NUCLEIC ACID COMPONENTS AND THEIR ANALOGUES. CXLVIII.* PREPARATION OF SOME NUCLEOTIDIC DERIVATIVES OF OROTIDINE

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Treatment of orotidine (*I*) with ethyl orthoformate afforded the 2',3'-O-ethoxymethylene derivative *II*, the reaction of which with triphenyl phosphite followed by an acidic removal of the protecting group led to orotidine 5'-phosphite (*III*). Phosphorylation of compound *II* with 2-cyanoethyl phosphate in the presence of N,N'-dicyclohexylcarbodiimide gave orotidine 5'-phosphate (*IV*) in a low yield. Phosphorylation of compound *I* with pyrophosphoryl chloride resulted in a selective formation of orotidine 2'(3')-phosphate (*V*) while the reaction with phosphorus oxychloride afforded orotidine 5'-phosphate as the predominant product. N^3-Methylorotidine (*VI*) was converted to the 2',3'-O-ethoxymethylene derivative *VII*, the phosphorylation of which with 2-cyanoethyl phosphate and N,N'-dicyclohexylcarbodiimide led after removal of the protecting group to N^3-methylorotidine 5'-phosphate (*VIII*). None of the above nucleotidic derivatives *III*, *IV*, and *VIII* represents a substrate for the snake venom 5'-nucleotidase.

In connection with investigations on the specificity of orotidylate-decarboxylase (EC 4.1.1.23) and snake venom 5'-nucleotidase (EC 3.1.3.5) we have been interested in the preparation of some 5'-nucleotidic derivatives of orotidine (6-carboxyuridine) (*I*). Thus, orotidine 5-phosphite (*III*), differs from the naturally occurring substrate of orotidylate-decarboxylase, *i.e.*, from orotidine 5'-phosphate (*IV*) by dissociation under the formation of a monoanion only. Compound *III* was prepared according to the general method for the synthesis of ribonucleoside 5'-phosphites[1]. On treatment with ethyl orthoformate[2], orotidine (*I*) was converted to the 2',3'-O-ethoxymethylene derivative *II* which was not isolated. Reaction of compound *II* with triphenyl phosphite in the presence of triethylamine and the subsequent acidic hydrolysis afforded a fair yield of compound *III*.

The preparation of orotidine 5'-phosphate (*IV*) has been reported earlier[3]. The presence of a carboxylic function on the heterocyclic moiety of compound *I* interferes with condensations in which the phosphomonoester (2-cyanoethyl phosphate) is activated by N,N'-dicyclohexylcarbodiimide since this agent interacts also with the carboxylic function. Consequently, orotidine

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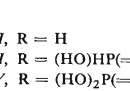
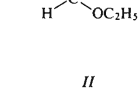
was used in the esterified form (as methyl ester). The preparation of this ester, however, requires a relatively great quantity of the starting *I* and the yield is not satisfactory. For this reason, we have reinvestigated the synthesis of the phosphate *IV* with a special respect to the small-scale preparation. The low yield of compound *IV* in the direct phosphorylation in the presence of N,N'-dicyclohexylcarbodiimide might be due, *inter alia*, to the lability of the nucleosidic bond in compound *I* in acidic media. We have therefore attempted the preparation of compound *IV* from the 2',3'-O-ethoxymethylene derivative *II*, the protecting group of which on the *cis*-diol system may be removed under far milder conditions than in the case of the previously used[3] 2',3'-O-isopropylidene derivative.

The pyridinium salt of compound *II* reacts with 2-cyanoethyl phosphate in pyridine very badly in the presence of N,N'-dicyclohexylcarbodiimide. After hydrolysis of the reaction mixture first in alkaline and then in weakly acidic media, the yield of the phosphate *IV* was 5–8%. The yield did not improve with the use of 2,4,6-triisopropylbenzenesulfonyl chloride[4] as the activating agent for the 2-cyanoethyl phosphate. The phosphate *IV* was isolated by chromatography on DEAE-cellulose and purified by paper chromatography. The product was free of any isomers and differed from the 2'(3')-isomer *V* obtained by an independent route[5]. The alkaline phosphatase *Escherichia coli* degradation led to orotidine (*I*) as the sole product.

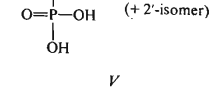
Recently, two novel phosphorylation methods for ribonucleosides have been reported[6,7] which do not require the protection of the *cis*-diol system of the nucleoside and should afford selectively ribonucleoside 5'-phosphates. We have now applied these methods to orotidine (*I*). Thus, the reaction with pyrophosphoryl chloride in acetonitrile[6] resulted in a complete conversion of orotidine (*I*) to a nucleotidic derivative. As shown on comparison with authentic nucleotidic derivatives *IV* and *V*, the phosphorylation of orotidine (*I*) with pyrophosphoryl chloride led in contrast to other ribonucleosides to the exclusive formation of the 2'(3')-monophosphate *V*. On the other hand, the phosphorylation of the cyclohexylammonium salt of compound *I* with phosphorus oxychloride in triethyl phosphate[7] afforded a mixture



I, R = H
III, R = (HO)HP(=O)—
IV, R = $(HO)_2P(=O)$—

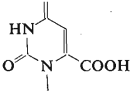


II



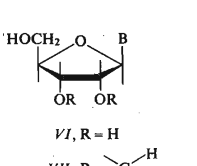
V

In formulae *I*–*V* B =



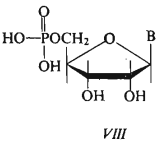
of mononucleotides containing roughly 90% of the 5'-isomer *IV*. This mixture was separated by preparative paper chromatography in a system containing boric acid. Orotidine 5'-phosphate (*IV*) was isolated in 30–40% yield, based on the starting nucleoside *I*. The latter method is thus suitable for the preparation of compound *IV*. In small scale preparations, the novel method is to a certain degree more advantageous than the original method[3]. The occurrence of the 2'(3')-isomer *V* in phosphorylations of orotidine (*I*) with phosphorus oxychloride[7] and especially with pyrophosphoryl chloride[6] is obviously due to the presence of the carboxylic function on the heterocyclic moiety of compound *I* and its influence on the character of hydroxylic functions attached to the sugar portion of the molecule; the difference in nucleophilicity of these functions (leading with other nucleosides[6,7] to the selective reaction at position 5') is lowered.

N^3-Methylorotidine 5'-phosphate (*VIII*) was prepared from N^3-methylorotidine[3] (*VI*) *via* the 2',3'-O-ethoxymethylene derivative *VII* by phosphorylation with 2-cyanoethyl phosphate in the presence of N,N'-dicyclohexylcarbodiimide and removal of protecting groups first in alkaline and then in weakly acidic media. Compound *VIII* was isolated by paper chromatography as a homogeneous substance. The yield was as low as in the preparation of compound *IV*. Alkaline phosphomonoesterase *E. coli* degradation of compound *VIII* led to N^3-methylorotidine (*VI*) as the sole product.

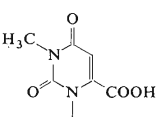


VI, R = H

VII, R =



VIII

In formulae *VI*–*VIII*, *B* = 

As shown elsewhere[8], the presence of a carboxylic function at position 5 or 6 of the heterocyclic moiety exerts considerable influence on the affinity of some nucleolytic enzymes towards the corresponding nucleotidic derivatives. It is of interest in this connection that neither orotidine 5'-phosphate (*IV*) nor N^3-methylorotidine 5'-phosphate (*VIII*) are substrates for the snake venom 5'-nucleotidase. A preliminary report on negative results obtained with orotidine 5'-phosphite (*III*) and 5'-phosphate (*IV*) has appeared earlier[9]. These observations along with those obtained with analogous 5-carboxyuridine derivatives[8] are in accordance with the idea that the acidic function on the heterocyclic moiety interferes with the interaction between the heterocyclic base and the enzyme molecule, which interaction is necessary in the case of the snake venom 5'-nucleotidase for the complex formation between the enzyme and the substrate[10,11].

EXPERIMENTAL

Unless stated otherwise, the solutions were taken down on a rotatory evaporator at 30—35°C/15 Torr. The pyridine solution of the pyridinium salt of 2-cyanoethyl phosphate was prepared from the barium salt (Calbiochem, Los Angeles, U.S.A.) with the use of pyridinium Dowex 50 ion exchange resin, dried by coevaporation with pyridine at 30°C/0·1 Torr, and diluted to the value of 0·5M.

Methods. Paper chromatography was performed by the descending technique on paper Whatman No 1 (preparative runs on paper Whatman No 3 MM) in the solvent systems S_1, 2-propanol–concentrated aqueous ammonia–water (7 : 1 : 2); S_2, ethanol–1M ammonium acetate (5 :2); and S_3, 1-propanol–concentrated aqueous ammonia–0·1M triethylammonium borate pH 7·5 (55 : 5 : 40). Paper electrophoresis was performed by the technique of Markham and Smith[12] on Whatman No 3 MM in the buffer solutions E_1, 0·1M triethylammonium hydrogen carbonate (pH 7·5), and E_2, 0·05M sodium hydrogen citrate (pH 3·5) at 40 V/cm (60 min). For R_F values and electrophoretic mobilities see Table I. Enzymatic degradations were performed as follows: the substance (1 μmol) in 50 μl of 0·05M-Tris-HCl (pH 9·0) contg. 0·005M-$MgSO_4$ was incubated at 37°C for 4 hours with *a*) 10 μl of a solution of alkaline phosphatase *E. coli* in ammonium sulfate (Worthington), *b*) 60 μg of *Crotalus adamanteus* 5′-nucleotidase (Worthington), and *c*) 100 μg of the crude snake venom *Vipera russelli*.

Orotidine 5′-Phosphite (*III*)

A mixture of the cyclohexylammonium salt of orotidine (*I*; 100 mg; 0·26 mmol), dimethylformamide (2 ml), ethyl orthoformate (0·7 ml), and 6M hydrogen chloride in dimethylformamide (0·1 ml) was stirred until a solution was obtained and then kept at room temperature for 3 days. Sodium hydrogen carbonate (0·5 g) was then added, the mixture stirred for 30 min, the solid filtered off, and washed with dimethylformamide (2 ml). The filtrates were combined and evaporated at 40°C/0·1 Torr. The residual compound *II* was diluted with dimethylformamide (5 ml) and the solution treated with triethylamine (0·75 ml) and triphenyl phosphite (1·5 ml). The mixture was kept at room temperature for 3 days, treated with dilute (1 : 4) aqueous ammonia (10 ml), kept for additional 2 hours, and washed with two 5 ml portions of ether. The aqueous phase was evaporated to dryness at 40°C/0·1 Torr and the residue dissolved in 10% aqueous acetic acid (5 ml). The solution was kept at room temperature for 6 hours, diluted with pyridine (10 ml), and evaporated to dryness under diminished pressure. The residue was then coevaporated with two 10 ml portions of pyridine. The final residue was chromatographed on two sheets of paper Whatman No 3 MM in the solvent system S_1. Bands of the product were eluted with dilute (1 : 100) aqueous ammonia (20 ml), the eluate evaporated to a small volume, and freeze-dried. Yield, 80 μmol of the ammonium salt of compound *III* (31%), homogeneous on chromatography and electrophoresis.

Orotidine 5′-Phosphate (*IV*)

A. *Using* N,N′-*dicyclohexylcarbodiimide*. Orotidine cyclohexylammonium salt (*I*; 2·5 mmol) was converted to the 2′,3′-O-ethoxymethylene derivative *II* analogously to the preparation of compound *III*. A solution of the residual compound *II* in 30% aqueous pyridine (10 ml) was applied to a column (20 ml) of pyridinium Dowex 50 X 8 ion exchange resin and the column eluted with 30% aqueous pyridine (100 ml). The eluate was concentrated to the volume of about 10 ml, the concentrate treated with the stock solution of 2-cyanoethyl phosphate pyridinium salt (10 mmol), and the whole mixture dried by repeated coevaporations with pyridine (six 20 ml portions) at 30°C/0·01 Torr. The final residue was dissolved in pyridine (20 ml), the solution shaken with N,N′-dicyclohexylcarbodiimide (10 g), and then kept at room temperature for 7

days. Water (5 ml) and triethylamine (1 ml) were added, the solution kept at room temperature for 1 hour, diluted with additional water (100 ml), and washed with ether (50 ml). The aqueous phase was evaporated to dryness and the residue heated at 50°C in 100 ml of 0·5M-NaOH for 2 hours. The mixture was neutralised with pyridinium Dowex 50 X 8 ion exchange resin, the solid material filtered off, and washed with 30% aqueous pyridine. The filtrates were combined, made alkaline with aqueous ammonia, and evaporated to dryness. The residue was heated at 50°C in 50% aqueous acetic acid (50 ml) for 30 min, the solution evaporated to dryness, and the residue chromatographed on Whatman No 3 MM in the solvent system S_1. Bands at the start line were eluted with dilute (1 : 50) aqueous ammonia (100 ml) and the eluates evaporated to dryness. Yield (determined spectrophotometrically at pH 2), 0·39 mmol (15·5%) of the crude *IV*, contaminated with orotidine polyphosphate derivatives.

B. *Using 2,4,6-triisopropylbenzenesulfonyl chloride.* Orotidine cyclohexylammonium salt (*I*; 7·8 mmol) was converted to compound *II* according to the above procedure (see preparation of compound *III*). The condensation mixture was prepared from 30 mmol of 2-cyanoethyl phosphate as given in paragraph *A*. The mixture was dried by coevaporation with pyridine, the residue dissolved in pyridine (40 ml), the solution shaken with 2,4,6-triisopropylbenzenesulfonyl chloride (9 g), the whole kept at room temperature overnight, and processed analogously to paragraph *A*. After chromatography and elution with dilute aqueous ammonia there was obtained 0·12 mmol (1·5%) of the ammonium salt of compound *IV*, homogeneous on paper chromatography and electrophoresis.

Phosphorylation of Orotidine (*I*)

A. *With pyrophosphoryl chloride in acetonitrile.* A suspension of orotidine cyclohexylammonium salt (*I*; 1 mmol) in acetonitrile (70 ml) was treated at 0°C under stirring with 50 ml of 0·4M triethylammonium hydrogen carbonate (pH 7·5) and 10 ml of triethylamine. The mixture was

TABLE I
Paper Chromatography and Electrophoresis

Compound	R_F			E_{Up} [a]	
	S_1	S_2	S_3	E_1	E_2
Uridine	0·50	0·70	—	0	0
Uridine 3′-phosphate	0·12	0·30	0·20	1·00	1·00
I	0·34	0·58	—	0·60	1·12
II	0·65	—	—	0·58	—
III	0·20	0·42	—	1·15	1·07
IV	0·02	0·16	0·16	1·30	1·92
V	0·02	0·14	0·19	1·48	2·15
VI	0·58	—	—	0·65	—
VII	0·79	—	—	0·62	—
VIII	0·06	0·26	—	1·07	1·85

[a] Electrophoretical mobility referred to uridine 3′-phosphate.

evaporated to dryness under diminished pressure and the residue applied (in 20 ml of water) to a 80 × 4 cm column of DEAE-cellulose (Cellex D, standard capacity, HCO_3^-). The elution was performed with the use of a linear gradient of triethylammonium hydrogen carbonate, pH 7·5 (2 l of water in the mixing chamber, 2 l of 0·5M buffer solution in the reservoir) at the rate of 3 ml per min, the fractions being withdrawn in 10 min intervals. The ultraviolet absorption of eluates was measured continuously on a Uvicord apparatus. The fractions were evaporated, the residues coevaporated with methanol, and the yield determined spectrophotometrically at pH 2 for ε_{260}^{pH2} 9 600 (ref.[13]). The 0·40—0·42M fraction afforded 0·14 mmol (14%) of a mixture of compounds *IV* and *V* which was separated by chromatography on Whatman No 3 MM in the solvent system S_3. Yield, 42 μmol (4·2%) of the 5′-isomer *IV* and 85 μmol (8·5%) of compound *V*. The 0·42—0·50M fraction contained 0·47 mmol of the pure 2′(3′)-isomer *V* which was converted to the diammonium salt on a 20 ml column of ammonium Dowex 50 X 8 ion exchange resin. Overall yield of orotidine 2′(3′)-phosphate diammonium salt (*V*), 55·5%. The product was homogeneous on paper chromatography and electrophoresis, and identical with a specimen prepared by an independent route[5]. For N/P calculated: 4·0; found: 4·18.

B. *With phosphorus oxychloride in triethyl phosphate.* A suspension of orotidine cyclohexylammonium salt (*I*; 55 mg; 143 μmol) in triethyl phosphate (1 ml) was treated at 0°C under stirring with phosphorus oxychloride (50 μl; 84 mg; 550 μmol), the mixture stirred at 0°C for 3 hours, diluted with ether (100 ml), and extracted with three 25 ml portions of water. The aqueous phase was made alkaline (pH 9) with 10% aqueous lithium hydroxide, heated at 70°C for 15 min, and concentrated. The concentrate was chromatographed on one sheet of paper Whatman No 3 MM in the solvent system S_1. The band at the start line was eluted with dilute (1 : 100) aqueous ammonia (20 ml), the eluate concentrated, and the concentrate rechromatographed for 48 hours on 2 sheets of the above paper in the solvent system S_3. The main ultraviolet-absorbing band of product *IV* was eluted as above, the eluate evaporated to dryness, and traces of the borate buffer were removed by coevaporation with three 20 ml portions of methanol. The residual product *IV* (diammonium salt) was homogeneous on paper chromatography and electrophoresis, and identical with an authentic specimen; yield, 38%. The 2′(3′)-isomer *V* was isolated in 6% yield.

N^3-Methylorotidine 5′-Phosphate (*VIII*)

A mixture of N^3-methylorotidine ammonium salt[3] (*VI*; 2 mmol), dimethylformamide (6 ml), ethyl orthoformate (3 ml), and 6M hydrogen chloride in dimethylformamide (0·5 ml) was kept at room temperature for 2 days (as shown by chromatography in the solvent system S_1, the reaction was quantitative). Triethylamine (2 ml) was then added, the mixture filtered, the filtrate evaporated to dryness at 35°C/0·1 Torr, the residue coevaporated with 50% aqueous pyridine and then with four 20 ml portions of pyridine, and the final residue (compound *VII*) diluted with a pyridine solution (6 ml) of 2-cyanoethyl phosphate pyridinium salt (3 mmol). The resulting mixture was dried by coevaporation with six 20 ml portions of pyridine at 35°C/0·1 Torr and the final residue was disolved in pyridine (10 ml). N,N′-Dicyclohexylcarbodiimide (2·5 g; 12·5 mmol) was added to the solution and the mixture kept at room temperature for 5 days. Water (5 ml) and triethylamine (0·5 ml) was then added, the mixture kept for additional 1 hour, evaporated almost to dryness, the residue diluted with aqueous (1 : 1) ammonia (50 ml), and heated at 60°C for 2 hours. The mixture was then diluted with water (100 ml), washed with two 50 ml portions of ether, and the aqueous phase filtered through a layer of Celite. The filtrate was evaporated almost to dryness, the residue dissolved in 10% aqueous acetic acid (25 ml), the solution heated at 50°C for 1 hour, evaporated, the residue coevaporated with two 25 ml portions of water, the final residue dissolved in water (25 ml), the solution adjusted to pH 8 by the addition of aqueous ammonia, and processed as above on a column of DEAE-cellulose analogously to the preparation of com-

pound *IV* by phosphorylation of compound *II* with 2-cyanoethyl phosphate. The peak corresponding to compound *VIII* was chromatographed on Whatman No 3 MM in the solvent system S_1. Elution of the ultraviolet-absorbing band of compound *VIII* with dilute (1 : 100) aqueous ammonia (25 ml) afforded 18 μmol (0·9%) of the ammonium salt of compound *VIII*, homogeneous on paper chromatography and electrophoresis. From the main fraction after chromatography on DEAE-cellulose, there was recovered 1·7 mmol (85%) of the starting nucleoside derivative *VI*.

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