Note

UDC 577.1:547.857.7'456-118.5.07

Tohru Ueda and Eiko Ohtsuka : Synthesis of Guanosine 5'-Diphosphate.

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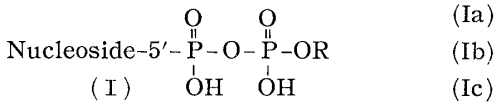
Recently the importance of the study of naturally occurring nucleotides, which exist as a fundamental skeleton of the nucleic acid and also as "nucleotide co-enzyme," the co-enzymes related to metabolism of carbohydrate and lipid, increased. The so-called nucleotide co-enzyme has the structure of nucleoside 5'-diphosphate (I), as shown below:



(I)

(Ia) R : H
(Ib) R : PO_3H_2
(Ic) R : sugar

In 1957, it was established by Ochoa, *et al.*[1] that the nucleoside diphosphate (Ia) is the direct precursor for the biosynthesis of polynucleotide. Triphosphate (Ib), ATP[*2] for instance, works as the energy-transfering substance in the living cell. On the other hand, nucleoside diphosphate sugar (Ic), as shown by UDPG[*2] or GDPM,[*2] is the co-enzyme associated with carbohydrate metabolism.

The present paper deals with the synthetic study of guanosine 5'-diphosphate (V), one of the precursors for biosynthesis of nucleic acid.

GDP[*2] was synthesized first by Khorana[2] using dicyclohexyl carbodiimide (DCC) in a yield of 25%. This reaction, however, is proceeded by the production of GTP[*2] and this proved to be a rather novel route to GTP-synthesis. Todd, *et al.*[3] synthesized ADP[*2] by a reaction of benzyl hydrogen phosphoramidate (II) with AMP, while Khorana[4] synthesized UDPG and FAD[*2] by the nucleoside-phosphoramidate method. This method may afford the most excellent procedure for the synthesis of pyrophosphates.

A synthetic route for GMP (III) according to Khorana's investigation[5] was adopted in the present case, in which guanosine[6] was converted to 2',3'-O-isopropylideneguanosine and phosphorylated with tetrakis(*p*-nitrophenyl) pyrophosphate, followed by alkaline and acid hydrolyses.

Benzyl hydrogen phosphoramidate (II) was obtained by Todd's method[7] with slight modification in a yield of 90%.

The reaction of (II) with (III) to (IV) was examined in a small scale as described in Table I, which shows the extent of reaction in dimethylformamide (DMF) was ca. 50%, both in the presence and absence of water at 37° and 70°. Prolonged reaction or additional phosphoramidate has no effect on the extent of the reaction. Upon close investigation a precipitate was observed, consisting mainly of the ammonium salt of GMP, which

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*2 Abbreviatiations used : .ATP adenosine 5'-triphosphate; UDPG uridine 5'-diphosphate glucose; GDPM guanosine 5'-diphosphate mannose; GDP guanosine 5'-diphosphate; GTP guanosine 5'-triphosphate; ADP adenosine 5'-diphosphate; AMP adenosine 5'-monophosphate; FAD flavin adenine diphosphate; GMP guanosine 5'-monophosphate.

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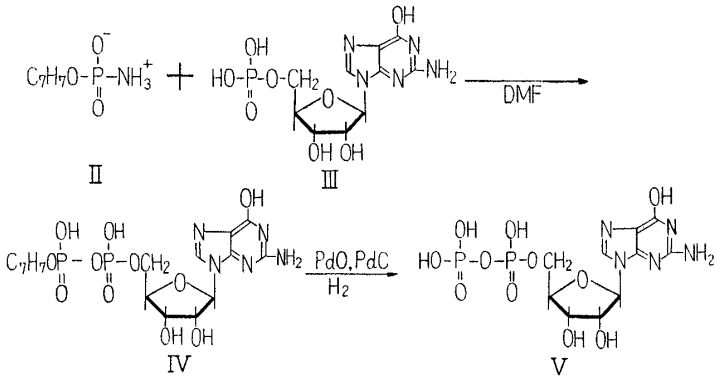

reaction proceeded 50%. Large-scale experiments, as shown in Table II, also gave similar results. Reaction mixture was hydrogenated in the presence of PdO-Pd/C catalyst and GDP (V) was separated from GMP by the aid of anion exchanger (Amberlite IRA-400, 100~200 mesh, Cl'-form), which gave Ca-salt of GDP in a yield of 38%. GDP thus obtained was hydrolyzed by *N* HCl at 100° for 7 minutes giving GMP and inorganic phosphate. From these findings together with Rf value obtained with paper chromatography, it was concluded that this substance contains labile phosphorus and has the structure of guanosine 5'-diphosphate.

The yield of this reaction did not seem to be satisfactory and must be increased somewhat by using N-alkyl amidate as the reagent, because of its higher solubility. Furthermore, the solvent system should be altered to obtain a uniphased solution dissolving ammonium salt of GMP. The synthesis of GDPM is now in progress along these lines and the results will be reported elsewhere.

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Experimental*3

Paper Chromatography—

Substances	Rf		
	Solvent (1)	Solvent (2)	Solvent (3)
GDP	0.10	0.82	0.09
GMP	0.23	0.70	0.33
GDP-benzyl	0.44	—	—
Benzyl hydrogen phosphoramidate	0.82	—	—
Guanosine	—	—	0.58
Ribose 5-phosphate	—	—	0.66
Phosphorus	—	—	0.40
Ribose	—	—	0.73
Guanine	—	—	0.48

Solvent system
(1) *iso*-PrOH : 1% $(NH_4)_2SO_4$=2:1 (descending)[5]
(2) *iso*-AmOH : 5% KH_2PO_4 (ascending)[5]
(3) *n*-PrOH : ammonia:water=6:3:1 (descending)[8]

Detection—
Base : UV Absorption.
Phosphorus : $HClO_4$-MoO_4.[8]
Sugar : HIO_4-benzidine.[9]

*3 UV absorptions were taken by Beckman DK-II and Shimadzu QR spectrophotometer.

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Reaction of GMP with Benzyl Hydrogen Phosphoramidate—0.1 m. mole of GMP-pyridine salt and 0.3 m. mole of benzyl hydrogen phosphoramidate[7] were reacted in 2 cc. of DMF in various conditions (cf. Table I). P^1-Guanosin-5′-yl-P^2-benzyl pyrophosphate (GDP-benzyl) was detected by UV-absorbing spot on paper chromatogram (Solvent (1)). The spot was cut off and eluted with 0.5*N* ammonia. The elute was hydrogenated in the presence of PdO and 10% Pd-C to remove the benzyl group. A spot, which shows a higher Rf value than GMP, vanished and a spot of small Rf value (GDP) was detected.

P^1-Guanosin-5′-yl-P^2-benzyl Pyrophosphate (GDP-benzyl)—0.6 g. of GMP-Ba·$8H_2O$ was added to 40 cc. of water containing appropriate amount of cation exchanger (Amberlite IR-120, H^+-form), mixed well by shaking, and filtered. The filtrate and washings were combined and excess of pyridine added. Evaporation and drying over P_2O_5 of above solution gave GMP-pyridine salt. 280 mg. of this salt was dissolved in 40 cc. of DMF containing 1% of water. Into the slightly turbid solution, 400 mg. of benzyl hydrogen phosphoramidate was added and heated for 1 hr. at 40°, but no remarkable change was observed. After an additional heating for 1 hr. at 70°, the initial colorless solution became pale yellow and a precipitation appeared, which was confirmed as the salt of GMP by paper chromatography. To dissolve the precipitate thus obtained an amount of water, 5% of the original volume, was added. A part of the precipitate first disappeared, but increased during reaction. Table II shows results obtained during prolonged reaction at 40° and addition of excess amidate into reaction mixture. These reagents were added three times, until product ceased to increase. Reaction was stopped at the point where no increase was observed by further additions of reagents. The solvent was evaporated under reduced pressure below 40°.

Table I.

Time (hr.)	Temp. (°C)	Yield of benzyl ester[a] DMF (%)	DMF(10% H_2O) (%)	DMF(5% H_2O) (%)	Additional amidate[b] (mg.)(mole)
0.5	room	detected	detected		
1.5	〃	〃	〃		
3.5	70	52	63		
4.5	70	57	55		
9.5	70	55	57		
15.5	70	50	52		
6.0	room			30	
30	37			35	
54	37			52	15(1.7)
78	37			53	15(1.7)

Table II.

Time (hr.)	Temp. (°C)	Yield of benzyl ester[a] (%)	Water added (%)	Additional amidate[b] (mg.)(mole)
0.1	room	8.5	1	
1.0	40	11	1	
2.0	70	12	1	
21	40	25	5	200(1.8)
70	40	23	5	
215	40	50	5	100(0.9)
264	70	53	5	
279	40	53	5	100(0.9)

[a] Aliquots were extracted each time and submitted to paper chromatography. (Solvent (1)). Spots of GMP and of GDP-benzyl were extracted with 5 cc. of 0.1*M* phosphate buffer (pH 7) and estimated by absorbance.

[b] Reaction mixture was heated at 70° for 1 hr. after each addtion.

Guanosine 5′-Diphosphate—The residue obtained in above reaction was dissolved in water, adjusting the pH to 4~5 with pyridine and AcOH. Debenzylation was carried out by the hydrogenation in the presence of 60 mg. of PdO and 10 mg. of 10% Pd-C. After the reaction, catalysts were removed by filtration and the filtrate was adsorbed on a column of anion exchanger (1.8×7 cm., Amberlite IRA-400, Cl^--form, 100~200 mesh) at a flow rate of 1 cc./min. The column was washed with water until the optical density of the eluant became 0.2. Elution with 0.003*N* HCl+0.015*N* $CaCl_2$ gave GMP, 0.003*N* HCl+*N* $CaCl_2$ gave GDP, and 0.003*N* HCl+0.2*N* $CaCl_2$ gave GTP, and this was followed by washing with 2*N* HCl (flow rate, 2 cc./min.).

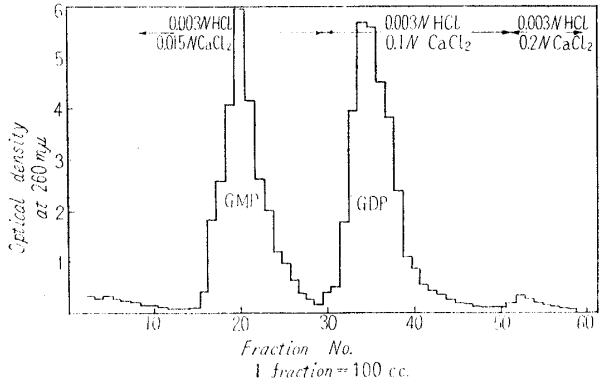

Fig. 1.
Ion Exchange Chromatogram

The results are shown in Fig. 1, in which optical density at 260 mμ is on the ordinate and the fraction number (100 cc. each) is scaled on the abscissa. Fractions showing constant ratio of absorbance at 260 mμ vs. 280 mμ were collected. GMP- and GDP-fractions were obtained at pH 7 with $Ca(OH)_2$, evaporated to a small volume, and freeze-dried. Residual mixture of $CaCl_2$ and Ca-salt of nucleotide were washed with EtOH-Et_2O (1:1 v/v) to remove $CaCl_2$.[10] GDP·$^3/_2$Ca was washed once with EtOH-Et_2O, twice with Et_2O, and dried over P_2O_5 *in vacuo* (yield, 220 mg.).

Paper chromatography: Solvent (1) GDP Rf 0.10, GMP 0.23; solvent (2) GDP 0.82, GMP 0.70. Purity calculated phothometrically from the ε-value, 13.5×10^3 (at λ_{max} 250 mμ in a phosphate buffer of pH 7) was 54.6%. The ratio of base:P was 1:2.0 analyzed by Allen's method.[11] Hydrolysis with *N* HCl at 100° for 10 min. showed the spot of GMP, inorganic P, and ribose 5-P. Rf's in solvent (3) were 0.33, 0.40, and 0.66, respectively. Neutralization of the hydrolysate gave a precipitation of guanine, while the hydrolysis of guanosine under the same conditions gave the spot of guanosine (0.58), guanine (0.48), and ribose (0.73).

Summary

Guanosine 5′-diphosphate was synthesized from guanosine 5′-monophosphate by the reaction of benzyl hydrogen phosphoramidate, followed by the catalytic hydrogenolysis, in a yield of 38%.

The effect of water in the reaction media and correlation of the yield with the amount of reagents are discussed.

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