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Nucleosides and Nucleotides. VIII.[1] Formation of Spongocytidylyladenosine in the Synthesis of Cytidylyladenosine from Cytidine 2',3'-Cyclic Phosphate and Adenosine

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Recently, we have described a convenient method for the synthesis of diribonucleoside monophosphates, starting from adenosine- and uridine-2',3'-cyclic phosphate and an unprotected nucleoside.[3]

In this report, we describe the formation of spongocytidylyladenosine (araCpA) (VI), in addition to cytidylyl-(2'-5')-adenosine ($C\overset{*}{p}A$) (Va) and cytidylyl-(3'-5')-adenosine (CpA) (Vb), from the reaction of 5'-O,N^4-diacetylcytidine 2',3'-cyclic phosphate(5'-O,N^4-diacetyl-2',3'-cyclic CMP) (I) with adenosine (II).

The cyclic phosphate (I) and 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) were allowed to react with II in anhydrous N,N-dimethylformamide (DMF) at room temperature and the products were, after the work up (see Experimental), applied to a DEAE-cellulose anion exchange column. Subsequently, a fraction corresponding to dinucleoside monophosphates was applied to a Dowex-1 column for further separation of the phosphodiester linkage isomers. This was separated into three fractions. The each fraction could be purified by further fractionation on Dowex-1 column.

The structural confirmation of cytidylyladenosines (Va, Vb, VI) in each fraction was performed by alkaline and enzymic hydrolysis, paper chromatography and ultraviolet (UV) absorption spectrum (Table I).

Table I. Properties of Dinucleoside Monophosphates

Dinucleoside monophosphate	$\lambda_{max}^{H_2O}$ (nm)	$\lambda_{max}^{H^+}$ (nm)	*Rf*[a]	Relative[b] mobility ($R_{2',3'\text{-CMP}}$)	Hydrolysis: alkaline	RNase A	PDase	Molar ratio[c]
$C\overset{*}{p}A$	259	262.5	0.16	0.57	+	−		1.00:1.01
CpA	259.5	263	0.15	0.56	+	+	+	1.00:0.95
araCpA	260.5	263	0.15	0.55	−	−	+	1.00:1.03

a) solvent system; EtOH–1M NH_4OAc (pH 7.0) (5:2), v/v) ascending
b) buffer; 0.05M triethylammonium bicarbonate (pH 8.0), 700 volts
c) Molar ratio are shown as cytidylate: adenosine for $C\overset{*}{p}A$ and CpA and for araCpA as araC: 5'-adenylate.

On paper chromatography each fraction showed the same *Rf*-value and so did mobility in paper electrophoresis. UV absorption spectra were all quite similar and periodate test was positive. The nucleotide Va in the first fraction was hydrolyzed to cytidine 2'(3')-phosphate(2'(3')-CMP) and adenosine with alkali but was resistant to ribonuclease A (RNase A), and compound Vb in the second fraction was hydrolyzed to cytidylate and adenosine with both alkaline and RNase A treatment. While compound VI in the third fraction was resis-

1) Part VII: T. Ueda and H. Ohtsuka (née Nishino), *Chem. Pharm. Bull.* (Tokyo), **21**, 1530 (1973).
2) Location: *Kita-12, Nishi-6, Kita-ku, Sapporo.*
3) K. Miura and T. Ueda, *Chem. Pharm. Bull.* (Tokyo), **19**, 2567 (1971).

tant to alkali and RNase A, it was hydrolyzed to spongocytidine (araC) and 5′-adenylic acid by venom phosphodiesterase (PDase).[4]

From the above results it was determined that the dinucleoside monophosphates, Va, Vb and VI, were C$\overset{*}{p}$A, CpA and araCpA, respectively.

Nagyvary has reported that treatment of 2′,3′-cyclic CMP with trimethylsilylchloride at 80° gave 2,2′-O-anhydrocytidine 3′-phosphate[5] and 5′-O,N^4-diacetyl-2′,3′-cyclic CMP was converted to 5′-O,N^4-diacetyl-2,2′-O-anhydrocytidine 3′-phosphate even at low temperature which was converted to 5′-O,N^4-diacetylarabinosylcytosine 3′-phosphate in mild alkaline condition.[6] Ukita and co-workers have suggested the formation of spongocytidylylguanosine in the synthesis of cytidylylguanosine by Michelson's method.[7]

It seemed that the reaction of I and II with TPS produced analogous intermediate IV *via* III which was hydrolyzed to VI, in which the configuration of 2′-hydroxyl group in cytidine moiety has been inversed (Chart 1). Because of the higher nucleophilic charactor of C_2-carbonyl group in cytidine it is reasonable that the intermediate III could be converted to unstable anhydronucleotide IV even at room temperature. Therefore the condition for the

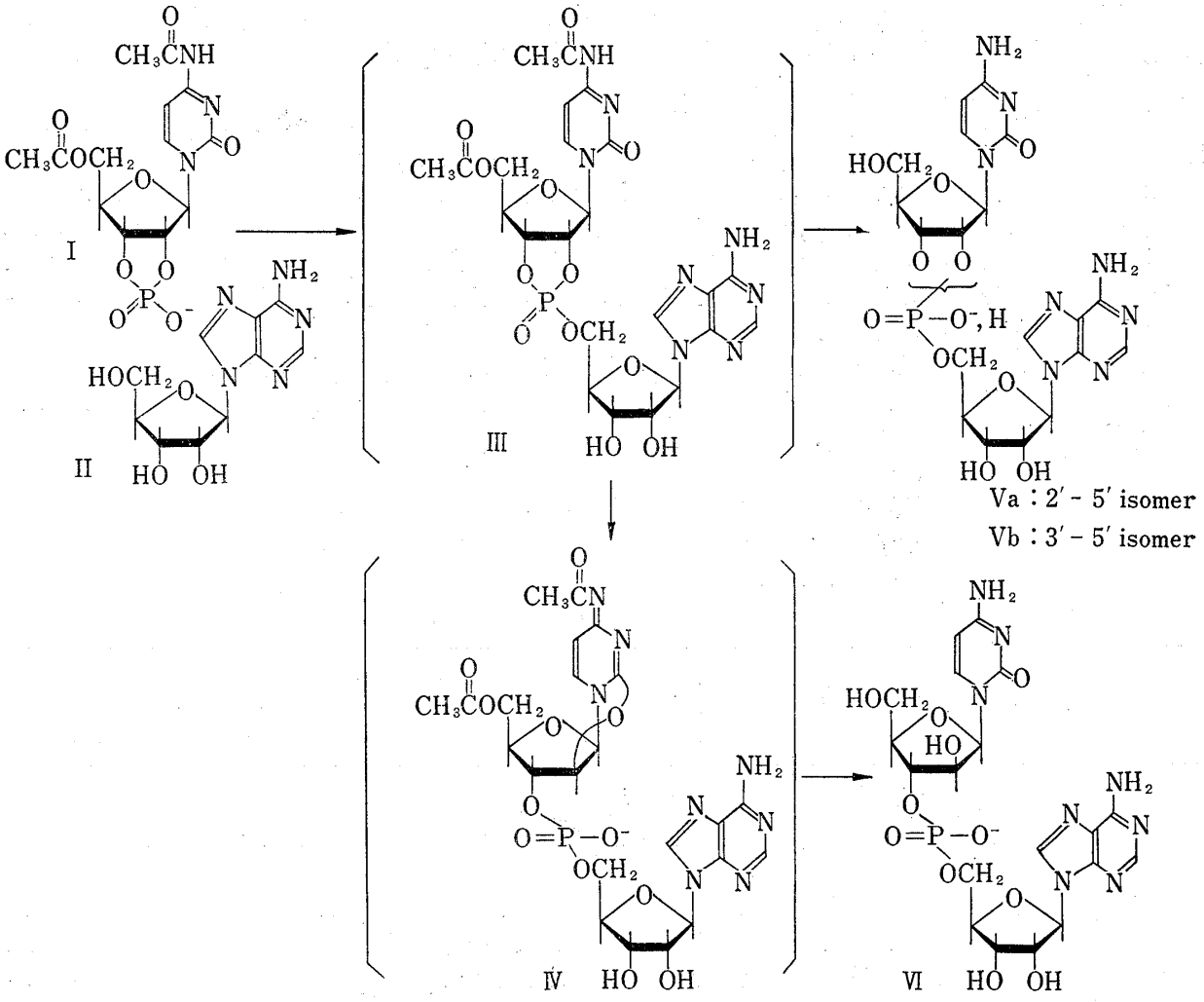

Chart 1

4) W.J. Wechter, *J. Med. Chem.*, **10**, 762 (1967).
5) J. Nagyvary, *J. Am. Chem. Soc.*, **91**, 5409 (1969).
6) J. Nagyvary and C.M. Tapiero, *Tetrahedron Letters*, **1969**, 3481.
7) T. Ukita, M. Irie, M. Imazawa, Y. Furuichi, H. Nishimura, and T. Sekiya, *Seikagaku* (*in Japanese*), **40**, 363 (1968).

Table II. Effect of Temperature for the Formation of AraCpA

Condition		Ratio of products (%)			Total yield (%)
Temperature	Reaction time (hr)	CpA*	CpA	araCpA	
0°	2	53	47	0	36
0°	6	55	45	0	57
Room temperature	15	49	39	12	45
50°	2	38	36	26	44
70°	2	16	21	63	36

formation of compound VI as a function of temperature was investigated and the results are summarized in Table II. It can be noticed from Table II that araCpA (VI) becomes the major product at elevated temperature, whereas the reaction at 0° produced no VI and the yield of dinucleoside monophosphates (Va and Vb) increased on prolongation of the reaction time.

It is to be noted here that although the synthesis of araCpA (VI) has been achieved by the condensation of a protected arabinosylcytosine 3′-phosphate with a protected adenosine by Wechter[4] the method described here is more convenient as compared with that reported.

Experimental

The alkaline and enzymic hydrolysis for structural determination of dinucleoside monophosphates was performed at 37° for 17 hr. The incubation mixture was examined by paper chromatography and electrophoresis and the molar ratio of the hydrolyzate was measured by UV absorption. For alkaline hydrolysis a dinucleoside monophosphate (10—20 OD units at 260 nm) was allowed to stand in 0.3N KOH. For enzymic hydrolysis a dinucleoside monophosphate and RNase A [EC 2.7.7.16] (purchased from Worthington Biochemical Corporation) (10 μl/ 1 mg in 1 ml of water) were allowed to stand in 0.05M tris-HCl buffer (pH 7.5). Hydrolysis by venom PDase [EC 3.1.4.1] (purchased from Worthington Biochemical Corporation) was performed according to the procedure by Wechter.[4]

5′-O,N^4-Diacetyl-2′,3′-cyclic CMP was prepared by the treatment of tri-*n*-butylammonium 2′,3′-cycic CMP[8] with acetic anhydride and tri-*n*-butylamine in DMF-dioxane.[9]

Preparation of Dinucleoside Monophosphates (V and VI)——To a solution of 5′-O,N^4-diacetyl-2′,3′-cyclic CMP tri-*n*-butylammonium salt (I) (prepared from 1 mmole of 2′(3′)-CMP) in 5 ml of DMF was added 3 mmoles of TPS and the solution was stirred for 10 min at room temperature followed by addition of 2 mmoles of adenosine. The reaction mixture was stirred for 2 hr at room temperature avoided from moisture. To the reaction mixture was added water (10 ml) and tri-*n*-butylamine (2 ml) and the solution was stirred for 30 min. After more 50 ml of water was added the mixture was subjected to ether extraction to remove excess amine and the water layer was concentrated to dryness *in vacuo*. The residue was treated with methanolic ammonia (100 ml) overnight at room temperature and the solvent was evaporated. The residue was dissolved in water and the solution was applied to a column of DEAE-cellulose (HCO^-_3 form).

The second eluate(7900 OD units at 260 nm)was subjected to rechromatography of Dowex-1 × 2($HCOO^-$ form) column (column size; 1.8 × 30 cm). Elution was performed with the use of a linear concentration gradient of sodium formate buffer (pH 4.0) (1 liter of 0.01M buffer in the mixing chamber and 1 liter of 0.1M buffer in the reservoir, one fraction; 10 ml/20 min). Final elution was performed with 0.2M buffer. The first fraction (tube No. 78—109, 2000 OD units at 260 nm) was combined and desalted by absorption-elution with the use of active charcoal to give Va containing a small amount of Vb and 2′,3′-cyclic CMP. Further purification was achieved by the repeated column chromatography or by the preparative paper chromatography. The second fraction (tube No. 110—178, 4100 OD units at 260 nm) was combined, desalted and solvent was evaporated. And a part of the residue (2300 OD units at 260 nm) was applied to Dowex-1 × 2 ($HCOO^-$ form) column (column size; 1.2 × 20 cm) and eluated with a linear gradient of sodium formate buffer (pH 4.0) (750 ml of 0.01M buffer in the mixing chamber and 750 ml of 0.1M buffer in the reservoir, one fraction; 10 ml/20 min). The residue was separated into three fractions, F-1: tube No. 48—55, 147 OD units at 260 nm; F-2: tube No. 60—68, 804 OD units at 260 nm; F-3: tube No. 75—93, 449 OD units at 260 nm.

8) H.G. Khorana, "Methods in Enzymology," ed. by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York, Vol. 6, 1963, p. 649.
9) A.M. Michelson, *J. Chem. Soc.*, **1959**, 3655.

The structural determination of dinucleoside monophosphate in each fraction was performed by alkaline and enzymic hydrolysis, paper chromatography, paper electrophoresis and UV absorption measurement (Table I). From that results the products were determined as $C\overset{*}{p}A$, CpA and araCpA, respectively.

Effect of Temperature on the Reaction of 5′-O,N⁴-Diacetyl-2′,3′-cyclic CMP and Adenosine with TPS for the Formation of AraCpA——To a solution of tri-*n*-butylammonium 5′-O,N⁴-diacetyl-2′,3′-cyclic CMP (0.1 mmole) in DMF (0.7 ml) was added 0.3 mmoles of TPS and the reaction mixture was stirred for 10 min followed by addition of adenosine (0.2 mmoles) at 0°, room temperature, 50° and 70°, respectively and kept for 2 to 15 hr.

After the work up as mentioned above the products were subjected to Dowex-1 × 2 ($HCOO^-$ form) column chromatography (column size; 1.3 × 14 cm, elution: a linear gradient of sodium formate buffer (pH 4.0)). The total yield and the ratio of the products were determined by UV spectra as summarized in Table II.