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# Polynucleotides. XX.[1] Synthesis of an AUG Analog containing 8,5′-O-Cycloadenosine

Morio Ikehara, Takeo Nagura and Eiko Ohtsuka

*Faculty of Pharmaceutical Sciences, Osaka University*[2]

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8,5′-Anhydro-8-oxyadenosine was phosphorylated using cyanoethylphosphate and dicyclohexylcarbodiimide (DCC) in dimethylformamide–pyridine. Treatment of the products with a mixture of 50% aqueous pyridine–triethylamine and DEAE-cellulose chromatography gave 2′,3′-cyclic phosphate in a yield of 48%. 8,5′-Anhydro-8-oxyadenosine 3′-phosphate was obtained by RNase M digestion of the cyclic phosphate.

After protection with benzoic anhydride in tetraethylammonium benzoate buffer, the 3′-phosphate was condensed with 2′-O-benzoyluridylyl-(3′→5′)-N², 2′,3′-O-triisobutyrylguanosine by the use of DCC. DEAE-cellulose, Sephadex G-25 and G-15 column chromatography gave 8,5′-anhydro-8-oxyadenylyl-(3′→5′)-uridylyl-(3′→5′)-guanosine. The trinucleotide was characterized by degradation to cyclo Ap, Up and G with RNase M.

Trinucleotide ApUpG[3] is known to act as an amino acid code for methionine in bacterial and mammarian protein biosynthesis.[4] However, exact mechanism how AUG is transcribed as an initiation or internal methionine codon is not known as yet.

Purine cyclonucleosides[5] have bases fixed at certain angle by virtue of anhydro linkages and serve as the model of natural nucleosides. Oligonucleotides containing cyclonucleosides have been synthesized and shown to have unique conformations.[6–8]

In this paper we describe synthesis of 8,5′-anhydro-8-oxyadenylyl-(3′→5′)-uridylyl-(3′→5′)-guanosine (ÅpUpG) (I), in which the adenine base is fixed at $\phi_{CN}=-42°$.[9] The use of this triplet in the protein synthetic system may shed light on the role of the first letter in AUG.

We now wish to report the synthesis of an analog of AUG containing 8,5′-O-cycloadenosine residue (Å).

The method for the synthesis of ÅpUpG follows essentially as described by Khorana and his colleagues.[10] A starting material, 8,5′-O-cycloadenosine 3′-phosphate (II) was obtained from 8,5′-anhydro-8-oxyadenosine (III) and β-cyanoethylphosphate[11] by the use of DCC as a condensating reagent. Although the method was originally developed for introducing phosphomonoester groups either in 2′, 3′ or 5′-OH, we attempted to isolate directly 2′,3′-cyclic phosphate (IV), which will give 3′-phosphate by the cleavage with RNases. When compound (III) was phosphorylated in anhydrous DMF–pyridine mixture in the presence of two equivalents cyanoethylphosphate and excess dicyclohexylcarbodiimide (DCC) at 30° for 2 days, almost quantitative conversion of III to 2′ or 3′-cyanoethylphosphate (V) was observed by

1) Part XIX of this series: E. Ohtsuka, S. Morioka and M. Ikehara *J. Amer. Chem. Soc.*, **95**, 8437 (1973).
2) Location: *6-1-1, Toyonaka, Osaka.*
3) Abbreviations are as suggested by IUPAC-IUB combined commission, *J. Biol. Chem.*, **241**, 531 (1966).
4) J. Lucas-Lenard and F. Lipman, *Ann. Rev. Biochem.*, **40**, 409 (1971).
5) M. Ikehara, *Accounts Chem. Res.*, **2**, 47 (1969).
6) M. Ikehara, S. Uesugi and M. Yasumoto, *J. Amer. Chem. Soc.*, **92**, 4735 (1970); S. Uesugi, M. Yasumoto, M. Ikehara, K.N. Fang and P.O.P. Ts'o, *J. Amer. Chem. Soc.*, **94**, 5480 (1972).
7) M. Ikehara and S. Uesugi, *J. Amer. Chem. Soc.*, **94**, 9189 (1972).
8) M. Ikehara, S. Uesugi and J. Yano, *Nature New Biol.*, **240**, 16 (1972).
9) K. Tomita, reported at 4th Intern. Congress of Crystallography (Kyoto) 1972.
10) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka and H.G. Khorana *J. Amer, Chem. Soc.*, **88**, 819 (1966).
11) G.M. Tener, *J. Amer. Chem. Soc.*, **83**, 159 (1961).

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thin-layer chromatography (TLC). After treatment of the products with a mixture of 50% aqueous pyridine–triethylamine at room temperature, compound (V) converted to the cyclic phosphate (IV). The omission of triethylamine gave only the 2′- or 3′-phosphate. Appropriate work-up and chromatography (Fig. 1) on DEAE-cellulose column gave compound IV in peak 2 and the yield was 48%. In peak 4 was obtained 2′- or 3′-phosphate (II) in a yield less then 10%.

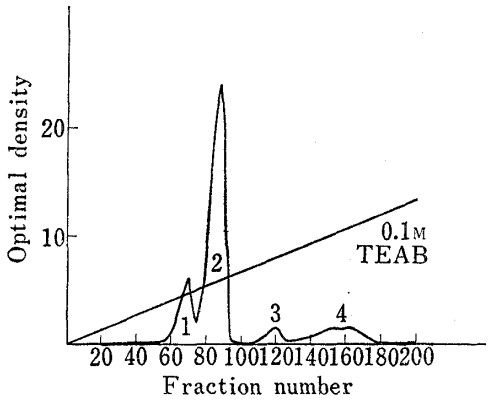


Fig. 1. DEAE-cellulose Column Chromatography of 8,5′-Anhydro-8-oxyadenosine 2′,3′-Cyclic Phosphate

Peaks 1 and 3 contained substances having $\lambda_{max}^{H_2O}$ 261 and 308 nm and were thought to be $N^6$-dimethylaminomethylene compounds,[12] which arose from the reaction of DMF with compound (III).

Compound (IV) was analyzed to have a correct structure as follows. It migrated in paper electrophoresis as fast as 2′,3′-cyclic AMP and gave 3′-phosphate exclusively by the digestion with RNase M.[13] An acid hydrolysis of IV gave the 2′- and 3′-phosphate in the ratio of nearly 1:1.

The mechanism of the cyclization of 2′- or 3′-cyanoethylphosphate (V) to 2′,3′-cyclic phosphate (IV) may be as reported previously[14] in the case of 2′- or 3′-phosphoromorpholidate.

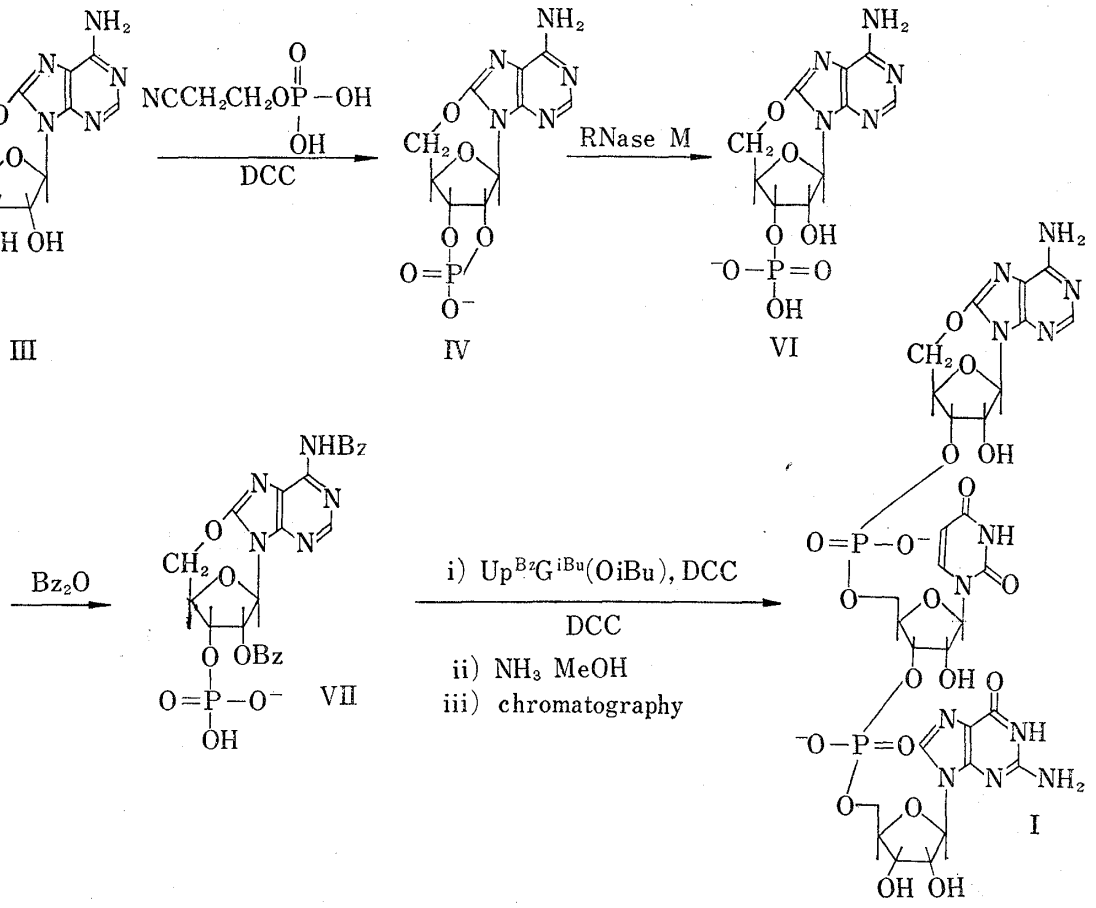


Chart 1

12) J. Zemlicka and A. Holy, *Collec. Czech. Chem. Comm.*, **92**, 645 (1964).
13) M. Irie, *J. Biochem.*, **62**, 509 (1967).
14) M. Ikehara and I. Tazawa, *J. Org. Chem.*, **31**, 819 (1966).

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The mild alkaline treatment cleaved cyanoethyl as a leaving group and 2′- or 3′-OH situating in a favorable position attacks concertedly P atom to make cyclic phosphate. By using this method nucleoside 2′,3′-cyclic phosphate is obtained in one step and in relatively high yield.

Compound (IV) was then hydrolyzed in 0.1M ammonium acetate buffer (pH 6.0) with RNase M. 8,5′-Anhydro-8-oxyadenosine 3′-phosphate (VI) was obtained by precipitation with $CaCl_2$–ethanol. After conversion of calcium salt to pyridinium salt, the yield was 85%. The substrate specificity of RNase M seems to be rather broad[13] judging from this result in which the base moiety was fixed still at $\phi_{CN}=-42°$.

The protection of 2′-OH of compound (VI) was done by benzoic anhydride in tetraethylammonium benzoate buffer at pH 7.0.[15] After the work-up $N^6$, 2′-O-dibenzoyl-8,5′-anhydro-8-oxyadenosine 3′-phosphate (VII) was obtained as powder having ultraviolet (UV) absorption properties $\lambda_{max}^{H_2O}$ 230 (sh) and 285.5 nm. The product migrated on paper electrophoreogram at pH 7.5 slower than AMP ($R_{AMP}=0.83$).

TABLE I

| Peak No. | Total OD at 260 nm | Identification[a] | Peak No. | Total OD at 260 nm | Identification[a] |
|---|---|---|---|---|---|
| NO. 1 | 310 | | NO. 5 | 310 | |
| 2 | 250 | Åp>[b] | 6 | 320 | ÅpUpG |
| 3 | 520 | UpG | 7 | 215 | |
| 4 | 230 | Åp | 8, 9 | 335 | |

*a*) Substances in peak 1—9 were identified by PEP, PPC (M) and hydrolyses with 0.3N KOH.
*b*) A°p refers 8,5′-anhydro-8-oxyadenosine 2′, 3′-cyclic phosphate.

The final condensation of compound VII with 2′-O-benzoyluridylyl-(3′→5′)-$N^2$,2′,3′-O-triisobutyrylguanosine ($Up_{Bz}G_{iBu}(OiBu)_2$) (VIII), which was synthesized previously from $MMTrUp_{Bz}$ and $G_{iBu}(OiBu)_2$,[16] was achieved by using DCC in pyridineat 30° for 5 days. After checking the reaction by paper electrophoresis and chromatography, the reaction mixture was worked up properly and treated in the usual manner. The products were precipitated by addition of ether–pentane. The products were separated by employing a DEAE-cellulose column chromatography as shown in Table I. The trinucleotide contained in peak 6 was concentrated and applied to a column of Sephadex G-25 and eluted with 0.05M triethylammonium bicarbonate buffer. The fraction containing ÅpUpG was collected and purified further by a gel filtration through Sephadex G-15 column (Fig. 2). ÅpUpG (I), thus obtained, showed UV $\lambda_{max}^{H_2O}$ 257.5 and a single spot on paper chromatograms in two solvent systems. Paper electrophoresis at pH 7.5 also showed one spot for compound (I).

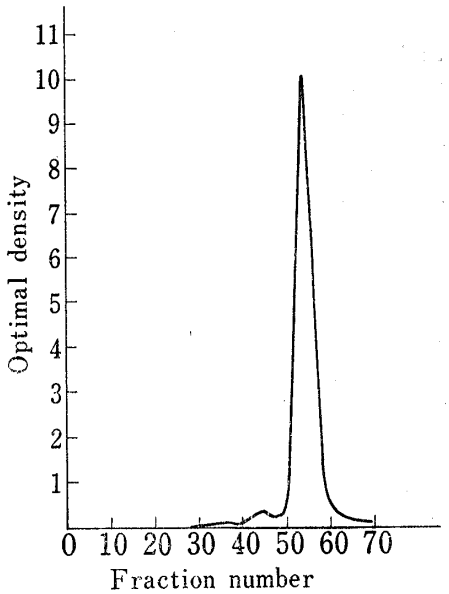


Fig. 2. Sephadex G-15 gel Filtration of 8,5′-Anhydro-8-oxyadenylyl-(3′-5′)-uridylyl-(3′-5′)-guanosine

The digestion of compound (I) by RNase M at 37° for 16 hr showed complete hydrolysis to Åp, Up and G in a ratio of 1.0: 0.95: 1.0. This fact clearly demonstrated that compound (I) was ÅpUpG having 3′→5′-phosphodiester bond in every internucleotide linkages.

15) D.H. Rammler, Y. Lapidot and H.G. Khorana, *J. Amer. Chem. Soc.*, **85**, 1989 (1963).
16) E. Ohtsuka, "Method in Nucleic Acid Res. (A)," Kyoritsu Shuppan Co. Ltd., 1972, p. 106.

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Behaviors of compound (I) in the binding experiment[17] will be reported elsewhere.

## Experimental[18]

**8,5′-Anhydro-8-oxyadenosine 2′,3′-Cyclic Phosphate (IV)**——8,5′-Anhydro-8-oxyadenosine[19] (150 mg, 0.567 mmole) was dissolved in DMF (6 ml) by slight heating. After cooling to room temperature cyanoethylphosphate (5 mmoles/10 ml pyridine) (2 ml, 1 mmole) and DCC (700 mg) were added. The reaction mixture was kept at 30° for 24 hr and another batch of DCC (300 mg) was added. After 24 hr the mixture was poured into cold 50% aqueous pyridine containing triethylamine (0.207 ml). Ether (10 ml) was added to the mixture, dicyclohexylurea was filtered, and DCC was extracted three times with ether (each 10 ml). Aqueous layer was diluted with water (200 ml) and applied to a column (2.0×41 cm) of DEAE-cellulose. Elution with 0.1M triethylammonium bicarbonate buffer (2 liter) by linear gradient (one fraction 20 g/30 min) gave a pattern shown in Fig. 1. Peak 1: 865 $OD_{260}$ ($\lambda_{max}^{H_2O}$ 261, 308 nm, probably $N^6$-dimethylaminomethylene compound), 2: 4428 $OD_{260}$ (compound IV), 3: 317 $OD_{260}$ ($N^6$-dimethylaminomethylene compound) and 4: 902 $OD_{260}$ (2′-or 3′-phosphate). Yield of IV was 48.2% ($\varepsilon$ was assumed as 16200[19]). PEP: $R_{pA}$ 0.67 (2′,3′-cyclic AMP $R_{pA}$ 0.72). PPC: *Rf* 0.34 (solvent C), *Rf* 0.60 (solvent B). Hydrolysis with 1N HCl gave 2′-(*Rf* 0.18 (solvent C)) and 3′-phosphate (*Rf* 0.14 (solvent C)) in ratio 0.80: 0.77.

**8,5′-Anhydro-8-oxyadenosine 3′-Phosphate (VII)**——2′,3′-Cyclic phosphate (IV) (4400 $OD_{260}$ units) was incubated with RNase M (1 mg/ml) (1.5 ml) in 1M ammonium acetate buffer (pH 6.0) (6.0 ml) at 39° for 4 days. The mixture was evaporated and the residue was dissolved in 20 ml of water. Addition of $CaCl_2$ $2H_2O$ (221 mg) dissolved in ethanol (40 ml) gave precipitates, which were collected by centrifugation. The precipitates were dissolved in $H_2O$ (10 ml) and precipitated again by adding ethanol (40 ml). This procedure was repeated three times. The powder was suspended in 50% pyridine, dissolved by adding Dowex 50×2 (pyridinium form), and passed through a column (1.1×10 cm) of Dowex 50×2 (pyridinium form) to obtain the pyridinium salt. Yield was 3800 $OD_{260}$ units. PEP: $R_{pA}$ 1.06. PPC: *Rf* 0.10 (solvent A), *Rf* 0.43 (solvent B), *Rf* 0.12 (solvent C).

**$N^6$,2′-O-Dibenzoyl-8,5′-anhydro-8-oxyadenosine (VII)**——8,5′-Anhydro-8-oxyadenosine 3′-phosphate (3500 $OD_{260}$ units, 0.134 mmole) was dissolved in a solution of tetraethylammonium benzoate (1.34 mmole) (prepared from benzoic acid (164 mg) in water brought to pH 7.0 with tetraethylammonium hydroxide). The mixture was evaporated four times with addition of pyridine. Traces of pyridine were coevaporated with anhydrous toluene. Benzoic anhydride (605 mg, 2.68 mmoles) was added into the residual caramel and the mixture was made homogeneous by heating at 40°. It was kept at 30° for 4 days. After addition of 50% aqueous pyridine (20 ml), the mixture was extracted three times with *n*-pentane (20 ml) and the water layer was extracted with $CHCl_3$ (20 ml+10 ml). The chloroform layer was washed with water (10 ml) and concentrated to dryness. The residue was dissolved in pyridine and the solution was concentrated to dryness for complete removal of moisture and finally dissolved in pyridine (10 ml). Pouring of the solution into ether–pentane (3: 2) caused precipitation. The powder, thus obtained, was dried by azeotropic distillation with pyridine and dissolved in pyridine (5 ml) and acetic anhydride (2.5 ml). After keeping at room temperature overnight, the solution was concentrated *in vacuo* and the residue was codistilled twice with added water. The residue was dissolved in 50% aqueous pyridine and kept overnight at room temperature. Solvent was evaporated, the residue dried by codistillation with added pyridine and finally dissolved in pyridine (10 ml). Dibenzoyl 3′-phosphate (3100 $OD_{285}$ units) was precipitated by addition of ether–pentane (3: 2). PEP: $R_{pA}$ 0.83, PPC: *Rf* 0.38 with a thin spot at *Rf* 0.71 (solvent D, UV $\lambda_{max}^{H_2O\text{-}EtOH}$ nm: 230 (sh), 285.5; $\lambda_{max}^{H^+}$ 229 (sh), 287 nm; $\lambda_{max}^{OH^-}$ 305 nm.

**8,5′-Anhydro-8-oxyadenylyl-(3′—5′)-uridylyl-(3′—5′)-guanosine (I)**——Compound (VII) (tetraethylammonium saly, 2500 $OD_{285}$, 0.139 mmole) was converted to pyridinium salt by passing through a column (1.0×3 cm) of Dowex 50×2 (pyridinium form). 2′-O-Benzoyluridylyl-(3′—5′)-N,2′,3′-triisobutyrylguanosine (VIII) (triethylammonium salt, 0.16 g, 1000 $OD_{260}$, 50 mmoles) was converted also to pyridinium salt similarly. Both solutions were combined and evaporated with added pyridine four times to remove traces of water. The residue was dissolved in pyridine (3 ml) and DCC (170 mg, 0.82 mmole) was added. The mixture was concentrated to *ca.* 1 ml and kept at 30° for 5 days. The reaction was quenched by the addition of 50% aqueous pyridine (20 ml) and the solution was kept overnight. After dicyclohexylurea was

17) Unpublished experiments of K. Shimokawa.

18) Paper chromatography (PPC) was performed by the descending technique by using Toyo Filter Paper No. 51A: solvent A, 2-propanol–concentrated ammonia–$H_2O$, 7: 1: 2; solvent B, 1-propanol–concentrated ammonia–$H_2O$, 55: 10: 35; solvent C, saturated ammonium sulfate–$H_2O$–2-propanol, 79: 19: 2; solvent D, ethanol–1M ammonium acetate, 7: 3 v/v (pH 7.5). Paper electrophoresis (PEP) was performed in 0.05M triethylammonium bicarbonate (pH 7.5) at 900 V/40 cm. $R_{pA}$ stands for migration ratio relative to adenosine 5′-phosphate.

19) M. Ikehara, M. Kaneko and R. Okano, *Tetrahedron*, **26**, 5675 (1970).

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filtered off, the solution was extracted twice with *n*-pentane (10 ml) and the water layer was evaporated. The residue was dried by coevaporation with added pyridine and dissolved in pyridine (10 ml). The solution was poured into ether–pentane (4: 1) and the precipitates were collected and dried in a desiccator *in vacuo*. The dried powder was dissolved in anhydrous methanol saturated with ammonia at 0° (70 ml) and kept at 25° for 20 hr. Ammonia–methanol was evaporated off, pyridine containing 10% of water (200 ml) was added and the solution was applied to a column (2.8×49 cm) of DEAE-cellulose. Elution was carried out with 0.2M triethylammonium bicarbonate (pH 7.5) (3 liter.) and $H_2O$ (3 liter.) in a linear gradient. Fractions of 20 ml were collected at 30 min intervals. Results were summarized in Table I. Compounds in each peak were identified by PEP, PPC (solvent B) and hydrolysis with 0.3N KOH to the component mononucleotide and nucleoside.

Fractions in peak 6 were evaporated to a residue, which was evaporated twice with added methanol. The residue was dissolved in $H_2O$ (0.2 ml) and applied to a column (1.7×98 cm) of Sephadex G-25 column. Elution with 0.05M triethylammonium bicarbonate buffer gave a peak containing mainly ÅpUpG in fractions No. 60—80 (one fraction was 3 ml/30 min). This material was rechromatographed on Sephadex G-15 column (1.5×113 cm). Elution with 0.05M triethylammonium bicarbonate gave a symmetrical peak as shown in Fig. 2. Yield was 48 $OD_{260}$ units. UV $\lambda_{max}^{H_2O}$: 257.5 nm; $\lambda_{max}^{H^+}$ 259.5 nm, $\lambda_{max}^{OH^+}$ 259.5 nm. PPC: *Rf* 0.24 (solvent B), *Rf* 0.02 (solvent A).

**Digestion of ÅpUpG with RNase M**——ÅpUpG (I) (*ca.* 3 $OD_{260}$ units) and RNase M (1 mg/ml) (20 λ) were incubated in ammonium acetate buffer (pH 5.0, 0.05M) (400 λ) at 37° for 16 hr. The mixture was concentrated *in vacuo* and applied on a TLC plate (cellulose). Development by isobutyric acid–0.5N $NH_4OH$ (5: 3) gave Åp (*Rf* 0.65); Up (*Rf* 0.37) and guanosine (*Rf* 0.53). Starting material (*Rf* 0.36) was not detected. The ratio: Åp (ε 16200): Up (ε 9900): G (ε 11400)=1.0: 0.95: 1.0 (theoretical value 1: 1: 1).

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