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Syntheses of 2,3-Cyclic Ribonucleotides and their Properties as Substrate of Ribonuclease

In a previous communication,[1] the present writers reported on syntheses of methyl α- and β-ribofuranoside 2,3-cyclic phosphates and showed that both of these phosphates failed to serve as the substrate of RNase-I*[1] in giving the corresponding methyl ribofuranoside 3′-phosphates. These results gave evidences that in these types of cyclic ribotide, the minimum structural requirement for the enzymatic activity might exist in the 2,3-cyclic ribotide containing a more specially arranged atom grouping than O-methyl group at 1-position of D-ribose moiety.

The present communication describes the syntheses of two 2′,3′-cyclic ribonucleotides containing pyrimidine bases; 3-(β-ribofuranosyl)thymine 2′,3′-cyclic phosphate (V) and 5-bromouridine 2′,3′-cyclic phosphate (VIII), and their properties as substrate for RNase-I•A.[2]

Although both 3-(β-ribofuranosyl)thymine (ribothymidine) (I) and its 2′- or 3′-phosphate (IV) were recently discovered in nature as minor constituents of ribonucleic acid from yeast,[3,4] wheat germ, and *Escherichia coli*,[3] no report has hitherto been made on the chemical synthesis of the latter.

Modifying Fox's procedure,[5] 3-(β-ribofuranosyl)thymine (I) was treated with trityl chloride in dehyd. pyridine to furnish 5′-tritylribothymidine (II), m.p. 162~165°. (II) was phosphorylated with dibenzyl chlorophosphonate in pyridine according to the method reported by Brown.[6] The syrupy product thereby obtained was detritylated by refluxing in 80% acetic acid for 20 minutes and followed by catalytic debenzylation using a mixture of palladium oxide and palladium-charcoal as a catalyst. The product, mixed 2′- and 3′-phosphates (IV) of ribothymidine which showed simple phosphorus spot (Rf_1*[2] 0.16, Rf_2 0.41, Rf_4 0.29, and Rf_5 0.53) on each paper chromatogram run with different solvents,*[2] was isolated as barium salt in 45% yield (*Anal.* Calcd. for $C_{10}H_{13}O_9N_2BaP \cdot 6H_2O$: C, 20.64; H, 4.29; N, 4.81; P, 5.33. Found: C, 20.73; H, 4.38; N, 4.64; P, 5.66. Mcp_1*[3] 1.18, Mcp_2 1.00). (IV) was decationized with Amberlite IR-120 (H^+) and dehydrated with DCC in pyridine to 2′,3′-cyclic phosphate (V) which was isolated as its ammonium salt of m.p. 190~200°(decomp.) (*Anal.* Calcd. for $C_{10}H_{16}O_8N_3P$: N, 12.45; P, 9.28. Found: N, 12.63; P, 8.85. Mcp_1 1.26, Mcp_2 0.85). This phosphate (V) showed typical properties of a cyclic phosphate in its mobility in paper electrophoresis,[7] giving a minor mobility at

*1 Abbreviation: RNase-I•A, ribonuclease-I•A; DCC, dicyclohexylcarbodiimide (1,3-dicyclohexylurea); NBS, N-bromosuccinimide.

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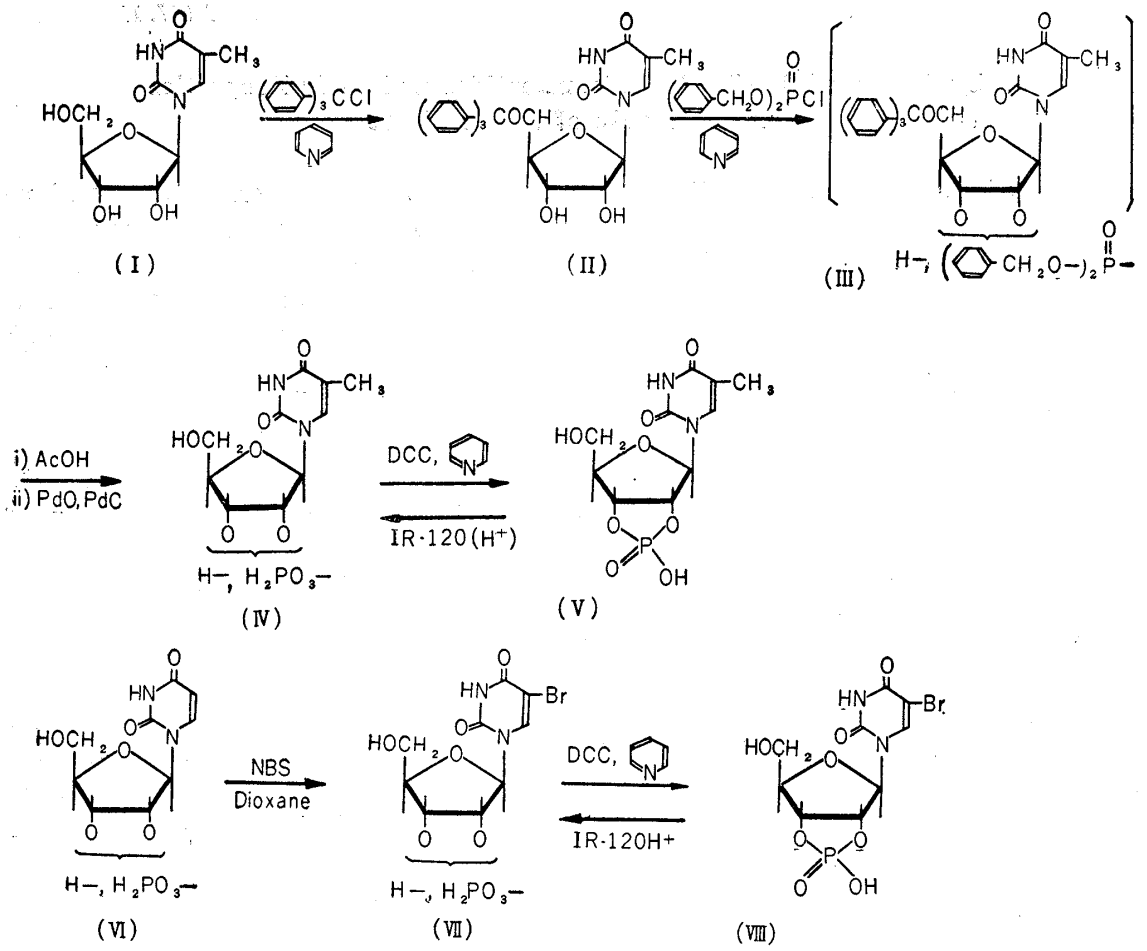
2) C. H. W. Hirs, S. Moore, W. H. Stein: J. Biol. Chem., **200**, 493(1953).
3) J. W. Littlefield, D. B. Dunn: Biochem. J., **70**, 642(1958); Nature, **181**, 254(1958).
4) F. F. Davis, A. F. Carleuci, I. F. Roubein: J. Biol. Chem., **234**, 1525(1959).
5) J. J. Fox, N. Yung, A. Bendich: J. Am. Chem. Soc., **79**, 2775(1957).
6) D. M. Brown, A. R. Todd: J. Chem. Soc., **1952**, 44.

*2 Paper chromatography was performed on Toyo Roshi No. 51, using the following solvent systems: (1) *iso*-PrOH:conc. NH_4OH:H_2O (7:1:2); (2) AcOH : BuOH:H_2O (1:4:5); (3) isobutyric acid:0.5*N* NH_4OH (10:6); (4) *tert*-AmOH:90% HCOOH:H_2O (3:1:3); (5) *iso*-PrOH:H_2O (2:1) saturated with ammonia vapor. The Rf values for these five solvent systems are respectively represented by Rf_1, Rf_2, Rf_3, Rf_4, and Rf_5.

*3 Paper electrophoresis was performed on Toyo Roshi No. 51 at 700 v/15 cm. for 1 hr., using the following buffer solutions: (1) Pyridine : AcOH : BuOH:H_2O (10:2:20:500); (2) the buffer solution (1) adjusted to pH 9.0 by addition of 4*N* NH_4OH. The mobilities of the compounds tested in respective buffer solutions of (1) and (2) are represented by Mcp_1 and Mcp_2 taking that for the mixed cytidine 2′- and 3′-phosphate as standard ($M=1.00$).

7) G. M. Tener, H. G. Khorana, R. Markham, E. H. Pol: J. Am. Chem. Soc., **80**, 6223(1958).



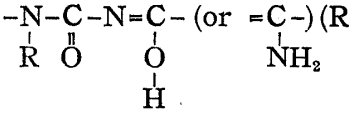
pH 9 and larger in its mobility at pH 6 than those observed for the corresponding doubly dissociable phosphate (IV). (V) was easily hydrolyzed to (IV) by treatment of its aqueous solution with Amberlite IR-120 (H^+) at room temperature.

In accordance with Michelson's bromination method[8] for the preparation of 5-bromouridine 5′-phosphate from uridine 5′-phosphate, the mixture of 2′- and 3′-uridylic acid (VI) was brominated with NBS to give a mixed 5-bromouridine 2′- and 3′-phosphates (VII) which was isolated as its calcium salt in 80~90% yield (*Anal.* Calcd. for $C_9H_{10}O_9N_2BrCaP \cdot 4H_2O$: C, 21.05; H, 3.50; N, 5.46; P, 6.04. Found: C, 21.41; H, 3.67; N, 5.44; P, 5.82. (Rf_1 0.19, Rf_2 0.22, Rf_3 0.41, Rf_4 0.29, Rf_5 0.55. Mcp_1 1.18, Mcp_2 1.07). The UV absorptions of this product closely resembled those of 5-bromuridine and 5-bromouridine 5′-phosphate, respectively reported by Roberts[9] and by Michelson.[8] After decationization, (VII) was treated with DCC in pyridine, as in the case of conversion of (IV) to (V). The product (VIII) with Rf_1 0.36 was isolated as ammonium salt, m.p. 165° (decomp.) (*Anal.* Calcd. for $C_9H_{13}O_8N_3BrP$: N, 10.42; P, 7.71. Found: N, 10.28; P, 7.28. Mcp_1 1.25, Mcp_2 0.86). (VIII) also showed typical properties of a cyclic nucleotide in its mobility in paper electrophoresis run at both pH 9.0 and 6.0.

These synthesized cyclic nucleotides (V and VIII) were checked on their activity as substrate for RNase-I•A. On their incubation with the enzyme in an acetate buffer (pH 6.0), the two compounds gave a respective hydrolysis product, with Rf_1 value of 0.16 and 0.19, and these were identified with (IV) and (VII), respectively, by paper chromatography.

8) A. M. Michelson: J. Chem. Soc., **1958**, 1957.
9) M. Roberts, D. W. Visser: J. Am. Chem. Soc., **74**, 668(1952).

Thus, RNase-I•A was found to hydrolyze the cyclic phosphate ring of these synthetic nucleotides giving the corresponding presumable 3′-monophosphates.

Recently, Witzel[10] proposed, without detailed experimental description, that a grouping of  (R=ribose moiety) in the pyrimidine part of componental 3′-ribonucleotide might be required for the hydrolytic cleavage of 3′,5′-internucleotide linkage and this assumption was deduced from observations that RNase-I did hydrolyze the above-mentioned linkage when the 3′-ribonucleotide part contained 4,5-dihydrouracil and did not hydrolyze it when its ribose moiety had no substituent, had ureidopropionate in its 1-position, or when it was reduced to ribitol type.

As far as the present results are concerned, they are in accord with Witzel's assumption and both cyclic nucleotides tested this time contain pyrimidine bases which include the atom grouping suggested by him.

Griffin and Todd[11] reported that ribothymidine 5′-pyrophosphate is active as a substrate for polynucleotide phosphorylase prepared from *Azotobacter vinelandii* or *Escherichia coli*. On hydrolysis of the produced polyribothymidylic acid by RNase, they found an intermediate hydrolysis product, detected on paper chromatogram, and proposed 2′,3′-cyclic ribothymidylic acid (V) for it.

In contrast to the relation between ribothymidine 5′-pyrophosphate and (V), the 5-bromouridine 5′-pyrophosphate, which has the same pyrimidine base as that in cyclic nucleotide (Ⅷ), was reported by Ochoa[12] to have no property as the substrate of polynucleotide phosphorylase, while (Ⅷ) was found to be hydrolyzed by RNase-I.

It is of interest that the structural requirement for a substrate in the 5-substitution of pyrimidine nucleotides is different for these two kinds of enzymes.

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