SYNTHESIS OF PHOSPHORAMIDATE ANALOGS OF RIBODINUCLEOSIDE PHOSPHATES*

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Cytidine 5'-phosphate (*Ia*) reacts with 1,1'-carbonyldiimidazole under the formation of 2',3'-O-carbonylcytidine 5'-phosphorimidazolidate (*IIa*) which affords (by the action of 3'-amino-3'-deoxyadenosine (*IIIa*)) 3'-deoxyadenosine-3'-amidophosphoryl-(3'→5')-cytidine (*IVa*) and (by the action of 3'-amino'-3-deoxy-N^6-dimethyladenosine (*IIIc*)) 3'-deoxy-N^6-dimethyladenosine-3'-amidophosphoryl-(3'→5')-cytidine (*IVc*). Starting from adenosine 5'-phosphate (*Ib*) and 3'-amino-3'-deoxycytidine (*IIIb*) or the substance *IIIc*, 3'-deoxycytidine-3'-amidophosphoryl-(3'→5')-adenosine (*IVb*) or 3'-deoxy-N^6-dimethyladenosine-3'-amidophosphoryl-(3'→5')-adenosine (*IVd*) were prepared. 6-Azauridine (*V*) was transformed, by the action of triphenylphosphine, lithium azide and carbon tetrabromide, followed by the action of triphenylphosphine and ammonia, to 5'-amino-5'-deoxy-6-azauridine (*VI*). The substance *VI* was transformed by the action of 2',3'-O-carbonyl-adenosine 5'-phosphorimidazolidate (*IIb*), to adenylyl-(5'→5')-5'-amino-5'-deoxy-6-azauridine (*VII*). The compound *IVb* is not degraded by snake venom and spleen phosphodiesterases and is degraded by pancreatic ribonuclease to adenosine and the compound *IIIb*. The compound *VII* is degraded by snake venom phosphodiesterase to adenosine and the compound *VI*.

The utility of ribooligonucleotides for biochemical studies is seriously hampered by their susceptibility to enzymatic degradation. This disadvantage may be overcome by using analogs possessing some degree of resistance towards nucleolytic enzymes. Such features were found within oligonucleotide analogs bearing phosphoramidate internucleotidic bonds[1,2]. To date there were described synthetic approaches to oligonucleotides bearing $O_{3'}$—$N_{5'}$ internucleotidic phosphoamidate bond exclusively in deoxyribo-series. The phosphoramidate bond was created by use of phosphochloridates[1,2], by the activation of phosphoryl group by triphenylphosphine–dipyridyl disulfide couple[3] or by the reaction of phosphite with azido group[4].

In pursuing the problems of ribooligonucleotide synthesis we started to explore the possibilities of the creation of phosphoramidate internucleotidic bond in *ribo-*

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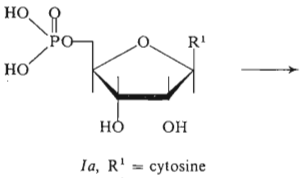
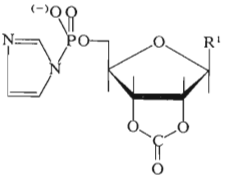
-series. At first we focussed our interest on compounds which would contain $N_{3'}—O_{5'}$ internucleotidic bond. For the synthesis of this type of linkage we examined the transamidation reaction of ribonucleoside 5'-phosphorimidazolidates with 3'-amino--3'-deoxynucleosides. This approach promised to eliminate the necessity of protecting groups. The preparation of phosphorimidazolidates from phosphoric acid monoesters and 1,1'-carbonyldiimidazole was extensively studied by Cramer and coworkers who discovered that phosphorimidazolidates reacted with amines under the formation of phosphoroamidates[5-8].

The reaction of nucleoside 5'-phosphates *Ia, b* with excess of 1,1'-carbonyldiimidazole in dimethylformamide at 80–100°C was finished within few minutes. The resulting solution of 2',3'-O-carbonyl-nucleoside 5'-phosphorimidazolidates[9-11] *IIa, b* was treated with water to destroy the reagent. Two equivalents of 3'-amino -3'-deoxynucleoside (*IIIa–c*) were then added and the transamidation reaction

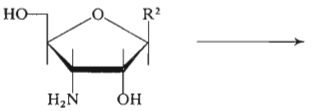


Ia, R^1 = cytosine
Ib, R^1 = adenine

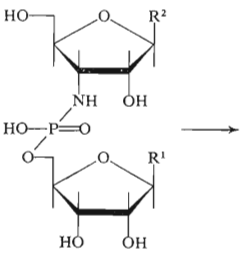


IIa, R^1 = cytosine
IIb, R^1 = adenine

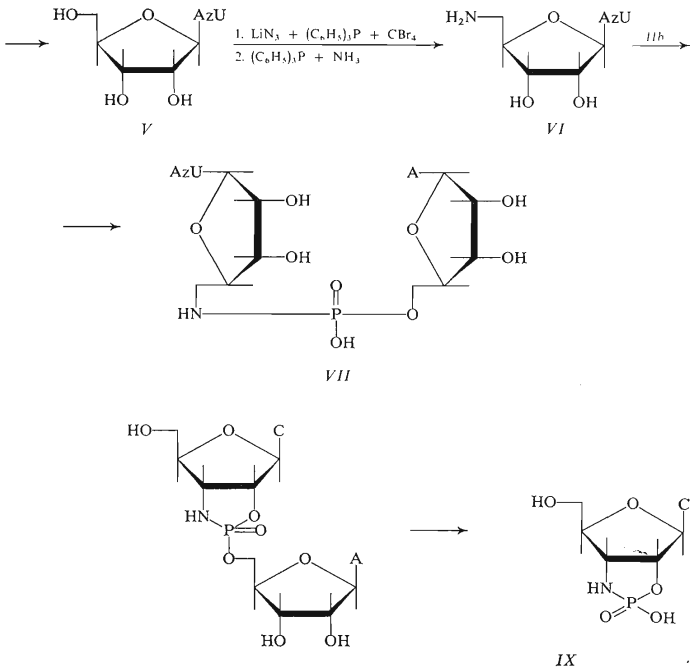
II +



IIIa, R^2 = adenine
IIIb, R^2 = cytosine
IIIc, R^2 = N^6-dimethyladenine



IVa, R^1 = cytosine, R^2 = adenine
IVb, R^1 = adenine, R^2 = cytosine
IVc, R^1 = cytosine, R^2 = N^6-dimethyladenine
IVd, R^1 = adenine, R^2 = N^6-dimethyladenine



carried out at 60°C. The resulting mixture was separated by ion exchange chromatography on DEAE-cellulose column. The 2',3'-cyclic carbonates were splitted down during the transamidation and isolation. The products *IVa–d* were isolated as triethylammonium salts in 55–86% yields.

For the comparison in stability studies, an N_5—$O_{5'}$ analog, adenylyl-(5' → 5')--5'-amino-5'-deoxy-6-azauridine (*VII*) was prepared from 5'-amino-5'-deoxy-6-azauridine (*VI*). Aminonucleoside *VI* was synthetized from 6-azauridine (*V*) which was converted by the reaction with triphenylphosphine–lithium azide–tetrabromomethane mixture, according to Hata[12], to 5'-azido derivative, and then, by reaction with

triphenylphosphine and ammonia to *VI*. Degradation of compound *VII* by 80% aqueous acetic acid at 100°C proceeded exclusively on P—N bond giving a mixture of adenosine 5'-phosphate and aminonucleoside *VI*. Snake venom diesterase degradation, proceeding on P—O bond, afforded a mixture of adenosine and aminonucleoside *VI*, resulting from nonenzymic hydrolysis of enzymatically formed 5'-deoxy-6-azauridine 5'-amidophosphate (according to ref.[1]).

On the other hand, the degradation of the diribonucleoside phosphate analog, bearing $N_{3'}$—$O_{5'}$ internucleotidic bond, showed more complex nature, resulting from the presence of vicinal $C_{(2')}$ hydroxyl function. In this study we explored the degradation of CpA analog *IVb*, where a possibility existed for pancreatic ribonuclease action. 80% Aqueous acetic acid at 100°C gave, along with the expected products of P—N bond cleavage (adenosine 5'-phosphate and aminonucleoside *IIIb*), 20% of material which was formed by cleavage of P—O bond, leading to adenosine and a cytidine nucleotide. Chromatographic and electrophoretic properties of this nucleotide suggested the structure of 3'-amino-3'-deoxycytidine 2'-phosphate. The cleavage of P—O bond could be explained by intermediary formation of cyclic phosphoramidate diester *VIII*. Longer half-time of *VIII* might be expected in more dilute acetic acid. Heating of *IVb* in 20% aqueous acetic acid gave approximately 40% of P—O cleavage products. Along with adenosine, a cytidine nucleotide of low electrophoretic mobility (E_{Up} 0·23) was observed, possibly 3'-amino-3'-deoxycytidine ($N_{3'}$—$O_{2'}$) cyclic phosphoramidate (*IX*). It is interesting that the product of P—N cleavage of intermediate *VIII*, 3'-amino-3'-deoxycytidylyl-(2' → 5')-adenosine, was not observed. The same intermediate, the substance *VIII*, may be assumed for the degradation of *IVb* by pancreatic ribonuclease. After short incubation period, the substance *VIII* disappeared under the formation of adenosine and two electrophoretically distinct cytidine nucleotides. One of them was identical with cytidine nucleotide formed by 20% acetic acid (*IX*), the second with higher electrophoretic mobility is probably 3'-deoxycytidine 3'-amidophosphate. Prolonged incubation gave equimolar amounts of adenosine and aminonucleoside *IIIb* resulting from non-enzymatical cleavage of amidophosphate. Snake venom and spleen diesterases did not affect the substance *IVb* under the conditions used. The susceptibility of the CpA analog *IVb* to pancreatic ribonuclease diminished the chance for use of $N_{3'}$—$O_{5'}$ phosphoramidate analogs in ribonucleases containing systems. The $O_{3'}$—$N_{5'}$ analogs of ribooligonucleotides might be more promissing.

EXPERIMENTAL

TLC was performed on ready-for-use Silufol UV 254 (Kavalier Glasworks, Votice, Czechoslovakia) silica gel sheets in the solvent systems S_1, 2-propanol–conc. ammonia–water (7 : 1 : 2), S_2, chloroform–methanol (85 : 15). Electrophoresis was performed on paper Whatman No 1 dipped in carbon tetrachloride, in 0·05M triethylammonium hydrogen carbonate buffer pH 7·5. Column chromatography was performed on macroporous silica gel (produced by Service Labora-

tories of this Institute). UV spectra were recorded on the apparatus Specord UV VIS (Carl Zeiss, Jena).

3′-Amino-3′-deoxy-N^6-dimethyladenosine (*IIIc*) was prepared from puromycine[13]. 3′-Amino--3′-deoxyadenosine (*IIIa*) and 3′-amino-3′-deoxycytidine (*IIIb*) were prepared according[14]. Pancreatic ribonuclease (product of Calbiochem, San Diego, USA) was dissolved in water (5 mg/ml). Snake venom diesterase (glycerol solution) and spleen diesterase (suspension) were products of Boehringer, Mannheim, Germany.

Phosphoramidate Analogs of Diribonucleoside Phosphates

1′1′-Carbonyldiimidazole (0·8 mmol) is added to a suspension of ribonucleoside 5′-phosphate (0·1 mmol) in dimethylformamide (2 ml), the mixture is heated to 80—100°C for 5 min, and cooled to 20°C. Water (0·1 ml) and, after 5 min, 3′ amino-3′-deoxynucleoside (0·2 mmol) are added, and the mixture heated to 60°C for 4 h. The mixture is diluted with water (10 ml) and applied to a column (4 × 18 cm) of DEAE-cellulose (HCO_3^-). The column is eluted with the use of linear gradient (2 l of water, 2 l of 0·1M triethylammonium hydrogen carbonate). The UV-absorbing peak (eluted at 0·035M for compounds *IVa—d* and at 0·1M for compound *VII*) is evaporated, the residue coevaporated with three 30 ml portions of ethanol, dissolved in water (5 ml) and lyophilized. For yield, spectra, chromatographic and electrophoretic properties see Table I.

Degradation of *IVb* by Aqueous Acetic Acid

Solutions of *IVb* (2 mg) in 80% and 20% aqueous acetic acid (50 µl) were heated for 10 min and 60 min, respectively. Samples were checked by TLC in S_1, followed by ninhydrin spray, and by electrophoresis. Electrophoretic spots were eluted by 0·05M hydrochloric acid and UV spectra of eluates recorded. 80% Acetic acid gave 80% equimolar amounts of adenosine 5′-phosphate and nucleoside *IIIb*. One fifth of material was transformed to adenosine and a cytidine nucleotide ($R_F(S_1)$ 0·18, E_{Up} 0·70). 20% Acetic acid gave a mixture of adenosine 5′-phosphate, nucleoside *IIIb*, adenosine and a cytidine nucleotide (R_F (S_1) 0·50, E_{Up} 0·23),

TABLE I

Yields and Properties of Phosphoamidate Analogs

Compound	Yield %	$R_F(S_1)$	E_{Up}	UV spectrum (water)	
				λ_{max}, nm	λ_{min}, nm
IVa	40	0·41	0·35	262	232
IVb	64	0·37	0·41	262	232
IVc	86	0·38	0·38	272	242
IVd	77	0·45	0·31	261, 283 sh	232
VII	55	0·45	0·65	260	229

Enzymatic Degradation of *IVb*

Samples of *IVb* (1 mg) were incubated at 37°C in *a*) 0·1M Tris-HCl buffer pH 8 (50 µl) with solution of pancreatic ribonuclease (10 µl), *b*) the same buffer (50 µl) with glycerol solution of snake venom diesterase (10 µl), *c*) 0·1M ammonium acetate buffer pH 6·5 (50 µl) with spleen diesterase suspension (10 µl). The resulting solutions were checked by the same methods as degradations by acetic acid. Pancreatic ribonuclease afforded after 1 h incubation a mixture of adenosine and two cytidine nucleotides (ratio 1 : 0·6 : 0·4). Chromatographic and electrophoretic properties of these nucleotides indicated structures of 3'-deoxycytidine 3'-amidophosphate (R_F (S_1) 0·20, E_{Up} 0·50) and 3'-amino-3'-deoxycytidine ($N_{3'}$—$O_{2'}$)-cyclic phosphoramidate (R_F (S_1) 0·53, E_{Up} 0·23). After 16 h incubation a mixture of adenosine and aminonucleoside *IIIb* (ratio 1 : 1) was obtained. Phosphodiesterases (snake venom and spleen) did not cause any detectable degradation of *IVb*.

Degradation of *VII* by 80% Aqueous Acetic Acid and Snake Venom Diesterase

Experiments were carried out analogously as with *IVb*. 80% aqueous acetic acid (100°C, 20') gave equimolar amounts of adenosine 5'-phosphate and *VI*. Snake venom diesterase afforded adenosine and *VI*.

5'-Amino-5'-deoxy-6-azauridine (*VI*)

A suspension of 6-azauridine (2·45 g) and lithium azide (1 g) in toluene (10 ml) is evaporated. To the residue, dimethylformamide (50 ml) and triphenylphosphine (2·68 g) are added, the stirred mixture is cooled in ice bath, and tetrabromomethane (3·38 g) is added. After 30 min the ice bath is removed and stirring is continued 20 h. The solvent is evaporated (55°C, 15 Toorr), the sirupy residue is extracted with two 50 ml portions of ether, dissolved in a mixture of chloroform and methanol (1 : 1; 50 ml) and applied to a column (4·5 × 45 cm) of silica gel. The column is eluted with methanol–chloroform (1 : 5). Fractions containing 5'-azido-5'-deoxy-6-azauridine ($R_F - S_2$ 0·95) are evaporated and the residue is dissolved in pyridine (20 ml). Triphenylphosphine (3·14 g) is added and, after 20 h, the mixture is diluted with conc. ammonia (10 ml). After 5 h, the solution is evaporated and the residue is triturated with ether (150 ml). The crude product *VI* is dissolved in 0·2M hydrochloric acid (50 ml) and applied to a column (50 ml) of Dowex-50 (H^+). The column is eluted with water till neutrality and the aminonucleoside is eluted with 5M aqueous ammonia. The eluate is evaporated, the residue dissolved in water (5 ml) and ethanol (50 ml) is added. After 5 days at 0°C, the crystallized substance is collected, washed with ethanol and ether and dried under diminished pressure. Yield 540 mg of *VI*, $R_F - S_1$ 0·32, E_{Up} 0·28. UV spectrum (pH 1) λ_{max} 263 nm, λ_{min} 235 nm. For $C_8H_{12}N_4O_5$ (244·2) calculated: 39·35% C, 4·49% H, 22·94% N; found: 38·96% C, 4·65% H, 22·63% N.

Analysis was performed in the Analytical Department (Dr J. Horáček, Head) of this Institute.

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