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Studies on Transfer Ribonucleic Acid and Related Compounds. XIII. Synthesis of Trinucleoside Diphosphates, A-U-A and εA-U-A *via* a Triester Intermediate[1]

Eiko Ohtsuka, Toshiki Tanaka, and Morio Ikehara

Faculty of Pharmaceutical Sciences, Osaka University[2]

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ApUpA (XI) and its 1,N^6-ethenoadenosine analog, εApUpA (XII) were synthesized by condensation of the protected mononucleotides, 5′-O-monomethoxytrityl-N,2′-O-dibenzoyladenosine 3′-phosphate (VIII) or 5′-O-monomethoxytrityl-2′-O-benzoyl-1,N^6-ethenoadenosine 3′-phosphate (IX) with the triester, 2′-O-benzoyluridylyl-(3′-5′)-N,N-2′,-3′-O-tetrabenzoyladenosine (VI), in yields of 29% and 24%, respectively. The triester (VI) was prepared by two appraoches. Best results were obtained by condensation of 5′-O-monomethoxytrityl-2′-O-benzoyluridine 3′-phosphate (I) with N,N-2′,3′-O-tetrabenzoyladenosine (III) in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride and subsequent treatment with β-cyanoethanol. Deprotected trinucleoside diphosphates, ApUpA (XI) and εApUpA (XII), were isolated by chromatography on diethylaminoethyl cellulose and the fluorescence quenching of εApUpA was observed by measurement of the spectra before and after enzymatic digestion of the trimer.

Synthesis of short ribooligonucleotides by stepwise condensation has been investigated by several workers.[3–6] Trinucleoside diphosphates from the four major nucleotides were synthesized *via* phosphodiester intermediates using monomethoxytrityl for protecting the 5′-hydroxyl, and acyl groups for protecting the 2′-hydroxyl and heterocyclic amino groups.[3a] Phosphotriesters of oligonucleotides having β-cyanoethyl[7] or β,β,β-trichloroethyl[5] groups have been used for the elongation of chain and were found to be more stable intermediates against condensing reagents. Although stepwise condensation of mononucleotides *via* diester intermediates has afforded a pentanucleotide,[6] it was thought that triesters bearing same protecting groups as the diester intermediate could be used for the synthesis of some trinucleoside diphosphates. In this paper ApUpA[8] and its 1,N^6-ethenoadenosine-containing analog, εApUpA, were synthesized using a triester intermediate 2′-O-benzoyluridylyl-(3′—5′)-N,N-2′,3′-O-tetrabenzoyladenosine [p^1-(β-cyanoethyl)ester], U(OBz)-p(CE)-A^{Bz}(OBz)$_2$ (VI). The 1,N^6-ethenoadenosine containing dinucleoside monophosphates were found to be subject to extensive quenching of the fluorescence.[9] Some tRNA's contain fluorescent bases, *e.g.* Y, and these bases were used as fluorescent probes of the molecule.[10] It was thought that some information about the unstacking of ApUpA could be obtained from εApUpA.[11] Tolman,

1) Part XII: E. Ohtsuka, T. Miyake, and M. Ikehara, *Nucleic Acids Res.*, 3, 653 (1970).
2) Location: *1–133 Yamadakami, Suita, Osaka, 565 Japan.*
3) *a*) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H.G. Khorana, *J. Am. Chem. Soc.*, **88**, 819 (1966); *b*) J. Smrt, *Collect. Czechion. Chem. Commun.*, **33**, 1462 (1968); *c*) B.E. Griffin and C.B. Reese, *Tetrahedron*, **24**, 2537 (1968).
4) E. Ohtsuka, H. Tagawa, and M. Ikehara, *Chem. Pharm. Bull.* (Tokyo), **19**, 139 (1971).
5) T. Neilson and E.S. Werstiuk, *Can. J. Chem.*, **49**, 3004 (1971).
6) E. Ohtsuka, K. Fujiyama, M. Ohashi, and M. Ikehara, *Chem. Pharm. Bull.* (Tokyo), **24**, 570 (1970).
7) J. Smrt. *Collection. Czech. Chem. Commun.*, **38**, 3189 (1973).
8) Abbreviations are principally as have been suggested by IUPAC-IUB commission, see *Eur. J. Biochem.*, **15**, 203 (1970). εA refers to 1,N^6-ethenoadenosine. For protected nucleotides, see ref. 13 and 14.
9) G.L. Tolman, J.R. Barrio, and N.J. Leonard, *Biochem.*, **13**, 4869 (1974).
10) *a*) K. Beardsley and C.R. Cantor, *Proc. Nat. Acad. Sci. U.S.A.*, **65**, 39 (1970); *b*) C. Yang and D. Söll, *ibid.*, **71**, 2838 (1974).
11) D.M. Gray, I. Tinoco, Jr. and M.J. Chamberlin, *Biopolymers*, **11**, 1235 (1972).

et al. introduced the fluorescent 1,N^6-ethenogroup into preformed dinucleoside phosphates.[9] The present approach involved chemical condensation of the protected fluorescent nucleotide to yield the trinucleoside diphosphate. This synthetic method can be applied to syntheses of oligonucleotides with 1,N^6-ethenoadenosine in desired positions.

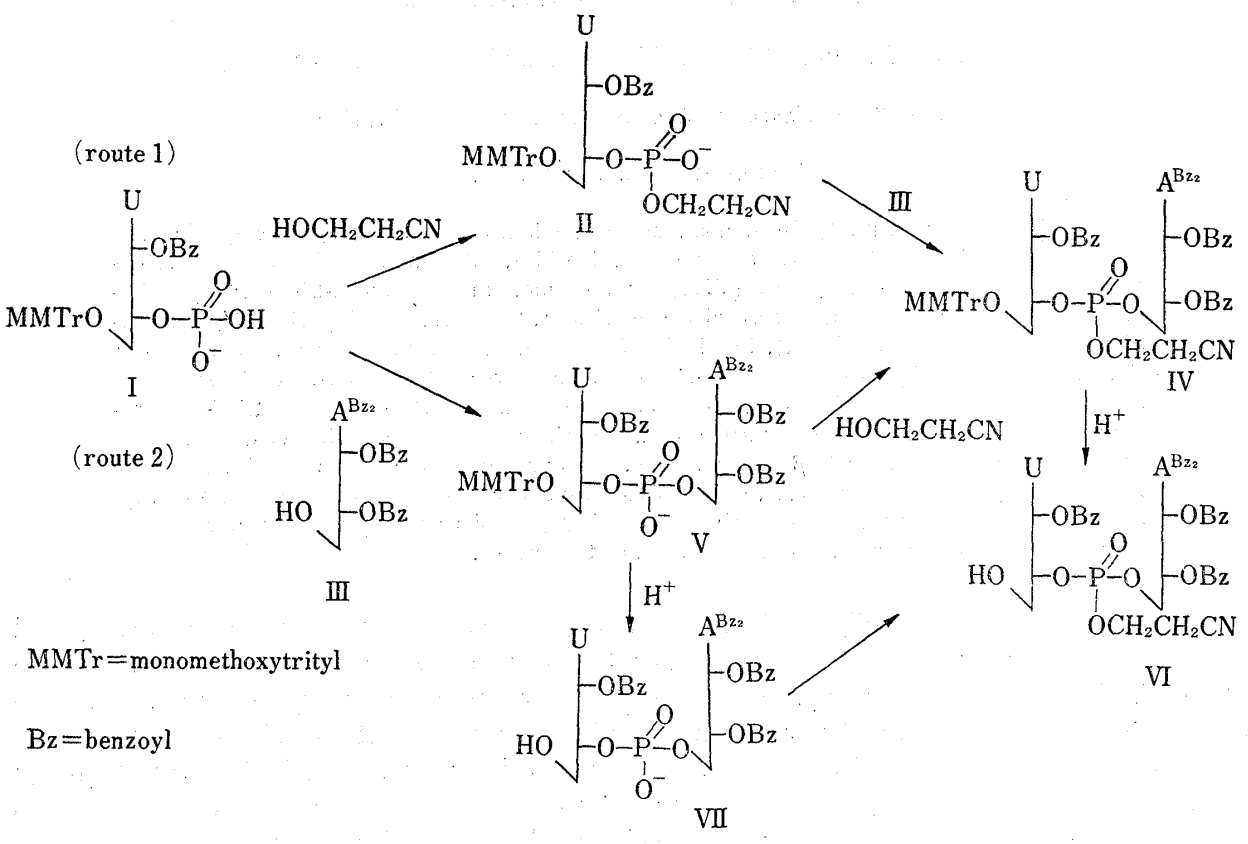

Chart 1

Synthesis of U(OBz)-p(CE)-$A^{Bz2}$$(OBz)_2$ (VI)

As shown in Chart 1 the triester (VI) was synthesized by two different routes. The first approach (route 1) involved a condensation of the nucleoside (III) with the diester (II) which was prepared from the mononucleotide (I) and β-cyanoethanol. DCC (dicyclohexylcarbodiimide) and TPS (2,4,6-triisopropylbenzenesulfonyl chloride) were used for the syntheses of II and IV, respectively. The triester (IV) was isolated by preparative thin-layer chromatography (TLC) in a yield of 30%. A side product which traveled faster than IV was detected in TLC and it gave adenosine and a derivative of uridine 3'-phosphate after deblocking. Since this approach was not satisfactory, the mononucleotide (I) was first condensed with the nucleoside (III) (route 2). TPS was used as the condensing reagent and the intermediate (V) was further treated with β-cyanoethanol without isolation. The reaction was stopped when no tritylated compounds were detected at the origin in TLC. The product (IV) was isolated by preparative TLC and further purified after removal of the monomethoxytrityl group. The overall yield of the detritylated triester (VI) was 46%. An attempted synthesis of VI by condensation of the detritylated diester (VII) with β-cyanoethanol and TPS was unsuccessful. It seems that the 5'-hydroxyl group can react with activated intermediates even in the presence of a large excess of the smaller alcohol. The yield of the detritylated diester (VII) after condensation of I and III with DCC, detritylation and extraction was slightly higher than that of VI. It may be concluded that extraction of the acylated diesters is the simplest method for the synthesis of ribodinucleoside monophosphates which have usually been isolated by chromatography on TEAE (triethylamimoethyl)-cellulose.

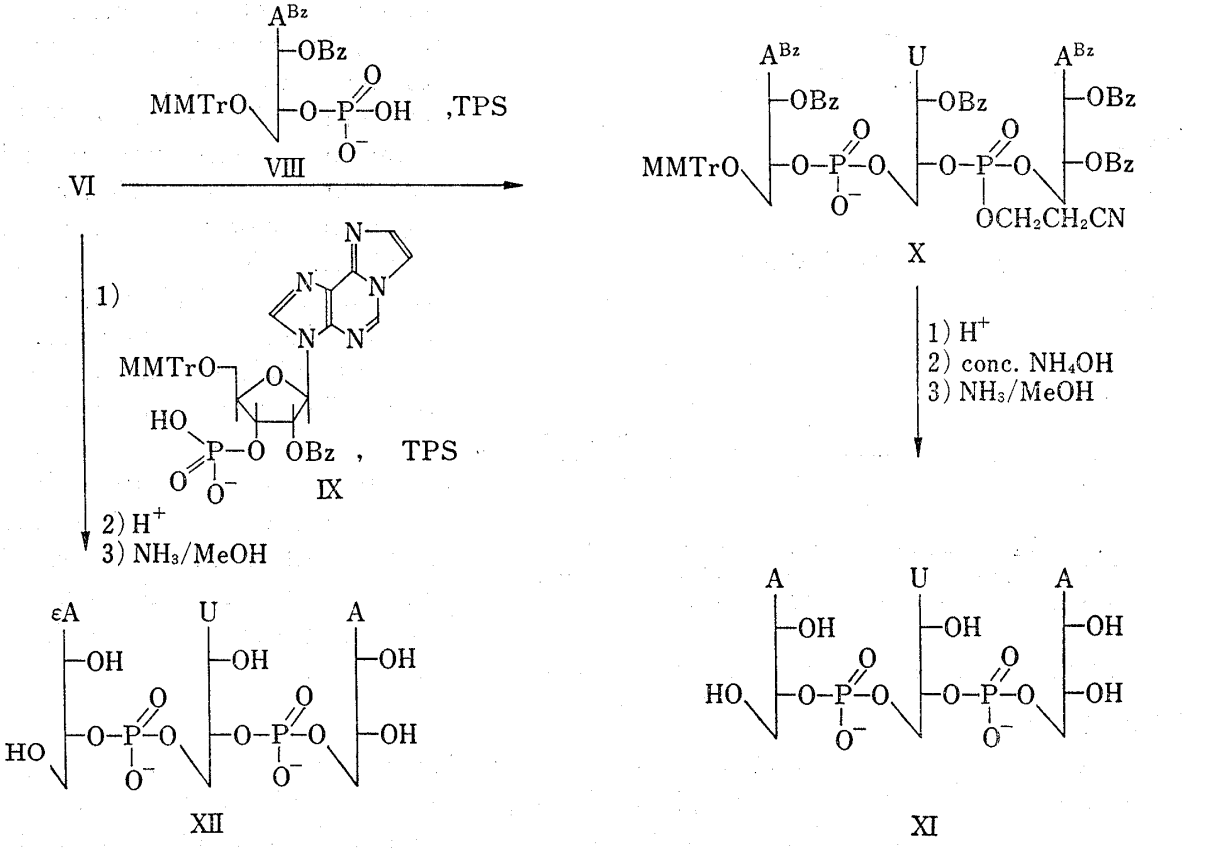

Chart 2

Synthesis of ApUpA (XI) and εApUpA (XII)

MMTr-A^{Bz}(OBz)-p (VIII) or MMTr-εA(OBz)-p (IX) was allowed to react with the triester (VI) using TPS as the condensing reagent for 5 or 6 hr at 10°. The trinucleoside diphosphate (*e.g.* X) was detritylated, then treated with ammonium hydroxide to remove the cyanoethyl group. Alternatively removal of the cyanoethyl group could be effected at the same time as debezoylation by the use of methanolic ammonia. The deprotected mixtures were subjected to chromatography on DEAE-cellulose column using the volatile buffer TEAB (triethylammonium bicarbonate). The peaks containing the two respective products were identified and the products isolated. The elution pattern for the synthesis of εApUpA is shown in Fig. 1. Judging from the chromatographic profile, degradation compounds such as εApUp were not formed during condensation. The yield was 24%. The product was characterized by hydrolysis with RNase M and snake venom phosphodiesterase. RNase M, however, hydrolyzed εApUpA (XII) incompletely under conditions in which ApUpA (XI) was completely degraded. Fluorescent spectra of εApUpA (XII) and its components are shown in Fig. 2. The increase

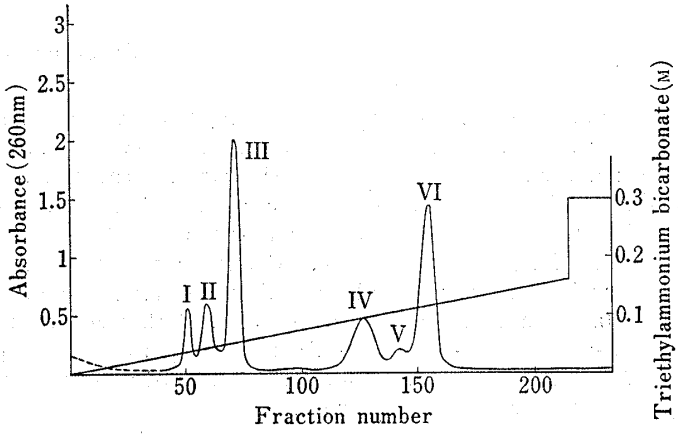

Fig. 1. Chromatography of the Product in the Synthesis of εApUpA on a Column (1.7×49 cm) of DEAE-cellulose

Elution was carried out using a linear gradient of triethylammonium bicarbonate (0—0.2 M). The total volume was 4 liter. 15 ml fractions were collected every 10 min.

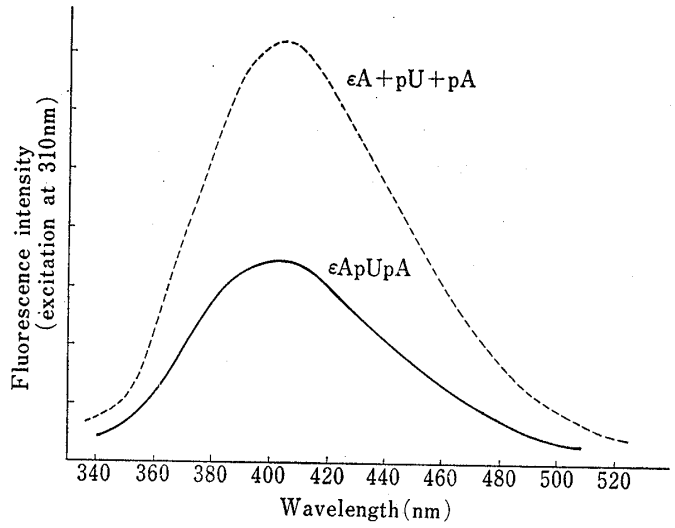

Fig. 2. The Fluorescence Emission Spectra of the Trimer εApUpA before (———) and after (·····) Venom Phosphodiesterase Hydrolysis

of fluorescent intensity after digestion of the trimer with venom phosphodiesterase was not as large as in the case of the dimer εApU.[9] This may mean that the structural properties of εApU are more favorable for quenching of the fluorescence than those of εApUpA. RNase A digestion of εApUpA would yield further decrease of the fluorescent intensity. The observed difference in the quenching of the fluorescence of εApUpA and εApU was expected on the basis of the previous finding from CD data that ApUpA was not as stacked as ApU.[11] Although 1,N^6-ethenoadenosine may not give the same backbone structure as adenosine, the present results also suggest that ApUpA is less stacked than ApU.

General Comments

The triester intermediate (VI) seems to have similar reactivity to the diester (VII) and the yields of the trinucleoside diphosphates (XI and XII) were not higher than those obtained in the diester method.[6] Since chain cleavages at the triester site were not detected in the present condensations, longer chains bearing β-cyanoethyl esters should be superior reactants for condensations using TPS. Esterification with β-cyanoethanol after formation of the internucleotide linkage was found to be preferable to the condensation of cyanoethylnucleotides with a nucleoside 5′-hydroxyl group. This observation is consistent with previous results of condensations involving derivatives with 2′-O-tetrahydropyranyl protecting groups.[12] One of the problems which might arise from this type of condensation would be the isolation of products from starting materials, when chain lengths become long. Condensation of triester intermediates of type VI with oligonucleotide blocks will be published shortly and the elongation of oligonucleotide chains in stepwise fashion with repeated accompanying cyanoethylation, is under investigation.

Experimental

General Methods——Paper chromatography was performed by the descending technique in the following solvents: A, 2-propanol–concentrated ammonia–water (7: 1: 2, v/v); B, ethanol–1 M ammonium acetate, pH 7.5 (7: 3, v/v). Paper electrophoresis was performed at 900 V/40 cm using 0.05 M triethylammonium bicarbonate. Fluorescent spectra were measured by Hitachi Spectrofluorometer MPE-2. Venom phosphodiesterase was phurchased from Worthington Biochemical Co. and RNase M was donated by Prof. M. Irie of Hoshi Pharmaceutical College. For enzymatic digests, oligonucleotides (*ca.* 3 A_{260}) were incubated at 37° for 4 hr with either venom phosphodiesterase (10 μg) in 0.1 M triethylammonium bicarbonate or RNase M (2 μg) in 0.1 M ammonium acetate (pH 5.0). Other general methods were described previously.[13,14] MMTr-U(OBz)-p,[3a] MMTr-A^{Bz}(OBz)-p,[14] and $A^{Bz_2}(OBz)_2$[15] were prepared as described. An extinction coefficient of 38700 at 260 nm was used for ApUpA according to the calculation[16] and 28800 for εApUpA assuming 5% hypochromicity using 5000 for εA.

12) J. Smrt, *Collection. Czech. Chem. Commun.*, **38**, 3189 (1973).
13) E. Ohtsuka, M. Ubasawa, S. Morioka, and M. Ikehara, *J. Am. Chem. Soc.*, **95**, 4725 (1973).
14) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Am. Chem. Soc.*, **92**, 3441 (1970).
15) R. Lohrmann and H.G. Khorana, *J. Am. Chem. Soc.*, **86**, 4188 (1964).
16) C.R. Cantor and I. Tinoco, Jr., *Biopolymers*, **5**, 821 (1967).

MMTr-εAp——1,N^6-Ethenoadenosine 3′-phosphate was prepared from adenosine 3′-phosphate by a similar method to that described by Secrist III, *et al.*[17] and isolated by desalting with DEAE-cellulose using triethylammonium bicarbonate. Triethylammonium 1,N^6-ethenoadenosine 3′-phosphate (0.41 mmole) was rendered anhydrous by coevaporation with pyridine and treated with monomethoxytrityl chloride (162 mg, 0.52 mmole) in DMF (4.7 ml) for 1 hr. Ice cooled 1 N NH_4OH (3 ml) was added to the reaction mixture and the mixture was evaporated to *ca.* 1 ml. Ammonium hydroxide (1 N, 5 ml) was added and the monomethoxy tritanol was extracted with ether. After removal of the bistritylated compound by extraction with ethyl acetate (2 ml) repeatedly, the aqueous phase was evaporated and the residue was dissolved in 1: 1 ethyl acetate–*n*-butanol. The organic layer was washed with water to remove the unchanged starting material and pyridinium hydrochloride. The product, MMTr-εAp (680 A_{275}, 0.094 mmole, 23%), was characterized by paper chromatography and electrophoresis (Table I). The spectral properties are $\lambda_{max}^{H_2O}$ (nm) 230, 258 (sh), 266, 276, 300 (sh); $\lambda_{max}^{H^+}$ 228 (sh), 276; $\lambda_{max}^{OH^-}$ 228 (sh), 258 (sh), 266.5, 276, 300 (sh).

Table I. Paper Chromatography and Paper Electrophoresis

Compound	Paper chromatography		Paper electrophoresis
	Solvent A	Solvent B	pH 7.5
U	0.48	0.70	0.0
Up	0.12	0.31	1.0
Up	0.32	0.57	0.67
εAp	0.19	0.29	0.86
MMTr-εAp	0.66	0.69	0.56
MMTr-εA(OBz)-P	—	0.86	0.35
εA(OBz)-P	—	0.56	0.68
UpA	0.24	0.40	0.29
ApUpA	0.10	0.13	0.43
εApUpA	0.11	0.18	0.43
A	0.58	0.61	−0.23
Ap	0.17	0.23	0.85

MMTr-εA(OBz)-p (IX)——MMTr-εAp (0.056 mmole) was dissolved in 50% pyridine and passed through a column (1×7 cm) of pyridinium Dowex 50 w×2. Tetraethylammonium benzoate (0.56 mmole) was added and the solution was rendered anhydrous by evaporation with pyridine five times. Traces of pyridine were removed with toluene similarly and then the mixture was treated with benzoic anhydride (269 mg) at 50° for 1 hr then at 27° for 2 days. After checking the extent of reaction of an aliquot, aqueous pyridine (50%, 5 ml) was added to the mixture under cooling and benzoic anhydride was removed with *n*-hexane. The benzoylated product was extracted with chloroform (2 ml) 3 times, washed with water and precipitated with a 1: 3 mixture of hexane–ether from its solution in dry pyridine. The benzoyl groups on phosphate were acetolyzed with acetic anhydride (2 ml) in pyridine (4 ml) for 24 hr. Aqueous pyridine (50%, 6 ml) was added in an ice bath and the solution was passed through a column (1×7 cm) of pyridinium Dowex 50w×2. The column was washed with 25% pyridine and the solution was evaporated with pyridine after standing for 4 hr. The product was precipitated with ether (30 ml) from its solution in pyridine (1 ml). The yield was 210 A_{275} (0.026 mmole), 46%.

MMTr-U(OBz)-p(CE) (II)——The pyridinium salt of MMTr-U(OBz)-p (I) (0.2 mmole) and β-cyanoethanol (0.15 ml, 2.1 mmoles) were treated with DCC (180 mg, 0.88 mmole) in the presence of triethylamine (0.025 ml, 1.8 mmoles) for 6 hr. The extent of the reaction was checked by TLC on silica gel ($CHCl_3$: MeOH= 5: 1) or paper electrophoresis after treatment with 80% acetic acid for 1 hr. Aqueous pyridine (50%, 4 ml) was added and the mixture was kept for 12 hr at room temperature. The filtered solution was extracted with hexane (2 ml) 3 times to remove DCC. The product was extracted with chloroform (3 ml) 4 times, washed with water, evaporated with pyridine and precipitated with a 2: 3 mixture of ether and hexane (40 ml) from its solution in pyridine (2 ml). The yield was 2060 A_{260} (0.137 mmole), 69%.

MMTr-U(OBz)-p(CE)-$A^{Bz2}$$(OBz)_2$ (IV) (route 1)——The pyridinium salt of II (0.05 mmole) and A^{Bz2}-$(OBz)_2$ (III) (0.15 mmole) were treated with TPS (0.15 mole) for 45 hr. The two trityl positive products (*Rf* 0.59 and 0.45 in $CHCl_3$: ethanol=19: 1) were detected and characterized by paper chromatography and electrophoresis after 80% acetic acid and methanolic ammonia treatment of aliquots. The faster traveling compound gave adenosine and a compound which had a negative charge (RUp=0.56). The slower moving compound gave UpA which was identified by digestion with RNase A. The product (IV) was collected from two plates (20×20 cm) of silica gel. The yield was 470 A_{260} (0.015 mmole), 30%. A side product which was

17) J.A. Secrist III, J.R. Barrio ,N.J. Leonard, and G. Weber, *Biochem.*, **11**, 3499 (1972).

estimated as the 5′-TPS derivative of III was found in TLC (*Rf* 0.89) and its spectral properties were λ_{max}^{EtOH} (nm) 232, 276.5, 283 (sh) and $A_{230}/A_{260}=2.52$.

VI (route 2)——The pyridinium salt of MMTr-U(OBz)-p (I) (0.46 mmole) and $A^{Bz2}(OBz)_2$ (III) (0.72 mmole) were rendered anhydrous by coevaporation with pyridine 5 times. The mixture was dissolved in pyridine (5 ml) and TPS (751 mg, 2.48 mmoles) was added. The solution was concentrated to *ca.* 3 ml and kept for 6 hr at room temperature. The mixture was then treated with β-cyanoethanol (0.35 ml, 5.11 mmoles) for 2.5 days until no trityl positive diester was detected in TLC ($CHCl_3$: MeOH=19: 1). The solution was evaporated with toluene to remove pyridine and the residue was subjected to thick layer chromatography on silicic acid (4 plates of 20×20 cm). The trityl positive band (*Rf* 0.67) was eluted with $CHCl_3$–MeOH (1: 1) and treated with 80% acetic acid for 1.8 hr. Detritylation was checked by TLC and the product was precipitated with 3: 2 ether–hexane (100 ml) from its solution in anhydrous pyridine (3 ml). The precipitate showed four spots in TLC ($CHCl_3$: MeOH=9: 1) and two slower spots (*Rf* 0.46, 0.39) gave UpA after methanolic ammonia treatment. These two bands were collected by preparative TLC (2 plate of 20×20 cm). The yield of U(OBz)-p(CE)-$A^{Bz2}(OBz)_2$ (VI) was 6300 A_{260} (0.21 mmole), 46%. The deprotected product was hydrolyzed with RNase A to give Up (0.342 A_{260}) and A(0.529 A_{260}), the ratio being 1.00=0.98.

U(OBz)-p-$A^{Bz2}(OBz)_2$ (VII)——The pyridinium salt of MMTr-U(OBz)-p (I) (0.69 mmole) and $A^{Bz2}(OBz)_2$ (III) (0.6 mmole) were rendered anhydrous by evaporation with pyridine 5 times and treated with DCC (2.5 mmoles) in pyridine (3 ml) for 5 days. Aqueous pyridine (50%, 5 ml) was added and DCC was extracted with hexane (5 ml) 3 times. The mixture was kept at room temperature for 4 hr and filtered. The solution was evaporated with added toluene to remove pyridine and the residue was treated with 80% acetic acid (20 ml) until no tritylated nucleotides were detected by TLC ($CHCl_3$: EtOH=15: 1). After 3 hr, acetic acid was removed, the residue was dissolved in butanol (30 ml) and the mononucleotide was extracted with 5% pyridine. Butanol was evaporated and the product was separated from the starting nucleoside by precipitation with ether (160 ml) from its solution in pyridine (3 ml). The precipitate was washed with ether 3 times. The yield was 8000 A_{260} (0.33 mmole), 55%.

ApUpA (XI)——U(OBz)-p(CE)-$A^{Bz2}(OBz)_2$ (VI) (1500 A_{260}, 0.048 mmole) and the pyridinium salt of MMTr-A^{Bz} (OBz)-p (VIII) (2700 A_{260}, 0.13 mmole) were coevaporated with pyridine 5 times. The residue was dissolved in pyridine (5 ml) and TPS added (84.7 mg, 0.28 mmole). A small amount of pyridine was removed and the mixture was kept at room temperature for 5.5 hr. Aqueous pyridine (50%, 4 ml) was added in an ice bath and the solution was kept at room temperature for 15 hr. Aqueous pyridine was removed by evaporation with toluene and the residue was treated with 80% acetic acid (5 ml) at 30° for 4 hr until no tritylated nucleotides were detected by TLC. After removal of acetic acid the mixture was dissolved in methanol (4 ml) and treated with 28% aqueous ammonia for 5 min to remove the cyanoethyl group. Volatile materials were evaporated and the residue was treated with methanolic ammonia (50 ml) for 15 hr. The nucleotides in 20% pyridine (105 ml) were applied to a column (1.7×49 cm) of DEAE-cellulose and eluted by a linear gradient of 0—0.2 M triethylammonium bicarbonate (6 liter). The product ApUpA (510 A_{260}, 0.013 mmole, 27%) was desalted by evaporation with water and characterized by paper chromatography and electrophoresis. RNase M digestion gave Up: Ap: A=1.08: 1.00: 1.04.

εApUpA (XII)——U(OBz)-p(CE)-$A^{Bz2}(OBz)_2$ (VI) (614 A_{260}, 0.021 mmole) and the pyridinium salt of MMTr-εA(OBz)-p (IX) (210 A_{275}, 0.026 mmole) were rendered anhydrous by coevaporation with pyridine 5 times. The mixture was treated with TPS (15.7 mg, 0.052 mmole) in pyridine (1 ml). After 6 hr, 50% pyridine (1 ml) was added in an ice bath and the mixture was kept at room temperature overnight. Pyridine was removed by evaporation with toluene and the trityl group was removed by treatment with 80% acetic acid for 2 hr. The extent of the reaction was checked by TLC and the evaporated residue was treated with ammonia as described for ApUpA. The product was isolated by chromatography on DEAE-cellulose using the same conditions as for ApUpA. The elution pattern is shown in Fig. 1. εApUpA was characterized by paper chromatography and electrophoresis. RNase M digestion gave adenosine, ApUp εAp cyclic, εAp and Up. Venom phosphodiesterase hydrolyzed εApUpA completely to give εA (0.43 A_{275}), pA (1.08 A_{260}) and pU (0.65 A_{260}), the ratio being 1.00: 1.01: 0.95. The yield was 144 A_{260} (0.005 mmole), 24%. $\lambda_{max}^{H_2O}$ (nm) 230, 262, 275 (sh) and 300; $\lambda_{max}^{H^+}$ 262.5; $\lambda_{max}^{OH^-}$ 228, 262, 275 (sh), and 300.

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