Studies on Transfer Ribonucleic Acids and Related Compounds. 20.[1] A New Versatile Ribooligonucleotide Block with 2′-(*o*-Nitrobenzyl) and 3′-Phosphorodianilidate Groups Suitable for Elongation of Chains in the 3′ and 5′ Directions[2]

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Abstract: 5′-*O*-Monomethoxytrityl-2′-*O*-(*o*-nitrobenzyl) derivatives of uridine, *N*-benzoyladenosine, *N*-benzoylcytidine, and *N*-isobutyrylguanosine have been phosphorylated with dianilidophosphorochloridate. Demonomethoxytritylation of the nucleotides (**3**) yielded the four key intermediates (**4**). Using these properly protected nucleotides as the terminal 3′ unit, a new type of oligonucleotide block bearing the terminal 3′-phosphorodianilidate and 2′-*O*-(*o*-nitrobenzyl) groups (**6**) has been synthesized. Acid treatment of **6** gave a dinucleotide with a free 5′-hydroxyl group (**7**), which could be condensed with mononucleotides to yield protected trinucleotides (**9**). Isoamyl nitrite treatment of **6a** gave protected dinucleotides with 3′-phosphomonoester groups (**8**) which could be activated with condensing reagents to elongate the chain in the 3′ direction. The partially protected oligonucleotides C-Unbzl-p,[3] A-A-Anbzl-p, A-G-Cnbzl-p, and G-Gnbzl-p were irradiated with UV light to remove the *o*-nitrobenzyl group. The completely deblocked oligonucleotides were characterized by enzymatic hydrolysis.

Protection of the 2′-hydroxyl group of ribonucleotides is one of the essential problems in the synthesis of ribooligonucleotides. We have recently synthesized photolabile 2′-*O*-(*o*-nitrobenzyl) derivatives of uridine,[4] adenosine,[5] cytidine,[5] and *N*-isobutyrylguanosine.[6] The 2′-(*o*-nitrobenzyl) ether has been proved to be a nonmigrating protecting group during phosphorylation[4,5] and the ether is also stable in acid and alkali. We have previously developed a method for the synthesis of protected ribooligonucleotides using aromatic phosphoromonoamidates as the protection for the 3′-phosphate.[7] The present paper describes a synthesis of a new type of ribonucleotide block (**6**) having the 2′-(*o*-nitrobenzyl)-3′-phosphorodianilidate (Chart I). Key intermediates (**4**) for the synthesis of this type of oligonucleotide have been derived from the four major nucleosides as shown in Chart I. Condensation of these 3′-phosphorodianilidates with properly protected mononucleotides yielded the dinucleotides (**5**). Since the terminal phosphorodianilidate had no anionic dissociation, the internucleotidic phosphate could be esterified after the linkage had been formed. This approach showed some advantages over that involving condensation of diesterified phosphates.[8,9] Removal of the amidate substitution from the terminal phosphate by isoamyl nitrite treatment of **6** provided triesterified blocks with 3′-phosphomonoesters (**8**), which were suitable for elongation of the chain in the 3′ direction. Acid treatment of **6** yielded the 5′-deprotected dinucleotides (**7**). The fully protected trinucleotide (**9**) was synthesized from **7c.** Thus the chain could also be elongated in the 5′ direction.

The properly protected oligonucleotides **6a, 6d,** and **9** are the starting materials for the synthesis of a 5′ fragment from the *E. coli* $tRNA_f^{Met}$. **6b** has been used in the synthesis of the partially protected trinucleotide ApApAnbzlp which has been subjected to a template-directed polymerization on poly(rU).[10]

5′[11]- and 3′[12]-phosphorodianilidates of deoxynucleotides have been used for the synthesis of oligodeoxynucleotides with terminal phosphates.

Synthesis of 2′-*O*-(*o*-Nitrobenzyl)nucleoside 3′-Phosphorodianilidates (4). 2′-*O*-(*o*-Nitrobenzyl)-5′-*O*-monomethoxytrityl derivatives of uridine (**2a**)[4] and *N*-benzoyladenosine (**2b**)[5] have been described previously. The corresponding cytidine derivative (**2c**) was prepared by treatment of 2′-*O*-(*o*-nitrobenzyl)-*N*-benzoylcytidine (**1c**) with monomethoxytrityl chloride. The guanosine derivative (**2d**) was synthesized from *N*-isobutyryl-2′-*O*-(*o*-nitrobenzyl)guanosine[6] by similar methods. These 5′-*O*-monomethoxytritylated compounds (**2**) were treated with dianilidophosphorchloridate[13] in pyridine at room temperature and the dianilidate (**3**) was isolated by extraction with organic solvents or by chromatography on silica gel. The monomethoxytrityl group was removed by treatment with 80% acetic acid and the products (**4**) were obtained as powders by precipitation with ether–hexane from their solutions in pyridine. The conditions used in the synthesis of **4** and yields are summarized in Table I. Phosphorylation occurred

Chart I

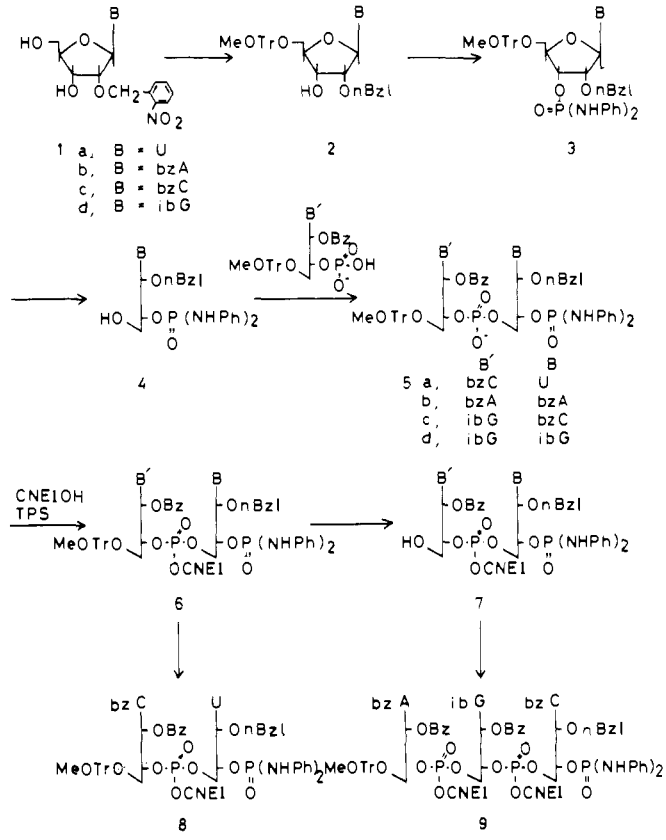

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Table I. Reaction Conditions for Phosphorylation in the Synthesis of **4** and Analyses of the Products

2, mmol	$P(NHPh)_2$-OCl, mmol	Pyridine, mL	Time, h	Product, mmol	Yield, %	UV in 95% EtOH λ_{max} ($\epsilon \times 10^{-4}$), nm	UV in 95% EtOH λ_{min} ($\epsilon \times 10^{-4}$), nm	Elemental anal. calcd (found) C	H	N
2a				**4a**				$C_{28}H_{28}N_5O_9P$, 609.52		
1.90	3.20	10	72	3.00	93	231 (2.53) 262 (1.62)	247.5 (1.22)	55.17 (55.05)	4.63 (4.70)	11.49 (11.49)
2b				**4b**				$C_{35}H_{33}N_8O_8P \cdot \frac{1}{2}H_2O$		
1.25	1.62	8	96	1.15	92	230 (3.90)[a] 279 (2.60)[a]	248 (1.49)[a]	745.67 57.98 (58.02)	4.60 (4.73)	15.03 (14.80)
2c				**4c**				$C_{35}H_{33}N_6O_9P$, 712.64		
2.70	4.03	8	20	2.65	98	231.5 (3.26) 262 (3.18) 303 (1.30)	245.5 (2.14) 292.5 (1.27)	58.98 (58.72)	4.67 (4.62)	11.79 (11.86)
2d				**4d**				$C_{33}H_{35}N_8O_9P \cdot \frac{3}{2}H_2O$		
4.60	11.0	20	36	3.10	67	232.5 (3.06) 261 (2.19) 283 (sh) (1.59)	246 (1.76)	745.77 53.14 (53.19)	5.15 (5.24)	15.03 (14.81)

[a] Measured in MeOH.

Table II. NMR Spectral Data for 2′-*O*-(*o*-Nitrobenzyl)nucleosides and Their 3′-Phosphorodianilidates

Compd	1′-H	2′-H	3′-H	4′-H	5′-H	CH_2Ar	2′-OH	3′-OH	5′-OH	5	6
1a	5.93 d 1 $J_{1'2'}$ = 5 Hz		3.85–4.30 m 3		3.65 bs 2	4.93, 5.02 AB q 2 J_{gem} = 15 Hz		5.27 d 1	5.08 t 1	5.58 q 1 J_{56} = 8 Hz	
4a	6.12 d 1 $J_{1'2'}$ = 7 Hz	4.31 m 1	5.08 m 1	4.31 m 1	3.61 bs 2	4.87 s 2			5.31 m 1	5.65 d 1 J_{56} = 8 Hz	7.83 d 1 J_{56} = 8 Hz
1b	6.28 d 1 $J_{1'2'}$ = 5 Hz	4.70 t 1 $J_{1'2'}$ = 5 Hz $J_{2'3'}$ = 5 Hz	4.49 t 1 $J_{2'3'}$ = 5 Hz $J_{3'4'}$ = 5 Hz	4.11 m 1	3.70 bs 2	5.10, 4.96 AB q 2 J_{gem} = 2.6 Hz					
4b	6.37 d 1 $J_{1'2'}$ = 7 Hz	4.97 m 1	5.32 m 1	4.48 bs 1	3.70 bs 1	4.92 s 2			5.32 m 1		
1c	5.98 d 1 $J_{1'2'}$ = 2 Hz		3.90–4.37 m 3		3.76 bs 2	5.13 s 2		5.20–5.40 m 2		7.29 d 1 J_{56} = 7 Hz	8.50 d 1 J_{56} = 7 Hz
4c	6.16 d 1 $J_{1'2'}$ = 4 Hz	4.39 m 1	5.05 m 1	4.39 m 1	3.72 bs 2	4.82, 5.04 AB q 2 J_{gem} = 14 Hz			5.38 bs 1	7.32 d 1 J_{56} = 7 Hz	8.47 d 1 J_{56} = 7 Hz
1d	6.04 d 1 $J_{1'2'}$ = 6 Hz	4.32–4.68 m 2		4.08 m 1	3.66 bs 2	4.88, 5.04 AB q 2 J_{gem} = 14 Hz	5.38 d 1		5.08 t 1		
4d	6.05 d 1 $J_{1'2'}$ = 8 Hz	4.75 m 1	5.27 m 1	4.34 m 1	3.60 bs 2	4.82, 4.93 AB q 2 J_{gem} = 14 Hz			5.30 m 1		

almost quantitatively except for isobutyrylguanosine. In the case of **2d** phosphorylation took place in the guanine ring as well as at the 3′-hydroxyl group. From NMR and UV data the ring phosphorylation seemed to occur at either N-1, N-2, or O-6 but not at N-7. The dianilidophosphoryl group on the ring could be removed by prolonged 80% acetic acid treatment. Thus **4d** was isolated in a yield of 67% by chromatography on silica gel after treatment with 80% acetic acid for 3 days. NMR data for **4** are shown in Table II.

Synthesis of a New Type of Fully Protected Dinucleotide (6) and Selective Deblocking to Yield 7 and 8. The dianilidates (**4a–d**) were used for the synthesis of the fully protected dimers (**6**). **6a** was prepared by condensation of **4a** with 5′-*O*-monomethoxytrityl-*N*,2′-*O*-dibenzoylcytidine 3′-phosphate[7a] using dicyclohexylcarbodiimide (DCC) as the condensing reagent followed by esterification of the internucleotide linkage using β-cyanoethyl alcohol and triisopropylbenzenesulfonyl chloride (TPS). The triesterified dinucleotide (**6a**) was isolated by chromatography on silica gel in a yield of 58%. **6b–d** were synthesized similarly and converted to the 5′ free dinucleotides by treatment with 80% acetic acid. **7b–d** were obtained in yields of 45, 42, and 46%. **7c** was further elongated in the 5′ direction as described below. To remove the protection from the 3′-phosphate **6a** was treated with isoamyl nitrite in pyridine–acetic acid overnight. The dinucleotide with the 3′-phosphomonoester (**8**) which was a suitable intermediate for elongation of the chain in the 3′ direction was isolated by precipitation in a yield of 83%. The removal of the dianilidate with isoamyl nitrite took longer than the corresponding deprotection of phosphomonoanilidates.[7a] The monoanilidate of **8** was not detected during isoamyl nitrite treatment. Presumably as soon as the first anilidate was removed the second group was de-

Table III. Paper Chromatography and Electrophoresis

Compd	Solvent A	Solvent B	Relative mobility pH 7.5
A	0.67	0.63	−0.15
G	0.51	0.46	0.13
C	0.62	0.56	0
U	0.63	0.56	0.21
A > p			0.48
G > p			0.62
C > p	0.46		0.63
U > p	0.45		0.81
Ap	0.39	0.42	0.92
Gp	0.23	0.28	1.00
Cp	0.35	0.39	1.00
Up	0.32		1.10
CpUnbzlp$(NHPh)_2$	0.81		0.38
ApAnbzlp$(NHPh)_2$	0.81		0.34
GpCnbzlp$(NHPh)_2$	0.82		0.32
GpGnbzlp$(NHPh)_2$	0.75		0.30
CpUnbzlp	0.36	0.45	0.97
ApAnbzlp	0.39		
GpCnbzlp	0.23	0.34	
GpGnbzlp	0.10	0.32	
CpUnbzl	0.70		
GpCnbzl	0.58	0.33	
CpUp	0.27		
ApAp	0.26		
GpCp	0.17	0.22	
GpGp	0.06	0.21	
ApGpCnbzlp$(NHPh)_2$	0.73		0.45
ApGpCnbzlp	0.10	0.25	0.93
ApGpCp		0.15	1.00

composed rather rapidly. R_f values in paper chromatography and relative mobilities in paper electrophoresis for partially deprotected compounds are shown in Table III. Completely deblocked dinucleotides were characterized by enzymatic hydrolysis.

Synthesis of the Protected Trinucleotide (9). The 5′-deblocked dinucleotide **7c** was condensed with 5′-*O*-monomethoxytrityl-*N*(*N*),2′-*O*-dibenzoyladenosine 3′-phosphate[14] using DCC. Attempts were made to isolate the partially diesterified trinucleotide by extraction but the trimer was contaminated with the mononucleotide. Gel filtration on Sephadex LH-20 did not resolve the trimer and the mononucleotides presumably due to anionic repulsion of the phosphomonoester group in the monomer. After β-cyanoethylation of both the internucleotide linkages of the trinucleotide and the 3′-phosphate of the monomer the product (**9**) was isolated in a yield of 56% by chromatography on silica gel and characterized by the methods used for **7** and **8**.

Conclusion

The synthesis of the 2′-*O*-(*o*-nitrobenzyl) derivatives from four major nucleotides allowed the introduction of the phosphorodianilidate to the 3′ position. These 3′-phosphorodianilidates were used for the synthesis of fully protected dinucleotides (**6**) which could be elongated in either the 3′ or 5′ directions by selective deblocking. Each dinucleotide contained a different nucleotide at the 3′ end. These four derivatives were subjected to condensation reactions. Removal of the protecting groups of the dinucleotides (**7**) by UV irradiation showed that no detectable photochemical reactions took place during the removal of the *o*-nitrobenzyl group. The trinucleotide (**9**) was synthesized from the 5′-deblocked dinucleotide (**7c**) and repetition of this reaction followed by isoamyl nitrite treatment would yield oligonucleotides with 3′-phosphates. Detailed studies on condensations of oligonucleotides having protected internucleotide linkages and terminal phosphomonoesters have yet to be performed. It is desirable to reduce the protection of internucleotide linkages when the oligonucleotide chain gets longer, since removal of the protecting group becomes difficult.[15] However, it is necessary to protect the phosphate to prevent undesired side reactions. An oligomer such as **8** would provide an example of activation of phosphomonoesters in triesterified oligonucleotides. Activation of the phosphomonoesters should be easier than that of the corresponding diesters. Results after the condensation of **8** with the 5′-deblocked dinucleotide (**7d**) will be reported shortly.

Experimental Section

General Methods. Paper chromatography was performed by the descending technique using the following solvent systems: A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); B, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v/v). Paper electrophoresis was performed using 0.05 M triethylammonium bicarbonate (pH 7.5) or 0.2 M morpholinium acetate (pH 3.5) at 900 V/40 cm. Thin layer chromatography (TLC) was performed on plates of silica gel (Merck HF 254) using a mixture of chloroform–methanol. For columns silica gel G (Merck) or AR (100 mesh, Mallinckrodt) was used. 5′-Monomethoxytrityl groups were removed by treatment with 80% acetic acid until all monomethoxytrityl color behaved as the carbinol on TLC as detected by spraying with 30% sulfuric acid. The β-cyanoethyl group was removed by treatment with aqueous ammonia (7 N) at 0 °C for 5 min or treatment with triethylamine in pyridine.[16] Acyl groups were removed by treatment with 15 N methanolic ammonia at 25–30 °C for 20 h. Removal of the *o*-nitrobenzyl group was performed in aqueous solution by irradiation with UV light through a Pyrex filter (2 mm thick) over a Pyrex test tube (1 mm thick) for 2 h using a photolysis apparatus bearing a 300-W high-pressure mercury lamp (Eikosha Co. Model PIH 300) and quarz water-circulating jacket.

Other general methods and enzymatic hydrolyses were as described previously.[7b]

2′-*O*-(*o*-Nitrobenzyl)-*N*-benzoylcytidine (1c). 2′-*O*-(*o*-Nitrobenzyl)cytidine[5] (1.10 g, 2.91 mmol) was dissolved in pyridine (21 mL) and treated with benzoyl chloride (1.3 mL, 11 mmol) with cooling in an ice bath. After the mixture had been stirred for 1 h at room temperature, the extent of the reaction was checked by TLC (30:1). The UV spectrum of the compound (R_f, 0.62) in 95% EtOH was λ_{max} 230, 261.5, and 301 nm. Water (20 mL) was added with cooling and the mixture was extracted with chloroform (20 mL, two portions). Chloroform was washed with 5% sodium bicarbonate (20 mL, two portions), then with water until the aqueous layer became neutral and the organic phase was concentrated. The syrup was dissolved in 95% ethanol (15 mL) and pyridine (10 mL). To the solution was added 2 N sodium hydroxide (20 mL) at 0 °C dropwise, and after standing at room temperature for 5 min pyridinum Dowex 50X2 (70 mL) was added. The resin was washed with 50% pyridine and the N-benzoylated compound **1c** was crystallized by evaporation of the filtrate and washings. The product was recrystallized with 99% ethanol. The yield was 1.32 g (2.74 mmol), 94%: mp 187–188 °C; TLC (10:1) R_f 0.44 [2′-*O*-(*o*-nitrobenzyl)cytidine, 0.05; the tribenzoyl derivative, 1]; λ_{max} (95% ethanol) 260, 303 nm. Anal. Calcd for $C_{23}H_{22}N_4O_8$ (482.44): C, 57.26; H, 4.60; N, 11.61. Found: C, 57.01; H, 4.52; N, 11.33.

5′-*O*-Monomethoxytrityl-2′-*O*-(*o*-nitrobenzyl)-*N*-benzoylcytidine (2c). **1c** (1.32 g, 2.74 mmol) was treated with monomethoxytrityl chloride (1.2 g, 3.89 mmol) in pyridine (25 mL) at room temperature under stirring. After 20 h, an aliquot was analyzed by TLC (15:1) to confirm the starting material (R_f, 0.20) to be monomethoxytritylated (R_f 0.74). Ice water (20 mL) was added, the mixture was extracted with chloroform (20 mL, three portions), and the organic layer was washed with water (10 mL, three portions) and evaporated. The product was precipitated with *n*-hexane (1 L) from its solution in pyridine (15 mL). The yield was 2.04 g (2.7 mmol, 98%).

5′-*O*-Monomethoxytrityl-2′-*O*-(*o*-nitrobenzyl)-*N*-isobutyrylguanosine (2d). **1d** (2.77 g, 5.7 mmol) was coevaporated with pyridine and treated with monomethoxytrityl chloride (2.5 g, 8.0 mmol) in pyridine (35 mL) at room temperature for 8 h. The reaction was checked by TLC (10:1) and ice water (30 mL) was added to the mixture with cooling. The product was extracted with chloroform (20 mL, three portions), and the organic layer was washed with water (10 mL, three portions). The residue was coevaporated with pyridine and precipi-

tated with ether–hexane (1:5, 500 mL) from its solution in ethyl acetate (30 mL). The yield was 4.20 g (5.5 mmol), 90%.

2′-*O*-(*o*-Nitrobenzyl)uridine 3′-Phosphorodianilidate (4a). A General Method for the Synthesis of 4a, 4b, and 4c. 2a (1.9 g, 2.9 mmol) was dried by coevaporation of pyridine and treated with dianilidophosphorochloridate (853 mg, 3.2 mmol) in pyridine (10 mL) at room temperature for 3 days. TLC (15:1) showed the complete conversion of **2a** (R_f 0.29). An aqueous solution (5%) of potassium acetate (15 mL) was added with cooling in an ice bath and the mixture was stirred for 1 h. **3a** was extracted with chloroform (10 mL) three times. The chloroform solution was washed with water (5 mL) four times and concentrated. The residue was coevaporated with toluene to remove pyridine and then treated with 80% acetic acid (100 mL) for 1.5 days. After demonomethoxytritylation was complete by TLC (10:1) acetic acid was evaporated and **2a** was precipitated with ether–hexane (1:1, 500 mL) from its solution in pyridine (15 mL). The yield was 1.8 g (3.0 mL, 93%). Conditions for the synthesis of **4b** and **4c** are summarized in Table I together with elemental analyses. NMR data are shown in Table II.

2′-*O*-(*o*-Nitrobenzyl)-*N*-isobutyrylguanosine 3′-Phosphorodianilidate (4d). 2d was treated with dianilidophosphorochloridate as shown in Table I by a similar procedure to that used for the synthesis of **4a.** Since the phosphorylated compound (**3d**) had the same mobility as **2d** in TLC, the reaction was checked after treatment of an aliquot with isoamyl nitrite (2 drops) in pyridine–acetic acid. When no starting material was found at 0.4 in TLC (15:1) and a trityl-positive spot remained at the origin the reaction was stopped with potassium acetate (5%, 25 mL) with cooling. The mixture was stirred for 1 h at room temperature and **3d** was extracted with chloroform. Acetic acid treatment was performed as described above and the monomethoxytrityl group was removed after 16 h to give two compounds (R_f 0.25 and 0.32 in TLC, 10:1). The slower moving material was identified as **4d** (Table II) and the faster moving compound showed another phosphorodianilidate substitution on the base as estimated by NMR data (Me_2SO-d_6): δ 8.55 (s, 1 H, C_8H), 6.19 (d, 1 H, C_1, H), 6.6–7.8 (m, 24 H, ArH). The substituted compound was converted to **4d** by treatment with 80% acetic acid for 16 h. Acetic acid was removed and the mixture was applied to a column (6.6 × 12.4 cm) of silica gel G (190 g). The product was eluted with 20:1 chloroform–methanol, passed through a column (1 × 3.0 cm) of pyridinium Dowex 50X2 to remove colored material in 50% aqueous pyridine, precipitated with *n*-hexane (350 mL) from its solution in pyridine (15 mL), and washed with *n*-hexane three times.

5′-*O*-Monomethoxytrityl-*N*,2′-*O*-dibenzoylcytidylyl-(3′–5′)-2′-*O*-(*o*-nitrobenzyl)uridine 3′-Phosphorodianilidate [P^1-(β-Cyanoethyl) Ester] (6a). The pyridinium salt of 5′-*O*-monomethoxytrityl-*N*,2′-*O*-dibenzoylcytidine 3′-phosphate (2.0 g) was passed through a column (1.4 × 12 cm) of pyridinium Dowex 50X2 in 50% pyridine to obtain 2.32 × 10^4 A_{302} units (1.9 mmol) and combined with **4a** (4.4 × 10^4 A_{260}, 2.7 mmol) passed through a column (1.7 × 3 cm) of pyridinium Dowex 50X2. The mixture was dried by coevaporation of pyridine, Dowex 50X2 (2.5 g) was added, and the drying (three times) was repeated; then the mixture was treated in pyridine (10 mL) with DCC (3.9 g, 19 mmol) at room temperature for 5 days. Aqueous pyridine (50%, 6 mL) and *n*-hexane (10 mL) were added with cooling. After standing at room temperature overnight the filtered aqueous layer was concentrated and the residue was dissolved in 1-butanol (50 mL). The butanol phase was washed with water and concentrated with pyridine. The dinucleotide (**5a**) was precipitated with ether (300 mL) from its solution in pyridine (20 mL), washed with ether three times, dried by evaporation of added pyridine, and treated with β-cyanoethanol (1.44 mL, 21 mmol) in pyridine (10 mL) using TPS (2.5 g, 8.3 mmol) for 16 h at room temperature. After the reaction had been checked by TLC (10:1), water (5 mL) was added with cooling. The product was extracted with chloroform (10 mL) twice, washed with water (5 mL) three times, and dried by evaporation with pyridine and then toluene. The residue was dissolved in a small amount of chloroform and applied to a column (7.0 × 5.8 cm) of silica gel G (100 g). Elution was performed using chloroform (60 mL), 40:1 chloroform–methanol (120 mL), and 30:1 chloroform–methanol. The trityl-positive fractions were combined and **4a** was precipitated with 1:1 ether–hexane (200 mL) from its solution in pyridine (6 mL). The yield was 1.66 g (4.58 × 10^4 A_{260}, 1.1 mmol), 58%. An aliquot was deblocked by successive treatment with isoamyl nitrite, 80% acetic acid, and 15 N methanolic ammonia. The 2′-*O*-(o-nitrobenzyl)dinucleotide was isolated by paper electrophoresis and deblocked by irradiation with UV light for 2 h. CpUp was subjected to paper chromatography in solvent B and transfered to paper electrophoresis (pH 7.5). R_f values and relative mobilities are shown in Table III. A single spot (ca. 3 A_{260}) was hydrolyzed by RNase A (60 μg) in 0.05 M triethylammonium bicarbonate (100 mL) at 37 °C for 6 h and the mononucleotides were separated by paper electrophoresis (pH 3.5). The ratio of Up and Cp was estimated spectrophotometrically in 0.01 N HCl as 1.03:1.00.

***N*,(*N*),2′-*O*-Di(tri)benzoyladenylyl-(3′–5′)-2′-*O*-(*o*-nitrobenzyl)-*N*-benzoyladenosine 3′-Phosphorodianilidate [P^1-(β-Cyanoethyl) Ester] (7b).** The pyridinium salt of 5′-*O*-monomethoxytrityl-*N*,(*N*),2′-*O*-di(tri)benzoyladenosine 3′-phosphate (8100 A_{280}, 0.426 mmol) was condensed with **4b** (431 mg, 0.585 mmol) in pyridine (3 mL) using DCC (4.3 mmol) in the presence of Dowex 50X8 (pyridinium form, 439 mg) as described for **6a.** After 3 days an aliquot was deblocked for checking the extent of reaction. After 5 days aqueous pyridine (50%, 18 mL) was added and DCC was removed with pentane. The aqueous layer was kept at 27 °C overnight and the dinucleotide **5b** was precipitated with 3:2 ether–pentane (190 mL) from its solution in pyridine. **5b** was contaminated with **4b** as judged by TLC. The mixture was treated with monomethoxytrityl chloride (59 mg) in pyridine at 33 °C for 21 h to convert **4b** to a more soluble derivative in ether and **5b** was reprecipitated as above. **5b** was still contaminated with a small amount of **4b** but esterified with β-cyanoethanol (0.3 mL) and TPS (518 mg) at 27 °C. After 2 days **5b** was detected by TLC and the same amount of the reagents was added. The reaction was completed after 1 further day and the mixture was treated with 50% pyridine at room temperature for 30 min. **6a** was extracted with methylene chloride (20, 15, 15 mL) and washed successively with water and 0.1 M triethylammonium bicarbonate (21 mL, each). The organic layer was dried with sodium sulfate and concentrated. The residue was coevaporated with toluene, dissolved in chloroform (10 mL), and applied to a column of silica gel (AR, 80 g). Elution was performed with a linear gradient of chloroform (1 L) and chloroform containing 9% methanol (1 L). **5b** containing N,N-dibenzoylated Ap was eluted first and the second fraction was contaminated with the dinucleotide N-monobenzoylated Ap (60%). Each fraction was treated with 80% acetic acid overnight and preparative TLC gave **7b. 7b** containing N,N-dibenzoylated Ap (0.0987 mmol) was obtained from the first fraction: UV (95% EtOH) λ_{max} 232, 276 nm, min 264; $\epsilon_{232/276}$ 1.46 $\epsilon(p)_{276}$ 22 800. The spectral properties of **7b** containing N-monobenzoylated Ap follow: λ_{max} 232, 278; λ_{min} 250 nm; $\epsilon_{232/276}$ 1.36 in 95% EtOH. The combined yield was 0.19 mmol (45%) calculated from 5′-*O*-monomethoxytrityl-*N*,(*N*),2′-*O*-benzoyladenosine 3′-phosphate (ϵ 17 800). Successive treatments of **7b** with isoamyl nitrite, aqueous ammonia, and methanolic ammonia gave ApAnbzlp after paper chromatography in solvent A. The *o*-nitrobenzyl group was removed as described in General Methods and ApAp was isolated by paper chromatography in solvent A. The dinucleotide was characterized by hydrolysis with RNase M after treatment with phosphatase. Ap (1.08 A_{260}) and A (1.04 A_{260}) were separated by paper electrophoresis (pH 7.5) and the undigested material was not detected.

2′-*O*-Benzoyl-*N*-isobutyrylguanylyl-(3′–5′)-2′-*O*-(*o*-nitrobenzyl)-*N*-benzoylcytidine 3′-Phosphorodianilidate [P^1-(Cyanoethyl) Ester] (7c). Pyridinium 5′-*O*-monomethoxytrityl-2′-*O*-benzoyl-*N*-isobutyrylguanosine 3′-phosphate (35 700 A_{260}, 1.8 mmol) was passed through a column (1.8 × 7.5 cm) of pyridinium Dowex 50X2 in 50% pyridine and combined with **4c** (1.89 g, 2.65 mmol). The mixture was dried with pyridinium Dowex 50X2 (1.3 g) by evaporation of pyridine three times and treated with DCC (4.13 g, 2.0 mmol) at 15–20 °C for 4 days. Aqueous pyridine (50%, 10 mL) and hexane were added and the filtered aqueous layer was evaporated after 6 h. The product **5c** was precipitated with ether (300 mL) from its solution in anhydrous pyridine (10 mL), dried over P_2O_5 in vacuo, coevaporated with pyridine twice, and treated with TPS (2.0 g, 6.6 mmol) in pyridine (8 mL) for 30 min; then β-cyanoethanol (1.2 mL, 17.6 mmol) was added. After 15 h the unreacted **5c** was detected by TLC (10:1, R_f 0.27). The same amounts of the reagent and the solvent were again added. After 6 h the reaction was completed and aqueous pyridine (50%, 8 mL) was added with cooling. The product **6c** was extracted with chloroform (10 mL) three times, washed with water (10 mL) three times, and applied to a short column (6.7 × 5 cm) of silica gel as above using 10:1 chloroform–methanol as the eluent. The triesterified dinucleotides showed two spots (R_f 0.36 and 0.30) in TLC (10:1) but yielded the same dinucleotide GpCp after deblocking. The yield was 36 700 A_{260}

(0.75 mmol, 42%). GpCp (2 A_{260}) was hydrolyzed with RNase M. The mononucleotides were separated by paper chromatography in solvent A and eluted with 0.01 M sodium cacodylate (pH 7.0, 1.5 mL). The ratio of Cp (0.49 A_{270}) to Gp (0.715 A_{252}) was 1.04:1.00. No undigested compounds were detected. Anal. Calcd for $C_{59}H_{58}O_{18}N_{12}P_2 \cdot 2H_2O$ (1321.166): C, 53.64; H, 4.73; N, 12.72. Found: C, 53.81; H, 4.61; N, 12.50. UV (95% EtOH) ϵ_{233} 53 800, ϵ_{262} 46 000.

2′-*O*-Benzoyl-*N*-isobutyrylguanylyl-(3′–5′)-2′-*O*-(*o*-nitrobenzyl)-*N*-isobutyrylguanosine 3′-Phosphorodianilidate [P^1-(β-Cyanoethyl) Ester] (7d). Pyridinium 5′-*O*-monomethoxytrityl-2′-*O*-benzoyl-*N*-isobutyrylguanosine 3′-phosphate was passed through a column (1.4 × 9.0 cm) of pyridinium Dowex 50X2 in 50% pyridine and the amount estimated (16 600 A_{260}, 0.84 mmol). This was combined with **4d** (756 mg, 1.05 mmol), dried by coevaporation of pyridine three times in the presence of Dowex 50X2 (pyridinium form, 527 mg), and treated with DCC (1.68 g, 8 mmol) in pyridine (4 mL) for 4 days. Aqueous pyridine (50%, 5 mL) and hexane (5 mL) were added with shaking. After 15 h the filtered aqueous solution was concentrated and **5d** was precipitated with ether from its solution in pyridine. The precipitate was washed with ether three times, dried by evaporation of pyridine (4.4 mL), and treated with TPS (1.1 g, 3.6 mmol) for 30 min. β-Cyanoethanol (0.64 mL, 9.4 mmol) was added and the mixture was kept overnight at 20–25 °C. After the reaction was checked by TLC (10:1, R_f 0.40) ice water (5 mL) was added to the mixture. **6d** was extracted with chloroform (5 mL) three times, washed with water (5 mL) twice, concentrated with toluene, dissolved in a small amount of chloroform, and applied to a column (6.6 × 9.8 cm) of silica gel G (150 g). Elution was performed uSING CHLOROFORM–METHANOL= 3]:1 (600 mL), 25:1 (600 mL). The monomethoxytrityl positive fractions were collected and treated with 80% acetic acid (80 mL) overnight. Demonomethoxytritylation was checked by TLC and the solvents were removed. The residue was dried by evaporation of pyridine and toluene, then dissolved in a small amount of chloroform and applied to a column (2.7 × 16.8 cm) of silica gel G. Elution was performed using a linear gradient of chloroform (550 mL) and 10:1 chloroform–methanol (550 ml). Diastereoisomers of **7d** were separated and the combined yield was 497 mg (0.385 mmol, 46%). An aliquot was deprotected and GpGp was characterized by hydrolysis with alkaline phosphatase followed by RNase M treatment. Gp (0.337 A_{252}) and G (0.329 A_{252}) were separated by paper electrophoresis (pH 7.5) and the ratio was found to be 1.03:1.00. R_f values of the partially protected product are shown in Table III.

5′-*O*-Monomethoxytrityl-*N*-2′-*O*-dibenzoylcytidylyl-(3′–5′)-2′-*O*-(*o*-nitrobenzyl)uridine 3′-Phosphate [P^1-(β-Cyanoethyl) Ester] (8a). **7a** (1.1 mmol) was treated with isoamyl nitrite (2.5 mL) in 1:1 pyridine–acetic acid (20 mL) overnight and the completion of the reaction was checked by TLC (10:1). Ice water (20 mL) was added. **8a** was extracted with chloroform (15 mL) three times, washed with water (10 mL) four times, and precipitated with ether (200 mL) from its solution in pyridine (7 mL). The yield was 35 400 A_{260} (0.91 mmol, 83%). The product was characterized as described for **7a.**

5′-*O*-Monomethoxytrityl-*N*,2′-*O*-dibenzoyladenylyl-(3′–5′)-2′-*O*-benzoyl-*N*-isobutyrylguanylyl-(3′–5′)-2′-*O*-(*o*-nitrobenzyl)-*N*-benzoylcytidine 3′-Phosphorodianilidate [P^1,P^2-(β-Cyanoethyl) Ester] (9). Pyridinium 5′-monomethoxytrityl-*N*,2′-*O*-dibenzoyladenosine 3′-phosphate (15 700 A_{280}, 0.71 mmol) was passed through a column (2.7 × 3.0 cm) of pyridinium Dowex 50X2, dried by evaporation with pyridine in the presence of the same resin (800 mg), and condensed with **7c** (562 mg, 0.43 mmol) in pyridine (4 mL) using DCC (1.44 g). After 4 days an aliquot was treated with 80% acetic acid and analyzed by TLC (10:1) for the absence of **7c.** Aqueous pyridine (50%, 10 mL) and hexane (100 mL) were added with cooling. The mixture was kept overnight after shaking and the partially diesterified trimer was precipitated with 2:1 ether–hexane (200 mL) from its solution in anhydrous pyridine (7 mL). The mixture was applied to a column (2.2 × 74.5 cm) of Sephadex LH-20 equilibrated with 95% ethanol to remove the mononucleotide. However, no resolution was obtained and the monomethoxytrityl group was partially lost. The combined materials were treated with monomethoxytrityl chloride (433 mg, 1.14 mmol) in pyridine (10 mL) overnight and the product was extracted with chloroform (10 mL) twice after addition of ice water (5 mL). The precipitated compound was dried by evaporation with pyridine twice and esterified with β-cyanoethanol (0.5 mL, 7.3 mmol) using TPS (847 mg, 2.8 mmol) overnight. After the reaction was checked by TLC (10:1) ice water (10 mL) was added, and the product (**9**) was extracted with chloroform (10 mL) twice, washed with water, evaporated with toluene, and applied to a column (3.2 × 19.3 cm) of silica gel G. The appropriate fractions (R_f 0.47 in 10:1 TLC) were combined and **9** was precipitated with 1:1 ether–hexane (100 mL) from its solution in pyridine (3 mL). The yield was 536 mg (15 300 A_{260}, 0.25 mmol), 56%. **9** was identified by paper chromatography and electrophoresis of the partially deblocked trinucleotides (Table III) and the base ratio was obtained by hydrolysis of ApGpC with RNase M followed by paper chromatography in solvent A. The ratio of Ap (0.576 A_{257}), Gp (0.417 A_{252}), and C (0.452 A_{280}) at pH 2 was 1.09:0.98:1.00.

Acknowledgment. The authors thank Dr. A. F. Markham for reading the manuscript.

References and Notes

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(3) Abbreviations follow principally recommendations of IUPAC–IUB Commission of Biochemical Nomenclature [*J. Biol. Chem.*, **245**, 5171 (1970); *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 2222 (1977)]. nbzl refers to *o*-nitrobenzyl.
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