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179. Morio Ikehara, Fumio Harada*1 and Eiko Ohtsuka*2 : Polynucleotides. III.*3 Synthesis of Four Trinucleoside Diphosphates containing Tubercidin (7-Deazaadenosine) and N^6-Dimethyladenosine.

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In recent years much attention has been drawn to the mechanism by which ribonucleic acids code for protein synthesis *in vitro* is translated. Synthetic polynucleotides have been shown to act as efficient messenger RNA's in directing the incorporation of amino acids into polynucleotides[1~3] and some polynucleotide analogs have been similarly investigated.[4,5]

The formation of an active complex of messenger RNA and soluble RNA (sRNA) with ribosomes is considered an essential step of protein biosynthesis.[6] The recent discovery of Nirenberg and Leder[7] that ribonucleoside diphosphates can direct the binding of amino acid specific sRNA to ribosomes has provided the assignment of the majority of the amino acid triplet codons.[8~10] This binding specificity is presumably due to hydrogen bond formation between the trinucleotide bases and the anticodon bases of the sRNA. It is therefore of interest to assess the binding ability of trinucleoside diphosphate analogs in which hydrogen bond formation may be affected. The following analogs of adenylyl-(3′→5′)-adenylyl-(3′→5′)-adenosin (ApApA) and adenylyl-(3′→5′)-cytidylyl-(3′→5′)-cytidine (ApCpC) were synthesized for this purpose : 7-deazaadenylyl-(3′→5′)-adenylyl-(3′→5′)→adenosine(TupApA), 7-deazaadenylyl-(3′→5′)-cytidylyl-(3′→5′)-cytidine (TupCpC), N^6-dimethyladenylyl-(3′→5′)-adenylyl-(3′→5′)-adenosine (DMApApA) and N^6-dimethyladenylyl-(3′→5′)-cytidylyl-(3′→5′)-cytidine (DMApCpC). N^6-Dimethyladenine was chosen as one base analog since poly N^6-dimethyladenylic acid did not form a stable hydrogen-bonded complex with polyuridylic acid.[11] It was anticipated that the introduction of this base residue into a trinucleotide might elucidate the necessity for hydrogen bonding for codon-anticodon recognition. For the same reason, the substitution of adenine with 7-deazaadenine might provide information concerning the role of the N^7-atom for the binding phenomenon. Moreover, 7-deazaadenosine is shown to be the antibiotic tubercidin[12] which inhibits the growth of mouse L-cells by the rapid

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*3 Part II. M. Ikehara, E. Ohtsuka : Biochem. Biophys. Res. Comm., **21**, 257 (1965).

1) M. Nirenberg, J.H. Mathaei : Pro. Nat. Acad. Sci. U.A., **47**, 1558 (1961).
2) J.F. Speyer, P. Lengyel, C. Basilio, S. Ochoa; *Ibid.*, **48**, 441 (1962).
3) *a*) S. Nishimura, D.S. Jones, E. Ohtsuka, H. Hayatsu, T.M. Jacob, H.G. Khorana : J. Mol. Biol., **13**, 283 (1965); *b*) S. Nishimura, D.S. Jones, H.G. Khorana : *Ibid.*, **13**, 302 (1965).
4) M. Grunberg-Manago, A.M. Michelson : Biochem. Biophys. Acta, **80**, 431 (1964).
5) A.M. Michelson, M. Grunberg-Manago : *Ibid.*, **91**, 92 (1964).
6) J.D. Watson : Sciences, **140**, 17 (1963).
7) M. Nirenberg, P. Leder : Sciences, **145**, 1399 (1964).
8) M. Nirenberg, P. Leder, M. Bernfield, R. Brimaconbe, J. Trupin, F. Rottman, C. O'Neal : Proc. Nat. Acad. Sci. U.A., **53**, 1161 (1965).
9) R. Brimaconbe, J. Trupin, M. Nirenberg, P. Leder, M. Bernfield, J. Jaouni : *Ibid.*, **54**, 954 (1965).
10) D. Soll, E. Ohtsuka, P. Lohrmann, H. Hayatsu, S. Nishimura, H.G. Khorana : *Ibid.*, **54**, 1378 (1965).
11) B.E. Griffin, W.J. Haslem, C.B. Reese : J. Mol. Biol., **10**, 353 (1964).
12) Y. Mizuno, M. Ikehara, K.A. Watanabe, S. Suzaki : J. Org. Chem., **28**, 3331 (1963).

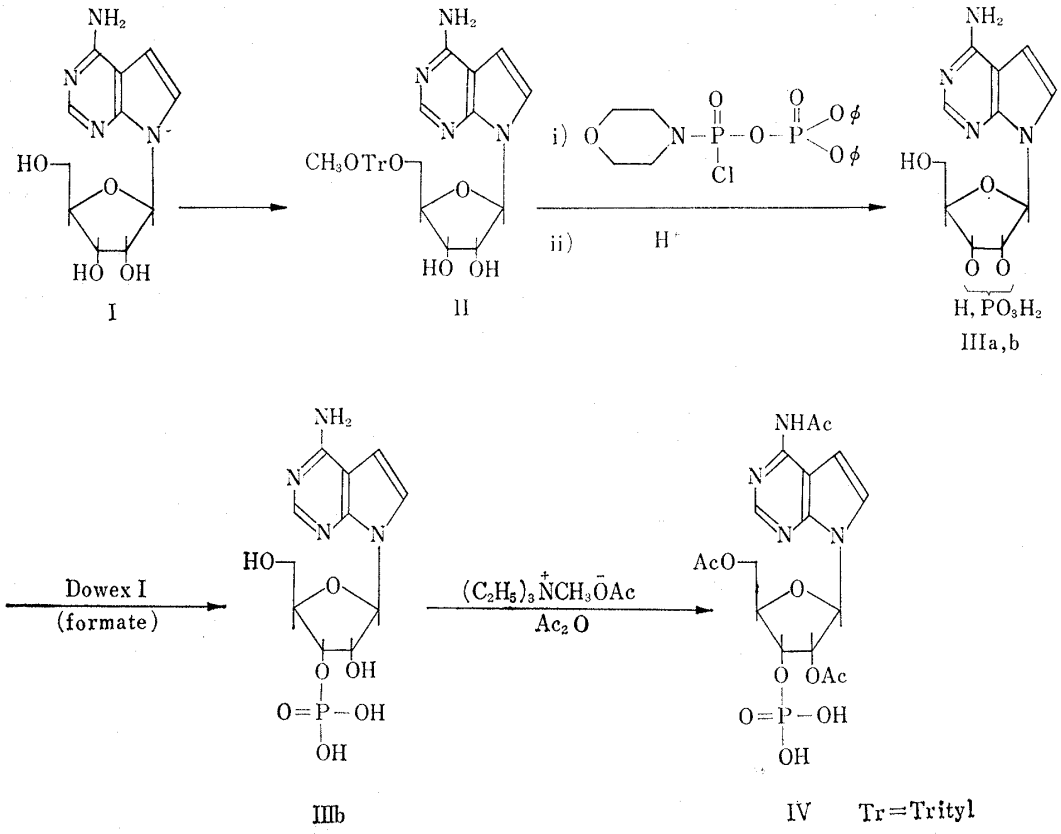
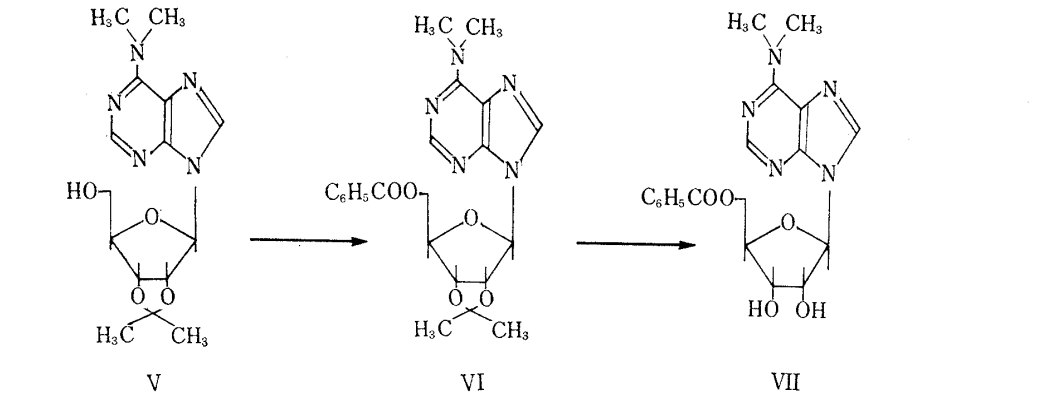


Chart 1.



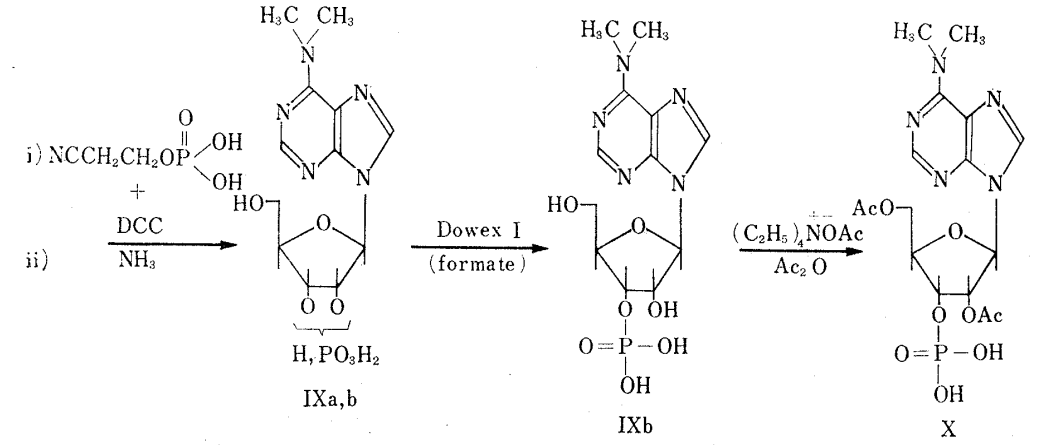


Chart 2.

inhibition of DNA, RNA and protein synthesis.[13] An investigation of the binding of aminoacyls RNA's to ribosomes stimulated by tubercidin-containing trinucleoside diphosphates might shed further light on the step in which protein synthesis is inhibited. The results of such binding experiments have been previously reported.*3

The synthesis of the trinucleoside diphosphates was achieved essentially following the method developed by Khorana and his collabolators[14] with minor modifications. Thus, a preformed suitably protected dinucleoside diphosphate was condensed with protected tubercidin 3′-phosphate or N^6-dimethyladenosine 3′-phosphate with dicyclohexylcarbodiimide (DCC). For the synthesis of N^6,2′,5′-triacetyltubercidin 3′-phosphate, tubercidin (I) was first reacted with monomethoxytrityl chloride[15] to give 5′-monomethoxytrityltubercidin (II) in 48% yield. Compound II was then phosphorylated by P^1-diphenyl-P^2-morpholinopyrophosphorochloridate[16] (DMPC) to give a 2′-and 3′-phosphate mixture of compound II, which was deprotected by acid treatment. Separation of tubercidin 2′- and 3′-monophosphates (IIIa, IIIb) was carried out by ion-exchange chromatography on a Dowex I (formate) column. Characterization of these phosphates was done by paper chromatography, paper electrophoresis and ultraviolet absorption. The 3′-monophosphate (IIIb) was then acetylated with acetic anhydride in the presence of triethylmonomethylammonium acetate to provide the N^6,2′,5′-triacetyl derivative (IV). The N-acetyl group seems to enter the N^6-position as occurrs with adenylic acid (ca. 10 mμ bathochromic shift of the ultraviolet absorption maximum).

2′,3′-O-Isopropylidene-N^6-dimethyladenosine (V)[17] was benzoylated to give 5′-O-benzoyl-2′,3′-O-isopropylidene-N^6-dimethyladenosine (VI), which was deprotected by acetic acid treatment to give 5′-O-benzoyl-N^6-dimethyladenosine (VII). Compound VII was then phosphorylated with cyanoethylphosphate and DCC;[18] deprotection of the 2′- and 3′-phosphate derivatives (VIIIa,b) by conc. ammonia gave a mixture of N^6-dimethyladenosine 2′- and 3′-phosphates (IXa, IXb), which were separated by column chromatography as described above. The yield of the 2′- and 3′-phosphates was 29% and 30% respectively. In this case, however, the concentration of the eluting buffer should be raised to 0.2*N* formic acid (Fig. 1). The structure of these monophosphates was

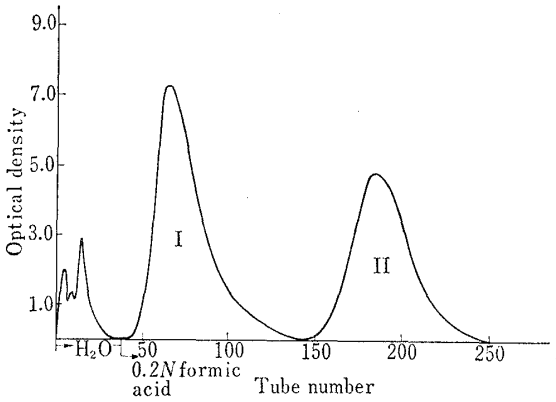


Fig. 1. Column Chromatography of N^6-Dimethyladenosine 2′- and 3′-phosphate

Peak I: N^6-dimethyladenosine 2′-phosphate, peak II: N^6-dimethyladenosine 3′-phosphate. Other conditions appeared in the text.

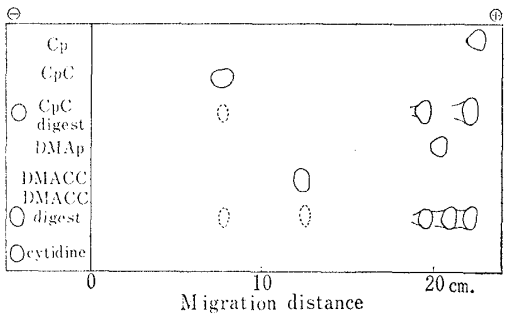


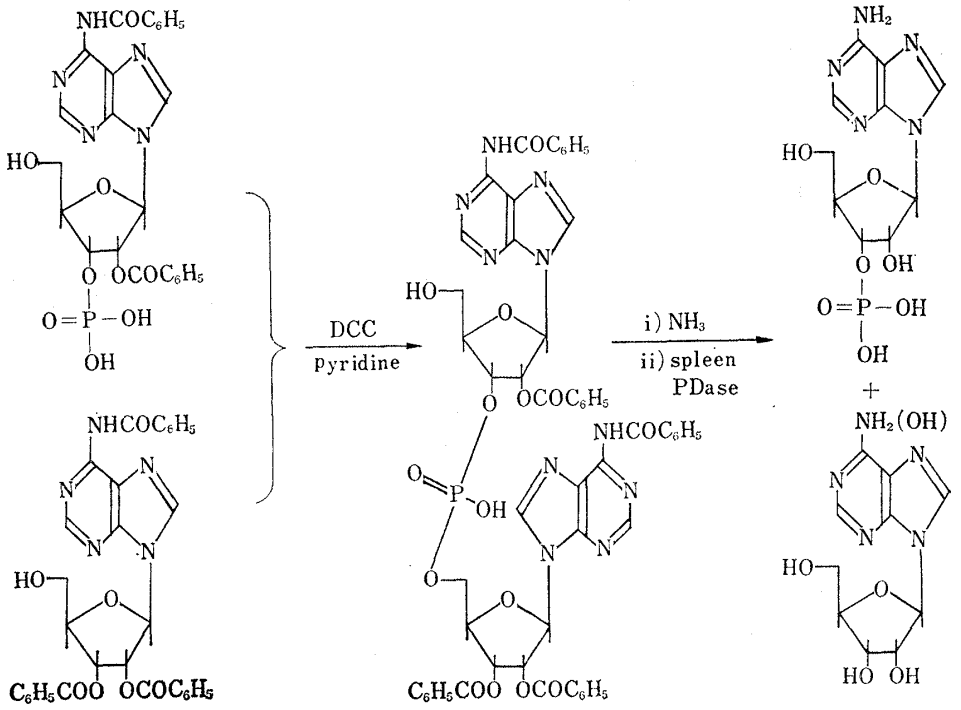
Fig. 2. Paper Electrophoresis of Spleen Phosphodiesterase Digestion of Cytidylyl-(3′-5′)-cytidine and N^6-dimethyladenylyl-(3′-5′)-cytidylyl-(3′-5′)-cytidine

Conditions appeared in the text.

13) G. Acs, E. Reich, M. Mori: Proc. Nat. Acad. Sci., **52**, 493 (1964).
14) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, H. G. Khorana: J. Am. Chem. Soc., 88, 619 (1966).
15) M. Smith, D. H. Rammler, I. H. Goldberg, H. G. Khorana: *Ibid.*, **84**, 430 (1962).
16) M. Ikehara, E. Ohtsuka: This Bulletin, **11**, 961 (1963).

confirmed by paper chromatography and paper electrophoresis. The 3′-monophosphate (Xb) was then acetylated with acetic anhydride in the presence of tetraethylammonium acetate to give 2′,5′-di-O-acetyl-N^6-dimethyladenosine 3′-phosphate (X) as a white powder which was characterized by paper chromatography and paper electrophoresis. The confirmation of 3′-phosphate (rather than 2′) structure was obtained by enzymatic digestion of the final trinucleoside diphosphates.

The protected dinucleoside phosphates, N,2′-dibenzoyladenylyl-(3′→5′)-N,2′,3′-tribenzoyladenosine (A^{Bz2} pA^{Bz3}) (XVI) and N,2′-dibenzoylcytidylyl-(3′→5′)-N,2′,3′-tribenzoylcytidine (C^{Bz2} pC^{Bz3}*4) (XVII), were synthesized by Khorana's procedure,[14] except that N,2′,3′-tribenzoyladenosine (starting material for (XI)) was obtained using benzoic acid-*p*-toluene sulfonic acid anhydride.[19] Condensation of "fully" acetylated tubercidin



XI

Chart 3.

3′-phosphate (IV) and N^6-dimethyladenosine 3′-phosphate (X) (35～55μmoles) with compound (XI) or (XII) (each 10 μmoles) was achieved by DCC in pyridine-DMF solution for 3～4 days at room temperature. The resulting trinucleoside diphosphates (XII a,b,c,d) were applied to preparative electrophoresis, which separated XIII from tubercidin-or N^6-dimethyladenosine monophosphates, as well as ApA and CpC. Subsequent paper chromatography separated XIII from the tubercidin- or N^6-dimethyladenosine 2′,3′-cyclic phosphates. The four trinucleoside diphosphates were characterized by ultraviolet absorption charactaristics, paper chromatography and by digestion with spleen phosphodiesterase[20], which showed in each case complete digestion as outlined in Fig. 2. As estimated from the results, all XIII analogs have less than 5% of 2′,5′-phosphodiester contamination.

*4 This Compound was generously provided by Dr. D. Söll.

17) M. Ikehara, H. Uno, F. Ishikawa: This Bulletin, **12**, 267 (1964).
18) G. M. Tener: J. Am. Chem. Soc., **83**, 159 (1961).
19) M. Ikehara, H. Tada, to be published.
20) L. A. Heppel, R. J. Hilmoe: "Method in Enzymology." Vol. II, 565 (1955), Academic Press, Inc., New York, N. Y.

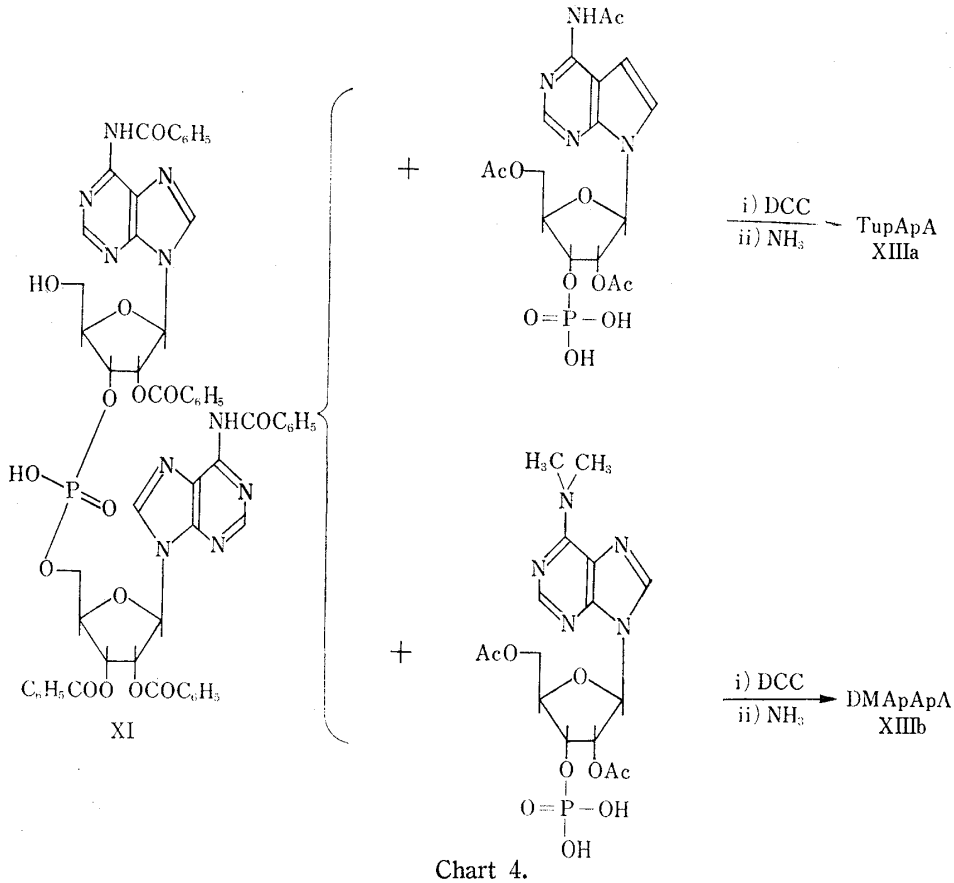


Chart 4.

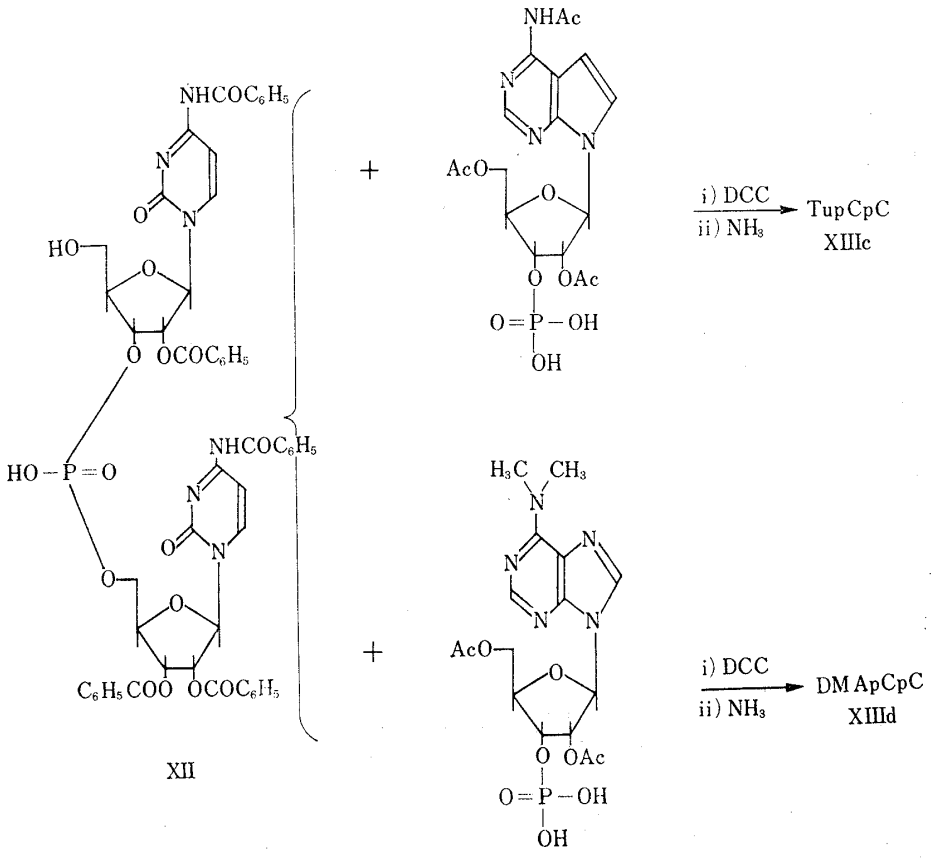


Chart 5.

Experimental

Paper Chromatography——All chromatography was performed by the descending technique. Solvent A : butanol-water, 86:14; Solvent B : isopropanol-ammonia-water, 7:1:2; Solvent C : saturated ammonium sulfate-0.1*M* sodium acetate-isopropanol, 79:19:2; Solvent D : ethanol-1*M* ammonium acetate, 7:3 (pH 7.5); Solvent E : ethanol-0.5*M* ammonium acetate, (pH 3.8).

Paper Electrophoresis——Condition I : 0.03*M* phosphate buffer (pH 7.1), 69 V/cm., 20 min. Condition II : 0.05*M* triethylammonium bicarbonate buffer (pH 7.5), 20 V/cm. for 1 hr.

5′-O-Monomethoxytrityltubercidin——Tubercidin[21] (2.0 g.) was bissolved in 40 ml. of dimethylformamide (DMF) followed by the addition of 2.8 g. (1.16 equivalent) of monomethoxytrityl chloride.[15] After standing for 3 days at 30°, the mixture was poured into ice-water containing 2% ammonia with stirring. The resulting precipitate was collected by filtration and recrystallized from ethyl acetate and ether. 5′-Monomethoxytrityltubercidin was obtained as a white powder, m.p. 160~170° (yield 1.94 g., 48%). *Anal.* Calcd. for $C_{31}H_{30}O_5N_4 \cdot H_2O$: C, 66.86; H, 5.79; N, 10.06. Found : C, 66.59; H, 5.43; N, 10.20. Ultraviolet absorption property : λ_{max}^{EtOH} 271 mμ. Paper chromatography : Rf (A) 0.88; Rf (B) 0.92; Tubercidin Rf (A) 0.28; Rf (B) 0.59. The comopund could be detected by a orange-yellow color by spraying with 5% perchloric acid.[22] Metaperiodate spray[23] showed the presence of vicinal hydroxyl. group.

Tubercidin 3′-Phosphate——5′-Monomethoxytrityltubercidin (538 mg., 1 mmole) was dissolved in 2 ml. of anhydrous dioxane (dried over calcium hydride and distilled freshly) followed by the addition of P^1-diphenyl-, P^2-morpholino-pyrophosphorochloridate freshly prepared from 500 mg. (4 mmole) of diphenyl phosphate, 408 mg. (2 mmole) of morpholinophosphorodichloridate[24] and 351 mg. (6 mmole) of 2,6-lutidine (dried over calcium hydride) dissolved in 1 ml. of dioxane. The reaction mixture was shaken at room temperature for 3 days taking care to exclude moisture. Water (30 ml.) was added into the mixture and the slightly turbid solution (pH 2) was heated at 100° for 45 min. After extraction with ether (3×20 ml.), the solution was adjusted to pH 7.5 with ammonia and extracted again with ether (3×20 ml.). The aqueous layer was applied to a column (2×30 cm.) of Dowex 1×8 (formate) and washed with water, which eluted the unreacted nucleoside (TOD_{270} *5 61, 0.7%). Elution with 0.1*N* formic acid (ca. 10 ml. fractions were collected) gave tubercidin 2′,3′-cyclic phosphate (TOD_{270} 157, 1.8%), tubercidin 2′-phosphate (TOD_{270} 689, 7.8%) and tubercidin 3′-phosphate (TOD_{270} 848, 9.5%). The remainder of the nucleosides were not precisely investigated. Tubercidin 2′- and 3′-phosphates were examined by paper electrophoresis and paper chromatography (see Table I). Ultraviolet absorption properties : 2′-phosphate $\lambda_{max}^{H_2O}$ 271 mμ, 3′-phosphate $\lambda_{max}^{H_2O}$ 272 mμ.

N^6,2′,5′-Triacetyltubercidin 3′-Phosphate——Tubercidin 3′-phosphate (848 O. D. units, 0.07 mmole) and triethyl-monomethylammonium acetate*6 (0.7 mmole) were rendered anhydrous by repeated evaporation with pyridine (3×5 ml.). To the residue was added toluene (5 ml.) and the solution was evaporated *in vacuo* to remove traces of pyridine. This process was repeated until no odor of pyridine remained. To the residual solid, acetic anhydride (0.4 ml., 4 mmole) was added. The reaction mixture was tightly stoppered and shaken at room temperature for 3 days. Into this mixture 0.2 ml. of pyridine and 0.8 ml. of methanol was added and the solution was kept for 20 min. Aqueous pyridine (10%, 10 ml.) was added into the mixture, which was set aside for 3 hr. at room temperature. Passing this solution through a column (1×15 cm.) of Dowex 1×2 (pyridinium) resin and evaporation of the effluent *in vacuo* gave a syrup, which was azeotropically dried with pyridine (5×5 ml.). Finally, the residue was taken up in 1 ml. of pyridine and added dropwise into anhydrous ether. The resulting fine white precipitate was collected by centrifugation. Purity was tested by paper electrophoresis, which showed one spot having $\lambda_{max}^{H_2O}$ 280 mμ.

2′,3′-O-Isopropylidene-5′-O-benzoyl-N^6-dimethyladenosine——2′,3′-O-Isopropylidene-N^6-dimethyladenosine (300 mg., 0.9 mmole) was dissolved in 5 ml. of pyridine followed by the addition of 250 mg. (ca. 2 mmole) of benzoyl chloride. The solution was kept overnight at room temperature. The reaction mixture was poured into ice-water (100 ml.), extracted with chloroform, washed with sodium bicarbonate and water, and dried over magnesium sulfate. Evaporation of the solvent *in vacuo* and recrystallization of the residue from ethanol gave the crystalline benzoyl derivative, m.p. 136~137°. *Anal.* Calcd. for $C_{22}H_{25}O_5N_5$: C, 60.17; H, 5.72; N, 15.94. Found : C, 60.02; H, 5.87; N, 16.21. Ultraviolet absorption property : λ_{max}^{EtOH} 274 mμ.

5′-O-Benzoyl-N^6-dimethyladenosine——Isopropylidene-benzoyldimethyladenosine (350 mg., 0.8 mmole) was dissolved in 30 ml. of 20% aqueous acetic acid and heated at 100° for 1.5 hr. The pale-yellow solution was evaporated *in vacuo* and traces of acetic acid were removed by repeated co-distillation with ethanol. The single spot of the residue on paper chromatogram (see Table I) showed the consumption of metaperiodate.

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*5 The absorption of the solution in a 1 cm. light path multiplied by the total volume (ml.) of the solution.

*6 Obtained by the reaction of triethylamine and methyl iodide, followed by the replacement of iodide with a hydroxyl ion by silver oxide treatment (Unpublished experiment by M. Ikehara).

21) S. Suzuki, S. Marumo : J. Antibiotics (Tokyo), **13A**, 360 (1960).
22) R. Lohrmann, H. G. Khorana : J. Am. Chem. Soc., **86**, 4488 (1964).
23) M. Viscontini, D. Hoch, P. Karrer : Helv. Chim. Acta, **38**, 642 (1955).
24) M. Ikehara, E. Ohtsuka : This Bulletin, **11**, 435 (1963).

Table I. Paper Chromatography of Different Compounds

Compound	Solvent				
	A	B	C	D	E
Tubercidin	0.28[a]	0.59[a]			
Methoxytrityltubercidin	0.88[a]	0.92[a]			
Tubercidin 3′-phosphate		1.11[b]	0.87[b]		
Tubercidin 2′-phosphate			1.50[b]	1.29[b]	1.31[b]
Tubercidin 2′,3′-cyclic phosphate		0.90[b]			
Isopropylidenedimethyladenosine	0.85[a]				
Benzoylisopropylidenedimethyladenosine	0.85[a]				
Benzoyldimethyladenosine	0.72[a]				
Dimethyladenosine 2′-phosphate			1.27[b]		1.92[b]
Dimethyladenosine 3′-phosphate		1.32[b]	0.78[b]		1.89[b]
Dimethyladenosine 2′,3′-cyclic phosphate				2.96[b]	
N^6,2′,3′-Tribenzoyl adenosine	0.90[a]				
ApA		1.58[b]			
CpC		1.55[b]			
TupApA		1.32[b]			
TupCpC		0.68[b]			
DMApApA		1.50[b]			0.53[b]
DMApCpC		0.89[b]			0.56[b]

a) Rf value *b*) Rf value divided by that of Ap

Table II. Paper Electrophoresis of Different Compounds

Compound	Condition	
	I	II
Tubercidin	−0.25[a]	
Tubercidin 2′-phosphate	0.90[b]	
Tubercidin 3′-phosphate	0.95[b]	0.99[b]
Triacetyltubercidin 3′-phosphate	0.98[b]	
Dimethyladenosine	−0.10[a]	
Dimethyladenosine 3′-phosphate	0.90[a]	0.94[b]
Dimethyladenosine 2′,3′-cyclic phosphate		0.60[b]
Diacetyldimethyladenosine 3′-phosphate	0.96[b]	
ApA		0.48[b]
CpC	0.35[a]	0.49
TupApA		0.79[b]
TupCpC	0.53[a]	0.74[b]
DMApApA		0.64[b]
DMApCpC	0.55[a]	0.62[b]

a) Migration distance divided by that of Cp. *b*) Migration distance divided by that of Ap.

N^6-Dimethyladenosine 3′-Monophosphate——The 5′-O-benzoyl derivative (1 mmole) obtained above, was dissolved in 5 ml. of pyridine and 2 mmole of cyanoethyl phosphate in 2 ml. of pyridine was added. Slight insolubles were dissolved by the addition of 1 ml. of DMF. After addition of 1.6 g. of DCC, the reaction mixture was shaken in the dark for 2 days at room temperature. After addition of 10 ml. of water, the reaction mixture was extracted with cyclohexane, filtered and the filtrate was evaporated *in vacuo* to a small volume. A gellatine-like precipitate was dissolved in pyridine, and 1 vol. of conc. ammonia was added. After heating at 55° for 1 hr. and allowing to cool overnight, the solution was filtered and concentrated to 25 ml. (TOD_{275}=7050). This solution was applied to a column (1.5×26 cm.) of Dowex 1×2 (formate) and eluted with 0.2*N* formic acid (8.5–11 ml. fractions were collected). Dimethyladenosine 2′-phosphate (TOD_{275}= 2030, 28.8%) was obtained as the first major peak and the 3′-phosphate was obtained as the next peak (TOD_{275} 2100, 29.8%) (see Figure). Purity of the sample was examined by paper chromatography and paper electrophoresis (see Table I and II).

2′,5′-Di-O-acetyl-N⁶-dimethyladenosine 3′-Phosphate——Dimethyladenosine 3′-phosphate (0.114 mmole), obtained above, was dissolved in pyridine and evaporated to give a hard syrup. Tetraethylammonium acetate (1.75 ml. of 0.65*M* aqueous solution) was added to the syrup and azeotropically dried with pyridine. The traces of pyridine was removed by co-distillation with toluene. Acetic anhydride (0.6 ml., 6 mmole) was then added and the mixture was shaken in the dark at room temperature for 3 days. Work-up of this mixture as described in the case of triacetyl tubercidin 3′-phosphate gave 2′,5′-di-O-acetyl-N⁶-dimethyladenosine 3′-phosphate as a white powder, which was tested for purity by paper electrophoresis (see Table II).

N⁶,2′,3′-O-Tribenzoyladenosine——5′-Monomethoxytrityladenosine[22] (540 mg., 1 mmole) was dissolved in 30 ml. of pyridine followed by the addition of a mixture of benzoic anhydride (1.0 g., 4.4 mmole) and *p*-toluenesulfonic acid (516 mg., 3 mmole) previously heated in 30 ml. of pyridine for 30 min. at 100°. After three days at room temperature in the dark, the reaction mixture was poured into ice-water. The resulting pink-white powder was dissolved in chloroform, the solution was washed with sodium bicarbonate solution and water, and dried over sodium sulfate. Evaporation of chloroform gave a heavy oil, which was freed of pyridine by distillation with toluene. To the residue 80% aqueous acetic acid (10 ml.) was added and the mixture was shaken for 4 hours at room temperature. Acetic acid was removed to give a solid, which was taken up in chloroform and dried over sodium sulfate. After evaporation of chloroform to a small volume, the solution was applied to a column (1.8×20 cm.) of silicic acid. A linear gradient elution with chloroform and chloroform containing 5% methanol gave N,2′,3′-tribenzoyladenosine as a glass (yield 505 mg.). *Anal.* Calcd. for $C_{31}H_{25}O_7N_5 \cdot H_2O$: C, 54.94; H, 4.03; N, 10.33. Found : C, 54.48; H, 4.32; N, 10.01. Utraviolet absorption properties : λ_{max}^{EtOH} 231, 280 mμ (shoulder). Paper chromatography (see Table I, spot does not consume metaperiodate[23]) also showed the existence of benzoyl groups on 2′ and/or 3′.

N⁶,2′-O-Dibenzoyladenylyl-(3′→5′)-N⁶,2′,5′-tribenzoyladenosine——Pyridinium 5′-monomethoxytrityl-N,2′-dibenzoyladenosine 3′-phosphate[14] (0.1 mmole) and N,2′,3′-tribenzoyladenosine (250 mg., 0.37 mmole), obtained as above, was dissolved in 2 ml. of pyridine, followed by the addition of DCC (206 mg., 1.0 mmole) and 30 mg. of dried Dowex 50×8 (pyridinium form) resin. The mixture was kept for 3 days at room temperature with occasional shaking. After pyridine-water (1 : 1, 10 ml.) was added, the mixture was extracted with cyclohexane, and the water layer was set aside for 4 hours. Dicyclohexylurea was filtered off and the filtrate was evaporated to remove the last traces of pyridine by the co-distillation with toluene. The residue was dissolved in 80% aqueous acetic acid (10 ml.) and kept at room temperature for 4 hours. Evaporation of the solvent gava a glass, which was taken up in 95% ethanol (100 ml.) and applied to a column (4.0×50 cm.) of DEAE-cellulose (acetateL) at 4°. Elution with a linear gradient of triethylammonium acetate (2.1. of 0.2*M* solution)-95% ethanol (2L.) gave the protected dinucleoside phosphate. Center fractions of main peak were pooled and evaporated below 10° with addition of pyridine. The anhydrous pyridine solution (2 ml.) was added dropwise to anhydrous ether. The resulting white precipitate was collected by centrifugation, washed with ether and dried over phosphorous pentoxide (yield 55 mg.). Deprotection of this material with methanol (saturated with ammonia) gave a compound, R_{Ap} (E) 0.69, which is degraded by spleen phosphodiesterase as described below.

Tubercidin-(3′→5′)-adenylyl-(3′→5′)-adenosine (TupApA)——Triacetyl-tubercidin 3′-phosphate (35 μmole) and N,2′-O-dibenzoyladenylyl-(3′→5′)-tribenzoyladenosine (10 μmole) were condensed in 0.2 ml. of pyridine in the presence of 30 μmole of DCC and 70 mg. of Dowex 50×8 resin as described in Ref. 14. The trinucleoside diphosphate was first purified by preparative paper electrophoresis as in condition II, the appropriate spot was excised, extracted with water and applied to paper chromatography in solvent B. (Rf values are summarized in Table I). Yield estimated spectrophotometrically on the basis of ε_{260} 41000 was 13 μmole (53 optical density units). Ultraviolet absorption properties : $\lambda_{max}^{H_2O}$ 260 mμ, $\lambda_{min.}^{H_2O}$ 234 mμ, 280/260= 0.49.

Enzymatic digestion of this specimen was carried out as follows : TupApA (2 O.D. units) in 50 μl of water was incubated with 20 μl. of spleen phosphodiesterase*7 (20 units/ml.) and 10 μl of 1*M* ammonium acetate (pH 5.9) at 37° for 4.5 hours. ApA (2 O.D. units), obtained above, was similarly incubated. Paper electrophoresis of the incubated mixture showed 2 spots corresponding to the nucleoside R_{Ap} 0.04 (presumably inosine, which was derived from adenosine by the contaminating deaminase) and nucleotides, R_{Ap} 1.01 (adenosine 3′-phosphate and tubercidin 3′-phosphate) in the ratio of 1.0 : 1.92. The control ApA was found to be digested to inosine and A_p in the ratio of 1.0:0.96. In both cases spots corresponding to TupApA (R_{Ap} 0.79) and ApA (R_{Ap} 0.48) were not detected.

N⁶-Dimethyladenylyl-(3′→5′)-adenylyl-(3′→5′)-adenosine (DMApApA)——The reaction was carried out in a similar manner as described for the synthesis of TupApA, except that diacetyl-N⁶-dimethyladenosine 3′-phosphate (55 μmole) was used instead of triacetyl-tubercidin 3′-phosphate and the solvent DMF (0.1 ml.) was used in addition to pyridine. Work-up of the reaction mixture as described above gave 33 O.D. units ($_{260}$ 41700) of DMApApA. Ultraviolet absorption properties : $\lambda_{max}^{H_2O}$ 260 mμ, $\lambda_{min}^{H_2O}$ 232 mμ; 280/260=0.50. Paper chromatography and paper electrophoresis both showed single spots (see Table I and II).

*7 Purchased from Worthington Biochemical Corporation, Freehold, N. J., U. S. A.

Tubercidinyl-(3′→5′)-cytidylyl-(3′→5′)-cytidine (TupCpC)——Triacetyltubercidin 3′-phosphate (35 μmole) and dibenzoylcytidylyl-(3′→5′)-tribenzoylcytidine (10 μmole) were condensed in 0.2 ml. of pyridine in the presence of 300 μmole of DCC and 70 mg. of Dowex 50×8 (pyridinum) resin for 3 days at room temperature. Work-up and purification similar to that described above gave 16 μmole (40 O.D. units) of TupCpC (ε_{260} 24800). Ultraviolet absorption properties : $\lambda_{max}^{H_2O}$ 271 mμ, $\lambda_{min}^{H_2O}$ 246 mμ; 280/260=1.02. Paper chromatography and electrophoresis data are summarized in Table I and II.

TupCpC (2 O.D. units) was incubated with spleen phosphodiesterase as described in the case of TupApA. A control digestion of CpC was also done. Paper electrophoresis of the incubation mixture showed complete digestion of TupCpC and CpC.

N⁶-Dimethyladenylyl-(3′→5′)-cytidylyl-(3′→5′)-cytidine (DMApCpC)——Diacetyl-N⁶-dimethyladenosine 3′-phosphate (55 μmole) and dibenzoylcytidylyl-(3′→5′)-tribenzoylcytidine[14] (10 μmole) were condensed by a procedure similar to that used for DMApApA. Work-up and purification by successive paper electrophoresis (condition II) and paper chromatography (solvent B) gave 31 O.D. units of DMApCpC (ε_{260} 25500). Ultraviolet absorption properties : $\lambda_{max}^{H_2O}$ 272 mμ, $\lambda_{min}^{H_2O}$ 243 mμ; 280/260=1.28. Rf's in paper chromatography and electrophoresis are presented in Tables I and II. Incubation of this sample (2 O.D. units) with spleen phosphodiesterase as described in the case of TupApA showed complete digestion to the monophosphates*8 and cytidine (see Figure).

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Summary

Tubercidin (7-deazaadenosine) was converted to the 5′-O-monomethoxytrityl derivative, which was phosphorylated with P¹-diphenyl-P²-morpholinopyrophosphorochloridate to afford the 2′- and 3′-monophosphates. The latter compound was acetylated to N,2′,5′-tri-O-acetyltubercidin 3′-phosphate. N⁶-Dimethyladenosine was converted to the 5′-benzoyl derivative which was phosphorylated with cyanoethyl phosphate and DCC to give the 2′- and 3′-monophosphates. N⁶-Dimethyladenosine 3′-phosphate was protected by acetylation in the 2′- and 5′-positions. These protected 3′-phosphates were subjected to condensation with N,2′-dibenzoyladenylyl-(3′→5′)-N,2′,3′-tribenzoyladenosine or N, 2′-dibenzoylcytidylyl-(3′→5′)→N, 2′,3′-tribenzoylcytidine by the DCC procedure. The trinucleoside diphosphates 7-deazaadenylyl-(3′→5′)-adenylyl-(3′→5′)-adenosine, 7-deazaadenylyl-(3′→5′)-cytidylyl-(3′→5′)-cytidine, N⁶-dimethyl-adenylyl-(3′→5′)-adenylyl-(3′→5′)-adenosine and N⁶-dimethyladenylyl-(3′→5′)-cytidylyl-(3′→5′)-cytidine were purified by paper chromatography and paper electrophoresis. Complete digestion of these trinucleoside diphosphates with spleen phosphodiesterase established the 3′→5′ phosphodiester linkages.

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*8 A slower moving U.V. absorbing spot just after the spot of monophosphate may be ascribed to protein-nucleotide complex, which had appeared in the case of enzymatic digests and has a characteristic phosphorescence.