RIBONUCLEASE A CATALYZED PREPARATIVE SYNTHESIS OF DINUCLEOSIDE MONOPHOSPHATES CONTAINING URIDINE ANALOGUES

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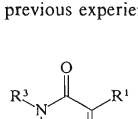
Preparative synthesis of dinucleoside monophosphates, catalyzed by ribonuclease A, is described. Uridine 2',3'-cyclic phosphate was used as a donor, the acceptors being uridine (*Ia*), N^3-methyluridine (*Ib*), 5-methyluridine (*Ic*), 6-methyluridine (*Id*), 3-(β-D-ribofuranosyl)uracil (*IIa*), 1-methyl-3-(β-D-ribofuranosyl)uracil (*IIb*), 6-azauridine (*III*) and 6-methyl-2'-deoxyuridine (*IV*). The obtained compounds of the type UpN (where N is the nucleoside moiety *I*—*IV*) were characterized by paper chromatography, electrophoresis and UV-spectra.

Recently, we started to investigate systematically how a modification of nucleosides affects their acceptor activity in the ribonuclease A catalyzed synthesis of internucleotide bond, with uridine 2',3'-cyclic phosphate as a phosphate donor[1,2]. Preliminary experiments have shown that most of the twenty hitherto studied uridine analogues differ from the parent uridine in the reaction rate as well as in the extent of formation of the corresponding dinucleoside monophosphate. In most cases the rate of the competitive donor hydrolysis to uridine-3'-phosphate substantially increases:

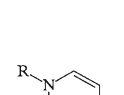
$$\begin{array}{ccc} \text{U} > \text{p} + \text{N} & \overset{\text{RNase}}{\rightleftharpoons} & \text{UpN} \\ \Big\downarrow \text{RNase} \mid \text{H}_2\text{O} & & \\ \text{Up} & & \end{array} \quad ,$$

where U > p is uridine 2',3'-cyclic phosphate, Up uridine 3'-phosphate, N nucleoside and UpN dinucleoside monophosphate. Since this reaction is irreversible, its enhancement leads to a substantial decrease in the actual donor concentration and thus to retardation of the synthesis and achieving the equilibrium; consequently, the total yield of the synthesis is lower. This complicates kinetic study of the reaction which is accompanied also by formation of oligomers due to donor polymerisation and its subsequent interactions with products of synthesis as well as hydrolysis.

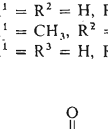
Therefore, we decided to obtain the kinetical parameters of the reverse reaction (splitting of the dinucleoside monophosphates UpN) as the additional data on the reactivity of modified nucleosides. This paper concerns the preparative synthesis of the desired dinucleoside phosphates (UpN), derived from the following nucleosides N which were selected on the basis of typical effects, observed in preliminary experiments: uridine (*Ia*), 3-methyluridine (*Ib*), 5-methyluridine (*Ic*), 6-methyluridine (*Id*), the isomeric 3-(β-D-ribofuranosyl)uracil (*IIa*) and its N^1-methyl derivative *IIb*, 6-azauridine (*III*) and 6-methyl-2′-deoxyuridine (*IV*). The reaction conditions used in this study were chosen on the basis of preliminary analytical experiments, performed according to previous experience.



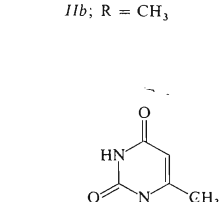
Ia; $R^1 = R^2 = R^3 = H$
Ib; $R^1 = R^2 = H$, $R^3 = CH_3$
Ic; $R^1 = CH_3$, $R^2 = R^3 = H$
Id; $R^1 = R^3 = H$, $R^2 = CH_3$



IIa; R = H
IIb; R = CH_3



III



IV

In formulae *I*–*III*, Rf = β-D-ribofuranosyl residue.

In one of our previous studies[3] we found that yield of products of reaction, catalyzed by ribonuclease A, increases with increasing absolute concentration of the starting components in the reaction mixture. A similar effect was observed also with other ribonucleases[4,5]. Since it is necessary to use an excess of the nucleoside relative to the acceptor in order to suppress the above-mentioned side reactions, the preparation of homogeneous reaction mixtures is limited by solubility of the nucleosides.

In our experiments, performed at 0°C, we kept the acceptor and donor concentration not higher than 1·24M and 0·31M, respectively (Table I). In cases where the nucleoside solubility was limited, the reaction was carried out in the frozen state which increased the yield and suppressed the concurrent hydrolysis[6,7]. The relatively dilute solutions were prepared at 0°C and the reactions were performed at

TABLE I

Synthesis of the Dinucleoside Monophosphates UpN

N	Concentration of starting components			Time h	Method[a]	Yield[b] %
	Donor M	Acceptor M	RNase A mg/ml			
Ia	0·31	1·24	240	48	A	30·0
Ib (ref.[11])	0·25	0·925	80	45	A	12·0
Ic (ref.[12])	0·06	0·26	250	120	B	23·2
Id (ref.[13])	0·25	0·88	135	22	A	41·9
IIa (ref.[14])	0·12	0·49	50	50	B	53·8
IIb (ref.[14])	0·05	0·19	45	48	B	20·4
III (ref.[15])	0·09	0·35	45	120	B	27·3
IV	0·12	0·42	50	30	B	34·2

[a] See Experimental; [b] based on U > p.

TABLE II

Properties of Dinucleoside Monophosphates UpN

UpN	UV-Spectra, pH 2					R_F[a]		E_{Up}[b]
	λ_{max}, nm	λ_{inin}, nm	*A* 250/260	*A* 270/260	*A* 280/260	S_1	S_2	
Ia	262	231	0·71		0·38	0·25	0·29	0·30
Ib	262	233	0·79	0·94	0·54	0·40	0·46	0·32
Ic	262	235	0·75	0·96	0·61	0·25	0·33	0·32
Id	263	232	0·72	0·86	0·37	0·27	0·29	0·31
IIa	263	231	0·74	0·85	0·40	0·30	0·35	0·30
IIb	267	237	0·70	1·04	0·72	0·39	0·45	0·32
III	265	233	0·71	0·95	0·51	0·36	0·35	0·31
IV	265	233	0·72	0·80	0·54	0·25	0·27	0·50

[a] R_F (S_1): Up = 0·18, U > p = 0·41, UpU > p = 0·28, UpUp = 0·09, R_F (S_2): $(Up)_2$ 0·15 U > p = 0·47 UpU > p = 0·12; [b] electrophoretic mobility, based on Up; U > p = 0·61, UpU > p = 0·80.

$-10°C \pm 2°C$. Concentration of the enzyme was chosen according to relative rates of synthesis and hydrolysis, found in preliminary experiments with the given nucleoside[2]. Lower concentrations were used if one of both reactions was too fast to ensure establishing the equilibrium. In the opposite case, a reverse hydrolysis of the product UpN prevailed which resulted in a lower yield. Longer time for equilibrium establishment was achieved also at lower temperatures (in the frozen state[7]). All the concentrations of components, given in Table I, refer to the reactions at 0°C. The reactions were performed invariably in water; the donor hydrolysis liberates the 3'-nucleotide which undergoes an additional dissociation ($pK_a = 5{\cdot}8$). Since the donor concentration is sufficiently high, the donor acts simultaneously as a buffer, maintaining the pH value of the reaction mixture between $6{\cdot}0-6{\cdot}2$. These experimental conditions are advantageous since it is not necessary to remove any salts from the reaction mixture during the work-up procedure.

One of the most important tasks of the work-up procedure is a rapid and complete removal of the enzyme. Previous experiments on synthesis of UpC have shown that this can be achieved by action of Dowex 50 in H^+-form[8] because the total charge of the protein is +4 at pH 7 and +8 at pH 5 (ref.[9]). The reaction mixture was therefore shaken with dry ion exchange resin, filtered, the filtrate neutralized with ammonia and subjected to paper chromatography. In the case of the nucleosides *Ib* and *IIb* the chromatographic mobility of the compounds UpN is identical (under the conditions employed for U > p). Therefore, the mixtures were separated by paper electrophoresis in a volatile buffer. This procedure proved to be suitable also for the compound *IV*, where the product R_F value is very close to that of U > p (Table II). After elution with water (pH 8), the obtained products UpN were transformed into the lithium salts on columns of Dowex 50 (lithium form) and lyophilized. This transformation is recommended because of low acidity of the phosphodiesters. Ammonia would be removed during the lyophilisation and the partially formed acidic form of UpN could undergo isomerisation and decomposition during storage.

The obtained products were homogeneous according to electrophoresis and paper chromatography in two systems. They are cleaved with RNase A to give equimolecular amounts of Up and N which proves not only the structure but also the isomeric purity of the internucleotidic (3' → 5')-phosphodiester bond. The UV spectra of compounds UpN (Table II) are composed of spectra of the two components. The absence of any contaminating traces of enzyme in the products was proved by their complete stability for 10 days at room temperature and for longer than three months at $2-5°C$.

Some of the prepared dinucleoside phosphates have not been hitherto described and it is obvious that their chemical synthesis would be very difficult. Undoubtedly, the described method is highly effective and suitable for the synthesis of oligoribonucleotides, required as model compounds for investigations of RNA, enzyme specificity, and for other purposes.

EXPERIMENTAL

Uridine and 6-azauridine were Calbiochem (Los Angeles, U.S.A.) products, RNase was purchased from Worthington (U.S.A.). Other nucleosides were prepared by described procedures (Table I). The UV spectra were measured on a Specord UV-VIS (Zeiss, Jena, G.D.R.) instrument. Paper chromatography (descending arrangement) was performed on papers Whatman No 3 MM or No 1 (the latter was used for analytical purposes) in the systems S1 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2), S2 ethanol–1M ammonium acetate pH 7·5 (7 : 3), paper electrophoresis on a paper Whatman No 3 MM according to ref.[10] (preparative experiments at 15 V/cm, 2 h, on Labor (Hungary) instrument. The compounds were detected in UV light (Chromatolight).

Preparation of Dinucleoside Phosphates UpN

Method A. An aliquot amount of a solution of ribonuclease A (1 mg/ml) in 0·1M phosphate buffer, pH 7·0, was added at 0°C to an aqueous solution of uridine 2′,3′-cyclic phosphate lithium salt and nucleoside. The mixture (Table I) was incubated at 0°C and the reaction course was followed by electrophoresis. The reaction was quenched by addition of Dowex 50 × 8 (H^+-form; 0·1—0·5 ml, to acidic reaction), prewashed with water and ethanol. After shaking for 5—10 min at room temperature, the solution was decanted und neutralized with ammonia. The ion exchange resin was washed (decantation) three times with a small volume of water. The combined solutions were chromatographed in the system S1 (about 1 mg of the nucleoside per 1 cm) or separated electrophoretically. The bands of the products were eluted with water (25—30 ml) the pH of which was adjusted to 8. The eluate was concentrated *in vacuo* to about 2 ml, applied to a column (10 ml) of Dowex 50X8 (H^+-form) and eluted with water. The UV-absorbing eluate was concentrated *in vacuo* and lyophilized.

Method B. The reaction mixture was prepared according to *A* (Table I). After addition of the enzyme the mixture was cooled rapidly to —30°C and allowed to stand at —10°C ± 2°C for the time required. Ion exchange resin was added (*vide supra*), the mixture thawed and worked up as described sub *A*. All the compounds prepared are characterized by their UV-spectra, R_F values and electrophoretic mobilities (Table I, II); the yields are given in Table I.

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