Aliphatic analogues of nucleotides: synthesis and affinity towards nucleases

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ABSTRACT

DL-1-(2,3-Dihydroxypropyl)thymine was prepared by Hilbert-Johnson reaction of 2,4-dimethoxy-5-methylpyrimidine with allyl bromide followed by the osmium tetroxide catalyzed hydroxylation of the 1-allyl-4-methoxy-5-methylpyrimidin-2-one obtained as an intermediate. The D-glycero enantiomer, R-1-(2,3-dihydroxypropyl)thymine and the corresponding 1-substituted uracil derivative were prepared from 3-O-p-toluenesulfonyl-1,2-O-isopropylidene-D-glycerine and sodium salt of 4-methoxy-5-methylpyrimidin-2-one or 4-methoxypyrimidin-2-one followed by treatment with hydrogen chloride in ethanol. The phosphorylation of the above 2,3-dihydroxypropyl derivatives with phosphoryl chloride in triethyl phosphate afforded the corresponding 3-phosphates which were transformed into the 2,3-cyclic phosphates by the condensation with N,N-dicyclohexylcarbodiimide. The latter compounds of the D-glycero configuration are split by some microbial RNases to the 3-phosphates.

INTRODUCTION

In the course of our investigation on the influence of nucleoside sugar modification upon the affinity of the compound towards nucleolytic enzymes we have been interested in the preparation and investigation of nucleotide analogues, the furanose or pyranose sugar moiety of which would be replaced by simple aliphatic three- or four-carbon-atom substituents corresponding partially to the natural ribo- or 2-deoxyribofuranose moiety. Similar compounds have recently aroused attention from different points of view[1-3] and various procedures of their synthesis have been reported.

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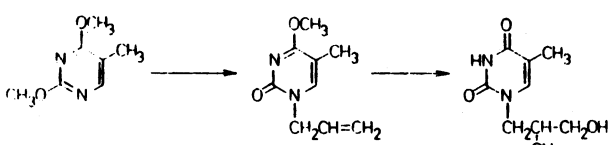
The present paper deals with an unequivocal synthesis of pyrimidine (uracil, thymine) derivatives bearing the 2,3-dihydroxypropyl residue in the position N^1 as an alternative to procedures described earlier[1,2].

SYNTHETIC PART

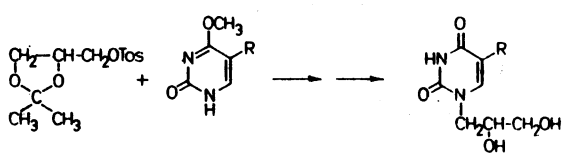
Route A (DL-Derivatives): In order to ensure the introduction of the substituent into the position N^1 of the pyrimidine ring, the principle of Hilbert-Johnson reaction has been applied. This reaction consists in treatment of 2,4-dimethoxypyrimidine derivatives unsubstituted or substituted at the C_5-position, with an alkyl (or glycosyl) halide and affords specifically 1-substituted 4-methoxypyrimidin-2-one derivatives (for ref., cf.[4]). Thus, 2,4-dimethoxy-5-methylpyrimidine[5] (I) and allyl bromide afforded 1-allyl-4-methoxy-5-methylpyrimidin-2-one (II) which, upon cis-hydroxylation with sodium chlorate in the presence of osmium tetroxide[6], was transformed into the 2,3-dihydroxypropyl derivative. During the hydroxylation reaction, the 4-methoxy group at the pyrimidine base was simultaneously demethylated, giving rise to the 1-(2,3-dihydroxypropyl)thymine (IIIb) as the final product:



Ib II IIIb

Route B (DL- and D-glycero derivatives): Since the 2'-carbon atom of the aliphatic substituent is an asymmetric one, the above method (Route A) results in a racemic derivative IIIb. Therefore, the conditions for a synthesis of the optically active compound III were sought, preferentially of the derivative with a D-glycero configuration which, as indicated by examination of molecular models could assume a conformation close to that of the naturally occurring pyrimidine ribonucleosides. For this purpose, a different route was chosen which consists

in the reaction of 3-O-p-toluenesulfonyl-1,2-O-isopropylidene--DL- or -D-glycerine[7] (IV) with the sodium salt of 4-methoxypyrimidin-2-one (Va) or its 5-methyl derivative (Vb). The reaction was performed by a prolonged reflux in methanolic solution followed by treatment with hydrogen chloride in ethanol which split off the isopropylidene group and, simultaneously, demethylated the 4-methoxy group affording the compound III. The reaction mixture was then deionized and the mixture of product III and uracil (or thymine) separated by chromatography on a silica layer. The yields of this method are somewhat lower than those of the Route A; however, both the racemate and the R-isomers of the D-glycero configuration of the uracil (IIIa) and thymine (IIIb) series have been synthetized by the Route B. The starting compounds V were obtained from 2,4-dimethoxypyrimidines I by reaction with acetyl chloride followed by the alkaline work-up of the reaction mixture[8]:



IV Tos = tosyl

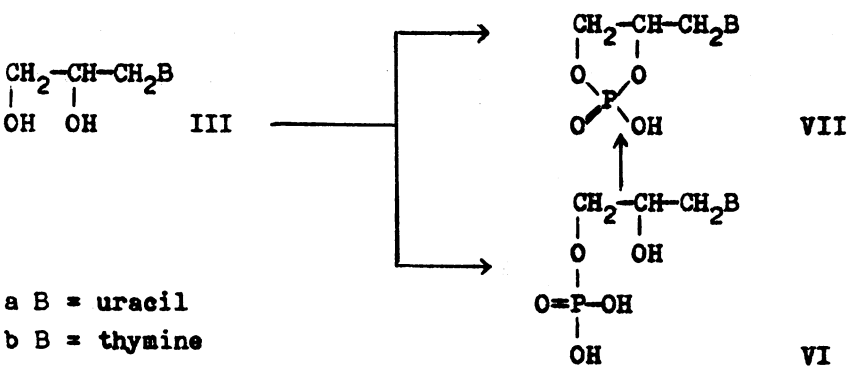
Va R=H
Vb R=CH_3

IIIa R=H
IIIb R=CH_3

The attempts to improve the reaction yields by replacement of the tosylate IV through the 3-iodo derivative formed in situ or separately from the tosylate IV and lithium iodide in acetonitrile were unsuccesseful, regardless of the solvents and reaction conditions used. Also, there is no direct Hilbert-Johnson-type reaction of the tosylate IV with a 2,4-dimethoxypyrimidine derivative I leading to the products required.

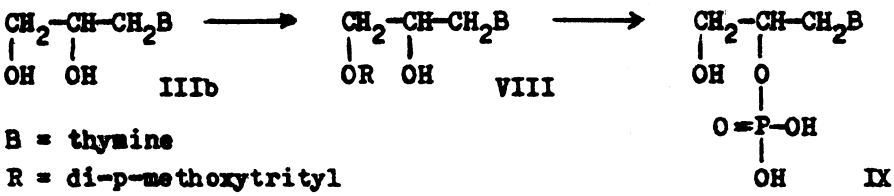
The phosphorylation of the above ribonucleoside analogues III was performed with the use of phosphoryl chloride in triethyl phosphate[9]; under the neutral buffered hydrolytical conditions, the 2',3'-cyclic phosphate VII can be obtained as the main reaction product. This situation is common for the application of the above phosphorylation reaction to all compounds

bearing a vicinal hydroxylic group cis with respect to the primary hydroxylic function at which the reaction begins[10]. However, owing to the difficulties accompanying the purification of the compound VII from traces of the starting compound III and some unknown compound, presumably a symmetric phosphoric acid diester of III, it is recommendable to perform the work-up of the reaction mixture under acidic conditions which lead unequivocally to the 3'-phosphate VI. After purification by paper or DEAE-cellulose column chromatography, the above nucleotide analogue can be transformed into the 2',3'-cyclic phosphate VII by an N,N'-dicyclohexylcarbodiimide (DCC) condensation under usual conditions[11]:



a B = uracil
b B = thymine

The synthesis of the 2'-isomer of the 3'-phosphate VI was performed indirectly: first, the primary 3'-hydroxylic group in the racemic compound IIIb was protected by the reaction with di-p-methoxytrityl chloride in pyridine. The substituted trityl derivative VIII was then phosphorylated with 2-cyanoethyl phosphate[12]; in the work-up of the mixture the 2-cyanoethyl group was first removed by an alkaline treatment and then the trityl group split off under mild acidic conditions. This procedure diminishes the danger of the 2'-phosphate isomerisation under acidic or alkaline conditions. In fact, the 2'-phosphate IX was obtained free of the 3'-isomer VI, as demonstrated by paper chromatography:



B = thymine
R = di-p-methoxytrityl

After the above unequivocal synthesis of the 2′-isomer IX it was possible to assign definitely the isomeric structure of the former 3′-phosphate VI obtained by direct phosphorylation of the nucleoside analogues III. It was also confirmed that the acidic hydrolysis of the 2′,3′-cyclic phosphodiester VII produces the 3′-isomer VI as the only compound. This finding is consistent with the earlier observations on analogous five-membered phosphoric acid cyclic diesters[2,13,14] which are hydrolyzed under acidic conditions producing the phosphomonoesters attached to the primary hydroxyl group as the predominant products.

ENZYMOLOGICAL STUDIES

As stated above, it was the principal aim of this study to find out whether the open-chain nucleotide analogues VI, VII and IX are substrates for the typical nucleolytic enzymes. For this purpose the racemic thymine derivatives DL-VIb, DL-VIIb and DL-IXb and some pyrimidine-specific and non-specific enzymes degrading ribonucleoside 2′,3′-cyclic phosphates, 5′-nucleotides and 3′-nucleotides were examined under the assay conditions usual for the appropriate enzymes (see Experimental). The results of this investigation are given in Table 1.

Table I. Splitting of DL-1-(2,3-Dihydroxypropyl)thymine Nucleotide Analogues by Some Nucleolytic Enzymes (Assay conditions see Experimental; + good substrate, ± traces of splitting, - no splitting)

Enzymes	DL-VIb	DL-VIIb	DL-IXb
Bovine pancreas RNase		-	
Bovine seminal fluid RNase		-	
Ribonuclease T2		-	
RNase Penicillium brevicompactum		+	
RNase P. chrysogenum		+	
RNase P. claviforme		+	
RNase Asp.clavatus		+	
Spleen cyclic phosphodiesterase		±	
Snake venom (Crotalus terr.)phosphodiesterase		-	
Snake venom (C.adamanteus) 5-nucleotidase	-		-
Bacterial alkaline phosphatase	+		+

The last item of the Table, bacterial alkaline phosphatase, need not be included into the detailed discussion. Its aim was only to prove the structure of compounds VIb and IXb by their dephosphorylation to the starting compound IIIb. As the enzyme is a non-specific phosphomonoesterase, splitting all phosphoric acid monoesters, there was no doubt of its response towards the compounds listed.

The other enzymes of the Table I are specific nucleases which require an interaction of the heterocyclic base of the substrate with the enzyme[15]. They can be divided into two groups with respect to their response towards the nucleotide aliphatic analogues. The most important one is the group of enzymes which has been checked with the 2',3'-cyclic phosphate VIIb and, more specifically, the ribonucleases. It is evident that the ability to split the compound VIIb is not general for the ribonucleases; the most usual pyrimidine-specific enzyme, the pancreatic ribonuclease, and the common non-specific enzyme, the T2-RNase, are unable to split the above compound. However, the other microbial ribonucleases of the species and strains very similar to the source of T2-enzyme, which have been recently investigated in this Laboratory[16], do split the compound DL-VIIb under the formation of the 3'-phosphate VIb as the sole product. It was found that the splitting does not exceed 50% of the total amount, the rest of which being enzyme-resistant even after prolonged incubation with an increased enzyme concentration. These and the following experiments were performed with non-specific ribonuclease Penicillium brevicompactum[16] as a representative of enzymes with the positive response towards the acyclic analogues VII:

(a) The enzyme resistant fraction of VIIb was quantitatively split by the action of dilute acid; thereby, the cyclic phosphate structure of the unchanged material was unequivocally proved, as the splitting product possesses the structure of the 3'-phosphate VIb.

(b) The 3'-phosphate VIb obtained by the enzymatic splitting was recyclized by the action of DCC and its stability towards the enzyme reexamined. This time, the extent of cleavage was over 90%.

(c) The circular dichroism spectra of compound VIb obtained both from the enzymatic splitting and from the acidic hydrolyses of

the enzyme-resistant fraction were compared (Fig.1). It is fully evident that the action of the enzyme results in the resolution of both enantiomers of the racemic compound VIIb and that one of the enantiomers is quantitatively split by the enzyme (the small difference observed above is due to the non-enzymatic hydrolysis which amounted to 7-10% under the experimental conditions used and which does not distinguish the enantiomers). On the other hand, the other enantiomer of compound VIIb is obviously quite resistant towards the action of the above ribonuclease. This situation is quite analogous to that of pyrimidine D- and L-ribonucleoside 2',3'-cyclic phosphates the latter of which are resistant towards the action of ribonucleases[17] including those which split the aliphatic analogue VIIb[16].

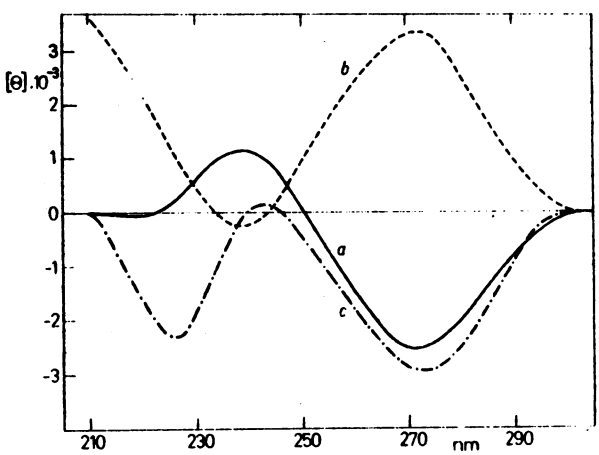


Fig.1. The CD-Spectra of 3'-Phosphate VIb obtained (a) by enzyme action, (b) by acid hydrolysis of the enzyme resistant fraction and, (c) of the synthetic D-VIb.

On inspection of the molecular model it was evident that one of the enantiomers might exist in a conformation very similar to that of the derivatives in the ribonucleoside series, whereas such situation in the other one would be impossible. It was deduced from the same observation that the former enantiomer ought to possess the D-glycero configuration. Therefore, the synthesis of thymine and uracil derivatives VII has been effected and their response towards RNase P.brevicompactum investigated. The splitting of the cyclic phosphate D-VIIa,b was near-

ly identical with that of the splitting product of DL-VIIb (Fig. 1).

Thus, the absolute configuration of the enantiomer VIb which is recognized by the RNase P.brevicompactum and some other microbial RNases was unequivocally assigned as D-glycero.

CONCLUSION

The above finding on the affinity of 1-(2,3-dihydroxypropyl)-uracil or thymine 2',3'-cyclic phosphates VII of the D-glycero configuration towards some ribonucleases and the resistance of the L-glycero derivatives of the same structure is quite consistent with the recent hypotheses on the necessity of the substrate molecules to assume a defined conformation which makes simultaneously possible both the binding of the phosphate group and the interaction of the heterocyclic base. Such a mutual orientation of the two groups mentioned can be achieved in the D-glycero configuration only though, in the alifatic analogues III or VII the conformational freedom is much greater than it is with ribofuranoside derivatives.

Furthermore, from the different response of various ribonucleases towards the D-enantiomer of VII, of those in which a functional group of the base takes part in the reaction (pancreatic RNase, seminal RNase), as well as of those which do not require the presence of any particular group at the base (RNase T2), it is possible to conclude that the lack of affinity observed with some enzymes is not due to a hindrance for some group to be localized in a proper conformation with respect to the enzyme. Rather, such enzymes evidently require the presence of the whole ribofuranose ring of the ribonucleoside 2',3'-cyclic phosphates, either to fix still more accurately the substrate conformation or, more probably, the presence of the sole ribofuranose ring oxygen atom to interact with. Thus, the nucleotide analogues derived from the acyclic compound type III might serve as a useful tool for a detailed nuclease classification.

The isosteric character of the compounds D-VII and pyrimidine D-ribonucleoside 2',3'-cyclic phosphates also follows from the fact that the splitting product, if any, is the 3'-phosphate D--VI. The O^2-P linkage is broken in both cases of compound types though in D-VII the electron density at this particular linkage

must be quite different from that of the latter type.

EXPERIMENTAL

Solutions have been taken down at 40°C/15 Torr on a rotatory evaporator if not stated otherwise. Melting points were determined on a Kofler apparatus and are uncorrected. Paper chromatography was performed on a Whatman No. 3 MM paper in solvent systems: S1, 2-propanol-conc.aqueous ammonia-water (7:1:2, v/v) and S2, 1-butanol-acetic acid-water (5:2:3, v/v). Thin-layer chromatography was performed on silica plates Silufol UV_{234} with fluorescent indicator in S3, chloroform, S4, chloroform--ethanol (9:1), S5, chloroform-ethanol (7:3). Paper electrophoresis was performed at 20 V/cm in 0.1M triethylammonium hydrogen carbonate, pH 7.5 (E1) (Table II).

1-Allyl-4-methoxy-5-methylpyrimidin-2-one (II): A mixture of 2,4-dimethoxy-5-methylpyrimidine (Ib)[5] (15.4 g, 0.1 mol) and allyl bromide (30 ml, 43 g, 0.36 mol) was refluxed for 8 hours, evaporated and separated on a column of silica (30-60 mesh, 200 g) in ethyl acetate. The elution by the same solvent afforded a small amount of starting material, followed by the pure product. After evaporation in vacuo, the residue was crystallized from cyclohexane. Yield, 11.0 g (61%), m.p. 76-77°C. For $C_9H_{12}N_2O_2$ (180.2) calc.: 60.00 % C, 6.71% H, 15.54% N; found: 60.36% C, 6.82% H, 1568% N.

DL-1-(2,3-Dihydroxypropyl)thymine (IIIb): To a solution of 9.0 g (0.05 mol) IIb and 5.3 g (0.06 mol) sodium chlorate in 100 ml water there was added 10 mg osmium tetroxide and the whole stirred for 4 days at room temperature. The TLC in S3 revealed afterwards a quantitative reaction. The mixture was then filtered through Celite and applied to an Amberlite IR 4 B (acetate) column (300 ml). The UV-absorbing material was eluted with water (3 ml/min, checked by the Uvicord apparatus), evaporated and applied on a Dowex 50 X 8 (100-200 mesh, H^+-form) column (300 ml). Again, the neutral UV-absorbing fraction was eluted with water under the same conditions and the eluate evaporated to dryness and crystallized from ethanol. Yield, 5.5 g (55%), m.p. 151°C. For $C_8H_{12}N_2O_4$ (200.2) calc.: 48.02% C, 6.03% H, 14.00% N; found: 47.71% C, 6.13% H, 13.53% N. UV-Spectrum (pH 2): λ_{max} 268 nm, ε_{max} 11000; (pH 12): λ_{max} 270 nm, ε_{max}8800.

NMR-Spectrum (d_6-dimethyl sulfoxide): 1.79 (d, 3H) 5-CH_3 ($J_{CH_3,H}$ = 1.2); 3.20 (compl.m., 5 H) 2 CH_2 + CH; 7.32 (d, 1H) 6-H; 11.04 (br s, 1H) NH.

Table II. Chromatography and Electrophoresis

Compound	R_F					El[a]
	S1	S2	S3	S4	S5	
Ia	--	--	0.62	--	--	--
Ib	--	--	0.70	--	--	--
IIb	0.87	--	0.55	--	--	--
IIIa	0.52	0.37	0.04	0.10	0.25	0
IIIb	0.68	0.42	0.08	0.16	0.35	0
IV	--	--	0.60	--	--	--
Va	0.70	--	0.04	0.48	--	--
Vb	0.75	--	0.08	0.56	--	--
VIa	0.20	0.30	--	--	--	0.95
VIb	0.27	0.33	--	--	--	0.95
VIIa	0.43	--	--	--	--	0.45
VIIb	0.48	--	--	--	--	0.45
IX	0.31	0.33	--	--	--	0.95
uracil	0.50	0.38	--	0.20	0.48	-0.10
thymine	0.65	0.45	--	0.40	0.60	-0.10
UMP	0.10	0.08	--	--	--	1.00

[a] referred to UMP

3-O-p-Toluenesulfonyl-1,2-O-isopropylidene-DL-glycerine (DL-IV) and 3-O-p-Toluenesulfonyl-1,2-O-isopropylidene-D-glycerine (D-IV) were prepared as described earlier[7]. In contrast to the reported data, the D-enantiomer was obtained in a crystalline form from diethyl ether-petroleum ether (3:2) at -40°C in the 40% yield from D-mannitol; m.p. 41-43°C, $[\alpha]_{25}^{D}$ -2.5° (c=2, dimethylformamide). For $C_{13}H_{18}O_5S$ (286.3) calc.: 54.54% C, 6.33% H, 11.19% S; found: 54.52% C, 6.17% H, 11.00% S.

4-Methoxypyrimidin-2-one (Va) and 4-methoxy-5-methylpyrimidin-2-one (Vb): A mixture of compound I (0.25 mol) and acetyl chloride (50 ml) was kept at room temperature under exclusion of moisture for 2 days under occasional stirring. The resulting suspension was taken down to dryness, codistilled with three 25-ml portions toluene and warmed with 260 ml 1.0M sodium methylate in methanol 2 h at 50°C. After neutralisation with acetic acid, the mixture was evaporated to dryness and extracted with three 100-ml portions of hot chloroform, filtered, the filtrate evaporated and crystallized from hot methanol. Yield, 45%

compound Va, m.p. 207-208°C (lit.[18] gives m.p. 206-208°C), or 43% compound Vb, m.p. 211-213°C (lit.[19] gives m.p. 182-183°C).

DL-1-(2,3-Dihydroxypropyl)uracil (DL-IIIa): To a mixture of 5.7 g (20 mmol) DL-IV and 2.5 g (20 mmol) 4-methoxypyrimidin-2-one (Va) there was added 22 ml 1M sodium methylate in methanol and 10 ml methanol. The mixture was then refluxed for 30 h under exclusion of moisture, neutralized with acetic acid, taken up to dryness and dissolved in 100 ml 10% hydrogen chloride in ethanol. After standing at room temperature overnight, the mixture was filtered and the precipitate (uracil) discarded. The filtrate was evaporated to dryness, stirred with methanol (50 ml), filtered again and the filtrate concentrated in vacuo. This solution was applied successively to columns of Amberlite IR4B (acetate) and Dowex 50X8 (100-200 mesh, H^+-form) (100 ml each) and the elution performed with water in both cases. The corresponding UV-absorbing eluates were taken down in vacuo. The final concentrate was dried by repeated codestillation with ethanol (3x25 ml), triturated with 25 ml methanol, filtered from the precipitated uracil and the filtrate applied to two layers of loose silica (35x15x0.3 cm) with fluorescent indicator. After developing the plates in system S5, the bands of compound IIIa were eluted with methanol (300 ml), evaporated and the residue crystallized from ethanol (ether added to cloudiness). Yield, 1.2 g (30%), m.p. 143-144°C (lit.[1] gives m.p. 143-144°C). UV-Spectrum (pH 2): λ_{max} 263 nm, ε_{max} 10000; pH 12: λ_{max} 260 nm, ε_{max} 7700. For $C_7H_{10}N_2O_4$ (186.2) calc.: 45.15% C, 5.41% H, 15.05% N; found: 45.18% C, 5.50% H, 15.24% N.

D-1-(2,3-Dihydroxypropyl)uracil (D-IIIa) was prepared exactly as described for the racemate DL-IIIa, except that D-IV was used, in the 27% yield. M.p. 141-142°C, $[\alpha]_{25}^{D}$ -22.3° (c=1, water). CD-Spectrum (H_2O): 267 nm (Ⓗ = -1700); 247 (0); 238 (+780); 206! (+1570).

D-1-(2,3-Dihydroxypropyl)thymine (D-IIIb) was prepared as described for DL-IIIa except 4-methoxy-5-methylpyrimidin-2-one was used instead of Va and D-IV instead of DL-IV. The final purification was performed on silica plates in system S4 and the product crystallized from ethanol-ether. Yield, 32%, m.p. 152°C,

$[\alpha]_{25}^{D}$ -19.3° (c=1, water). CD-Spectrum (H_2O): 273.5 (-1360); 253 (0); 238.5 (+910); 205! (+1210).

DL-1-(2,3-Dihydroxypropyl)thymine 3'-Phosphate (DL-VIb): A suspension of 2.15 g (10 mmol) DL-IIIb in 20 ml triethyl phosphate was cooled by ice water and 3.5 ml (5.9 g, 4.5 equiv.) phosphoryl chloride added. After stirring at room temperature for 1.5 h the reaction was quantitative (TLC in S5). Water (25 ml) was then added, the whole kept 1 h at 60°C, neutralized with triethylamine, concentrated in vacuo and applied to a column (80x4 cm) DEAE-cellulose (Cellex D, HCO_3^- form); the column was eluted with a linear gradient of triethylammonium hydrogen carbonate pH 7.5 (2 l water in the stirring chamber, 2 l 0.2M buffer in the reservoir (rate, 3ml/min, fractions were taken in 10 min intervals and checked by measurement on an Uvicord apparatus). The peak of the product was pooled, evaporated and freed from the buffer by codistillation with methanol. Yield (estimated spectrophotometrically): 6.2 mmol (62%) of the chromatographically homogeneous (S1, S2) fraction. An aliquot (1 mmol) was applied to 4 sheets of Whatman No. 3 MM paper and chromatographed in the system S1. The bands of product were eluted with dilute (1:100) ammonia (20 ml) and freeze-dried as the ammonium salt.

DL-1-(2,3-Dihydroxypropyl)thymine 2',3'-Cyclic Phosphate (DL-VIIb): The above triethylammonium salt of DL-VIb (5 mmol) was applied to a column of Dowex 50 X 8 (20 ml) and the UV-absorbing fraction eluted with water. The eluate was made alkaline with conc. ammonia and evaporated to dryness in vacuo. The residue in 50 ml 2 N ammonia, 40 ml dimethylformamide, and 40 ml tert.butanol was refluxed with 10 g DCC for 6 hours, cooled down, diluted with 250 ml water, extracted with two 50-ml portions of ether, the aqueous phase filtered and evaporated to dryness in vacuo. The residue in water (50 ml) was applied to a column of DEAE-cellulose (see above), and the elution performed with a 0-0.1M linear gradient of triethylammonium hydrogen carbonate pH 7.5 under the conditions given above. The 0.05-0.08M fraction which contained the product was pooled, evaporated and transformed into the lithium salt by passage through a 50-ml column of Dowex 50 X 8 (Li^+) followed by elution with wa-

ter until the UV-absorption dropped. The eluate was evaporated to dryness, taken up in water (2.5 ml) and a mixture of ethanol-ether (200 ml, 1:1) added. The precipitated lithium salt of DL-VIIb was collected with suction, washed with the same mixture and dried in vacuo. Yield, 1.0 g (75%), content (spectrophotom,): 95%. The material is chromatographically (S1) and electrophoretically (E1) homogeneous.

D-1-(2,3-Dihydroxypropyl)thymine 3'-Phosphate (D-VIb) and 2',3'-Cyclic Phosphate (D-VIIb): 428 mg (2 mmol) D-IIIb was phosphorylated analogously as described for DL-VIb. After TLC has shown a nearly complete disappearance of the starting compound, the reaction mixture was diluted with 20 ml 1M triethylammonium hydrogen carbonate and the pH adjusted to 9 with triethylamine. During the first half an hour of the hydrolysis the pH was controlled occasionally to keep within 8-9 by the addition of triethylamine. After 1 hour at room temperature, the mixture was taken down in vacuo and applied to 8 sheets Whatman No. 3 MM paper. After developing the chromatograms in the system S1 overnight, the major band of the 2',3'-cyclic phosphate D-VIIb and the minor band of the 3'-phosphate D-VIb were eluted with dilute (1:100) ammonia (50 ml), taken up to dryness and the residues precipitated from methanol (5 ml) with ether (100 ml). The products thus obtained were collected by centrifugation, washed with ether and dried in vacuo. Yield, 1.2 mmol (60%) D-VIIb, 0.4 mmol (20%) D-VIb. The cyclic phosphate VIIb was contaminated by less than 10% of an unknown material (E_{Up} = 0.40 in E1). To remove this material, the compound VIIb was heated with 1 N HCl (3 ml) 1 hour at 80°C, chromatographed on 4 sheets as above, the 3'-phosphate VIb eluted as described above and recyclized by the action of DCC under the conditions given for DL-VIIb. The compound D-VIIb can be obtained quite pure after final purification by paper chromatography in system S1 and methanol-ether precipitation (see above).

Alternatively, the hydrolysis of the whole mixture after phosphorylation of D-IIIb was performed as described for DL-VIb. In this case, D-VIb was obtained as the sole product by chromatography on 8 sheets of Whatman No. 3 MM in S1. Yield, 1.4 mmol (70%) ammonium salt of D-VIb precipitated from metha-

nol with ether (see above), homogeneous in S1 and E1.

D-1-(2,3-Dihydroxypropyl)uracil 3'-Phosphate (D-VIa) and 2',3'-Cyclic Phosphate (D-VIIa) were prepared analogously as described for the thymine derivatives D-VIb, D-VIIb. In this case, the 3'-phosphate was isolated by the acidic work-up of the mixture in the yield 65% as amorph ammonium salt, homogeneous in S1 and E1. 1 mmole of this compound was transformed into the compound D-VIIa by DCC-condensation and isolated by paper chromatography in 67% yield as ammonium salt.

DL-1-(2,2-Dihydroxypropyl)thymine 2'-Phosphate (DL-IX): A solution of DL-IIIb (107 mg, 0.5 mmol) and di-p-methoxytrityl chloride (0.2 g) in 2 ml pyridine was left to stand for 2 days at room temperature, diluted with ethyl acetate (50 ml), extracted with three 25-ml portion water, dried with magnesium sulfate and evaporated. TLC in S3 revealed the presence of compound VIII and traces of di-p-methoxytritylcarbinol. The crude residue was taken up in pyridine (20 ml), 2.5 mmol pyridinium 2-cyanoethylphosphate[21] in pyridine was added and the whole dried by codistillation with ten 20-ml portions pyridine at 30°C/0.1 Torr. The residue was then dissolved in pyridine (3 ml), 2,4,6-triisopropylbenzenesulfonyl chloride (1.2 g, 4 mmol) was added and the mixture left to stand overnight at room temperature under the exclusion of moisture. Water (2 ml) was added, after one-hour standing the mixture was evaporated to dryness in vacuo and pyridine removed by codistillation (5x20 ml) toluene in vacuo. The residue in 25 ml 50% methanol and 25 ml acetic acid was heated at 50°C 1 hour, evaporated in vacuo, the residue treated with water (50 ml) and ether (25 ml). The aqueous phase was extracted once more with 25 ml ether, evaporated to dryness, and treated with 50 ml 50% methanol and 10 ml triethylamine at 50°C 2 hours. Afterwards, the solution was evaporated in vacuo and the residue chromatographed on two sheets Whatman No. 3 MM paper in solvent S1. The bands of IX were eluted by dilute (1:100) ammonia (50 ml), evaporated to dryness and transformed into the lithium salt as described for DL-VIIb. Yield, 0.27 mmol (54%) chromatographically homogeneous (S1, S2) DL-IX (lithium salt).

Enzymatic Hydrolysis of DL-VIIb by RNase P.brevicompactum:

A solution of 295 mg (1 mmol) DL-VIIb (lithium salt) in 6 ml 0.1M sodium γ,γ-dimethylglutarate pH 6.0 was treated with 1500 eu RNase P.brevicompactum (cf.[20]) 48 hours at 37°C and chromatographed on six sheets Whatman No. 3 MM paper in system S1. The estimation of an aliquot revealed 48% starting material unchanged. The bands of starting material VIIb and product VIb were eluted with dilute ammonia (50 ml each) and evaporated. Yield, 146 µmol VIb, 158 µmol VIIb. An aliquot (5 µmol) of unchanged VIIb was digested with the enzyme under the assay conditions (see below) and found resistant towards the action of the enzyme. Hence, 100 µmol of this material was heated in 2 ml 1 N HCl for 1 hour at 80°C and the resulting VIb was isolated on 2 sheets of Whatman No. 3 MM as above. Yield, 78 µmol L-VIb.

The compound VIb formed by the enzymatic splitting (D-VIb) (100 µmol) was cyclized by DCC procedure as described above (2 ml 2N NH_3, 1.5 ml dimethylformamide, 1.5 ml tert-butanol and 1 g DCC was used, reaction time 4 hours) and the resulting D-VIIb was isolated by paper chromatography (2 sheets Whatman No. 3 MM, system S1). Yield, 64 µmol D-VIIb (ammonium salt). This material (5 µmol) when assayed by the above enzyme under standard conditions, gave 84% splitting after 3 hours.

Enzymatic Hydrolysis of DL-VIIb - Assay Conditions: The incubation mixture contained 3 µmol DL-VIIb in 100 µl 0.05M buffer solution and the appropriate enzyme. After 4 and 24 h at 37°C aliquots (50 µl) were chromatographed in S1 on Whatman No. 3 MM. The spots of VIb and VIIb were eluted with 0.01 N HCl (10 ml) and the A_{270} estimated. Control experiments were performed analogously, in the absence of enzyme. Following enzymes and buffers were used: bovine pancreas RNase A (Worthington) (50 µg, Tris-HCl pH 8.0); bovine seminal vesicle fluid RNase[15] (50 µg, Tris-HCl pH 8.0); RNase T2 (50 µg, γ,γ-dimethylglutarate pH 6.0; read DMG); snake venom (Crotalus terr.terr.) phosphodiesterase (Boehringer)(10 µg, Tris-HCl pH 9.0); spleen phosphodiesterase (Boehringer)(20 µg, DMG pH 6.0); RNase P.chrysogenum[21] (20 eu, DMG pH 6.0); RNase P.claviforme[22] (20 eu, DMG pH 6.0); RNase P.brevicompactum[20] (30 eu, DMG pH 6.0); RNase Asp. clavatus (10 eu, DMG pH 6.0); snake venom (Crotalus adamanteus) 5'-nucleotidase (Worthington) (25 µg, Tris-HCl pH 9.0); bacterial

alkaline phosphatase (Worthington) (10/ug, Tris-HCl pH 9.0). The control experiments of the non-enzymatic hydrolysis (3% after 4 h, 11-14% after 24 h) were taken into account in the estimation of splitting percentage (Table I).

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