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Polynucleotides. XXIII[1]. A synthesis of ribodinucleoside monophosphates using nucleoside 5'-phosphates

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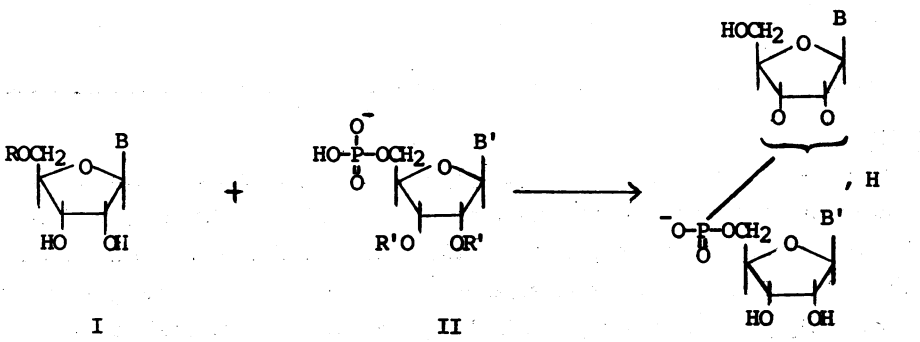
SUMMARY

Guanylyl-(3'-5')-cytidine and guanylyl-(2'-5')-cytidine were synthesized by condensing 5'-O-monomethoxytritylguanosine and N,2',3'-O-triacetylcytidine 5'-phosphate using dicyclohexylcarbodiimide. The reaction gave nearly quantitative yield and the isomers were separated by ion-exchange chromatography after removing protecting groups. Similarly isomers of adenylyladenosine were obtained from N,N',2',3'-O-tetrabenzoyladenosine 5'-phosphate and N,N', 5'-O-tribenzoyladenosine using 2,4,6-triisopropylbenzenesulfonyl chloride as the condensing reagent in a yield of 56%.

A ribonucleoside monophosphate is the simplest model of ribopolynucleotides. It is desirable to synthesize this oligonucleotide in quantity for chemical and physicochemical studies. A specific synthesis of 3'-5' linked ribooligonucleotides using nucleoside 3'-phosphate has been established[2-4] and a method of activation of ribonucleoside 2',3'-cyclic phosphates[5] to yield a mixture of 2'-5' and 3'-5' internucleotide linkages has been used for the synthesis of ribodinucleoside monophosphates[6-8]. Several approches for the specific protection of the 2'-hydroxyl function of the ribose moiety have been investigated[9,10]. However, for the synhtesis of dinucleoside monophosphates the 2'- or 3'-hydroxyl groups can be linked to the 5'-phosphate, as the isomers can be resolved by ion-exchange chromatography. The present paper describes a synthesis of dinucleoside monophosphates(III) (Chart I) starting from nucleoside 5'-phosphates(II) and nucleosides having free 2' and 3'-hydroxyl groups(I). Cytidine 5'-phosphate was acetylated with acetic anhydride in pyridine to give N,2',3'-O-triacetylcytidine 5'-phosphate(IIa) and condensed with 5'-O-monomethoxytritylguanosine[4,11] (Ia) using dicyclohexylcarbodiimide(DCC) as the condensing reagent. Essentially no starting materials were detected by paper chromatography in solvent A. The reaction mixture

was treated with ammonia and then with 80% acetic acid to remove the protecting groups. An isomeric mixture of guanylylcytidine(IVa) which was isolated by paper electrophoresis (Table I) gave two spots in paper chromatography in solvent C and 44% of the mixture was sensitive to RNase St[12]. The mixture was subjected to ion-exchange chromatography to separate the 2'-5'

Chart I



I

a, B = G
R = MMTr

b, B = A^{Bz2}
R = Bz

II

a, B'= C^{Ac}
R'= Ac

b, B'= A^{Bz2}
R'= Bz

III

a, B = G
B'= C

b, B = B' = A

and 3'-5' linked isomers. The latter peak which gave smaller Rf value in solvent C was identified as guanylyl-(3'-5')-cytidine by the enzymatic hydrolysis. When 5'-O-monomethoxytrityl-N-dimethylaminomethylene guanosine[11] was used instead of Ia, 49% of the isomeric mixture was hydrolyzed with the enzyme.

Similarly N,N',2',3'-O-tetrabenzoyladenosine 5'-phosphate(IIb) was allowed to react with N,N',5'-O-tribenzoyladenosine(Ib) which was synthesized from N,N',5'-O-tribenzoyl-2',3'-ethoxymethylideneadenosine[13]. 2,4,6-Triisopropylbenzenesulfonylchloride[14] (TPS) was used as the condensing reagent. After aqueous pyridine and ammonia treatment the dinucleoside monophosphate (IIIb), the pyrophosphate and the mononucleotide were detected by paper electrophoresis in the ratio of 56%, 16% and 28%, respectively neglecting hypochromicity. The isomeric mixture was digested with RNase M[15] to effect partial hydrolysis. The undigestable dinucleoside phosphate was hydrolyzed by alkali completely. Ion-exchange chromatography of the mixture showed almost equal amount of adenylyl-(2'-5')-adenosine and adenylyl-(3'-5')-adenosine.

Table I. Paper Chromatography and Paper Electrophoresis

Compound	Paper Chromatography Solvent			Paper Electrophoresis
	A	B	C	pH 7.5
C	0.63			0
pC	0.12	0.32	0.80	1.00
G	0.44		0.27	0.05
Gp2'	0.09		0.46	1.04
Gp3'	0.09		0.35	1.04
G2'pC	0.13		0.35	0.33
G3'pC	0.13		0.18	0.33
A	0.58			0
pA	0.11	0.15		1.00
A2'pA	0.21		0.10	0.30
A3'pA	0.21		0.08	0.30
$pC^{Ac}(OAc)_2$		0.68		
$pA^{Bz2}(OBz)_2$		0.73		

The present study showed that ribonucleoside 5'-phosphate are useful starting materials for the synthesis of ribodinucleoside monophosphates. The vicinal hydroxyl groups of the nucleoside were not protected because the desired 3'-5' isomer could be separated from the 2'-5' isomer by ion-exchange chromatography. The 5'-hydroxyl group, however, was protected to avoid a complication. The amino group of guanosine was protected in one case. The results of the condensations with and without protection of the amino group were almost the same. The phosphoramidate formation seemed not to be extensive under the condition used. The exact comparison of the reactions using DCC and TPS was not investigated in the present study. DCC seemed to complete the reaction nearly quantitatively. Trace of cytidine and guanylic acid were detected in paper chromatography. This could indicate that triester formation or direct cleavage between the 3'-phosphate and the 5'-hydroxyl group occured before or after the deprotection.

Ribonucleoside 5'-phosphates can be protected in a single step and the protection of the primary hydroxyl group of nucleosides provides a rather simple method for the synthesis of ribonucleoside monophosphates.

EXPERIMENTAL

General methods ----- Paper chromatography was performed by descending technique using solvent systems: A, isopropanol-concentrated ammonia-water (7:1:3, v/v); B, ethanol-1 M ammonium acetate, pH 7.5 (7:3, v/v); C, ammonium sulfate-water-isopropanol (66:100:2, w/v/v). Paper electrophoresis was performed using 0.05 M triethylammoniumbicarbonate at 900 V/40 cm. Other general methods are described previously[16].

RNase St[12] was generously provided by Dr. N. Yoshida. For the hydrolysis of oligonucleotides (2 A_{260} units) the enzyme (10 µg) was used in 0.1 M Tris-HCl, pH 7.4 at 37° for 4 hr.

Desalting of oligonucleotides was carried out using charcoal (for chromatography, Wako Co. Ltd.) and eluting nucleotides with 50% ethanol containing 2% ammonium hydroxide.

Pyridinium N,2',3'-O-triacetylcytidine 5'-phosphate ----- Disodium cytidine 5'-phosphate (2 g, 4.27 mmoles) were dissolved in 10% pyridine (20 ml) and passed through a column (1.3 x 10 cm) of pyridinium Dowex 50X2 (100-200 mesh) ion-exchange resin. The column was washed with 10% pyridine. The eluent and washings were evaporated to dryness at 30° and the residue was coevaporated with pyridine three times. The anhydrous compound was treated with acetic anhydride (15 ml) in pyridine (50 ml) for 18 hr at 25° with stirring and acetic anhydride was evaporated. The residue was dissolved in 50% pyridine with cooling in an ice bath and the solution was concentrated after 2 hr at room temparature. The residue was rendered anhydrous by coevaporation of pyridine and the anhydrous pyridine solution was added to ether with vigorous stirring. The precipitate was centrifuged and washed with ether three times. The yield was nearly quantitative. $\lambda_{max}^{H_2O}$ 248 and 299 nm, $\lambda_{max}^{H^+}$ 248 and 333 nm, $\lambda_{max}^{OH^-}$ 249 and 301 nm. Rf values in paper chromatography and mobilities in paper electrophoresis are shown in Table I. This preparation contained about 10% of the pyrophosphate (Rf 0.80 in solvent B), which could be used for the next condensation reaction.

N,N',2',3'-O-Tetrabenzoyladenosine 5'-phosphate ----- Pyridinium adenosine 5'-phosphate (1 mmole) was coevaporated with pyridine and suspended in pyridine (15 ml). Benzoylchloride (2.5 ml) was added in an ice bath and the mixture was kept at room temperature for 1 hr. The solution was added to ice water (50 ml) and extracted with chloroform (50 ml, 3 portions). The chloroform layer was washed with water and evaporated. The residue was rendered an-

hydrous with coevaporation of pyridine and treated with acetic anhydride (5 ml) in pyridine (15 ml) for 24 hr at 25°. Acetic anhydride was evaporated in vacuo and 50% pyridine (15 ml) was added in an ice bath. The solution was kept at room temperature for 2 hr and evaporated to make anhydrous pyridine solution. Coevaporation with pyridine was repeated three times and the pyridine solution (5 ml) was added to ether (150 ml) with vigorous stirring. The precipitate was centrifuged and washed with ether three times and then dried in a desicator over P_2O_5. The yield was 85%.

5'-O-Monomethoxytritylguanosine[4] ----- The compound was prepared from 5'-O-monomethoxytrityl-N-dimethylaminomethylene guanosine[11] by treating with a large excess of 15 N methanolic ammonia for 20 hr at 25°.

N,N',5'-O-Tribenzoyladenosine ----- N,N',5'-O-Tribenzoyl-2',3'-ethoxymethylideneadenosine[13] was treated with 80% acetic acid for 2 hr at 25° and acetic acid was removed in vacuo. The residue was coevaporated with aqueous ethanol and treated with 50% pyridine for 1 hr. Solvents were removed and the residue was subjected to a silicic acid column in chloroform and eluted with chloroform containing increasing amount of methanol. The overall yield from 2',3'-ethoxymethylideneadenosine (2 mmoles) was 44%.

Guanylyl-(2'-5')-cytidine and guanylyl-(3'-5')-cytidine ----- Pyridinium N,2',3'-O-triacetylcytidine 5'-phosphate(IIa)(1 mmole) was dissolved in 50% pyridine (10 ml) and passed through a column (1 x 8 cm) of pyridinium Dowex 50X2. The eluent and washings were made anhydrous by coevaporation of pyridine and the anhydrous pyridine solution (7 ml) was added to ether (200 ml). The precipitate was washed with ether and dried in vacuo over P_2O_5. The precipitated nucleotide(IIa) and 5'-O-monomethoxytritylguanosine (Ia)(610 mg, 1 mmole) were coevaporated with pyridine three times and the dry residue was dissolved in pyridine (6 ml). The mixture was treated with DCC (5 mmoles) for 48 hr at 30°. Water (5 ml) was added and the solution was kept for 12 hr at 30°. Cyclohexylurea was removed by filtration and the filtrate was extracted with n-pentane. The solution was concentrated and treated with 15 N methanolic ammonia (50 ml) for 16 hr at 30°. The volatile materials were removed by evaporation and the residue was treated with 80% acetic acid (50 ml) for 1 hr. Acetic acid was evaporated and the residue was dissolved in water and extracted with ether. Paper chromatography and paper electrophoresis showed mainly the dinucleoside phosphate. The aqueous solution (200 ml) was adjusted with ammonia to pH 8 and applied to a column (1.8 x 42 cm) of Dowex 1X2

(formate). The column was washed with water and eluted with a linear gradient of formic acid. The mixing chamber contained water (2 l) and the reservoir contained 0.08 N formic acid (2 l). The 2'-5' linked isomer was eluted with 0.06 N formic acid and the 3'-5' linked isomer was eluted at the concentration of 0.07 N. The former peak showed higher Rf value in solvent C and the latter peak was sensitive to RNase St. Guanylyl-(2'-5')-cytidine (10926 A_{260}) and guanylyl-(3'-5')-cytidine (7925 A_{260}) were isolated as ammonium salts after charcoal treatment.

Adenylyl-(2'-5')-adenosine and adenylyl-(3'-5')-adenosine ----- Pyridinium N, N',2',3'-O-tetrabenzoyladenosine 5'-phosphate (IIb, 0.83 mmole) and N,N',5'-O-tribenzoyladenosine (Ib, 0.80 mmole) were allowed to react with TPS (1.2 mmoles) in anhydrous pyridine at room temperature for 4 hr. Aqueous pyridine (50%, 2 ml) and triethylamine (0.3 ml) were added in an ice bath. After 16 hr at room temperature 5% of the mixture was evaporated and treated with 15 N methanolic ammonia for 16 hr at 25°. An aliquot of the mixture was subjected to paper electrophoresis and paper chromatography. Rf values are given in Table I. The isomeric mixture of adenylyladenosine (13.4 A_{260} units), adenosine 5'-phosphate (4.4 A_{260} units) and the pyrophosphate (3.8 A_{260} units) were detected by paper electrophoresis. The ammonia treated mixture (600A_{260} units) was desalted by charcoal treatment and the recovered materials (450 A_{260} units) were applied to a column (1.5 x 30 cm) of Dowex 1X8 (formate). The column was washed with water and eluted with a linear gradient of salt using 0.04 M (1.5 l) and 0.1 M (1.5 l) of ammonium formate. The 2'-5' linked isomer (105 A_{260} units) was eluted with 0.07 M salt and the 3'-5' isomer (96 A_{260} units) was eluted with 0.09 M salt.

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