[Chem. Pharm. Bull. 25(5) 949—959 (1977)]

UDC 547.963.32.04 : 547.539.3.04

Studies on Transfer Ribonucleic Acids and Related Compounds. XVI.[1] Synthesis of Ribooligonucleotides using a Photosensitive *o*-Nitrobenzyl Protection for the 2′-Hydroxyl Group

Eiko Ohtsuka, Shoji Tanaka, and Morio Ikehara

Faculty of Pharmaceutical Sciences Osaka University[2]

(Received July 29, 1976)

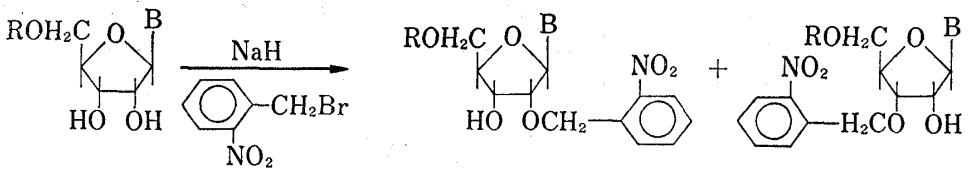
Previously 2′-O-(*o*-nitrobenzyl)uridine was synthesized *via* 2′,3′-O-(stannylene)uridine and used in the synthesis of UpA and UpU. 2′-O-(*o*-Nitrobenzyl)derivatives of cytidine and adenosine were synthesized with *o*-nitrobenzyl bromide in the presence of sodium hydride. 3′-O-(*o*-Nitrobenzyl)cytidine was also isolated. Using these 2′-protected nucleosides, partially protected trinucleoside diphosphates, CpA(*o*-nitrobenzyl)-pA and CpCpA(*o*-nitrobenzyl) were synthesized using a diester method or a triester method. These oligomers are candidates as suitable substrates of ribonucleic acid (RNA) ligase. Removal of the *o*-nitrobenzyl group was effected by irradiation with ultraviolet spectrum (UV) light (wavelength longer than 280 nm) and the completely deblocked oligonucleotides were characterized by enzymatic hydrolysis.

Keywords——2′-O-(*o*-nitrobenzyl)adenosine; 2′-O-(*o*-nitrobenzyl)cytidine; cytidylyl-(3′-5′)-(2′-*o*-nitrobenzyl) adenyl-(3′-5′) adenosine; cytidylyl-(3′-5′)-cytidylyl-(2′-*o*-nitrobenzyl)adenosine; UV; NMR; DEAE-cellulose chromatography; enzymatic digestion

Protection of the 2′-hydroxyl group of ribonucleosides has been a central problem in the chemical synthesis of ribopolynucleotides. Direct acylation of the 2′-hydroxyl group of nucleoside 3′-phosphates enabled useful intermediates for ribooligonucleotide synthesis to be synthesized.[3] Selective monoacylation of the vicinal 2′,3′-hydroxyl groups has been attempted by acid hydrolysis of orthoesters and the 2′-acyl group was found to migrate to the 3′-position.[4] These 3′-acyl nucleosides became starting materials for preparation of the 2′-acetal or ketal derivatives which have also been important intermediates.[5] Direct 2′-benzylations were reported earlier[6,7] and 2′-O-(*o*-nitrobenzyl)uridine which was prepared *via* 2′,3′-O-(stannylene)uridine[7a] was used for the synthesis of dinucleoside monophosphates.[8] Deprotection by irradiation with UV light of wave length longer than 280 nm was proved to be compatible with oligomers containing uridine and adenosine. *o*-Nitrobenzyl derivatives have also been reported as protecting groups for amino acids and carbohydrates.[9] Recently synthesis of 2′-O-(*o*-nitrobenzyl)derivatives of uridine, cytidine and adenosine using *o*-nitrophenyldiazomethane was reported in a preliminary communication.[10] In the present paper the synthesis of 2′-O-(*o*-nitrobenzyl)adenosine and cytidine using *o*-nitrobenzyl bromide in the presence of sodium hydride and their use in oligonucleotide synthesis

1) Part XV of this series: E. Ohtsuka, S. Nishikawa, M. Sugiura, and M. Ikehara, *Nucleic Acids Res.*, **3**, 1613 (1976).
2) Location: *133–1 Yamadakami, Suita, Osaka, 565, Japan.*
3) D.H. Rammler, Y. Lapidot, and H.G. Khorana, *J. Amer. Chem. Soc.*, **85**, 1989 (1963).
4) C.B. Reese and D.R. Trentham, *Tetrahed. Lett.*, **1965**, 2467.
5) *a*) B.E. Griffin and C.B. Reese, *Tetrahedron*, **25**, 4057 (1969); *b*) C.B. Reese and J.C.M. Stewart, *J.*H. van Boom, H.P.M. de Leeuw, J. Nagel, and J.M. de Rooy, *J. Chem. Soc.*, **934**, (1975).
6) K. Kikugawa, F. Sato, T. Tsuruo, N. Imura, and T. Ukita, *Chem. Pharm. Bull.* (Tokyo), **16**, 1110 (1968).
7) *a*) D. Wagner, J.P.H. Verheyden, and J.G. Moffatt, *J. Org. Chem.*, **39**, 24 (1974); *b*) L.F. Christensen and A.D. Brrom, *ibid.*, **37**, 3398 (1972).
8) E. Ohtsuka, S. Tanaka, and M. Ikehara, *Nucleic Acids. Res.*, **1**, 1351 (1974).
9) B. Amit, U. Zehavi, and A. Patchornik, *Israel J. Chem.*, **12**, 103 (1974).
10) D.G. Bartholomew and A.D. Broom, *Chem. Commun.*, **1975**, 38.

are reported. It is also shown that in deprotection of the *o*-nitrobenzyl derivative of CpCpA photohydration and photodimerization[11] did not occur in the sequence of CpC.



a : B=adenine, R=H
b : B=cytosine, R=H
c : B=cytosine, R=monomethoxytrityl

Chart 1

2′- or 3′-O-(*o*-Nitrobenzyl)derivative of Adenosine and Cytidine

The selective monoalkylation of adenosine and cytidine *via* the O-stannylene derivative was reported to be unsuccessful.[7a] Reaction of N^4-benzoyl-2′,3′-O-stannylenecytidine with benzyl bromide resulted in debenzylation.[7a] The vicinal hydroxyl functions were found to be dissociated preferentially by forming a hydrogen bond when ionized with sodium hydride.[12] The reaction of adenosine with *o*-nitrobenzyl bromide in the presence of sodium hydride in dimethyl formamide (DMF) gave the monoalkylated products (65%), the disubstituted material (11%) as estimated by UV spectroscopy and an unidentified compound (6.5%) after 1.5 hr, as determined by thin-layer chromatography (TLC) on silicic acid. The monosubstituted products (**2** and **3**) in Chart 1 were not resolved in TLC and the precipitate from water after 4 hr reaction contained **2a** and **3a** in a ratio of 3:2 as estimated from the integration of the nuclear magnetic resonance (NMR) signals of the H-1′ protons. The 2′-substituted compound (**2a**) was separated by crystallization from aqueous ethanol in a yield of 37%. **2a** was characterized by elemental analysis, mass spectrum (Mass), UV and NMR data. As shown in Table I, the ε values of **2a** in different media indicated a fairly large hypochromicity in water. The homogeneity of **2a** was shown by the H-1′ proton signal (δ 6.08, doublet). The location of the *o*-nitrobenzyl group was confirmed by decoupl-

Table I. UV Data for *o*-Nitrobenzyl Derivatives

	0.01N HCl		H_2O			0.01N NaOH		95% EtOH			
	λ_{max}	ε_{max}	λ_{max}	ε_{max}	ε_{320}	λ_{max}	ε_{max}	λ_{max}	ε_{max}	ε_{260}	ε_{320}
Adenosine			260	15400				260			
Cytidine			271	8900[a]				268	8200		
o-Nitrobenzyl alcohol					1270					5730	1420
2′-O-(*o*-Nitrobenzyl)-adenosine (**2a**)	258.5	18200	260	17800	1300	260	18000	260	20000		970
2′-O-(*o*-Nitrobenzyl)-cytidine (**2b**)	277	17000	268	13400[a]		268	13500	268	14200		
2′-O-(*o*-Nitrobenzyl)-cytidine·HCl (**2b**·HCl)	277	17300	268	13900[a]		268	13900	268	14700		
3′-O-(*o*-Nitrobenzyl)-cytidine·HCl (**3b**·HCl)	277	18300	268	14700[a]		268	14700	268	13800		

a) 0.01M sodium cacodylate

11) a) K.B. Freeman, P.V. Hariharan, and H.E. Jones, *J. Mol. Biol.*, **13**, 833 (1965); b) P.V. Hariharan and H.E. Jones, *Can. J. Chem.*, **46**, 912 (1968).
12) M. Ikehara and M. Kaneko, *Tetrahedron*, **26**, 4251 (1970).

ing experiments of the di-O-acetylated compound. One proton dd signal (δ 5.42) which became a doublet by irradiation at the C_4,-H (δ 4.44) was shifted downfield by acetylation and C_1,-H (δ 6.08) became a singlet by irradiation at C_2,-H.

In the case of cytidine the similar reaction with sodium hydride yielded the monosubstituted products (**2b** and **3b**) (67%) after 1.5 hr and no increase in the amount of products was detected after 2.5 hr. After the reaction the products were extracted with *n*-butanol and the starting material (**1b**) was removed by washing with water. The pure 2′-isomer (**2b**) was isolated by crystallization of its hydrochloride from aqueous ethanol in a yield of 26%

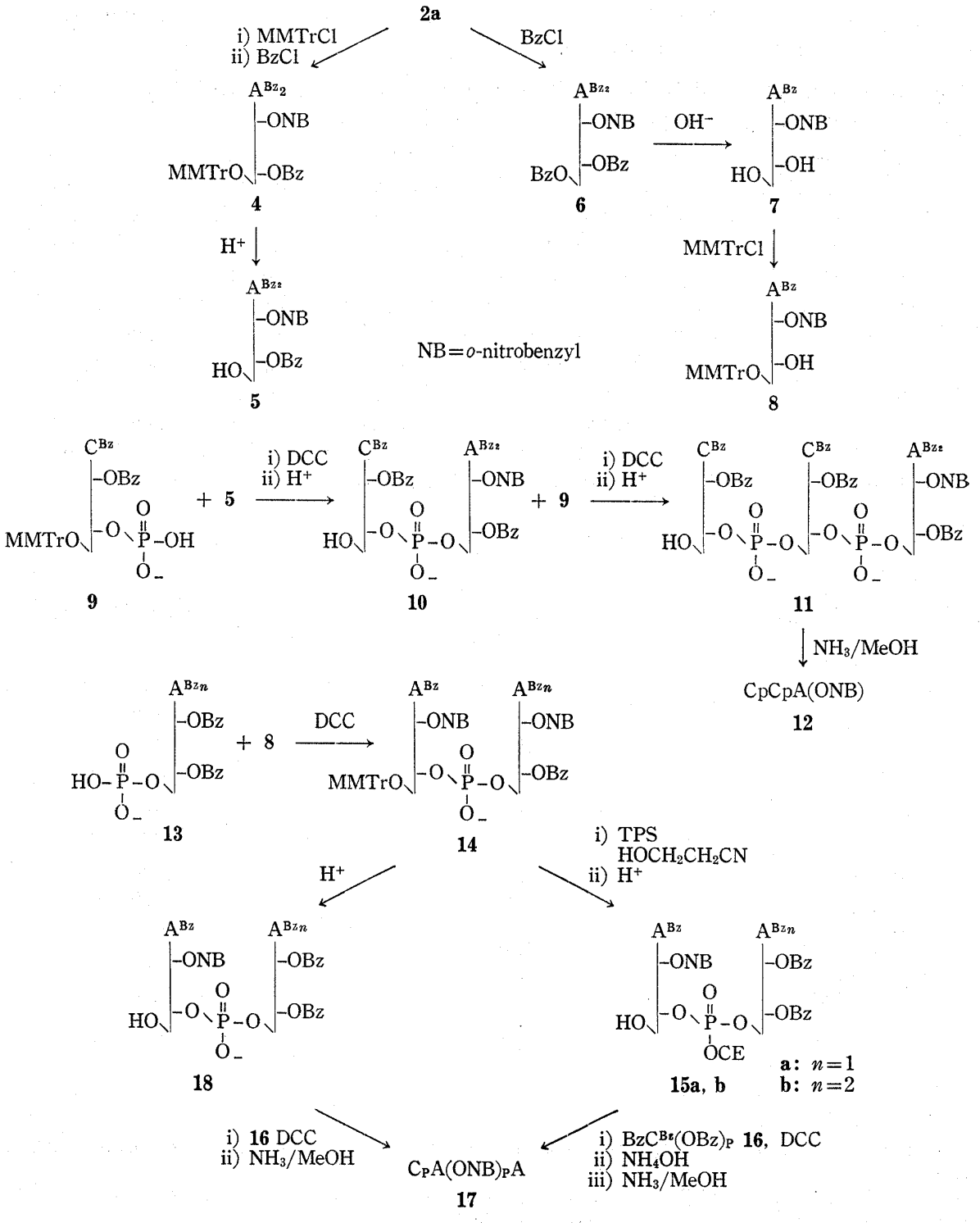


Chart 2

and the 3′-isomer (**3b**) was obtained from the mother liquor (6%). *o*-Nitrobenzylation of 5′-monomethoxytritylcytidine (**1c**) yielded **2c** and **3c** after application of the reaction mixture to a silica gel column. The site of the substitution was determined spectroscopically. The chemical shifts of C_1,-H for the 2′-substituted compound (**2b**) was at lower field than for the 3′-isomer (**3b**) and $J_{1',2'}$ for **2b** was smaller than for **3b**. These data were not contradictory to rules which were proposed by Reese.[13] ε values of **2b** and **3b** in Table I indicated a larger hypochromicity of the 2′-substituted compound (**2b**). This observation was consistent with previous data for O-benzyl substituted nucleosides.[7b] Further characterization of **2b** was performed by conversion of **2b** to 2′-O-(*o*-nitrobenzyl)uridine[8] by deamination. The product thus obtained showed identical mp, elemental analysis and NMR spectra to the specimen synthesized from uridine.

Cytidylyl-(3′-5′)-cytidylyl-(3′-5′)-2′-O-(*o*-nitrobenzyl)adenosine [CpCpA(oNB)] (12) and cytidylyl-(3′-5′)-2′-O-(*o*-nitrobenzyl) adenylyl-(3′-5′-adenosine [CpA(oNB)pA] (17)

The 2′-substituted product, **2a**, was utilized in the synthesis of the two partially *o*-nitrobenzylated oligonucleotides, **12** and **17** as shown in Chart 2. The *o*-nitrobenzyl group of CpCpA(oNB) (**12**) could play a role as a terminator in enzymatic joining using RNA ligase when phosphorylated at the 5′-hydroxyl group. It was of interest to determine whether or not these partially protected oligonucleotides could become substrates for RNA ligase[14] and nucleases.[15] It was thought that the 3′-hydroxyl group of **17** could be recognized by RNA ligase but that the 3′-exonuclease degraded material [CpA(oNB)] could not be joined with the enzyme. The joined **12** and **17** would yield the hexanucleoside pentaphosphate which had the same sequence as the 3′-terminus of *E. coli* $tRNA_f^{Met}$.[16]

The trimer (**12**) was synthesized by stepwise condensation of the mononucleotide (**9**) and the trimer (**17**) was synthesized using two different intermediates, the triester (**15**) and the diester (**18**) for comparison. For the synthesis of **12**, 2′-O-(*o*-Nitrobenzyl)adenosine (**2a**) was

Table II. Paper Chromatography and Paper Electrophoresis

Compound	Paper chromatography		Paper electrophoresis pH 7.5
	Solvent A	Solvent B	
C	0.52	0.58	0.0
Cp cyclic	0.43		0.60
Cp	0.14	0.41	1.0
CpA(oNB)	0.58		0.36
CpA	0.28		0.46
CpCpA(oNB) (12)	0.29		0.58
CpCpA	0.09		0.64
A	0.60	0.63	
Ap cyclic	0.55		0.55
Ap	0.11		0.92
pA	0.09	0.37	0.92
Ap(oNB)A	0.51		0.32
ApA	0.32		0.34
CpAp(oNB)A (17)	0.23	0.57	0.56
CpApA	0.11	0.38	0.57
pA(oNB)	0.35		
A(oNB) (2a)	0.80		

13) H.P.M. Fromageot, B.E. Griffin, C.B. Reese, J.E. Sulston, and D.R. Trentham, *Tetrahedron*, **22**, 705 (1966).
14) R. Silber, V.G. Malathi, and J. Hurwitz, *Proc. Nat. Acad. Sci.*, USA, **69**, 3009 (1972).
15) S.K. Niyogi and A.K. Datta, *J. Biol. Chem.*, **250**, 7307 (1975).
16) S.K. Dube, K.A. Marcker, B.F.C. Clark, and S. Cory, *Nature*, **218**, 232 (1968).

protected to give **5** as shown in Chart 2. Condensation of **5** with the 5′-monomethoxytritylated nucleotide (**9**) using dicyclohexyl carbodiimide (DCC) and subsequent detritylation yielded the protected dimer (**10**) in a yield of 75%. A similar condensation to the above gave the protected trimer (**11**) which was isolated by chromatography on TEAE-cellulose using similar conditions to those described in the synthesis of the acylated trimer.[17] The trimer (**11**) was contaminated with the pyrophosphate of N,2′-O-dibenzoylcytidine 3-phosphate and purified by extraction with organic solvents. The yield of the pure **11** was 20% and deacylation of **11** gave the o-nitrobenzylated product (**12**) which was deblocked with UV light with wavelength longer than 280 nm to give CpCpA in a yield of 95%. The rest of the compound (5%) showed the same mobility as the starting material (**12**) in paper chromatography. No other UV absorbing materials were detected. If the pyrimidine was hydrated, adenosine containing compounds should still have the absorption. *Rf* values in paper chromatography and relative mobilities in electrophoresis of the compounds are shown in Table II.

The other trimer (**17**) was synthesized by slightly different approaches as shown in Chart 2. The protected nucleoside (**8**) was allowed to react with the protected 5′-nucleotide (**13**) using DCC as the condensing reagent. The dimer (**14**) was obtained in a yield of 70% and characterized by digestion with RNase M after deblocking. For the next condensation, one portion of **14** was esterified with a 10 fold excess of β-cyanoethanol using 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) to yield the triester intermediate (**15**). Previously it was reported that cyanoethylated triesters were synthesized in better yield by cyanoethylation after activation of phosphomonoesters to form bulky dinucleoside monophosphate as the first step than condensation of cyanoethylated phosphodiesters.[18] Recently cyanoethylation of an isolated dinucleoside monophosphate was found to be more advantageous than the reaction without isolation of the intermediate.[19] The monomethoxytritylated triester was isolated by preparative TLC and **15** was obtained by subsequent acid treatment. The amino group of **13** seemed to be partially dibenzoylated, since two products were separated in TLC. The slower moving triester (**15a**) and the faster traveling triester (**15b**) were characterized by their neutral UV spectra and by methanolic ammonia treatment. The deacylation of **15a** and **15b** gave the same compound, 2′-(*o*-nitrobenzyl)adenylyl-(3′-5′)-adenosine. *Rf* values in paper chromatography and electrophoresis are shown in Table II. The combined yield of **15a** and **15b** was 41% and these compounds were used for the next reaction. Condensation of **15** with N,2′,5′-tribenzoylcytidine 3′-phosphate (**16**) using DCC yielded the trimer (**17**). The reaction mixture after 3 days showed almost complete conversion of the dimer (**15**) to the trimer (**17**) as checked by paper chromatography after ammonia treatment of an aliquot. The reaction was stopped by treatment with 50% aqueous pyridine as usual and the cyanoethyl group was removed with aqueous ammonia at 0°. The benzoyl group were removed by treatment with methanolic ammonia. The *o*-nitrobenzylated product (**17**) was isolated by chromatography on diethylaminoethyl(DEAE)-cellulose as shown in Fig. 1. Identification of the peaks are summarized in Table III and *Rf* values of the product are shown in Table II. The yield estimated from the recovered dimer and the product in column chromatography was 85%. However, the isolated yield was 59%. It is not clear where any loss of the product occurred, although the present yield was higher than that of **11** or that of the next condensation.

For comparison the same trimer (**17**) was synthesized by condensing **18** with **16**. The diester **18** was obtained by detritylation of **14**. Conditions for the reaction were essentially the same as above. The product (**17**) was also separated by chromatography on DEAE-cellulose. The conditions for elution were the same as described in Fig. 1. The yield of

17) E. Ohtsuka, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, **93**, 2296 (1971).
18) J. Smrt, *Collect. Czech. Chem. Commun.*, **38**, 3189 (1973).
19) E. Ohtsuka, T. Tanaka, and M. Ikehara, reported at 96th Annual Meeting of the Pharm. Soc., Japan at Nagoya, 1976.

17 in this case was 36% and more unchanged starting material (**18**) remained than in the case shown in Fig. 1.

TABLE III. Identification of Compounds in the Synthesis of CpA(oNB)pA (**17**) (Fig. 1)

Peak	Fractions pooled	A_{260}	Identification
I	26— 36	78	Cp cyclic
II	37— 40	32	unidentified
III	41— 50	157	A(oNB)pA
IV	60— 70	103	Cp
V	100—130	1247	CpA(oNB)pA

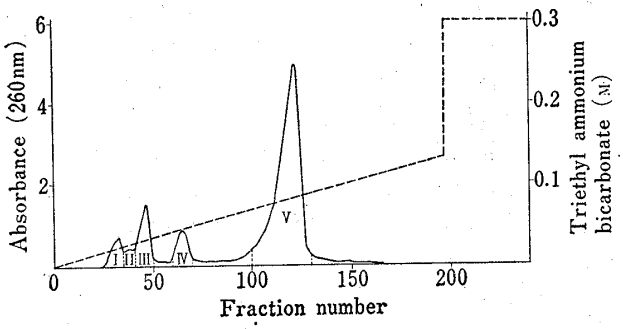


Fig. 1. Chromatography of the products in the synthesis of CpA(oNB)pA (**17**) on a column (1.7 × 50 cm) of DEAE-cellulose (HCO_3^-). Elution was carried out using a linear gradient of triethylammonium bicarbonate. (0—0.2M, total 6 liter). Fractions of 20 ml were collected every 19 min. Identification of compounds in each peak was summarized in Table III.

ApApApApApApA + *pCpCpA (*o*-nitrobenzyl)
↓ **19**
ApApApApApApA*pCpCpA (*o*-nitrobenzyl)
↓ **20**
*pApApApApApApA*pCpCpA (*o*-nitrobenzyl)
21

Chart 3

Conclusion

From these two reactions for the synthesis of **17** it seems that repulsion of charges of the phosphate dissociation might interrupt condensation reactions, since the triester intermediate (**15**) reacted more readily than **18**. Although it is dangerous to draw conclusions from very few examples, the present results are consistent with other experiments with oligonucleotides bearing different protecting groups.[20] Removal of the cyanoethyl groups on internucleotide phosphates may become difficult when the chain length increases. The synthesis of larger ribooligonucleotide with cyanoethyl protection in internucleotide linkages will be of interest.

o-Nitobenzyl derivatives of adenosine and cytidine have thus been shown to be useful intermediates for the synthesis of oligonucleotides. Together with the previous synthesis UpU and UpA using 2′-O-(*o*-nitrobenzyl)uridine,[8] it may be concluded that the deblocking conditions with UV light of wavelength longer than 280 nm are tolerable for pyrimidine containing oligonucleotides. Especially in the case of CpCpA synthesis, the CpC sequence would have been particularly susceptible to photochemical reactions.[11] Independent experiments using the four common nucleosides with irradiation under the same conditions for 5 hr showed no changes in the UV spectra. The present method provides 2′-substituted adenosine and cytidine. In the case of cytidine the 3′-substituted derivative was also obtained. The 3′-O-(*o*-nitrobenzyl)adenosine could probably be isolated after monomethoxytritylation of 5′-hydroxyl group.

20) J. Smrt, *Collect. Czech. Chem. Commun.*, **37**, 846 (1972).

The oligonucleotide with the terminal 2′-O-(*o*-nitrobenzyl)group (**12**) was shown to be recognized by RNA ligase[21] and joined with the 3′-hydroxyl of the oligoadenylate (A_7) after phosphorylation of the 5′-position by polynucleotide kinase[22] to give **20** (Chart 3). If the phosphorylated product (**21**) is not cyclized to yield the cyclic oligonucleotide by RNA ligase, the 2′-O-(*o*-nitrobenzyl)group can serve as a protecting group to prevent intramolecular reaction of the enzyme, which was found to be a favored reaction in oligonucleotides with chain lengths longer than eight.[23] If the 2′-substitution does not prohibit the cyclization reaction at the 3′-hydroxyl group, we propose to introduce the 3′-derivative for use at the terminus. The RNA ligase reaction using **17** has yet to be performed but the partially protected oligonucleotide may also be useful in studies on nuclease specificities. Joining of **17** and **19** would provide the starting 3′-terminal fragment for enzymatic joining of chemically synthesized fragments of *E. coli* $tRNA_f^{Met}$.

Experimental Section

General Methods——Paper chromatography was performed by the descending technique using solvent systems: A, isopropanol–concentrated ammonia–water (7: 1: 2, v/v), B, ethanol–1M ammonium acetate (pH 7.5) (7: 3, v/v); C, *n*-propanol–concentrated ammonia–water (55: 15: 35, v/v). Paper electrophoresis was performed using 0.05M triethylammonium bicarbonate (pH 7.5) at 900 V/40 cm. Photolysis apparatus had a 300 W high pressure mercury lamp (Eikosha Co. Model PIH 300) with a quartz water-circulationg jacket. Compounds were placed in a pyrex tube (1.5 mm thick) and irradiated through a pyrex filter (2 mm thick) inserted in the water jacket.

Molecular extinction values of nucleosides shown in Table I were measured with a Hitachi 124 spectrophotometer. Enzymatic hydrolyses and other general methods were as described previously.[24]

2′-O-(*o*-Nitrobenzyl)adenosine (2a)——Dried adenosine (20 mmoles) was dissolved in hot DMF (180 ml) and cooled to 0°. Sodium hydride (50% oil, 1.246 g, 26 mmoles, washed with dry benzene) was suspended in DMF and added under stirring. After 45 min stirring, *o*-nitrobenzyl bromide (6.48 g, 30 mmoles) in DMF (20 ml) was added to the emulsion at 0° and the pink colored solution was stirred for 3—4 hr at 0°. The solution was turned yellow during the reaction and was poured into ice water (2 liter) with stirring. The stirring was continued overnight and the precipitate was collected by filtration. The precipitate was washed with ethanol and recrystallization from ethanol-water (5: 3) (630 ml) gave 2.967 g (37%) of pure **2a**. mp>250° colored at 230°. *Anal.* Calcd. for $C_{17}H_{18}O_6N_6$ (402.36): C, 50.74; H, 4.51; N, 20.89. Found: C, 51.04; H, 4.45; N, 20.87. NMR (DMSO-d_6) δ 3.66 (m, 2H, $C_{5'}$–H_2), 4.05 (m, 1H, $C_{4'}$–H), 4.42 (m, 1H, $C_{3'}$–H), 4.58 (t, 1H, $J_{1'2'}$=6 Hz, $J_{2'3'}$=6 Hz, $C_{2'}$–H), 4.86 and 5.04 (ABq, 2H, J_{gem}=15 Hz, Ar–CH_2), 5.25—5.44 (m, 2H, $C_{5'}$–OH, $C_{3'}$–OH), 6.08 (d, 1H, $J_{1'2'}$=6 Hz, $C_{1'}$–H), 7.24 (brs, 2H, 6-NH_2), 7.3—7.98 (m, 4H, Ar), 8.04 (s, 1H, C_2–H or C_8–H), 8.30 (s, 1H, C_8–H or C_2–H); 6.08 became a singlet by irradiation at 4.58; 5.25—5.44 and 7.24 disappeared by addition of D_2O.

2′-O-(*o*-Nitrobenzyl)-3′,5′-O-diacetyladenosine——2′-O-(*o*-Nitrobenzyl)adenosine (50 mg, 0.125 mmole) was treated with acetic anhydride (0.4 ml) in pyridine (1.6 ml) at 0° for 4 hr. The completion of the reaction was checked by TLC ($CHCl_3$: EtOH=10: 1). The volatile materials were evaporated and the residue was coevaporated with aqueous ethanol. Recrystallization from ethanol gave 30 mg (50%): Mass Spectrum *m/e*: 486 M^+, mp 172—173° (168° wet); λ_{max} (95% EtOH) 260 nm (ε 19000), ε_{300} (95% EtOH, 1500). *Anal.* Calcd. for $C_{21}H_{22}O_8N_6$ (486.43): C, 51.85; H, 4.56; N, 17.28. Found: C, 51.80; H, 4.64, N, 17.08. NMR ($CDCl_3$) δ 2.10 and 2.13 (s, 6H, Ac), 4.44 (m, 3H, $C_{5'}$–H_2), 5.0 (m, 3H, Ar–CH_2, $C_{2'}$–H), 5.42 (dd, 1H, $C_{3'}$–H, $J_{2'3'}$=4 Hz), 6.08 (d, 1H, $C_{1'}$–H, $J_{1'2'}$=6 Hz), 6.20 (br s, 2H, NH_2), 7.3—8.3 (m, 6H, Ar, C_2–H, C_8–H). Decoupling: 5.42 (dd) became d with irradiation at 4.44 and 6.08 (d) became s with 5.0.

2′- and 3′-O-(*o*-Nitrobenzyl)cytidine Hydrochlorides——Dried cytidine (4.86 g, 20 mmoles) was dissolved in DMF (160 ml) and allowed to react with sodium hydride (50% oil, 1.26 g, 26 mmoles, washed with dry benzene) in DMF (40 ml) at 0° for 50—60 min under stirring. *o*-Nitrobenzyl bromide 6.48 g (30 mmoles) in DMF (40 ml) was added dropwise at 0° and stirring was continued for 4 hr. Water (a few drop) was added and the mixture was concentrated to an oil. The oil was dissolved in water (160 ml) and extracted with chloroform (20 ml, 2 portions). The aqueous solution was evaporated and the residue was dissolved in *n*-

21) E. Ohtsuka, A.F. Markham, S. Tanaka, T. Tanaka, T. Miyake, E. Nakagawa, S. Nishikawa, and M. Ikehara, *Nacleic Acids Res.*, (Special Publicatcn No. 2), S77 (1976).
22) C.C. Richardson, *Proc. Natl. Acad. Sci.*, USA, **54**, 158 (1965).
23) G. Kaufmann, T. Klein, and U.Z. Littauer, *FEBS Letters*, **46**, 271 (1974).
24) *a*) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, **92**, 3441 (1970); *b*) E. Ohtsuka, M. Ubasawa, S. Morioka, and M. Ikehara, *ibid.*, **95**, 4725 (1973).

butanol (160 ml). Cytidine was removed by extraction with water from the butanol solution. Both layers were checked for the presence of the product by TLC ($CHCl_3$: EtOH=2: 1) and the butanol phase was evaporated to a syrup. Ethanol (99%, 31 ml) and 1N HCl (19 ml) were added to the syrup. After refrigeration 1.204 g of the pure 2′-isomer crystallized. A second crop of the pure 2′-isomer was obtained by recrystallization of the evaporated mother liquor from ethanol (16 ml) and water (10 ml), 923 mg. The yield of the 2′-derivative was 5.15 mmoles (25.8%), mp 160—168°. The 3′-derivative was recrystallized from ethanol (26 ml) and water (4 ml), 290 mg, a second crop 187 mg, yield 1.15 mmoles 5.7%, mp 185—193°.

(a) **Characterization of the 2′-Derivative**——The sample was recrystallized from 95% ethanol mp 200—204°. *Anal.* Calcd. for $C_{16}H_{19}O_7N_4Cl$ (414.797): C, 46.33; H, 4.62; N, 13.22; Cl, 8.55. Found: C, 46.32; H, 4.36; N, 13.46; Cl, 8.80. NMR (DMSO-d_6) δ 3.73 (br s, 2H, $C_{5'}$–H), 3.9—4.28 (m, 3H, $C_{2'}$–H, $C_{3'}$–H, $C_{4'}$–H), 5.05 (s, 2H, Ar–CH_2), 5.87 (d, 1H, $J_{1'2'}$=3 Hz, $C_{1'}$–H), 6.18 (d, 1H, J_{56}=8 Hz, C_5–H), 7.4—8.1 (m, 4H, Ar), 8.31 (d, 1H, C_6–H), 8.79 (br s, 1H, N–H, disappeared with D_2O), 9.85 (br s, 1H, N–H, disappeared with D_2O). 3H (δ 3.5—6.0) disappeared with D_2O. For UV data, see Table I.

(b) **Characterization of the 3′-Derivative**——The sample was recrystallized from aqueous ethanol, mp 200—203° (wet 196°). *Anal.* Calcd. for $C_{16}H_{18}O_7N_4 \cdot HCl \cdot 1/2H_2O$(423.805): C, 45.34; H, 4.52; N, 13.22; Cl, 8.55. Found: C, 45.50; H, 4.79; N, 13.46; Cl, 8.40. NMR (DMSO-d_6) δ 3.68 (br s, 2H, $C_{5'}$–H_2), 4.06 (m, 2H, $C_{3'}$–H, $C_{4'}$–H), 4.33 (t, 1H, $J_{1'2'}$=4 Hz, $C_{2'}$–H), 4.95 and 5.03 (ABq, 2H, J_{gem}=14 Hz, Ar–CH_2), 5.70 (d, 1H, $J_{1'2'}$=4 Hz, $C_{1'}$–H), 6.15 (d, 1H, J_{56}=8 Hz, C_5–H), 7.4—8.10 (m, 4H, Ar), 8.28 (d, 1H, J_{56}=8 Hz, C_6–H), 8.80 and 9.88 (br s, 1H, NH, disappeared with D_2O). A doublet (5.70) and a triplet (4.33) were decoupled by irradiation at 4.33 and 5.70, respectively.

2′-O-(*o*-Nitrobenzyl)cytidine (2b)——The hydrochloride (133 mg, 0.32 mmole) was suspended in chloroform (7 ml) and stirred with triethylamine (0.1 ml, 0.7 mmole) for 24 hr at room temperature. Chloroform solutions were evaporated and recrystallization of the residue from water gave the product (85 mg), yield 70%, mp 234—235°. *Anal.* Calcd. for $C_{16}H_{18}O_7N_4$ (378.34): C, 50.79; H, 4.80; N, 14.81. Found: C, 50.81; H, 4.50; N, 14.57. NMR (DMSO-d_6) δ 3.67 (br s, 2H, $C_{5'}$–H_2), 3.94 (m, 2H, $C_{2'}$–H, $C_{4'}$–H), 4.10 (m, 1H, $C_{3'}$–H), 4.90—5.29 (m, 4H, Ar–CH_2, C_5–OH, $C_{3'}$–OH), 5.71 (d, 1H, J_{56}=7 Hz, C_5–H), 5.95 (d, 1H, $J_{1'2'}$=3 Hz, $C_{1'}$–H), 7.11 (br s, 2H, 4-NH_2), 7.38—8.10 (m, 5H, Ar, C_6–H); 5.95 became s by irradiation at 3.94.

Deamination of 2b——**2b** (250 mg, 0.6 mmole) was treated with sodium nitrite (1.64 g, 23.8 mmoles) in 30% acetic acid (8 ml) for 5 hr. TLC ($CHCl_3$: EtOH=4: 1) showed a faster travelling compound (*Rf* 0.14). The solution was kept at 4° overnight and the crystallized product was collected. 176 mg (78%) mp 204—205°. mp 203—205°.

2′-O-(*o*-Nitrobenzyl)-N,3′,5′-O-triacetylcytidine——**2b** (24 mg) was treated with acetic anhydride (0.4 ml) in pyridine (0.8 ml) at room temperature for 20 hr and the product was isolated as described for the synthesis of 2′-O-(*o*-nitrobenzyl)-3′,5′-diacetyladenosine. UV λ_{max} nm (95% EtOH) 252 and 298. NMR ($CDCl_3$) δ 2.09 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.29 (s, 3H, Ac), 4.30—4.65 (m, 4H, $C_{5'}$–H_2, $C_{4'}$–H, $C_{2'}$–H), 4.90 (q, 1H, $C_{3'}$–H), 5.07 and 5.25 (ABq, 2H, J_{gem}=14 Hz, Ar–CH_2), 6.01 (d, 1H, $C_{1'}$–H, $J_{1'2'}$=1 Hz), 7.2—8.1 (m, 7H, Ar, C_5–H, C_6–H, NH). The signal at 6.01 collapsed to a singlet by irradiation at 4.40 but did not change by irradiation at 4.90. Irradiation at 4.40 also caused collapse of the signal at 4.90 to a doublet.

5′-O-Monomethoxytrityl-2′-O-(*o*-nitrobenzyl)cytidine and the 3′-Isomer——5′-O-Monomethoxytrityl-cytidine (2.58 g, 5 mmoles) was dissolved in DMF (40 ml) and NaH (311 mg, 0.5 mmole, 50% oil, washed with benzene) in DMF (5 ml) was added at 0° with stirring. After evolution of hydrogen gas ceased (*ca.* 45 min), *o*-nitrobenzylbromide (1.4 g, 6.5 mmoles) was added at 0° with stirring. The extent of the reaction was checked by TLC ($CHCl_3$: EtOH=15: 2, the 2′-isomer, 0.34; the 3′-isomer, 0.29; the starting material, 0.11). The mixture was poured into water (500 ml) when the starting material did not decrease (*ca.* 3 hr) and stirred overnight at 0°. The precipitate was collected by filtration and 2 g out of 2.963 g of the product was applied to a column (3.1×28.5 cm) of silica gel (Mallincrodt, pH 4, 100 mesh). Elution was carried out using a linear gradient of chloroform (1 liter) and chloroform containing 12% ethanol (1 liter). Fractions of 10 ml were collected every 15 min. The 2′- and 3′-isomers were eluted in fraction 66—76 and 78—87. The yields were 450 mg and 133 mg respectively. The unchanged starting material was recovered (366 mg). The products were characterized by NMR spectra after detritylation.

N,N,3′-O-Tribenzoyl-2′-O-(*o*-nitrobenzyl)-5′-O-monomethoxytrityladenosine (4)——**2a** (402 mg, 1 mmole) was suspended in DMF (5.5 ml) and treated with monomethoxytrityl chloride (1.2 mmoles) at 27° under stirring. After 1 hr an homogeneous solution was obtained. Since the starting material was still detected after 3 days in TLC ($CHCl_3$: MeOH=10: 1), monomethoxytrityl chloride (0.2 mmole) was added and the mixture was kept for 2 further days. The solution was added to ice-water containing 5% NH_3 (100 ml) and the monomethoxytritylated compound was separated from the starting material (**2a**) by extraction of the precipitate with $CHCl_3$. The chloroform solution was washed with water and evaporated with benzene. The residue was dissolved in a small amount of benzene. and added to cyclohexane (100 ml) to remove monomethoxytritanol. The precipitate (376 mg), which had spectral properties of λ_{max}^{MeOH} nm 233, 259 and $A_{233/259}$=1.06, was coevaporated with pyridine 3 times and treated with benzoyl chloride (0.79 ml, 6.72 mmoles) in pyridine (4.6 ml) at 25—27° overnight. The mixture showed *Rf* 0.93 (no spot at 0.23) as checked by TLC ($CHCl_3$: EtOH=20: 1) and was stirred into ice water (16 ml). The product was extracted

with $CHCl_3$, washed with 5% $NaHCO_3$ and with water 3 times, respectively. The $CHCl_3$ solution was dried with Na_2SO_4 and evaporated. The residue was used for the next reaction without purification.

N,N,3′-O-Tribenzoyl-2′-O-(*o*-nitrobenzyl)adenosine (5)——The syrup (**4**) was evaporated with toluene to remove residual pyridine azeotropically and treated with 80% acetic acid (4 ml) at 30° for 6 hr. The detritylation was checked by TLC and the volatile materials were removed. The residue was coevaporated with aqueous *n*-butanol, dissolved in $CHCl_3$, washed with 5% $NaHCO_3$ (10 ml) and water 3 times respectively. The solution was dried with Na_2SO_4 and the product was precipitated with 2:3 *n*-pentane–cyclohexane (50 ml) from its solution in benzene. The yield was 301 mg (0.42 mmole), 42% from **2a**. The spectral properties were λ_{max} (95% EtOH) nm (232, 254, 275 (sh) and $A_{232/275}=1.41$.

N,2′-O-Dibenzoylcytidylyl-(3′—5′)-N,N,3′-O-tribenzoyl-2′-O-(*o*-nitrobenzoyl)adenosine (10)——Pyridinium 5′-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate (**9**) (3000 A_{305}, 0.273 mmole) was dissolved in 50% aqueous pyridine, passed through a small column (4 ml) of pyridinium Dowex 50×2 and the column was washed with 50% pyridine. The eluent and washings were rendered anhydrous by evaporation with pyridine together with **5** (290 mg, 0.405 mmole). The mixture was treated with DCC (720 mg, 3.5 mmoles) in pyridine (2 ml) for 2 days at 25°. An aliquot was checked for the extent of the reaction by paper electrophoresis after treatment with aqueous pyridine then methanolic ammonia. 5′-O-Monomethoxytritylcytidine 3′-phosphate disappeared mostly at this stage and after 4 days reaction 50% pyridine (10 ml) was added to the reaction mixture. Pyridine was added to obtain a homogeneous solution and DCC was removed with *n*-pentane (10 ml) 3 times. The aqueous solution was kept at room temperature overnight and the filtered solution was evaporated with toluene to remove the last traces of pyridine. The residue was treated with 80% acetic acid for 2.5 hr. Removal of the monomethoxytrityl could be checked by TLC and the product (**10**) was precipitated with ether (150 ml) from its solution in anhydrous pyridine after coevaporation with *n*-butanol. The nucleoside (**5**) was removed by this precipitation as checked by TLC but **10** was contaminated with the pyrophosphate of the mononucleotide. The precipitate was dissolved in *n*-butanol (40 ml) saturated with water and washed with water (6 ml) 3 times until nothing travelled from the origin in paper electrophoresis. The organic solution was evaporated and the residue was coevaporated with pyridine. **10** was precipitated with 3:2 ether–*n*-pentane (130 ml) from its solution in pyridine. The yield was 291 mg, 2944 A_{305}, 0.226 mmole using ε_{305} (95% EtOH) of 13000 (83%). Spectral properties of **10** were λ_{max} (95% EtOH) nm 230, 258, 305 (sh), $A_{230/258}=1.2$, $A_{258/305}=3.42$.

N,2′-O-Dibenzoylcytidylyl-(3′-5′)-N,2′-O-dibenzoylcytidylyl(3′-5′)-N,N,2′-O-tribenzoyl(*o*-nitrobenzyl)-adenosine (11)——The pyridinium salts of **9** (0.33 mmole) and **10** (0.22 mmole) were passed through a small column of pyridinium Dowex 50×2 as described above. The mixture was rendered anhydrous by coevaporation of pyridine and allowed to react with DCC (4.4 mmoles) in anhydrous pyridine (3 ml) for 2 days at 24°. The extent of the reaction was checked by paper chromatography after deblocking. Aqueous pyridine treatment (overnight) was given after 7 days and DCC were removed. The nucleotides were precipitated with 3:2 ether–pentane (300 ml) from their solution in anhydrous pyridine and the precipitate was dissolved in a small amount of CH_2Cl_2 after coevaporation with toluene. The solution was treated with 80% acetic acid (20 ml) for 2.5 hr and evaporated with aqueous *n*-butanol 3 times. The nucleotides were precipitated with 3:2 ether–pentane (300 ml) from their solution in pyridine, dissolved in pyridine (10 ml), diluted with 95% EtOH (500 ml) and applied to a column (3×48 cm) of triethylaminoethyl (TEAE)-cellulose (acetate). Elution was carried out using a linear gradient of triethylammonium acetate (0—0.225M) in 95% EtOH (6 liter). The product (**11**) was eluted with 0.155—0.2M salt but the last part of the peak was contaminated with a pyrophosphate of the mononucleotide which was removed by extraction with 50% pyridine from the solution in CH_2Cl_2. The yield of pure **11** was 1024 A_{305}, 0.045 mmole, 20% and the spectral properties of **11** were: λ_{max} (95% EtOH) nm 230, 260, 305 (sh), $A_{260/280}=1.66$, $A_{260/305}=3$.

Cytidylyl-(3′-5′)-cytidylyl-(3′-5′)-2′-O-(*o*-nitrobenzyl)adenosine (12)——**11** was treated with methanolic ammonia for 20 hr at 25° and **12** was isolated by paper chromatography in solvent A. The spectral properties of **12** were: $\lambda_{max}^{H_2O}$ nm 263, $\lambda_{max}^{H^+}$ 269 and ε_{260} was estimated as 30550. An aliquot of **12** (3.5 A_{260} in 1 ml of H_2O) was deblocked by irradiation of UV for 2 hr and 95% of **12** was found to be converted to CpCpA as checked by the same chromatographic system using a calculated ε value of 28050 for CpCpA.[25] The deblocked product was characterized by hydrolysis with RNase A. The completely degraded products were separated by paper electrophoresis, eluted with 0.01N HCl, measured at 280 nm and the ratio of Cp to A was found to be 2.04 to 1.00.

2′-O-(*o*-Nitrobenzyl)-N-benzoyladenosine (7)——**2a** (1.2 g, *ca.* 3 mmoles) was suspended in anhydrous pyridine and treated with benzoyl chloride (2.1 ml) at 0° for 2 hr then at room temperature overnight. The reaction was cheked by TLC ($CHCl_3$: EtOH=5:1) and **2a** (*Rf* 0.09) was found to be converted to a faster travelling compound (*Rf* 0.93). The mixture was stirred with ice water (3 ml), added to ice water containing $NaHCO_3$ (100 ml), extracted with $CHCl_3$ (50 ml) 3 times, washed with 5% $NaHCO_3$ then with water and the organic solution was dried with Na_2SO_4. The benzoylated product (**6**) thus obtained was used for the next reaction to yield **7**. The chloroform was evaporated and the residue was dissolved in 99% EtOH

25) C.R. Cantor and I. Tinoco, Jr., *Biopolymers*, **5**, 821 (1967).

(15 ml)–pyridine (8 ml). The solution was stirred with 2N NaOH (16 ml), made homogeneous by addition of 99% EtOH (32 ml)–pyridine (20 ml), kept at room temperature for 7 min then at 0° for 15 min. Sodium ions were removed by addition of an excess of pyridinium Dowex 50×8 and the solution was passed through a column (30 ml) of the same resin. TLC ($CHCl_3$: EtOH=10: 1) showed a single spot (*Rf* 0.10). The eluate and washings were concentrated and the residue was washed with ether, then dissolved in $CHCl_3$, washed with $NaHCO_3$ and with water. The solution was dried with Na_2SO_4 and the product **7** was precipitated with cyclohexane (200 ml)–pentane (50 ml) from its solution in CH_2Cl_2. The yield was 963 mg (64%). λ_{max} (95% EtOH) nm 280 and $\lambda_{max}^{OH^-}$ 313. NMR (DMSO-d_6) δ 3.7 (br s, 2H, $C_{5'}$–H), 4.11 (m, 1H, $C_{4'}$–H), 4.49 (m, 1H, $C_{3'}$–H), 4.70 (t, 1H, $J_{1'2'}=5$ Hz, $J_{2'3'}=5$ Hz, $H_{2'}$–H), 4.96 and 5.10 (ABq, 2H, $J_{gem}=12.6$ Hz, Ar–CH_2), 6.28 (d, 1H, $J_{1'2'}=5$ Hz, $C_{1'}$–H), 7.3—8.2 (m, 9H, Ar), 8.73 (s, 2H, C_8– and C_2–H), 11.13 (br s, 1H, N–H, disappeared with D_2O). A doublet (6.28) became a singlet by irradiation at 4.70.

2′-O-(*o*-Nitrobenzyl)-5′-O-monomethoxytrityl-N-benzoyladenosine (8)——**7** (506 mg, 1 mmole) was rendered anhydrous by evaporation of pyridine twice and treated with monomethoxytrityl chloride (370 mg, 1.2 mmoles) in pyridine (12 ml) for 20 hr at room temperature. Completion of the reaction was cheked by TLC ($CHCl_3$: EtOH=10: 1) (*Rf* 0.68) and 99% EtOH (2 ml) was added. After 45 min the mixture was poured into ice water (60 ml), extracted with $CHCl_3$, washed with 0.1M NH_4HCO_3 then with water 2 times. The solution was evaporated after drying with Na_2SO_4 and the residue was coevaporated with toluene 3 times. The product was precipitated with cyclohexane (60 ml)–*n*-pentane (60 ml) from its solution in benzene. The yield was 594 mg, 76%. λ_{max} nm (95% EtOH) 230 and 279. NMR (DMSO-d_6) δ 3.35 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3), 4.22 (m, 1H, $C_{4'}$–H), 4.58 (m, 1H, $C_{3'}$–H), 4.82 (m, 1H, $C_{2'}$–H), 5.02 (d, 2H, Ar–CH_2), 5.49 (d, 1H, $C_{3'}$–OH, disappeared with D_2O), 6.27 (d, 1H, $J_{1'2'}=4$ Hz, $C_{1'}$–H), 6.6—8.1 (m, 23H, Ar), 8.54 and 8.56 (s, 1H, C_8–H or C_2–H), 11.07 (s, 1H, 6-NH, disappeared with D_2O).

5′-O-Monomethoxytrityl-N-benzoyl-2′-O-(*o*-nitrobenzyl) adenylyl (3′-5′)-N,2′,3′-O-benzoyladenosine (14)——The pyridinium salt of **13**[26] (260 mg, 6400 A_{275}, 0.36 mmole) was passed through a column (*ca.* 3 ml) of pyridinium Dowex 50×8, rendered anhydrous by evaporation with pyridine and allowed to react with **8** (365 mg, 0.468 mmoles) using DCC (741 mg, 3.6 mmoles) in pyridine (2 ml) for 3 days at 27°. An aliquot was deblocked and the reaction extent was checked by paper electrophoresis. After aqueous pyridine treatment (overnight) the filtered solution was concentrated and the residue precipitated with ether from its solution in pyridine. The contaminating nucleoside in the product (**14**) was removed by reprecipitation with ether (160 ml). The yield was 362 mg, 9900 A_{275}, 0.252 mmole using an additive ε_{275} value of the component: $A^{Bz}2$, 17800; A^{Bz}, 17000; *o*-nitrobenzyl alcohol, 4540. The product was characterized by hydrolysis with RNase M[27] after deblocking. The hydrolysed products, Ap (0.675 A_{260}) and A (0.645 A_{260}) were separated by paper electrophoresis and the ratio was found to be 1.05 to 1.00.

N-Benzoyl-2′-O-(*o*-nitrobenzyl) adenylyl-(3′-5′)-N,2′,3′-benzoyladenosine [P-(β-cyanoethyl) Ester] (15)——**14** (4950 A_{275}, *ca.* 0.126 mmole) was allowed to react with TPS (151 mg, 0.496 mmole) in pyridine (1.5 ml) for 30 min and β-cyanoethanol (0.1 ml, 1.48 mmoles) was added. After 24 hr at 27°, TLC (CH_2Cl_2: MeOH=15: 1) showed two trityl positive spots and no starting material was detected at the origin. These two compounds gave an identical product by methanolic ammonia treatment. The reaction mixture was treated with aqueous pyridine (15 ml) for 30 min and the products were extracted with CH_2Cl_2 (15 ml) 3 times. The organic phase was washed with water 3 times, dried with Na_2SO_4, evaporated, coevaporated with added toluene 3 times and the residue was applied to preparative TLC (CH_2Cl_2: MeOH=15: 1). Each band was eluted with 10: 1 CH_2Cl_2–MeOH in a short column and treated with 80% acetic acid for 4 hr. The detritylated products, which were identified was **15a** (*Rf* 0.32) and **15b** (*Rf* 0.51) were subjected to preparative TLC (CH_2Cl_2: MeOH=10: 1) and precipitated with 3: 2 ether–pentane (100 ml) from their solution in CH_2Cl_2. The yield of **15a** was 860 A_{279}, using a sum of ε_{279} values of A^{Bz} (18300) and *o*-nitrobenzyl (3870), 0.021 mmole. The spectral properties of **15a** were λ_{max} (95% EtOH) nm 230, 279, λ_{min} 255, $A_{279/255}=1.41$, $A_{230/279}=1.28$. The amount of **15b** was 1240 A_{277}, 0.031 mmole calculated from a sum of ε_{277} values of $A^{Bz}2$ (17800), A^{Bz} (17900) and *o*-nitrobenzyl (4200). The spectral properties of **15b** were λ_{max} (95% EtOH) nm 230, 277, λ_{min} 265, $A_{277/265}=1.24$, $A_{230/277}=1.42$. The combined yield of **15a** and **15b** from **14** was 41%.

Cytidylyl-(3′-5′)-2′-O-(*o*-nitrobenzyl)adenylyl-(3′-5′)-adenosine (17)——i) Using **15**: A mixture of **15a** (0.021 mmole) and **15b** (0.031 mmole) was condensed with pyridinium N,2′,5′-O-tribenzoylcytidine 3′-phosphate (**16**) (640 A_{305}, 0.064 mmole) using DCC (136 mg) in pyridine (1 ml) at 27° for 3 days. An aliquot was deblocked for analysis and the reaction mixture was treated with aqueous pyridine overnight after 7 days. DCC and dicyclohexylurea were removed as described for the synthesis of **10**. The product, dissolved in a small amount of pyridine, was added to methanol (4 ml), treated with concentrated ammonia (1 ml) at 0° for 5 min, evaporated to a gum, coevaporated with pyridine and treated with methanolic ammonia for 23 hr at 30°. The volatile materials were removed and the residue was applied to a column of DEAE-cellulose. The elution pattern and identification of the products are shown in Fig. 1 and Table III. The product (**17**) (1247 A_{260}, 0.036 mmole calculated from ε_{260} of 40500 for **17**) was isolated in a yield of 59% from **15**. The

26) E. Ohtsuka, S. Nakamura, M. Yoneda, and M. Ikehara, *Nucleic Acids Res.*, **1**, 323 (1974).
27) M. Irie, *J. Biochem.*, **62**, 509 (1967).

spectral preperties of **17** were $\lambda_{max}^{H_2O}$ nm 260, $A_{280/260}=0.42$; $\lambda_{max}^{H^+}$ 260, $A_{280/260}=0.59$. Hydrolysis of **17** with venom phospho diesterase gave C (0.6 A_{271}), pA (oNB) (1.17 A_{260}), pA (1.10 A_{259}) in a ratio of 1.00: 0.97: 1.08. The product was further characterized by enzymatic hydrolyses after conversion of **17** to CpApA by irradiation with UV light for 2 hr. Cp (0.51 A_{271}) and ApA (1.41 A_{257}) obtained by RNase A hydrolysis in a ratio of 1.00 to 1.11 were separated by paper electrophoresis, and estimated in 0.01N HCl. ApA was further digested with RNase M to give Ap (0.638 A_{259}) and A (0.615 A_{259}) in a ratio of 1.04 to 1.00. Venom phosphodiesterase hydrolysis of CpApA gave C (0.525 A_{271}) and pA (1.82 A_{259}) in a ratio of 1.00 to 2.00.

ii) Using **18**: **18** was prepared by hydrolysis of **14** with 80% acetic acid for 2 hr. **18** (4800 A_{275}, 0.12 mmole) and **16** (1550 A_{305}, 0.155 mmole) were passed through a column of pyridinium Dowex-50×8. The nucleotides were rendered anhydrous by evaporation with pyridine and treated with DCC (319 mg, 1.55 mmoles) at 27° for 7 days. The reaction was stopped after being checked by deblocking of an aliquot. DCC and dicyclohexyl urea were removed as described for the synthesis of **10**. The nucleotides were made anhydrous by coevaporation of pyridine and treated with methanolic ammonia (40 ml) at 30° for 23 hr. The volatile materials were removed, the residue was dissolved in water (100 ml) and applied to a column (1.7×50 cm) of DEAE-cellulose. The conditions for elution were the same as described in Fig. 1 and **17** (1753 A_{260}) was obtained in a yield of 36%. The product was characterized by the same procedures as described above.

Acknowledgement This work was supported by a Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture.