Seven-membered cyclic phosphate: synthesis and properties of S-cycloadenosine 2', 5'-cyclic phosphate and cordycepin 2', 5'-cyclic phosphate. Studies of nucleosides and nucleotides. LXIV¹). Purine cyclonucleosides 25.

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ABSTRACT

8,3'-Anhydro-8-mercapto-9- β -D-xylofuranosyladenine (8,3'-S-cycloadenosine) was phosphorylated with cyanoethyl phosphate and DCC to 5'-phosphate. After 6-amino group was benzoyleated, the monophosphate was treated with DCC to give a cyclic phosphate (II). The structure of compound II was elucidated as 8,3'-S-cycloadenosine 2',5'-cyclic phosphate by UV, NMR and CD spectra, as well as enzymatic hydrolyses. When compound II was desulfurized with Raney nickel, cordycepin 2',5'-cyclic phosphate (III) was obtained. Although compound III could be obtained from cordycepin 5'-phosphate with DCC, the yield was extremely low.

Adenosine 3',5'-cyclic phosphate (I) is known to be a mediator of many important biological reactions². Although a number of structural analogues of cyclic AMP have been synthesized,³⁻¹⁵ no nucleotide having seven-membered cyclic phosphate has been reported. In this paper we report synthesis of 8,3'-anhydro-8-mercapto-9- β -D-xylofuranosyladenine 2',5'-cyclic phosphate (II) and cordycepin 2',5'-cyclic phosphate, which may be interesting as having seven-membered phosphate ring.

8,3'-S-Cycloadenosine¹⁶ (VI) was protected on 5'-OH by a monomethoxytrityl group and both on NH₂ and 2'-OH by ben-

zoyl groups. The trityl group was removed by the acetic acid treatment and resulting $N,N',2'$ -O-tribenzoylcycloadenosine was phosphorylated with cyanoethyl phosphate and DCC¹⁷. The benzoylated cycloadenosine 5'-phosphate was treated with 2N NaOH at 0° for 25 min to give N^6 -benzoyl-S-cycloadenosine 5'-phosphate (V) in a yield of 60%. The structure of (V) was

confirmed by paper chromatography, paper electrophoresis and UV absorption properties. Compound V was then heated in anhydrous pyridine in the presence of DCC (2 equiv.) for 1hr. After the usual work-up the product was applied to a column of DEAE-cellulose and eluted with 0.002-0.1M triethylammonium bicarbonate buffer in a linear gradient. 8,3'-S-Cycloadenosine 2',5'-cyclic phosphate was obtained in a well-resolved peak in a yield of 81%. This material was obtained in a crystalline form from methanol giving m.p. above 250°. The 2',5'-phosphate (II) showed UV absorption properties (Fig. 1b) having λ max at pH 7.0 in 230 and 285 nm, which were closely resembled with those reported for 8,3'-S-cycloadenosine.¹⁶ This fact suggested that the phosphorylation had occurred only

Fig 1. a. CD spectrum of 8,3'-S-cycloadenosine 2',5'-cyclic phosphate. b. UV spectrum of 8,3'-S-cycloadenosine 2',5'-cyclic phosphate.

on the carbohydrate moiety. CD spectrum (Fig. 1a) taken in 0.01M phosphate buffer, pH 7.0 was also showed a similar curve as that reported for 8,3'-S-cycloadenosine.¹⁸ This fact may suggest that the tertiary structure of this nucleotide(II) is almost same with that of nucleoside. NMR taken in d_6 -DMSO showed signals corresponding to 2'-H(5.35 τ), 3'-H(4.66 τ), 4'-H(4.83 τ) and 5'-H(4.00 δ). All these signals appeared in lower field relative to those of 8,3'-cycloadenosine. No signals for 2'- and 5'-OH were observed. These facts supported the structure of 2',5'-cyclic phosphate for II. The migration ratio in paper chromatography and paper electrophoresis suggested that the compound II had one negative charge in conformity with the cyclic phosphate structure.

When compound II was treated with 0.2M barium hydroxide at 100° for 2 hrs, no decomposition of the cyclic phosphate was observed. Compound II was also resistant to the hydrolysis with N HCl at 100° for 2 hrs. The cyclic phosphate ring of 3',5'-cyclic AMP was completely cleaved in this condition. The enhanced stability might be due to a cyclo-nucleoside structure of II and also to the seven-membered cyclic phosphate structure. The stability of seven-membered cyclic phosphate was previously shown in the case of butane-1,4-diol cyclic phosphate.¹⁹ When compound II was incubated with crude snake venom containing phosphodiesterase and 5'-nucleotidase, even after 8 hrs at 37° cleavage of the phosphate was not found. These features of compound II together with physical properties support the structure of 8,3'-S-cycloadenosine 2',5'-cyclic phosphate to be correct.

Since the Raney nickel desulfurization of 8,3'-S-cycloadenosine was known to afford cordycepin²⁰, compound II was treated with Raney nickel in refluxing methanol to give cordycepin 2',5'-cyclic phosphate(III). Migration of compound III in paper chromatography and electrophoresis showed that no change had occurred in the phosphate moiety. UV absorption spectrum (Fig. 2) showed that the base moiety of III should be adenine in agreement with cordycepin structure.²¹ TLC on Avicel AF gave R_f 0.68 which was higher than that of 3',5'-cyclic AMP as was observed in 2'-deoxy-3',5'-cyclic AMP. Heating of compound III in 0.2M barium hydroxide at 100° for 2.5 hrs showed only a slight decomposition to adenine. In contrast, acid hydrolysis of compound III in N HCl at room

Fig. 2. UV spectra of cordycepin 2',5'-cyclic phosphate. — in water, at pH 2, -.-.- at pH 12.

temperature showed decomposition of 25% giving adenine.

Raising the temperature to 100° in this condition gave rise to complete decomposition to adenine, 3-deoxyribose 2,5-cyclic phosphate and 3-deoxyribose phosphate. These hydrolytic properties suggested that compound III was cordycepin 2',5'-cyclic phosphate. This compound III was also obtainable from cordycepin 5'-phosphate (VII) by treating it with DCC. However the yield was extremely low, probably because of an unfavorable structure for cyclization reaction.

The reason for the easy cyclization of 8,3'-S-cycloadenosine 5'-phosphate (IV) to the 2',5'-cyclic phosphate (II) may be interpreted by an extreme 3'-endo conformation of compound IV, which was found by X-ray crystallography.²² The relatively

easy access of the 5'-phosphate to the 2'-hydroxyl group could also be demonstrated by a Corey-Pauling-Koltun model. The enhanced stability of the seven-membered cyclic phosphate in compound II and III should be ascribed to relatively less-hindered structure of the ring as was previously shown in the case of butanediol cyclic phosphate.¹⁹

Biological properties of these cyclic AMP analogues are under investigation and will be reported elsewhere.

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EXPERIMENTAL

General methods--- UV absorption spectra were taken with a Hitachi EPS-3T spectrophotometer, CD spectra were taken with a JASCO ORD/CD-5 spectropolarimeter equipped with a CD attachment. and NMR spectra were taken with a Hitachi R22 spectrometer operated at 90 MHz using tetramethylsilane as external standard. Electrophoresis was performed on Toyo PE-2 apparatus in 0.05M triethylammonium bicarbonate buffer (pH 7.5) at 35V/cm. Relative migration ratio (R_m) was represented by adenosine as 0.0 and 5'-AMP as 1.0. Paper chromatography was performed in the following solvent systems : A, isopropanol-conc. ammonia-water (7 : 1 : 2); B, ethanol-1M ammonium acetate (7 : 3); C, n-butanol-acetic acid-water (5 : 2 : 3).

5'-O-Monomethoxytrityl-8,3'-S-cycloadenosine --- i) Well-dried 8,3'-S-cycloadenosine (50.1 mg, 1.8 mmole) was dis-

solved in DMF (6.5 ml) and monomethoxytrityl chloride (680 mg, 2.2 mmoles) was added. The reaction mixture was kept in a dark place at room temperature for 3 days. The mixture was poured into a saturated aqueous solution of sodium bicarbonate (200 ml) and resulting precipitates were collected by filtration. The powder was washed with water, dried over P_2O_5 under vacuum, and dissolved in pyridine (5 ml). The solution was added dropwise to ether (200 ml) with stirring. Precipitates were collected by centrifugation, dried over P_2O_5 , and recrystallized from ethanol. Methoxytrityl-S-cycloadenosine was obtained as a crystalline solid, m.p. 274-275.5° (yield 48%). Anal. Calcd. for $C_{30}H_{27}O_4N_5S$: C, 65.05; H, 4.91; N, 12.73; S, 5.80. Found: C, 64.46; H, 4.72; N, 12.39; S, 5.94. U.V.: $\lambda_{max}^{95\%EtOH}$ 285 nm, λ shoulder 233, 278, 295 nm.

ii) 8-Bromo-3'-(2,4,6-triisopropylbenzenesulfonyl)adenosine (3.1 g, 5 mmoles) was dissolved in DMF (50 ml) and monomethoxytrityl chloride (2.5 g, 1.2 equiv) was added. The mixture was kept in a dark place at room temperature for 2 days. The reaction mixture was poured into water (500 ml) containing 2% ammonia. Resulting powder was collected by filtration and washed with water. This material showed U.V.: $\lambda_{max}^{50\%EtOH}$ 231, 265 nm; $\lambda_{max}^{OH^-}$ 230, 265 nm; $\lambda_{max}^{H^+}$ 230, 263 nm and TLC (in $CHCl_3$ -EtOH, 10:1): R_f 0.39 (8-bromo-3'-TPS-adenosine, 0.26). The powder was dissolved in DMF (150 ml) and bubbled through with N_2 gas. To the solution was added 50% NaSH (18 mmoles) freshly prepared from NaH and H_2S gas. The flask was tightly stoppered and heated at 70° for 24 hrs.

The reaction mixture was neutralized with N HCl and poured into water to cause precipitation of powder. This material was collected by filtration and recrystallized from ethanol. Yield was 1.16 g (2.1 mmoles, 42% from 8-bromo-TPS-adenosine). This sample was completely identical with that obtained above.

N,N',2'-O-Tribenzoyl-8,3'-S-cycloadenosine-----5'-O-Mono-methoxytrityl-8,3'-S-cycloadenosine (145 mg, 0.24 mmole) was dissolved in pyridine (3 ml) and benzoyl chloride (0.53 ml) was added. The reaction mixture was kept at room temperature for 2 hrs. Checking by TLC (ethanol-chloroform, 1:19) on silica gel showed disappearance of the starting material. The mixture was poured into a saturated sodium bicarbonate solution and stirred for 2 hrs. The solution was extracted with chloroform, the chloroform layer was washed thoroughly with water and dried over sodium sulfate. The solvent was evaporated in vacuo and the residue was dissolved in acetic acid (15 ml) and water (1.7 ml). The solution was kept at room temperature overnight. After checking the complete removal of trityl group by TLC, acetic acid was evaporated and the residue was coevaporated several times with water. Recrystallization of the residue from 95% ethanol gave crystalline tribenzoyl-8,3'-S-cycloadenosine (89 mg, 60%).

U.V.: (nm) $\lambda_{\text{max}}^{\text{pH}2}$ 273, 302; $\lambda_{\text{max}}^{\text{pH}7}$ 236.5, 301.5; $\lambda_{\text{max}}^{\text{pH}10}$ 314; $A_{236.5}^{\text{pH}7} / A_{301.5}^{\text{pH}7} = 1.75$. TLC (CHCl₃-EtOH, 19:1) : R_f 0.41; PPC (B), R_f 0.65 (5'-AMP, 0.14, Adenosine, 0.50).

8,3'-S-Cycloadenosine 5'-Phosphate-----Tribenzoyl-cyclo-nucleoside (213 mg, 0.5 mmole) and cyanoethyl phosphate (pyridinium salt, 2 mmoles) were rendered anhydrous by

repeated evaporation with pyridine. The residue was dissolved in pyridine (5 ml) and DCC (618 mg, 2.5 mmole) was added. After the reaction mixture was kept at room temperature for 2 days, 50% aqueous pyridine (5 ml) was added into the mixture and kept at room temperature for 3 hrs. Dicyclohexylurea was removed by filtration, the filtrate was washed three times with n-pentane, and the pyridine layer was evaporated in vacuo. Residue was rendered anhydrous by evaporation with pyridine, dissolved in 9N methanolic ammonia, and kept at 40° for 3 days. The reaction mixture was carefully evaporated, the residue was dissolved in pyridine, and dropped into dry ether. The resulting powder was dissolved in water, adjusted to PH3 with N-HCl, and applied to a column (2X4 cm) of charcoal. After washing with water (450 ml) the column was eluted with 50% ethanol containing 2% ammonia. Effluents were evaporated in vacuo to give 8,3'-S-cycloadenosine 5'-phosphate (6450 OD₂₈₀, 0.3 mmole, 58%).

N⁶-Benzoyl-8,3'-cycloadenosine 5'-phosphate --- 8,3'-S-Cycloadenosine 5'-phosphate (11700 OD₂₈₂, 0.53 mmole) was rendered anhydrous by repeated evaporation with pyridine and finally dissolved in pyridine (5 ml). Benzoyl chloride (1.6 ml, 5 equiv.) was added in the solution, which was kept at room temperature for 3 hrs with stirring. Into the mixture was added chloroform (20 ml) and water (20 ml) and chloroform layer was separated. The chloroform layer was washed with water (10 ml X 3) and chloroform was evaporated. Residue was dissolved in 70% pyridine (10 ml) and cooled at 0° in an ice-salt bath. Into the cooled solution was added 2N NaOH (10 ml)

which was pre-cooled to 0°. The reaction mixture was brought to room temperature and kept for 25 min. The solution was neutralized with excess Dowex 50W (pyridinium form) resin and eluted with 30% aqueous pyridine. Eluents were evaporated in vacuo and the residue was dissolved in pyridine. Dropping it into ether gave N-benzoyl-8,3'-S-cycloadenosine 5'-phosphate (220 mg, 70%). U.V.: $\lambda_{\text{max}}^{\text{pH}2}$ (nm) 237, 302; $\lambda_{\text{max}}^{\text{pH}7}$ 237, 302; $\lambda_{\text{max}}^{\text{pH}12}$ 314 nm. PPC: Rf (B) 0.42. This material had fluorescence under U.V. lamp irradiation.

8,3'-S-Cycloadenosine 2',5'-cyclic phosphate-----N-Benzoyl-
 8,3'-S-cycloadenosine 5'-phosphate (pyridisium salt, 54 mg, 0.1 mmole) and 4-morpholino-N,N'-dicyclohexylcarboxamidine (29.3 mg, 0.1 mmole) were dissolved in pyridine (1.5 ml) and water (0.5 ml). Solvent was evaporated in vacuo and the residue was dissolved in pyridine (30 ml) and DCC (41.2 mg, 0.2 mmole) was added. The reaction mixture was refluxed for 1 hr. under exclusion of moisture. After cooling, water (50 ml) was added to the mixture, which was kept at room temperature for 3 hrs. Dicyclohexylurea was filtered off and the filtrate was evaporated to dryness. The residue was rendered anhydrous by repeated evaporation with pyridine. The residue was dissolved in 9N methanolic ammonia (10 ml) and kept at room temperature for 3 days. Ammonia and methanol was carefully evaporated, the residue was dissolved in water, and applied to a column (1.5X 26 cm) of DEAE-cellulose. The column was eluted with 0.002M-0.1M triethylammonium bicarbonate in a linear gradient. 8,3'-S-Cycloadenosine 2',5'-cyclic phosphate was obtained from the second peak as the major product.

The third peak contained a pyrophosphate of 8,3'-S-cyclo-adenosine, unreacted 5'-phosphate was obtained from the fourth peak. Fractions obtained in the second peak were evaporated to dryness and further evaporated with water to remove traces of triethylamine. The residue was dissolved in 50% ethanol (0.4 ml) and adjusted to pH 2.0 with N-HCl. Keeping the solution in a refrigerator for 2 days gave a precipitate. The crystalline precipitates were collected, washed with ethanol and dried in vacuo (27.5 mg, 80%). M.p. 265° (colorized at 265°). U.V. and C.D. spectra of this compound were shown in Fig 1a,b. R_f value in PPC and R_m value in paper electrophoresis were listed in Table I. N.M.R. signals were listed in Table II, together with those of 8,3'-S-cyclo-adenosine. 8,3'-S-Cycloadenosine 2',5'-cyclic phosphate was also obtained from 8,3'-S-cycloadenosine 5',5'-pyrophosphate by treatment with DCC in a yield of 20%.

Cordycepin 2',5'-cyclic phosphate -----1) 8,3'-S-Cycloadenosine 2',5'-cyclic phosphate (5.7 mg, 15.8 μ moles) was dissolved in water (2 ml) and refluxed for 2 hrs in the presence of Raney nickel W-2 (ca.1 ml). Catalyst was filtered and washed with hot water. Washings and filtrate were combined and concentrated solution was applied to paper chromatography in solvent A. The band migrated at 0.59 was cut off and extracted with water. Cordycepin 2',5'-cyclic phosphate was obtained in a yield of 50 OD₂₆₀ (10%). U.V.: $\lambda_{\text{max}}^{\text{pH}2}$ (nm) 258; $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 260.5; $\lambda_{\text{max}}^{\text{pH}13}$ 260.5. Migration ratios in PPC, and PEP were listed in Table I. TLC (Avicell SF in solvent A): R_f 0.68 (cordycepin 0.71, 5'-AMP 0.02, 3',5'-cyclic AMP 0.33,

Table 1

Rf and Rm values on paper chromatograms and electrophoreograms.

Solvent	PPC Rf values			PEP (pH 7.5)
	A	B	C	Rm (5'-AMP-Adenosine)
8,3'-S-Cycloadenosine	0.50	0.56	0.57	0.0
8,3'-S-Cycloadenosine 5'-phosphate	0.05	0.09	0.28	0.84
8,3'-S-Cycloadenosine 2',5'-cyclic phosphate	0.46	0.52	0.57	0.55
Adenosine	0.51	0.57	0.52	0.0
Adenosine 5'-phosphate	0.06	0.13	0.23	1.0
Adenosine 3',5'-Cyclic phosphate	0.37	0.41	0.29	0.60
Cordycepin-2',5'-Cyclic phosphate	0.67	0.72	0.68	0.60
Cordycepin	0.71	0.70		0.0

Table 2

NMR chemical shift of the 8,3'-S-cycloadenosine-2',5' cyclic phosphate and 8,3'-S-cycloadenosine.

	8,3'-S-Cycloadenosine (ppm)	8,3'-S-Cycloadenosine- 2',5'-cyclic phosphate. (ppm)	(ppm)
2-H	8.05	8.03	+0.02
6-NH ₂	7.07	7.03	+0.04
2'-OH	6.36	—	
1'-H	5.81	5.80	+0.01
5'-OH	4.87	—	
2'-H	4.85	5.35	-0.50
4'-H	4.63	4.83	-0.20
3'-H	3.92	4.66	-0.74
5'-H	3.70	4.0	-0.30

adencsine 0.54 and adenine 0.46).

11) Cordycepin (50.2 mg, 0.2 mmole) was dissolved in triethyl phosphate (0.2 ml) and POCl_3 (28 ml) dissolved in 0.5 ml triethyl phosphate was added at 0° . Stirring was maintained at this temperature for 3 hr. Ether (10 ml) was added to the mixture, and white precipitates were collected by centrifugation. The powder was washed with ether and dried. The powder was dissolved in water (20 ml) and adjusted to PH 9 with conc. ammonia. After stirring at room temperature for 30 min, the solution was applied to a column (1X8 cm) of Dowex 1X2. By the water unreacted cordycepin was eluted. Elution with 0.08N HCOOH gave cordycepin 5'-phosphate (1100 OD₂₆₀, 37%). The 5'-phosphate was applied to a column of active charcoal and eluted with 50% EtOH containing 2% ammonia. The ammonium salt was converted to pyridinium salt through a column of Dowex 50wx8 (pyridinium form). The pyridinium salt of cordycepin 5'-phosphate was dissolved in 3% aqueous pyridine (2 ml) and 4-morpholino-N,N'-dicyclohexylcarboxamidine (24.8 mg, one equivalent) was added. The solution was evaporated and the residue was rendered anhydrous by repeated evaporation with pyridine. The residue was finally dissolved in pyridine (25 ml) and DCC (35 mg) was added. The solution was refluxed for 2 hr under exclusion of moisture. After usual work-up, the nucleotides were applied to a column (0.6X 22 cm) of Dowex 1X2 (formate form). Elution with 0.05 N HCOOH gave cordycepin 2',5'-cyclic phosphate at a peak eluting from fraction No. 111-135 (one fraction was 3.1 ml). Yield was 58 OD₂₆₀ (5.7%). The main product was cordycepin 5',5'-pyrophosphate (180 OD₂₆₀)

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