.70	6	0.56	23a	73	0.55	0.72
21a	0.43	16	0.34	23b	86	0.51	0.72
21c	0.30	16	0.24	23c	70	0.52	0.71
4a	1.00	5	0.90	25a	81	0.58	0.72
18a	0.25	19	0.22	25c	72	0.70,[c] 0.74	0.78
25a	0.30	6	0.27	26a	78	0.50	0.68
25a	0.30	16	0.27	26b	70	0.48	0.66
25c	0.17	16	0.15	26c	75	0.59	0.72

[a] After purification, based on the 5'-hydroxy component. [b] On silica gel plates in system A and B, respectively; see Experimental Section. [c] Pair of diastereoisomers. [d] Obtained from **21b** by removal of the levulinyl group.

in the presence of TPSNT, afforded the fully protected dimer **25a** ($R^8 = CH_2CCl_3$). Repetition of this two-step procedure, i.e., reductive cleavage of the trichloroethyl group from **25a** to give **25b** (R^8 = H), followed by condenstion with **6** in the presence of TPSNT, gave the fully protected trimer **26a** (R^1 = MTHP; R^6, R^7 = methoxymethylene). In the same way, trimer **26b** (R^1 = MTHP; $R^6 = CH_3$) was prepared by condensing dimer **25b** (R^8 = H), in the presence of TPSNT, with the 3'-*O*-methyl derivative **16**. Finally, the fully protected trimer **26c** ($R^1 = R^6 = CH_3$) was synthesized. Conversion of **18a** ($R^8 = CH_2CCl_3$) into **18b** (R^8 = H), followed by condensation with **19**, with TPSNT as the condensing agent, afforded the fully protected dimer **25c** ($R^8 = CH_2CCl_3$). After removal of the trichloroethyl group from **25c**, condensation of the obtained 2'-phospho diester **25d** with **16** afforded trimer **26c** ($R^1 = R^6 = CH_3$). Yields and analytical data on the synthesis of the fully protected oligomers are given in Table I.

Conversion of the Fully Protected Trimers 23a–c and 26a–c into Completely Deblocked 2-5A Derivatives 27–32. The conversion of the 5'-phosphorylated and fully protected trimers **23a–c** into the required 5'-mono-, di-, and triphosphate derivatives is visualized in Scheme V. The first step in this process consisted, in all cases, of treating the fully protected compounds **23a–c** with activated zinc in the presence of TPSOH. The 5'-phosphoromorpholidate derivates thus obtained could be converted by ammonolysis followed by acid treatment into the corresponding 5'-monophosphate derivatives **27a**, **28a**, and **29a**, respectively. Reaction of the intermediates which were obtained from **23a–c** after the first deblocking step with tri-*n*-butylammonium phosphate[36] (step 2 in Scheme V, R = OH), followed by ammonia and acid treatment, gave the corresponding 5'-diphosphate derivatives **27b**, **28b**, and **29b**, respectively. The corresponding 5'-triphosphates **27c**, **28c**, and **29c** were obtained by using bis(tri-*n*-butylammonium) diphosphate[36] (R = $OPO(OH)_2$) in the second step of the reaction procedure (Scheme V). Finally, the synthesis of the modified 5'-triphosphate derivative **27d** was accomplished by using bis(tri-*n*-butylammonium) methylenediphosphonate (R = $CH_2PO(OH)_2$) in step 2 of the reaction procedure.

(36) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 83, 663 (1961).

Table II. Yield and High-Performance LC Data of the Deprotected Trimers 27–32

no.	yield, %[a,b]	R_t, min	no.	yield, %[a]	R_t, min
27a	60	10.4[c]	29a	53	9.5[c]
27b	45	13.5[c]	29b	45	12.9[c]
27c	40	16.3[c]	29c	17[e]	15.9[c]
27d	40	15.3[c]	30	61	11.5[d]
28a	50	9.8[c]	31	61	13.1[d]
28b	41	13.2[c]	32	78	14.9[d]
28c	26[e]	16.1[c]			

[a] Isolated yield based on purified fully protected trinucleotide. [b] Percentage of yield obtained after zinc treatment, coupling, and deblocking steps. [c] Retention time on high-pressure LC system II. [d] Retention time on high-pressure LC system III. [e] Yield was not optimized.

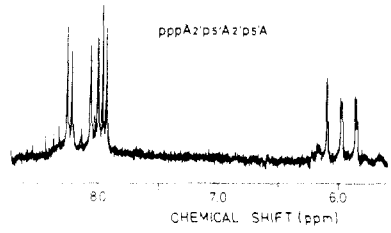


Figure 1. Low-field region of the 360-MHz ^1H NMR spectrum of compound **27c**. Chemical shifts are given in parts per million (δ) relative to Me_4Si.

The deblocking of the fully protected trimers **26a–c** is depicted in Scheme VI. Deblocking was performed by treatment with TBAF in THF/pyridine/water,[26] followed by aqueous ammonia and acid, giving the "core" trimers **30–32**, respectively.

Purification and Analysis of Trimers 27–32. The natural and modified **2-5A** derivatives **27–32** obtained above were purified by DEAE-Sephadex A25 anion-exchange chromatography. The homogeneity of the purified trimers was checked by high-performance LC. Yields and high-performance LC data for the **2–5A** derivatives are given in Table II. All deprotected trimers **27–32** were characterized by ^1H and ^{31}P NMR spectroscopy and by enzymatic methods.

A common feature of the ^1H NMR spectra (360 MHz) of compounds **27–32** was the presence of six singlets corresponding to three sets of H_2 and H_8 protons of the three adenine residues. Furthermore, three well-resolved doublets were found which, on the basis of the expected chemical shift values and coupling constants, could be ascribed to three $H_{1'}$ protons of the three ribose groups. These features are exemplfied in the partly reproduced spectrum of **27c** shown below in Figure 1. The presence of one *O*-methyl group in the trimers **28** and **31** as well as three O-methylated residues in the trimers **29** and **32** was indicated by the fact that one of three well-resolved singlets, respectively, was found in the high-field region of the ^1H NMR spectra of these compounds.

Whereas the presence of the correct nucleoside building blocks in the trimers was established by ^1H NMR spectroscopy, the identity of the internucleotide and 5'-terminal phosphate functions was corroborated by ^{31}P NMR spectroscopy. The ^{31}P NMR spectra of **27c,d** shown in Figures 2 and 3 reveal the presence of singlets corresponding to the internucleotide phosphate groups together with the correct pattern of signals corresponding to the 5'-triphosphate functions. ^{31}P NMR data for all trimers **27–32** are given in Table III.

Additional structural evidence was obtained from enzymic digestion data for all trimers. The identities of the

Table III. ^{31}P NMR Data of the Deprotected Trimers 27–30

no.	chemical shift,[a] ppm: internucleotide phosphates	α-P	β-P	γ-P
30	−0.99, −1.35			
27a	−1.03, −1.27	+1.01		
27b	−1.02, −1.25	−11.18 (d, J = 20.8)	−9.64 (d, J = 21.0)	
27c	−1.18, −1.22	−11.17 (d, J = 18.6)	−22.36 (dd, J = 18.4)	−10.39 (d, J = 18.1)
27d	−1.26	−10.91 (d, J = 25.6)	+9.38 (dd, J = 8.3, 25.4)	+14.87 (d, J = 8.3)
31	−1.10, −1.19			
28a	−1.01	+1.74		
28b	−1.12	−11.43 (d, J = 22.0)	−9.39 (d, J = 21.7)	
28c	−1.25, −1.44	−11.32 (d, J = 18.8)	−22.56 (dd, J = 18.5)	−10.51 (d, J = 18.3)
32	−1.60			
29a	−1.51	+1.83		
29b	−1.56	−11.21 (d, J = 20.3)	−9.88 (d, J = 20.3)	
29c	−1.56, −1.66	−11.25 (d, J = 17.8)	−22.45 (dd, J = 17.8)	−10.58 (d, J = 17.8)

[a] J values are given in hertz.

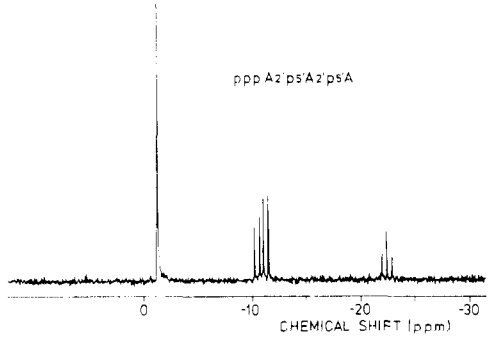


Figure 2. ^{31}P NMR spectrum of compound **27c**. Chemical shifts are given in parts per million (δ) relative to 85% H_3PO_4 as an external standard.

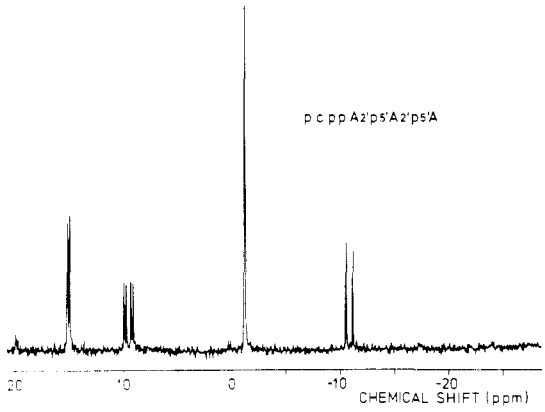


Figure 3. ^{31}P NMR spectrum of compound **27d**. Chemical shifts are given in parts per million (δ) relative to 85% H_3PO_4 as an external standard.

2′–5′ internucleotide phospho diester linkages of trimers **27**, **28**, **30**, and **31** were corroborated by their resistance to degradation with T_2 takadiastase.[37] Digestion with bacterial alkaline phosphatase converted the 5′-phosphorylated trimers **27a–c**, **28a–c**, and **29a–c** into the corresponding "core" trimers **30–32**, respectively. The modified triphosphate derivative **27d** was no substrate for the latter enzyme. Finally, the correct structure of all trimers was once more confirmed by complete conversion to the expected monomers by digestion with venom exonuclease.

(37) The observed resistance of **29** and **32** to digestion with the latter enzyme could be ascribed to the presence of *O*-methyl functions in all ribose residues of these trimers.

The biological activity of the synthetic trimers was extensively investigated. All triadenylates were tested for endonuclease activation in cell extracts and for inhibition of protein synthesis in intact cells. The results of these studies will be published elsewhere.[38]

Conclusion

The results described in this paper show that a range of **2-5A** derivatives can easily be obtained by chemical synthesis. The synthesized derivatives include the following compounds: natural triadenylates with a variable number of 5′-phosphates (i.e., compounds **27a–c** and **30**), a 5′-triphosphate derivative with a β,γ-methylene group (i.e., compound **27d**), and finally derivatives with one (i.e., compounds **28a–c** and **31**) or three 3′-*O*-methyl functions (i.e., compounds **29a–c** and **32**). The introduction of these well-defined modifications in the **2-5A** molecule illustrates the versatility of the applied chemical approach.

Features of the synthetic system are as follows: (a) convenient selective procedures were used for the preparation of properly protected adenosine and 3′-*O*-methyladenosine building blocks; (b) well-established and effective phospho triester methods were applied to obtain fully protected 2′–5′-linked oligoadenylate intermediates; (c) for the preparation of the desired 5′-phosphate functions a masked 5′-phosphate group was incorporated; conversion of fully protected 5′-phosphorylated derivatives (i.e., compounds **23a–c**) into the corresponding 5′-phosphoromorpholidates provided an easy access to all required phosphate derivatives.

The availability of well-defined modified derivatives of **2-5A** opens the way for a more detailed analysis of the function and structural requirements of this important natural compound.

Experimental Section

General Methods and Materials. UV absorption spectra were measured with a Cary C14 recording spectrophotometer. ^{1}H NMR spectra of the protected nucleosides(tides) were measured at 100 MHz with a JEOL JNMPS 100 spectrometer; shifts are given in parts per million (δ) relative to tetramethylsilane as internal standard. ^{13}C NMR and ^{31}P NMR spectra were measured at 25.15 and 40.48 MHz, respectively, with a JEOL JNMPFT 100 spectrometer equipped with an EC-100 computer and operating in the Fourier transform mode. Proton noise decoupling was used unless otherwise stated. ^{13}C Chemical shifts are given in parts per million (δ) relative to Me_4Si as internal standard and ^{31}P chemical shifts in parts per million (δ) relative to 85% H_3PO_4 as an external standard. Samples of the deprotected trimers for ^{31}P

(38) C. Baglioni, et al., *J. Biol. Chem.*, in press.

NMR were obtained by preparing a 10–20-mM solution in 99.75% D_2O and adjusting the pD to 7.0 by adding a trace of DCl or NaOD. For **27c**, **27d**, **28c**, and **29c** the pD was adjusted to 4.0. To all samples was added ethylenediaminetetraacetic acid (EDTA). ^1H NMR spectra of the deprotected trimers were measured at 360 MHz with a Bruker HX-360 spectrometer equipped with a BCN-12 computer and operating in the Fourier transform mode. Chemical shifts are given in parts per million (δ) relative to Me_4Si. Samples for ^1H NMR were treated with Dowex cation-exchange resin (Na^+ form) and lyophilyzed three times with 99.75% D_2O. A ca. 25-mM solution was prepared in 99.9% D_2O and pD adjusted to 7.0.

High-performance liquid chromatography (LC) was carried out on a Hupe & Busch/Hewlett-Packard 1010 A liquid chromatograph equipped with a gradient mixing system and UV absorption detector (254 nm). The photometer output was displayed on a Kipp & Sons flat-back recorder; peak areas were recorded by a Becker 7021 integrator. High-performance anion-exchange chromatography was performed with the strong anion-exchange resin Permaphase AAX (Du Pont) dry packed into a stainless-steel column (1 m × 2.1 mm). Isocratic elution of mononucleotides was effected by buffer A (0.005 M KH_2PO_4, pH 4.1) at 20 °C with a flow of 1 mL/min at a pressure of 70 kPa/cm^2 (system I). Buffer B was composed of 0.05 M KH_2PO_4 and 0.5 M KCl (pH 4.5) and buffer C of 0.1 M KH_2PO_4 and 1.0 M KCl (pH 4.5). Gradient elution was performed by building up a linear gradient, starting with buffer A, and applying 3% of buffer C per minute (system II). In system III, high-performance anion-exchange chromatography was performed on a column (25 cm × 4.6 mm) packed with Partisil PXS 10/25 SAX (Whatman). Gradient elution was performed by building up a linear gradient, starting with buffer A and applying 3% of buffer B per minute at a flow of 1 mL/min and a pressure of 45 kPa/cm^2. Retention times were measured relative to the injection peak.

Short-column chromatography was performed on Merck Kieselgel 60 (230–400 mesh ASTM). DEAE-Sephadex A25 and Sephadex G25 were purchased from Pharmacia. Schleicher & Schull DC Fertigfolien F 1500 LS 254 was used for TLC in solvent system A (chloroform–methanol, 92:8 v/v) and system B (chloroform–methanol, 88:12 v/v).

Dioxane, acetonitrile, tetrahydrofuran, triethylamine, 1,2-dimethoxyethane, and pyridine were dried by being refluxed with CaH_2 for 16 h and then distilled. Dimethylformamide was stirred with CaH_2 for 16 h and distilled under reduced pressure (15 mmHg). Pyridine, used in phosphorylation and condensation reactions, was redistilled from *p*-toluenesulfonyl chloride (60 g/L). Methanol was dried by being refluxed with magnesium methoxide, distilled, and stored over 3-Å molecular sieves. Dioxane and tetrahydrofuran were stored over 5-Å molecular sieves, 1,2 dimethoxyethane over sodium wire, and the other solvents over 4-Å molecular sieves. Levulinic acid and 1-methylimidazole were purchased from Aldrich and distilled before use. 4-(Dimethylamino)pyridine, *tert*-butyldimethylsilyl chloride, and 1,4-diazabicyclo[2.2.2]octane were purchased from Aldrich, 1,2,4-triazole from Fluka, and adenosine from Waldhof.

Methylenediphosphonic acid was obtained by hydrolysis in concentrated hydrochloric acid (16 h, reflux) of its tetraethyl ester.[39] After evaporation, the resulting solid was powdered, suspended in methyl ethyl ketone, filtered off, washed with ether, and dried in vacuo (P_2O_5): yield 88% (based on the ester); ^1H NMR (D_2O) 2.58 ppm (t, J = 21 HZ); ^{31}P NMR (D_2O) (not proton decoupled) 17.79 ppm (t, J = 21 Hz). Tri-*n*-butylammonium phosphate and bis(tri-*n*-butylammonium) diphosphate were prepared as published,[36] except that the final residue was dissolved in anhydrous DMF (solution A and B). In the same way, bis(tri-*n*-butylammonium) methylenediphosphonate was obtained from methylenediphosphonic acid and dissolved in anhydrous DMF (solution C).

3′-*O*-(4-Methoxytetrahydropyran-4-yl)-6-*N*-*p*-anisoyladenosine 5′-*O*-[(2,2,2-Tribromoethyl) morpholinophosphonate] (2). To a solution of compound **1**[25] (515 mg, 1 mmol) in acetonitrile (8 mL) and dioxane (2 mL) were added 1-methylimidazole (0.36 mL, 4 mmole) and phosphorochloridate **7**[22] (901 mg, 2 mmol). After 2 h, another portion of phosphorochloridate **7** (2 mmol) and 1-methylimidazole (4 mmol) was added. After 4 h, TLC analysis (system A) showed the reaction to be complete. The reaction mixture was diluted with chloroform (200 mL) and washed with 10% aqueous $NaHCO_3$ (100 mL) and water (100 mL). The organic layer was dried ($MgSO_4$), concentrated to an oil, and triturated with petroleum ether (40–60 °C, 200 mL). The precipitate was redissolved in chloroform (3 mL) and transferred to a column (3.5 cm × 7 cm^2) of Kieselgel (10 g). Elution of the column with chloroform–methanol (97:3 v/v) and evaporation of the appropriate fractions gave pure **2** as a glass. The latter was redissolved in chloroform (10 mL) and added dropwise with stirring to petroleum ether (40–60 °C, 200 mL). The precipitate of **2** was filtered off and dried in vacuo: yield 754 mg (81%); R_f 0.35 (system A); UV (95% EtOH) λ_{max} 288 nm (ϵ 31 400), λ_{min} 241 (7300); ^1H NMR ($CDCl_3$, mixture of diastereoisomers) 9.16 (NH, s), 8.76 (H_8, s), 8.28 and 8.23 (H_2, s), 6.06 (H_1', d, J = 3.5 Hz), 4.64 (CH_2CBr_3, d, J = 5 Hz), 3.60 and 3.16 (morpholine, m). Anal. Calcd for $C_{30}H_{38}Br_3N_6O_{11}P$ (mol wt 931.00): C, 38.70; H, 4.08; N, 9.02; P, 3.33. Found: C, 38.85; H, 3.72; N, 8.88; P, 3.41.

5′-*O*-Levulinyl-3′-*O*-(4-methoxytetrahydropyran-4-yl)-6-*N*-*p*-anisoyladenosine (3). To a stirred suspension of 2-chloro-1-methylpyridinium iodide[27] (511 mg, 2 mmol) in acetonitrile (3 mL) were added a solution of **1** (515 mg, 1 mmol) in dioxane (12 mL), levulinic acid (464 mg, 4 mmol), and 1,4-diazabicyclo[2.2.2]octane (538 mg, 4.8 mmol). After 2 h the pyridinium iodide was filtered off and the filtrate diluted with chloroform (150 mL). The solution was washed with 10% aqueous $NaHCO_3$ (100 mL) and water (100 mL), and both aqueous layers were extracted with chloroform (2 × 50 mL). The organic layers were dried ($MgSO_4$), concentrated to an oil, and triturated with petroleum ether (40–60 °C, 200 mL). The precipitate was redissolved in chloroform and the solution evaporated under reduced pressure to give crude **3** as a glass. Short-column chromatography gave no separation of **3** (55% of the reaction mixture) and the 2′-*O*-levulinyl derivative of **1** (5%). To overcome this problem, we specifically silylated the latter by addition of dry pyridine (15 mL) and *tert*-butyldimethylsilyl chloride (302 mg, 2 mmol) to crude **3**. After 5 h the reaction mixture was diluted with chloroform (150 mL) and washed with 10% aqueous $NaHCO_3$ (75 mL) and water (75 mL). The organic layer was dried ($MgSO_4$), concentrated to an oil, and triturated with petroleum ether (40–60 °C, 200 mL). The precipitate was redissolved in chloroform (3 mL) and transferred to a column (3.5 cm × 7 cm^2) of Keiselgel (10 g). The column was eluted with chloroform–methanol (97.5:2.5 v/v) and, after evaporation of the appropriate fractions, pure **3** was precipitated from petroleum ether (40–60 °C, 200 mL). The solid was filtered off and dried in vacuo (KOH): yield 245 mg (40%); R_f 0.39 (system A), 0.57 (system B); UV (95% EtOH) λ_{max} 278 nm (ϵ 28 200), λ_{min} 237 (6200); ^1H NMR ($CDCl_3$) 8.68 (H_8, s), 8.32 (H_2, s), 6.12 (H_1', d, J = 2.5 Hz), 2.70 and 2.56 (CH_2CH_2, t, J = 6 Hz), 2.16 (CH_3, s). Anal. Calcd for $C_{29}H_{35}N_5O_{10}$ (mol wt 613.29): C, 56.79; H, 5.71; N, 11.42. Found: C, 57.43; H, 6.64; N, 10.84.

5′-*O*-Levulinyl-3′-*O*-(4-methoxytetrahydropyran-4-yl)-6-*N*-*p*-anisoyladenosine 2′-*O*-(2-Chlorophenyl 2,2,2-trichloroethyl phosphate) (4). A solution of phosphorochloridate **8**[21] (394 mg 1.1 mmol) in acetonitrile (1 mL) was added to a cooled (ice–water bath) solution of **3** (613 mg, 1 mmol) in acetonitrile (10 mL) and 1-methylimidazole (0.16 mL, 2 mmol). After 15 min, another portion of phosphorochloridate **8** (1 mmol) and 1-methylimidazole (2 mmol) was added. After 30 min the reaction mixture was concentrated to an oil, which was dissolved in chloroform (150 mL) and washed with 10% aqueous $NaHCO_3$ (75 mL) and water (75 mL). The organic layer was dried ($MgSO_4$) and evaporated to an oil, which was triturated with petroleum ether (40–60 °C, 2 × 150 mL). The precipitate was dissolved in chloroform (2 mL) and transferred to a column (3 cm × 7 cm^2) of Kieselgel (8 g). The column was eluted with chloroform–methanol (99:1 v/v), and, after evaporation of the appropriate fractions, **4** was precipitated from petroleum ether (40–60 °C, 200 mL). The solid was filtered off and dried in vacuo (KOH): yield 804 mg (86%); R_f 0.62 (system A), 0.70 (system B); UV (95% EtOH λ_{max} 278 nm (ϵ 31 100), λ_{min} 238 (6300); ^1H NMR ($CDCl_3$, mixture of diastereoisomers) 9.46 (NH), 8.74 (H_8, s) 8.33 and 8.24

(39) G. M. Kosolapoff, *J. Am. Chem. Soc.*, **75**, 1500 (1953).

Table IV. NMR Data (ppm)

	atom	6-*N*-*p*-anisoylladenosine	3′-*O*-methyl derivative (14)	2′-*O*-methyl derivative
^{1}H NMR ($(CD_3)_2SO$–D_2O)	H_1'	6.06 (J = 5.5 Hz)	6.08 (J = 5.6 Hz)	6.18 (J = 4.5 Hz)
^{13}C NMR ($(CD_3)_2SO$)	C_2'	73.8	73.1	82.6
	C_3'	70.4	79.6	68.6

(H_2, s), 7.3 (2-chlorophenyl, m), 6.44 and 6.32 (H_1', d, J = 1.5 Hz), 5.84 (H_2', m), 4.74 (CH_2CCl_3, d, J = 7 Hz), 2.66 and 2.48 (CH_2CH_2, t, J = 6 Hz), 2.12 (CH_3, s). Anal. Calcd for $C_{37}H_{40}Cl_4H_5O_{13}P$ (mol wt 935.14): C, 47.52; H, 4.28; N, 7.49; P, 3.31. Found: C, 47.26; H, 4.53; N, 7.56; P, 3.42.

3′-*O*-(4-Methoxytetrahydropyran-4-yl)-6-*N*-*p*-anisoyladenosine 2′-*O*-(2-Chlorophenyl 2,2,2-trichloroethyl phosphate) (5). A solution of hydrazine hydrate (0.5 mL, 10 mmol) in pyridine–acetic acid (3:2 v/v, 10 mL) was added to a solution of **4** (935 mg, 1 mmol) in pyridine (10 mL). After 4 min at room temperature, pentane-2,4-dione (2 mL 20 mmol) was added to the reaction mixture, and the reaction flask was immersed into an ice-water bath. After a further 2 min the solution was added to chloroform (100 mL) and water (100 mL). The organic layer was separated and washed with 10% aqueous $NaHCO_3$ (100 mL) and water (100 mL). The dried ($MgSO_4$) chloroform layer was evaporated to an oil which was triturated with petroleum ether (40–60 °C, 200 mL) and redissolved in chloroform (2 mL). This solution was applied to a column (3 cm × 7 cm^2) of Kieselgel (8 g). The column was eluted with chloroform–methanol (98.5:1.5 v/v), and, after evaporation of the appropriate fractions, pure **5** was precipitated from petroleum ether (40–60 °C, 200 mL). The solid was filtered off and dried in vacuo: yield 770 mg (92%); R_f 0.53 (system A), 0.65 (system B); UV (95% EtOH) λ_{max} 278 nm (ϵ 30 700), λ_{min} 238 (6200); ^{1}H NMR ($CDCl_3$, mixture of diastereoisomers) 9.90 (NH, s), 8.76 (H_8, s), 8.37 and 8.24 (H_2, s), 7.4 (2-chlorophenyl, m), 6.34 and 6.27 (H_1', d, J = 6 Hz), 4.60 and 4.50 (CH_2CCl_3, d, J = 6.75 Hz). Anal. Calcd for $C_{32}H_{34}Cl_4N_5O_{11}P$ (mol wt 837.09): C, 45.91; H, 4.06; N, 8.36; P, 3.70. Found: C, 44.03; H, 3.85; N, 8.96; P, 3.59.

3′-*O*-Methyl-2′,5′-diacetyladenosine (13). *N*-Nitroso-*N*-methylurea (100 g) was added to a cooled (ice–water bath) mixture of 40% aqueous KOH (250 mL) and 1,2-dimethoxyethane (500 mL), in four portions of ca. 25 g in 1 h under vigorous mechanical stirring. After an additional 30 min the organic layer was separated by decantation and the water solution extracted with 1,2-dimethoxyethane (3 × 100 mL). The collected organic fractions were dried over KOH pellets (100 g), and the solution was filtered directly into a dropping funnel.

The brown-yellow solution (610 mL) of diazomethane was added dropwise in 2 h to a stirred suspenion of adenosine (**9**; 10 g, 37.5 mmol) in anhydrous methanol (1000 mL) containing $SnCl_2$ (10^{-3} M) at room temperature. The addition of diazomethane was stopped when TLC (system B) showed that no starting material remained. The solutions was evaporated to a glass under reduced pressure. ^{1}H NMR analysis showed that the latter material was a mixture of two isomers, i.e., 2′-*O*-methyladenosine (**10**) and 3′-*O*-methyladenosine (**11**) in a ratio of 2:3.

The thus obtained glass (10.5 g) was dissolved in pyridine (150 mL), and acetic anhydride (80 mL) was added. After 45 min the solution was cooled and the reaction stopped by addition of methanol (80 mL). Pyridine was removed by coevaporation with ethanol (3 × 100 mL). From a solution of the solid residue in warm ethanol (180 mL) pure crystalline **13** was selectively obtained: yield 7.39 g (two crops, 54%); mp 190–191 °C; R_f 0.37 (system A), 0.50 (system B); UV (95% EtOH) λ_{max} 258 nm (ϵ 14 100), λ_{min} 226 (1200); ^{1}H NMR ($CDCl_3$) 8.26 (H_8, s), 8.12 (H_2, s), 6.16 (H_1', d, J = 4 Hz), 5.88 (H_2', dd), 3.45 (CH_3, s), 2.18 and 2.10 (2 CH_3, acetyl, 2 s). Anal. Calcd for $C_{15}H_{19}N_5O_6$ (mol wt 365.15): C, 49.34; H, 5.20; N, 19.17. Found: C, 49.05; H, 5.97; N, 19.03.

When the mother liquor obtained by filtration of **13** was evaporated to a glass, ^{1}H NMR ($CDCl_3$) of this glass showed the following signals of **12**: 8.28 (H_8, s), 8.04 (H_2, s), 6.12 (H_1', d, J = 5 Hz), 5.48 (H_3', dd), 4.82 (H_2', dd), 3.48 (CH_3, s), 2.19 and 2.14 ppm (2 CH_3, acetyl, 2 s). On the basis of the intensity of the H_1' signals, the ratio of the isomers **12** and **13** was estimated to be 95:5.

3′-*O*-Methyl-6-*N*-*p*-anisoyladenosine (14). To a solution of **13** (7.39 g, 20.25 mmol) in pyridine (50 mL) was added dropwise *p*-anisoyl chloride (4.6 mL) at 20 °C. After 16 h the reaction was stopped by addition of water (10 mL). The solution was concentrated to an oil which was diluted with chloroform (150 mL) and washed with 10% aqueous $NaHCO_3$ (100 mL) and water (100 mL). The organic layer was dried ($MgSO_4$) and evaporated to an oil which was triturated with petroleum ether (40–60 °C, 2 × 200 mL). The precipiate was redissolved in chloroform (50 mL) and evaporated to a glass (9.3 g). The latter was dissolved in methanol–dioxane (150 mL, 1:1 v/v) and treated with sodium methanolate (1 M, 75 mL). After 15 min the reaction was stopped by the addition of a slight excess of cation-exchange resin (Dowex 50W X8, 100–200 mesh, pyridinium form). The resin was filtered off, and the filtrate was concentrated under reduced pressure to an oil, which was triturated with petroleum ether (40–60 °C, 2 × 200 mL). The precipitate was dissolved in chloroform–methanol (50 mL, 1:1 v/v) and evaporated. The resulting solid was crystallized from ethanol: yield 5.04 g (60%); mp 143 °C; R_f 0.31 (system A), 0.43 (system B); UV (95% EtOH) λ_{max} 289 nm (ϵ 27 600), λ_{min} 240 (3000); ^{1}H NMR ($(CD_3)_2SO$–D_2O) 8.72 (H_8, s), 8.68 (H_2, s), 8.05 and 7.10 (anisoyl, 2 d, J = 8.25 Hz), 6.08 (H_1', d, J = 5.6 Hz), 4.96 (H_2', dd), 3.93 (CH_3, *p*-anisoyl, s), 3.34 (CH_3, s). Anal. Calcd for $C_{19}H_{21}N_5O_6$ (mol wt 415.20): C, 54.96; H, 5.05; N, 16.86. Found: C, 54.60; H, 4.97; N, 16.70. The identity of **14** was also confirmed by comparison of the relevant ribose signals in its ^{1}H and ^{13}C NMR spectra with those of the isomeric 2′-*O*-methyl derivative (see Table IV).

5′-*O*-(*tert*-Butyldimethylsilyl)-3′-*O*-methyl-6-*N*-*p*-anisoyladenosine (15). To a solution of **14** (415 mg, 1 mmol) in pyridine (15 mL) was added *tert*-butyldimethylsilyl chloride (301 mg, 2 mmol), and the mixture was left at 20 °C for 16 h. After addition of some ice, the mixture was concentrated, and the residual oil was dissolved in chloroform (100 mL). The solution was washed with 10% aqueous $NaHCO_3$ (75 mL) and water (75 mL), dried ($MgSO_4$), and concentrated to an oil. Repeated coevaporation with ethanol (2 × 40 mL) gave a glass which was crystallized from acetonitrile–water (1:1 v/v, 40 mL) to give pure **15**: yield 466 mg (88%); mp 114–117 °C; R_f 0.55 (system A), 0.66 (system B); UV (95% EtOH) λ_{max} 289 nm (ϵ 28 600), λ_{min} 240 (4800); ^{1}H NMR ($CDCl_3$) 8.73 (H_8, s), 8.43 (H_2, s), 6.15 (H_1', d, J = 3.75 Hz), 3.51 (CH_3, s), 0.91 (*t*-Bu, s), 0.11 (2 CH_3, s). Anal. Calcd for $C_{25}H_{35}N_5O_6Si$ (mol wt 529.34): C, 56.72; H, 6.61; N, 13.22. Found: C, 56.60; H, 6.80; N, 13.10.

2′-*O*-Levulinyl-3′-*O*-methyl-6-*N*-*p*-anisoyladenosine (16). To a solution of **15** (529 mg, 1 mmol) in dioxane (10 mL) was added levulinic anhydride[31] (321 mg, 1.5 mmol), triethylamine (0.42 mL, 3 mmol), and 4-(dimethylamino)pyridine (12 mg, 0.1 mmol). After 40 min TLC (system A) showed the reaction to be complete. The mixture was concentrated to an oil and dissolved in chloroform (75 mL). The solution was washed with 10% aqueous $NaHCO_3$ (75 mL) and water (50 mL), dried ($MgSO_4$), and concentrated to a glass. The latter was dissolved in dry tetrahydrofuran (5 mL), and a solution of tetra-*n*-butylammonium fluoride in dry tetrahydrofuran (0.5 M TBAF, 5 mL) was added. After 30 min the mixture was diluted with chloroform (100 mL), washed with 10% aqueous $NaHCO_3$ (75 mL) and water (50 mL), dried ($MgSO_4$), and concentrated to an oil. After trituration with petroleum ether (40–60 °C, 150 mL), the precipitate was dissolved in chloroform (3 mL) and transferred to a column (3 cm × 7 cm^2) of Kieselgel (8 g). The column was eluted with chloroform–methanol (97.5:2.5 v/v), and, after evaporation of the appropriate fractions, pure **16** was precipitated from petroleum ether (40–60 °C, 200 mL). The solid was filtered off and dried in vacuo (KOH): yield 447 mg (87%); R_f 0.50 (system A), 0.60 (system B); UV (95% EtOH) λ_{max} 289 nm (ϵ 31 700), λ_{min} 243 (4000); ^{1}H NMR ($CDCl_3$) 9.39 (NH, s), 8.87 (H_8, s), 8.18 (H_2, s), 6.12 (H_1', d, J = 6.75 Hz), 5.82 (H_2', dd), 3.47 (CH_3, s), 2.62 and 2.66 (CH_2CH_2, t, J = 6 Hz),

2.14 (CH_3, s). Anal. Calcd for $C_{24}H_{27}N_5O_8$ (mol wt 513.24): C, 56.16; H, 5.26; N, 13.64. Found: C, 55.24; H, 5.65; N, 13.31.

3′-O-Methyl-6-N-p-anisoyladenosine 5′-O-[(2,2,2-Tribromoethyl) morpholinophosphonate] (17). Compound **17** was prepared by phosphorylation of **16** (513 mg, 1 mmol) with **7** followed by removal of the levulinyl group. Phosphorylation was performed as described for the synthesis of **2** and removal of the levulinyl group as described for the synthesis of **5**. Purification of the intermediate was omitted, and in the final purification step the column was eluted with chloroform–methanol (97:3 v/v): yield 663 mg (80%); R_f 0.44 (system A), 0.57 (system B); UV (95% EtOH) λ_{max} 289 nm, λ_{min} 240; ^{1}H NMR ($CDCl_3$, mixture of diastereoisomers) 8.74 (H_8, s), 8.30 and 8.26 (H_2, s), 6.08 (H_1', d, J = 4.5 Hz), 5.00 (H_2', dd, J = 4.5, 5.5 Hz), 4.66 (CH_2CBr_3, d, J = 5.25 Hz), 3.60 and 3.28 (morpholine, m), 3.54 (CH_3, s). Anal. Calcd for $C_{25}H_{30}Br_3N_6O_9$ (mol wt 828.95): C, 36.22; H, 3.62; N, 10.13; P, 3.74. Found: C, 36.02; H, 3.69; N, 10.27; P, 3.71.

5′-O-(tert-Butyldimethylsilyl)-3′-O-methyl-6-N-p-anisoyladenosine 2′-O-(2,2,2-Trichloroethyl 2-chlorophenylphosphate) (18). Compound **15** (529 mg, 1 mmol) was phosphorylated with **8** as described for the synthesis of **4**. After being washed, the organic layer was dried ($MgSO_4$), evaporated to an oil, and coevaporated with ethanol (2 × 50 mL) to give a glass. The latter was dissolved in chloroform (2 mL) and transferred to a column (3.5 cm × 7 cm^2) of Kieselgel (10 g). After elution of the column with chloroform–methanol (99:1 v/v), evaporation of the appropriate fractions gave a glass of pure **18**: yield 638 mg (75%); R_f 0.74 (system A), 0.77 (system B); UV (95% EtOH) λ_{max} 289 nm (ϵ 29 200), λ_{min} 239 (2600), ^{1}H NMR ($CDCl_3$, mixture of diastereoisomers) 9.05 (NH, s), 8.77 and 8.73 (H_8, s), 8.36 and 8.31 (H_2, s), 7.5–7.1 (2-chlorophenyl, m), 6.39 and 6.27 (H_1', d, J = 3.5 Hz), 4.76 (CH_2CCl_3, d, J = 6.4 Hz), 3.53 and 3.39 (CH_3, s), 0.91 (t-Bu, s), 0.13 (2 CH_3, s). Anal. Calcd for $C_{33}H_{40}Cl_4N_5O_9PSi$ (mol wt 851.19): C, 46.56; H, 4.70; N, 8.22; P, 3.64. Found: C, 46.15; H, 4.94; N, 7.99; P, 3.81.

3′-O-Methyl-6-N-p-anisoyladenosine 2′-O-(2,2,2-Trichloroethyl 2-chlorophenylphosphate) (19). To a solution of **18** (851 mg, 1 mmol) in tetrahydrofuran (5 mL) were added acetic acid (80 mL) and water (20 mL). After 2 days at 20 °C the reaction mixture was concentrated to an oil, which was dissolved in chloroform (150 mL) and washed with 10% aqueous $NaHCO_3$ (75 mL) and water (50 mL). The organic layer was dried ($MgSO_4$) and evaporated to an oil, which was triturated with petroleum ether (40–60 °C, 2 × 150 mL). The precipitate was redissolved in chloroform (3 mL) and transferred to a column (3 cm × 7 cm^2) of Kieselgel (8 g). The column was eluted with chloroform–methanol (98:2 v/v) and, after evaporation of the appropriate fractions, **19** was precipitated from petroleum ether (40–60 °C, 200 mL). The solid was filtered off and dried in vacuo: yield 561 mg (80%); R_f 0.58 (system A), 0.67 (system B); UV (95% EtOH) λ_{max} 289 nm (ϵ 30 500), λ_{min} 239 (5000); ^{1}H NMR ($CDCl_3$, mixture of diastereoisomers) 8.65 (H_8, s), 8.20 and 8.14 (H_2, s), 7.4–7.0 (2-chlorophenyl, m), 6.20 and 6.12 (H_1', d, J = 6.4 Hz), 4.58 (CH_2CCl_3, d, J = 6.4 Hz), 3.48 and 3.42 (CH_2, s). Anal. Calcd for $C_{27}H_{28}Cl_4N_5O_9P$ (mol wt 701.01): C, 46.25; H, 3.71; N, 9.99; P, 4.42. Found: C, 46.13; H, 3.72; N, 9.42; P, 4.40.

General Procedure for the Preparation of the Fully Protected 5′-Phosphorylated Oligonucleotides 21 and 23. A solution of 1,2,4-triazole (414 mg, 6 mmol), triethylamine (0.84 mL, 6 mmol) and 2-chlorophenyl phosphorodichloridate[40] (737 mg, 3 mmol) in tetrahydrofuran (20 mL) was stirred at 20 °C. After 30 min the triethylamine HCl salt was removed by filtration. The filtrate was collected in a round-bottomed flask containing a pyridine solution of the 2′-hydroxy (oligo)nucleotide (1 mmol) which had been dried by coevaporation with pyridine. After addition of pyridine to the filtrate, the latter was evaporated to a small volume. After 15 min at 20 °C, when TLC (system B) showed the phosphorylation to be complete, the reaction was stopped by the addition of 1 M triethylammonium bicarbonate (TEAB, pH 7.5, 5 mL). The mixture was diluted with chloroform (150 mL) and washed with TEAB (0.2 M, 5 × 40 mL). The organic layer was concentrated to an oil which was transferred to a smaller flask containing the 5′-hydroxy nucleoside (0.8 mmol). The mixture was dried by repeated coevaporation with anhydrous pyridine (3 × 20 mL), and TPSNT[33] (304 mg, 0.8 mmol) was added to the resulting solution. An extra amount of TPSNT (0.8 mmol) was added after 1 h. When TLC (system A or B) showed the reaction to be complete (2–3 h), the mixture was diluted with chloroform (150 mL) and washed with 10% aqueous $NaHCO_3$ (75 mL) and water (50 mL). The organic layer was dried ($MgSO_4$), concentrated to a small volume, and triturated with petroleum ether (40–60 °C, 200 mL). The precipitate was dissolved in chloroform (4 mL) and transferred to a column of Kieselgel (10–15 g/g of crude oligonucleotide). The column was eluted with chloroform–methanol (98:2 to 95:5 v/v), and, after evaporation of the appropriate fractions, the pure oligonucleotides were precipitated from petroleum ether (40–60 °C, 200 mL), filtered off, and dried in vacuo (P_2O_5). Data relevant to the synthesis of these compounds are given in Table I.

Removal of the Levulinyl Group from 21b. The removal of the 2′-O-levulinyl group from the fully protected dinucleotide **21b** and subsequent workup of the product **21c** were performed as described for the synthesis of compound **5**. In the purification step the column was eluted with chloroform–methanol (96:4 v/v). The yield and analytical data are given in Table I.

General Procedure for the Synthesis of the Fully Protected Oligonucleotides 25 and 26. The 2′-O-(2,2,2-trichloroethyl 2-chlorophenyl phosphate) derivatives (1 mmol) and 2,4,6-triisopropylbenzenesulfonic acid (TPSOH, 85 mg, 0.3 mmol) were dissolved in pyridine (10 mL), and activated[35] zinc (ca. 15 mmol) was added to the magnetically stirred solution. The course of the reaction was monitored by measuring the evolution of heat with a thermocouple. After 30–40 s the tempterature of the mixture rose sharply to ca. 35 °C, and, after 3 min, the mixture was filtered to remove excess zinc. TLC (system B) of the filtrate showed complete conversion of the starting compound into base-line material. The filtrate was diluted with chloroform (200 mL) and washed with 1 M TEAB (2 × 15 mL). The organic layer was concentrated to an oil and transferred to a smaller flask containing the 5′-hydroxy nucleoside(tide) (0.9 mmol). Coupling of both components and workup of the reaction mixture were performed as described for the synthesis of the 5′-phosphorylated oligonucleotides. Data relevant to the synthesis of these compounds are given in Table I.

General Procedure for the Introduction of the 5′-Terminal Phosphate Groups of Trinucleotides 23 and Complete Removal of Protection Groups. Zinc treatment of **23** (0.1 mmol) and subsequent workup of the reaction mixture were performed as described for the synthesis of **25** and **26**. The oil resulting from concentration of the organic layer was transferred to a flask containing DMF (2 mL) and one of the following solutions: (A) 0.5 M tri-n-butylammonium phosphate in DMF (2 mL) (to give **27–29b**), (B) 0.5 M bis(tri-n-butylammonium) diphosphate in DMF (2 mL) (to give **27–29c**), (C) 0.5 M bis(tri-n-butylammonium) methylenediphosphate in DMF (2 mL) (to give **27d**). The mixture was dried by repeated coevaporation with pyridine (3 × 10 mL) and left at 50 °C for 16 h after addition of phosphate (A) or diphosphonate (C) and at 40 °C for 6 h after addition of diphosphate (B).

Direct removal of all protecting groups from the product obtained after zinc treatment of **23** resulted in the formation of **27–29a**. Deblocking was performed as follows. The reaction mixture was diluted with dioxane (4 mL) and stirred magnetically, and a few drops of 25% aqueous ammonia were added. More ammonia (20 mL) was added carefully in the course of 10 h. After 60 h the mixture was concentrated until neutral, the residue was dissolved in 0.01 N HCl (10 mL), and the pH was adjusted to 2.0 by the addition of 0.1 N HCl. After 2 h at 20 °C the solution was carefully neutralized (pH 8) with 0.5 M ammonia and lyophilized. For **29b,c** the acid treatment was omitted. The crude product was transferred to a column (25 cm × 6 cm^2) of DEAE-Sephadex A25 (HCO_3^- form) suspended in 0.1 M TEAB. The column was eluted with a linear gradient of 0.1 → 1.0 M TEAB for 24 h at a flow of 36 mL/h. Fractions of 6 mL were taken. After high-performance LC analysis (system II) the appropriate fractions were collected, concentrated to a small volume, coevaporated with water (3 × 50 mL), and lyophilized. The products were purified from possible inorganic and nucleotide impurities by a column

(40) G. R. Owen, C. B. Reese, C. J. Ransom, J. H. van Boom, and J. D. H. Herscheid, *Synthesis*, 704 (1974).

(1 m × 3 cm^2) of Sephadex G25 suspended in 0.05 M TEAB. Elution occurred with the same buffer at a flow of 14 mL/h. Fractions of 3 mL were taken. The appropriate fractions were collected and lyophilized. All products were brought into the ammonium form by running them through a column (15 cm × 2 cm^2) of Dowex 50W cation-exchange resin (100–200 mesh, ammonium form). The resulting aqueous solution was relyophilized. High-performance LC data of the products are given in Table II.

Deblocking of the Fully Protected Trinucleotides 26. To a solution of the fully protected trinucleotide (0.1 mmol) in THF (16 mL) was added a solution of tetra-*n*-butylammonium fluoride (0.25 M TBAF, 4 equiv/phospho triester moiety) in pyridine–water (1:1 v/v, 4 mL). After 16 h at 20 °C, Dowex 50W cation-exchange resin (100–200 mesh, ammonium form, 10 g/mmol of TBAF) was added. The resin was filtered off, and a few drops of 2 M TEAB buffer were added to the filtrate. The solution was concentrated, and 25% aqueous ammonia (20 mL) was added. After 50 h at 20 °C the mixture was concentrated until neutral, the residue was dissolved in 0.01 N HCl (10 mL), and the pH was adjusted to 2.0 by the addition of 0.1 N HCl. After 2 h (8 h for **26c**) at 20 °C the solution was neutralized with ammonia and lyophilized. The crude product was transferred to column (25 cm × 6 cm^2) of DEAE-Sephadex A25 (HCO_3^- form) suspended in 0.01 M TEAB. The column was eluted with a linear gradient of 0.01 → 0.45 M TEAB for 16 h at a flow of 36 mL/h. Fractions of 6 mL were taken. After high-performance LC analysis (system III) the appropriate fractions were collected, concentrated to a small volume, coevaporated with water (2 × 50 mL), and lyophilized. The products were brought into the ammonium form and relyophilized as described above. High-pressure LC data of the products are given in Table II.

Enzymic Hydrolysis of the Trinucleotides and Determination of Hypochromicity and Yield. A quantity of 0.5 mg of each trinucleotide was dissolved in water (50 μL).

(a) Takadiastase T_2. A 5-μL sample of the stock solution was incubated with Takadiastase T_2 (Calbiochem, 2 μL of a solution containing 200 u/mL in 0.1 M NaCl) in 0.1 M NH_4OAc buffer (pH 4.5, 50 μL) at 37 °C for 2 h. High-performance LC analysis (system II, **27–29**; system III, **30–32**) showed the expected resistance of all trinucleotides to the enzyme.

(b) Bacterial Alkaline Phosphatase (BAP). A 5-μL sample of the stock solution was incubated with BAP (Sigma, 1 μL of a suspension containing 260 u/mL in 2.5 M $(NH_4)_2SO_4$) in 0.2 M Tris–HCl buffer (pH 8.0, 100 μL) at 37 °C for 2 h. High-performance LC analysis (system II, **27–29**; system III, **30–32**) showed the expected resistance of **30–32** and **27d** to the enzyme. The trimers **27a–c** were converted to **30**, **28a–c** to **31**, and **29a–c** to **32**.

(c) Venom Phosphodiesterase. A 5-μL sample of the stock solution was incubated with snake venom phosphodiesterase (Crotalis terr. terr., Boehringer, 2 μg) in a buffer (50 μL containing 25 mM Tris–HCl (pH 9.0) and 5 mM $MgCl_2$ at 37 °C for 2 h. For complete digestion of **28a–c**, **29a–c**, **31**, and **32** prolonged incubation with more phosphodiesterase was necessary. High-performance LC analysis (system I) showed the complete conversion of **27a–d** to adenosine 5′-phosphate (pA), of **29a–c** to 3′-*O*-methyladenosine 5′-phosphate (pA3′m), and of **28a–c** to a mixture of pA and pA3′m. Trimers **30–32** also showed complete digestion to the expected nucleoside and nucleotide products.

For the trimers **27b–d** the hypochromicity was determined[33] by addition of a solution of guanosine 3′-phosphate (Gp; 1 mg/mL, 15 μL) to the venom incubation. The quantity of the trimer was related, taking Gp as an internal standard, to the amount of pA released by the enzymic digestion (high-performance LC, system II). The resulting value of the hypochromicity, 23% (±3%), was used as a standard value for all trimers.

For determination of the yields of pure lyophilized trinucleotides (in the NH_4^+ form) they were weighed. A quantity of 0.5 mg of all trinucleotides was dissolved in water (25 μL). By measuring the OD_{254} of an appropriate dilution of this stock solution and taking into account the hypochromicity of the compounds, one can determine the quantity and yield of the trinucleotides. Yields are given in Table II.

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Registry No. **1**, 76998-92-0; **2** (isomer 1), 77057-81-9; **2** (isomer 2), 77057-82-0; **3**, 76991-44-1; **4a** (isomer 1), 77057-83-1; **4a** (isomer 2), 77057-84-2; **4b**, 76998-93-1; **5** (isomer 1), 77057-85-3; **5** (isomer 2), 77057-86-4; **6**, 16628-95-8; **7**, 57575-15-2; **8**, 59819-52-2; **9**, 58-61-7; **10**, 2140-79-6; **11**, 10300-22-8; **12**, 72560-74-8; **13**, 72560-72-6; **14**, 72351-35-0; **15**, 76991-47-4; **16**, 76991-48-5; **17** (isomer 1), 77057-87-5; **17** (isomer 2), 77057-88-6; **18a** (isomer 1), 77011-20-2; **18a** (isomer 2), 76998-94-2; **18b**, 76998-95-3; **19** (isomer 1), 77057-89-7; **19** (isomer 2), 77057-90-0; **20a**, 72351-26-9; **20b**, 77000-49-8; **21a**, 72351-29-2; **21b**, 76991-51-0; **21c**, 76991-52-1; **22a**, 76998-96-4; **22b**, 77000-50-1; **23a**, 72351-30-5; **23b**, 76991-53-2; **23c**, 76991-54-3; **24**, 72351-28-1; **25a**, 76991-55-4; **25b**, 76991-56-5; **25c**, 76991-57-6; **25d**, 76991-58-7; **26a**, 76991-59-8; **26b**, 77000-51-2; **26c**, 76991-60-1; **27a**, 61172-40-5; **27b**, 76991-64-5; **27c**, 65954-93-0; **27d**, 76991-67-8; **28a**, 76998-97-5; **28b**, 76991-65-6; **28c**, 72351-34-9; **29a**, 76991-63-4; **29b**, 76998-98-6; **29c**, 76991-66-7; **30**, 70062-83-8; **31**, 76991-61-2; **32**, 76991-62-3; 2′-*O*-levulinyl-3′-*O*-(4-methoxytetrahydropyran-4-yl)-6-*N*-*p*-anisoyladenosine, 76998-99-7; 2′-*O*-methyl-6-*N*-*p*-anisoyladenosine, 76999-00-3; levulinic acid, 123-76-2; levulinic anhydride, 40608-06-8; 1,2,4-triazole, 288-88-0; 2-chlorophenyl phosphorodichloridate, 15074-54-1.