[Chem. Pharm. Bull. 21(2) 264—269 (1973)]

UDC 547.854.81'456.04 : 547.541.5.04

Studies of Nucleosides and Nucleotides. LV.[1] Reaction of Cytidine 5'-Monophosphate with *p*-Toluenesulfonyl Chloride

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(Received May 30, 1972)

Cytidine 5'-monophosphate was reacted with *p*-toluenesulfonyl chloride in dioxane-sodium hydroxide solution. Ion exchange column chromatography of the nucleotides and analysis of each fraction by paper chromatography and paper electrophoresis showed presence of 5'-cytidine 5'-monophosphate, 2,2'-cyclo-arabinosylcytosine 5'-monophosphate, arabinosylcytosine 5'-monophosphate, 2,2'-cyclo-3'-tosylarabinosylcytosine 5'-monophosphate, 2,3'-cyclo-lyxosylcytosine 5'-monophosphate, 2',3'-epoxylyxofuranosylcytosine 5'-monophosphate and lyxofuranosylcytosine 5'-monophosphate. 2,2'-Cyclo-arabinosylcytosine 5'-monophosphate was isolated in 33% yield.

We have recently reported on the selective tosylation of adenosine 5'-monophosphate.[3] In this case toluenesulfonyl group entered selectively to the 2'-OH group and the subsequent bromination and thiolation at position 8 lead to 8,2'-S-cyclo AMP. We attempted to apply this reaction to other nucleoside 5'-monophosphates and found that the reaction of cytidine 5'-monophosphate (CMP) gave rise to variously transformed 5'-nucleotides of cytosine. In connection with known anticancer and antiviral activities of arabinosylcytidine,[4] these products might be interesting in their biological activity.

In the case of AMP, it was found that dropwise addition of the nucleotide dissolved in N sodium hydroxide into a dioxane solution of tosyl chloride gave 2'-tosylate exclusively. We employed this condition to CMP (I). When the reaction mixture was examined by paper chromatography before and after the neutralization (see Table I and II), there appeared more than five compounds and only one having *Rf* 0.63 in solvent G had a tosyl group. One compound appeared at *Rf* 0.26 must be 2,2'-cyclocytidine 5'-MP (II) from its ultraviolet (UV) absorption maxima at 232 and 262 nm.[5] Therefore tosylated compounds may be further converted to other derivatives in this reaction condition. The reaction mixture was applied to a column of Dowex 1×8 (formate form) and eluted with 0.01—0.1N formic acid and 0.01—1 M ammonium formate. Elution pattern was as shown in Fig. 1. The absorxtion ratios A_{270}/A_{260} were changed from fraction to fraction showing that many types of compounds existed. Accordingly, the structure of each compound was determined by evaporating each peak to a powder, which was dephosphorylated with alkaline phosphatase and analysed by alkaline Dowex column chromatography,[6] paper chromatography and paper electrophoresis.

Peak I was eluted by the water-wash and nucleotides were obtained in 55% yield. Dephosphorylation of the powder obtained from fractions of peak I was performed by incubation with alkaline phosphatase.[7] The incubation mixture was applied to a column of Dowex 1×2 (OH^- form) and eluted with 30% methanol and 0.1 M ammonium carbonate, successively.

1) Part LIV: M. Ikehara and Y. Ogiso, *Tetrahedron*, **28**, 3695 (1972).
2) Location: *6-1-1, Toneyama, Toyonaka, Osaka.*
3) M. Ikehara and S. Uesugi, *Tetrahedron Letters*, **1970**, 713; *idem.*, *Tetrahedron*, **28**, 3687 (1972).
4) J.S. Evans, E.A. Musser, G.D. Mengel, K.R. Forsblad, and J.H. Hanter, *Proc. Soc. Exptl. Biol. Med.*, **106**, 350 (1961).
5) I.L. Doerr and J.J. Fox, *J. Org. Chem.*, **32**, 1462 (1967).
6) J.B. Gin and C.A. Dekker, *Biochemistry*, **7**, 1413 (1968).
7) A. Torriani, *Biochim. Biophys. Acta*, **38**, 460 (1960).

Nucleosides were eluted in three peaks 1—3 as shown in Fig. 2-a. Compound obtained in peak 2 consumed periodate on paper chromatogram in several solvent systems, but those in peak 1 and 3 did not. Analysis by the paper electrophoresis in borate buffer[8] showed that

Table I. Distribution of Tosylation Products before Neutralization

Rf[a]		λ_{max} (nm)	λ_{min} (nm)	TOD_{max}	Compound
0.92					TsOH
0.80	H^+	267, 272 (sh.)	248	3.8	$pCTs_2$
	H_2O	262, 272 (sh.)	249		
	OH^-	267, 272 (sh.)	252		
0.63	H^+	261	245	8.0	pCTs
	H_2O	261	245		
	OH^-	268, 256, 262, 271	252		
0.32		256		0.5	?
0.26	H_2O	232, 262	220, 243.5	6.4	pC°[b]
0.20	H_2O	272	249	6.7	pC

a) Performed in solvent G. b) pC° stands for 2,2′-cyclo-CMP.

Table II. Distribution of Tosylation Products after Neutralization

Rf[a]		λ_{max} (nm)	λ_{min} (nm)	TOD_{max}	Compound
0.80	H^+	274.5	249	4.8	$pCTs_2$
	H_2O	263—267, 258, 274	252		
	OH^-	264, 257, 274	254		
0.68		262:257, 267 (sh.), 271 (sh.)			TsOH
0.50	H^+	257, 262.5 (sh.)	245	1.1	?
	H_2O	257, 262.5 (sh.)	245		
	OH^-	257, 264			
0.28	H^+	232, 262.5	243	17.9	pC°[b]
	H_2O	232, 262.5	243		
	OH^-	273	250		
0.20	H^+	280.5			
	H_2O	276	242	16.1	pC
	OH^-	272			

a) Performed in solvent G. b) pC° stands for 2,2′-cyclo-CMP.

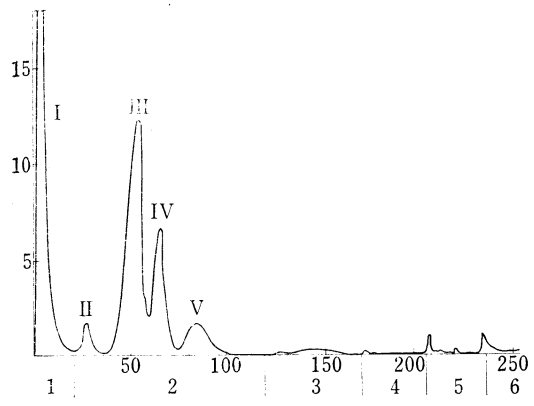

Fig. 1. Chromatography of Products in the Tosylation of CMP

1: H_2O, 2: 0.01N HCOOH, 3: 0.05N HCOOH, 4: 0.10N FmOH, 5: 0.01N FmOH, 6: 0.1N FmOH, 0.01M NH_4OFm, 0.5M NH_4OFm, 1M NH_4OFm

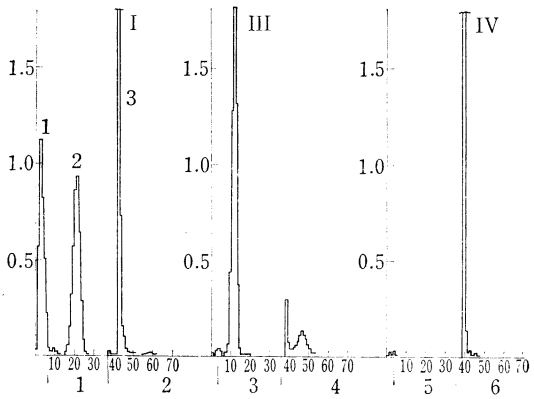

Fig. 2. Dekker Column Chromatography of Dephosphorylated Products

1: 30% MeOH, 2: 0.1M NH_4HCO_3, 3: 30% MeOH, 4: 0.1M NH_4HCO_3, 5: 30% MeOH, 6: 0.1M NH_4HCO_3

8) M.P. Jordan, O.M. Intrieri, and G.B. Brown, *J. Am. Chem. Soc.*, **80**, 5161 (1958).

the compound from peak 1 and 3 migrated slower toward the anode, but the compound from 2 migrated faster than cytidine.

UV absorption properties of the compound from 1 showed a maximum around 226 with a shoulder at 272 nm which was resembled to that of 2′,3′-anhydrocytidine,[9] and the structure of 2′,3′-anhydronucleoside of cytosine was suggested. Since this compound is not identical with an authentic sample of 2′,3′-anhydrocytidine,[9] it seemed to be 2′,3′-anhydro-1-β-D-lyxofuranosylcytosine (IIIa). The compound from peak 2 had UV absorption properties closely resembled to those of cytidine, but it had different *Rf* values compared to cytidine. Since this compound had vicinal OH groups in carbohydrate moiety by criteria of the periodate consumption, the structure of 1-β-D-lyxofuranosylcytosine was assigned to compound IVa. This was confirmed further by comparison with an authentic sample of lyxofuranosylcytosine.[10]

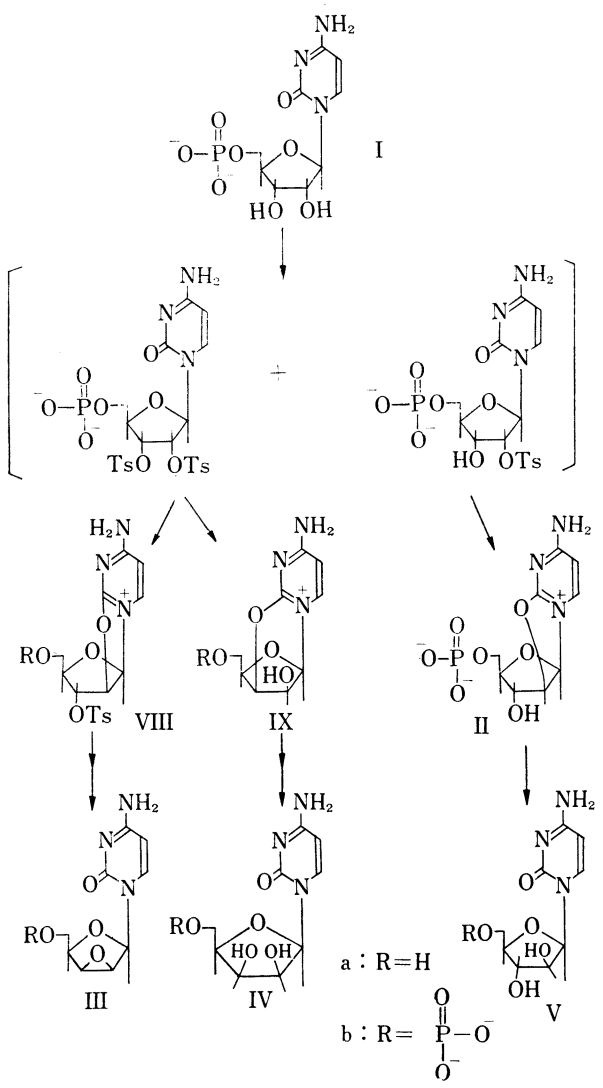

Chart 1

The third compound obtained from peak 3 had the cytidine chromophore but no vicinal OH groups. Comparison with an authentic sample showed that the structure of Va was 1-β-D-arabinofuranosylcytosine. Therefore nucleotides obtained from the first peak in the formate column chromatography were, 2′,3′-anhydro-1-β-D-lyxofuranosylcytosine 5′-monophosphate (IIIb), 1-β-D-lyxofuranosylcytosine 5′-monophoshhate (IVb), and 1-β-D-arabinofuranosylcytosine 5′-monophosphate (Vb). This fact suggests that the nucleotide obtained in peak I should be 2,2′-cyclocytidine 5′-monophosphate (II), because from the compound II formation of III—V was expected. Isolation of compound II was performed by the charcoal absorption, and elution with 50% ethanol containing 2% conc. ammonia. Evaporation of the eluants gave 2,2′-cyclocytidine 5′-monophosphate in a yield of 33%.

The nucleotide obtained in peak III of the formate column chromatography was dephosphorylated by phosphatase as above. Resulting nucleoside was applied to a Dekker column and eluted with 30% methanol and 0.1M ammonium bicarbonate, successively. From its

9) T. Kanai, unpublished experiments.
10) T. Kanai and M. Ichino, *Chem. Pharm. Bull.* (Tokyo), **16**, 1814 (1968).

UV absorption spectra and the comparison with an authentic sample the nucleoside obtained was determined to be cytidine. Therefore, nucleotide obtained in peak III must be cytidine 5′-phosphate (I). A nucleotide obtained in peak IV of the formate column was dephosphorylated. Nucleoside obtained had $A_{280}/A_{270}=0.75$ and *Rf* 0.18, 0.53 and 0.49 in solvent B, F, and G, respectively. The spot corresponding to this nucleoside did not consume periodate. By the paper electrophoresis in borate buffer of pH 6.0 it showed migratory behavior slower than cytidine. Direct comparison of this nucleoside with 1-β-D-arabinofuranosylcytosine (ara-C) (Va) showed complete identity. Therefore, the nucleotide was ara-CMP (Vb).

Nucleotide obtained in peak V was then analyzed. This peak contained two major nucleotides, VIb and VIIb having *Rf* 0.40 and 0.28 in solvent G, respectively. Dephosphorylation with *E. coli* alkaline phosphatase gave nucleosides VIa and VIIa. These compounds had UV absorption properties resembleld to those of 2,3′-cyclocytidine (IXa).[11] Treatment of both nucleosides with conc. ammonia at room temperature for 3 hr gave one compound, which was identical with 1-β-D-lyxofuranosylcytosine (IVa).[10] Therefore compound VIa and VIIa may be derivatives of 2,3′-anhydro-1-β-D-lyxofuranosylcytosine and VIb and VIIb are their 5′-phosphates.

Finally, nucleotides obtained in peak II was analyzed by paper chromatography in solvent G. From the UV absorption properties of each spots in the chromatography, the compound having *Rf* 0.70 was assigned to 2,2′-cyclo-3′-tosylcytidine 5′-monophosphate (VIII), that of *Rf* 0.24 to 2,3′-cyclolyxofuranosylcytosine 5′-monophosphate (IXb), and that of *Rf* 0.08 to the 5′-pyrophosphate of 2′,2′-cyclocytidine, respectively.

Considering these results, tosylation of CMP in an alkaline solution occured first selectively on the 2′-OH group and the successive cyclization gave 2,2′-cyclo-CMP, which was then converted to arabinosylcytosine 5′-monophosphate. Further attack of excess tosyl chloride gave rise to 2′,3′-ditosylate, which was cyclized to give either 2,2′-cyclo-3′-tosyl-CMP or 2,3′-cyclo-lyxosylcytosine 5′-monophosphate. The former was transformed to 2,3′-anhydro-lyxosylcytosine 5′-MP and the latter to lyxosylcytosine 5′-MP, respectively. Alkaline tosylation of other nucleotides are under investigation.

Experimental[12]

Tosylation of Cytidine 5′-Monophosphate——Cytidine 5′-monophosphate (free acid, 385 mg, 1 mmole) was dissolved in 1N NaOH (2 ml) and evaporated to dryness *in vacuo*. The residue was dissolved in 1N NaOH (1.5 ml) and added dropwise into a stirred solution of *p*-toluenesulfonyl chloride (760 mg, 4 mmoles) in dioxane (4.5 ml). The addition continued to 30 min and the mixture was further stirred until it became translucent. Examination of this solution by paper chromatography (G) showed six spots as shown in Table I. When the spot appeared at *Rf* 0.63 was extracted with water and applied again to paper chromatography (G), another spot appeared at *Rf* 0.22, which had UV absorption properties: $\lambda_{max}^{H^+}$: 232, 262 nm, $\lambda_{max}^{H_2O}$: 232, 262 nm, $\lambda_{max}^{OH^-}$: 273 nm. This UV absorption resembled to those of 2,2′-cyclocytidine, which may be converted from 2′-tosyl-CMP. Acidic solution was carefully neutralized with sodium carbonate solution. After the neutralization composition of the products changed to those appeared in Table II. It appeared that almost all of 2′-tosyl-CMP converted to 2,2′-cyclo-CMP by the alkaline treatment.

Column Chromatography of the Reaction Mixture——The reaction mixture was diluted with H_2O to 100 ml, kept at 40° for 2 hr, and then stored at room temperature overnight. Total optical density in this stage was 9070 at 260 nm and A270/A260 was 0.95. Column was filled with Dowex 1×8 (formate form, 100—200 mesh) in 1.2 cm(diameter)×30 cm. One fraction was 20 ml and the flow rate was 80 ml/hr. Elution was performed with H_2O (fr. No. 1—22), 0.01N H·COOH (No. 23—119), 0.05N H·COOH (No. 120—169), 0.01N H·COOH+0.01M H·COO NH_4 (No. 170—201), 0.01N H·COOH+0.5M H·$COONH_4$ (No. 202—230), and 0.1N H·COOH+1.0M H·$COONH_4$ (No. 231—). Elution pattern was shown in Fig. 1 and results were summarized in Table III.

11) Y. Mizuno and T. Sasaki, *Tetrahedron Letters*, **1965**, 4579.

12) UV absorption spectra were taken with Hitachi 124 and EPS-3T spectrophotometer. Paper chromatography was performed on Toyo filter paper No. 51A in solvent B (*n*-BuOH–H_2O, 86:14), G(*n*-BuOH–AcOH–H_2O, 5:2:3), F(EtOH–1MNH_4OAc, 7:3) and C(iso-PrOH–NH_3–H_2O, 7:1:2) by the descending technique.

Table III. Dowex (formate) Chromatography of Reaction Products

Peak No.	Fr. pooled	TOD_{260}	$TOD^{H_2O}_{max}$ a)	A_{270}/A_{260}
I	2— 21	4180		0.87
II	29— 35	66		1.07
III	43— 59	2330	1890	1.73
IV	62— 73	574	818	1.75
V	77— 98	272	388	1.60
Minor fractions				
	137—157			1.52
	203—213	62		0.89
	232—244	88		0.89
	251—263			1.25

a) Calculated from ε of cytidine, $A^{H^+}_{280}/A^{H^+}_{260}=2.10$, $\varepsilon^{H_2O}_{max}=9.1\times10^3$, $\varepsilon^{H^+}_{max}=13.4\times10^3$.

Dephosphorylation by Alkaline Phosphatase——i) Peak I: Substrate (20.5 mg, TOD_{270} 428), alkaline phosphatase[13] (0.4 mg protein/ml, 1.2 ml), 0.5M NH_4HCO_3 (2 ml), and water (16 ml) were incubated at 37°, overnight.

ii) Peak IV: Substrate (5 mg), enzyme (0.25 ml), 0.5M NH_4HCO_3 (0.5 ml) and water (4 ml) were incubated at 37°, overnight.

iii) Peak IV: Substrate (4 mg), enzyme (0.5 ml), 0.5M NH_4HCO_3 (0.5 ml) and water (4 ml) were incubated at 37°, overnight.

Column Chromatography of Dephosphorylated Products——Above incubation mixtures were evaporated, dissolved in water (50 ml), and applied to a column (1×19 cm) of Dowex 1×2 (OH^- form), 100—200 mesh.

Table IV. Dekker Column Chromatography of Reaction Products

Peak	Fr. No.	TOD_{270}	A_{280}/A_{270}	Compound	*Rf* B	*Rf* C	*Rf* G
I-1	2— 6	65	0.60	2′,3′-anhydro-lyxofuranosylcytosine		0.58	0.55
I-2	17—25	78	0.81	lyxofuranosylcytosine	0.09	0.36	0.46
I-3	43—46	156	0.77	arabinofuranosylcytosine	0.11	0.44	0.50
III	9—15	106	0.81	cytidine	0.09	0.46	0.46
IV	40, 41	70	0.75	arabinofuranosylcytosine	0.11		0.49

Table V. UV Absorption Properties of Nucleotides in Peak V

Rf a)		λ_{max}	λ_{min}	$TOD^{H_2O}_{max}$
0.57	H^+	280 mμ	242	2.5
	H_2O	272	248	
	OH^-	273	252	
0.46	H^+	278 mμ	242.5	3.1
	H_2O	228(sh.), 269	249	
	OH^-	270	250	
0.40	H^+	280 mμ	242.5	14.1
	H_2O	227(sh.), 272	249	
	OH^-	272	250	
0.28	H^+	278 mμ	240	21.6
	H_2O	270	249	
	OH^-	270	250	
0.11	H^+	263, 277 mμ	243	0.4
	H_2O	264		
	OH^-	264		

a) Performed in solvent G.

13) Purchased from Boehringer Mannheim Corporation.

Table VI. Properties of Compound-appeared in Peak V analyzed by PPC

	Rf[a]	Rcy[b]	$\lambda_{max}^{H_2O}$ (nm)	λ_{min} (nm)	$TOD_{max}^{H_2O}$
V-3-1	0.63	−1.3	270	248	4.9
V-3-2	0.55	−1.3	228(sh.) 269	248	1.7
V-3-3	0.35		268	250	0.15
V-4-1	0.63		270		0.18
V-4-2	0.53	−1.1	228(sh.) 269.5	249	3.9
V-4-3	0.33		268		
V-4-4	0.19		271		0.24

a) Performed in solvent C.
b) Migration ratio in paper electrophoresis performed in borate buffer at pH 6.0, cytidine as 0.0.

Table VII. UV Absorption properties and Distribution of Compounds appeared in Peak II

	Rf[a]	λ_{max} (nm)	λ_{min} (nm)	$TOD_{max}^{H_2O}$	Compound
0.70	H^+	229.5 262.5	212.5 246.5		2,2′-cyclo-3′-tosyl-CMP
	H_2O	229.5 262.5	212.5 246.5	5.8	
	OH^-	268.5	249		
0.62	H^+	224(sh.) 258	248		?
	H_2O	224(sh.) 258	248	0.85	
	OH^-	262.5	247		
0.24	H^+	263	244		2,2′-cyclolyxosylcytosine
	H_2O	263	246	1.4	5′-MP
	OH^-	271	254		
0.08	H^+	271.5	243		p^1,p^2-di-2,2′-cyclocytidine
	H_2O	226(sh.) 267.5	243.5	11.4	5′-pyrophosphate
	OH^-	271.5	251		

a) Performed in solvent G.

TOD's were 428, 139 and 90 for I, III, IV, respectively. Peaks obtained in the chromatography were analyzed by paper chromatography and paper electrophoresis performed in a borate buffer.[8] Elution pattern of the column chromatography was shown in Fig. 2. Results and elution buffers were summarized in Table IV.

Analysis of Peak V——The solvent was evaporated and the residue was applied to the paper chromatography in solvent G. Results were summarized in Table V. Dephosphorylation of nucleotides having *Rf* 0.40 and 0.28 with *E. coli* alkaline phosphatase were performed at 37° for 4 hr. Products were analyzed on paper chromatography (C) and paper electrophoresis. Results were summarized in Table V and VI.

Analysis of Peak II——The solvent was evaporated and the residue was analyzed by paper chromatography (G). Results were summarized in Table VII.

Isolation of 2,2′-Cyclo-CMP——Peak I (fraction No. 2—21) were collected and evaporated. Residue was dissolved in water, slightly acidified with 1N HCOOH, and adsorbed on activated charcoal. After the water-wash, the nucleotide was eluted with 50% EtOH containing 2% conc. NH_3 (300 ml×3) TOD_{270}= 2958. The solvent was evaporated, the residue was dissolved in water and filtered to remove insoluble material. EtOH was added to a concentrated solution to precipitate nucleotides, which were collected by centrifugation. Yield was 123 mg (0.33 mmole). *Rf* values and UV absorption properties appeared in Table I and II. Phosphate analysis showed base:phosphate=1.0: 0.95.

Acknowledgement Authors are indebted to Dr. Tei Kanai for the gift of reference compounds. We thank to Ministry of Education for the financial support by a Grant-in-aid for Scientific Research.