PREPARATION OF DERIVATIVES OF INOSINE 5'-PHOSPHOROTHIOATE FOR USE IN AFFINITY CHROMATOGRAPHY

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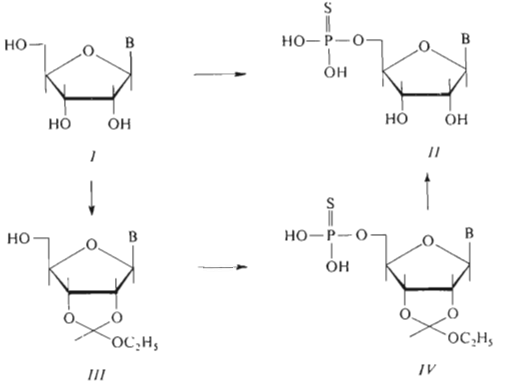
In the paper the known methods of preparation of ribonucleoside 5'-phosphorothioates *II* are evaluated and an improved method of their synthesis is described, based on the reaction of 2',3'-O-ethoxymethyleneribonucleosides *III* with thiophosphoryl chloride in the presence of one equivalent of pyridine, and subsequent hydrolysis. Inosine 5'-phophate and inosine 5'-phosphorothioate (*IIc*) were converted to 2',3'-O-cyclic ketals of levulinic acid *Vb*, *VIb* on reaction with ethyl levulinate and ethyl orthoformate and subsequent alkaline hydrolysis. O-(4-Aminophenyl)inosine 5'-phosphorothioate (*IX*) was prepared from 2',3'-O-isopropylideneinosine (*VII*) with O-(4-nitrophenyl)thiophosphoryldiimidazolide and subsequent catalytic hydrogenation of the O-(4-nitrophenyl) ester *VIII* formed.

In connection with the study of affinity chromatography of guanylspecific ribonuclease from *Streptomyces aureofaciens* we have described in our preceding communication[1] the preparation of derivatives of inosine 2'(3')-phosphate and inosine 2'(3')-phosphorothioate containing a group capable of bonding to an affinity support. In view of the known fact that even 5'-nucleotides[2,3] and 5'-phosphorothioates of nucleosides, or their esters[4,5], can be effective inhibitors or suitable ligands for affinity chromatography of ribonucleases, we also investigated the preparation of derivatives of inosine 5'-phosphate, inosine 5'-phosphorothioate and aromatic esters of inosine 5'-phosphorothioate which have a group capable of bonding to affinity supports in the positions 2' and 3' of the sugar residue.

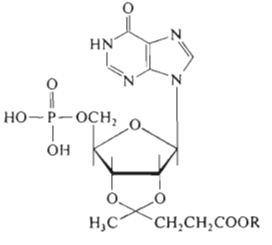
For this purpose 2',3'-cyclic ketals of levulinic acid (4-oxopentanoic) seem most suitable, because they can be introduced into the nucleoside molecule easily on reaction of free ribonucleoside with the ester of levulinic acid in the presence of ethyl orthoformate[6,7] and subsequent alkaline hydrolysis of the ester function. These derivatives are sufficiently stable even at lower pH values. Their phosphorylation to corresponding nucleotides[7] is also described in the literature. Since the corresponding thiophosphorylation takes place less easily[1] we considered it more suitable to carry out the conversion to ketals of levulinic acid from the inosine 5'-phosphate or 5'-phosphorothioate prepared beforehand.

A number of methods has been described in the literature for the preparation of nucleoside 5'-phosphorothioates from nucleosides, among which the most important reactions are those with the tributylammonium salt of monothiophosphoric acid[8], thiophosphoryl chloride in triethyl phosphate[9,10], triimidazolide of thiophosphoric acid[11], and finally sulfuration of silyl derivatives of nucleoside 5-'phosphites[12]. Since we needed, in connection with this study, to prepare also pure inosine 5'-phosphorothioate and derivatives of other 5'-phosphorothioates *II* for studies of enzymatic inhibition and the stability of the P=S bond under conditions of binding

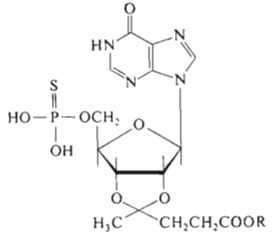


I *II* *III* *IV*

In formulae *I*—*IV*, *a* B = uracil-1-yl, *b* B = adenin-9-yl, *c* B = hypoxanthin-9-yl residue



Va R = C_2H_5
Vb R = H



VIa R = C_2H_5
VIb R = H

to the affinity support, we compared – similarly as in the preceding paper[1] – individual preparation methods from the point of view of the yield, possibility of isolation and purity of the product (content of desulfurated 5'-ribonucleotide).

The reaction of free ribonucleosides *I* with tri-n-butylammonium salt of thiophosphoric acid affords a mixture of isomeric phosphorothioates with prevailing 5'-isomer[8]; therefore we applied this reaction to 2',3'-O-isopropylideneinosine (*VII*) and afterwards cleaved the protecting groups by boiling with acetic acid. The disadvantage of this method is the high content of salts, requiring repeated fractional precipitation of the salts of the nucleotide fraction. When lithium salts were used, almost pure product *IIc* could be obtained, which gave a positive reaction for the P=S bond and which did not contain too much inosine-5'-phosphate as a contaminant. Nonetheless, the yields of the product obtained by this method are too low.

Thiophosphorylation of ribonucleosides *I* with thiophosphoryl chloride in triethyl phosphate[9,10] should afford the 5'-isomer *II* almost exclusively. In comparison with phosphorus oxychloride[13] this reaction takes place much more slowly, so that an excess of the reagent must be used and the reaction time prolonged. However, by this both the risk of contamination with further isomers and the danger of desulfuration are increased. Analytical experiments, the results of which are summarized in Table I, document both the low conversion of nucleosides and the proportion of 5'-nucleotide in the reaction mixture, which increases with time.

Therefore we selected as an alternative the reaction of 2',3'-O-ethoxymethylene derivative of ribonucleosides *III* with thiophosphoryl chloride (in the presence of an equimolar amount of pyridine necessary for binding hydrogen chloride formed). After elimination of the protecting group in weakly acid medium and chromatographic separation of the mixture, 5'-phosphorothioates of uridine and adenosine (*IIa*, *IIc*) were obtained in not too high yields, but sufficiently pure (Scheme 1).

According to our experience, the described[11] thiophosphorylation of protected ribonucleosides *III* with triimidazolide of thiophosphoric acid does indeed take place with a high conversion degree, giving a product of type *IV*. Even in this case, however, the reaction mixture contains a high amount of salts which impair isolation on a larger scale. Here we attempted to apply desalting on active charcoal; however, similarly as in 2'(3')-phosphorothioates[1] quantitative desulfuration of product *II* to the corresponding 5'-ribonucleotide took place in this process. Control experiments showed that desulfuration does not take place either under the effect of the low pH during adsorption, or the effect of the elution medium of the desorption. Hence, it seems that the surface of the sorbent is the desulfuration agent.

For a preparative synthesis of 5'-phosphorothioates *II* either thiophosphorylation reactions with thiophosphoryl chloride in the presence of an equivalent of base, or the sulfuration of nucleoside 5'-phosphites are most suitable. Both these methods require the preparation of a protected nucleoside (see[14]), the second of them also the isolation of 5'-phosphite, which, however, can be prepared easily[14]. Their advantages

are the purity of product *II* and lesser difficulties during the isolation than in other methods.

The conversion of inosine 5'-phosphate and inosine 5'-phosphorothioate (*IIc*) to cyclic ketals of levulinic acid takes place extremely slowly. In contrast to the reaction of free ribonucleosides[6,7] the formation of the ketal with the ester of levulinic acid takes several days, but even then it is not quite complete. In spite of this the products *Va* and *VIa* were isolated from the reaction mixture (blocking of the hydroxyl groups lipophilizes the molecule to such and extent that a separation on silica gel can be made use of) and converted on alkaline hydrolysis to the final products *Vb*, *VIb*. In the case of 5'-phosphorothioate *VIb* a contamination with compound *Vb* was not observed. The products are chromatographically homogeneous and their electrophoretic behaviour corresponds to the assumed structure (the presence of two dissociable functions in the molecule).

TABLE I

Time-Course of the Reaction of Uridine with Thiophosphoryl Chloride

$PSCl_3$[a] / Urd	Uridine 5'-Phosphorothioate, %[b]				
	2·5 h	5·0 h	7·0 h	24 h	92 h
6·0	7·7	10·1	11·2	19·4	—
	—	(4·0)	(4·8)	(10·0)	
10·0	—	—	—	35·6	60·9
				(8·0)	(14·5)

[a] Ratio of reactants; [b] uridine 5'-phosphate, % in parentheses.

TABLE II

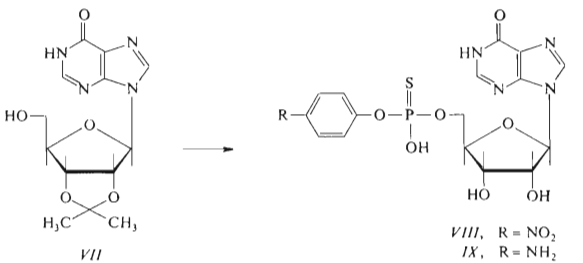
Paper Chromatography and Electrophoresis

Compound	R_F[a]	E_{Up}[b]	Compound	R_F[a]	E_{Up}[b]
Ia	0·50	—	*Va*	0·50	0·71
Ib	0·57	—	*Vb*	0·07	1·06
Ic	0·40	0·08	*VIa*	0·35	0·76
IIa	0·20	0·95	*VIb*	0·10	1·18
IIb	0·23	0·84	*VIII*	0·54	0·48
IIc	0·10	1·00	*IX*	0·29	0·40

[a] In S1; [b] referred to uridine 3'-phosphate.

In view of the planned bonding of compounds *Vb* and *VIb* to an affinity support mediated by the carboxyl function of the aliphatic residue, the stability of the P=S bond in compound *IIc* in the presence of carboxyl-activating reagents was also studied in this connection. The P=S bond is stable for 3 days at pH 8·0, but partial desulfuration takes place in the pH range from 1 to 5 in aqueous solutions at 37°C. In the presence of the water-soluble carbodiimide and at pH 6 complete desulfuration takes place within a few minutes, similarly as in the case of 2′(3′)-phosphorothioates. In contrast to this the formation of mixed anhydride with ethyl chloroformate in the presence of tri-n-butylamine in aqueous medium does not cause desulfuration (after evaporation of the mixture and mild alkaline hydrolysis unchanged *IIc* could be recovered).

O-(4-Aminophenyl)inosine 5′-phosphorothioate (*IX*) was prepared by the reaction sequence shown in Scheme 2: 2′,3′-O-isopropylideneinosine (*VII*) afforded on reaction with thiophosphoric acid O-(4-nitrophenyl)ester diimidazolide (prepared by *in situ* reaction of the dichlorothiophosphoric acid 4-nitrophenyl ester with imidazole) and subsequent hydrolysis O-(4-nitrophenyl)inosine 5′-phosphorothioate (*VIII*). Hydrogenation of this compound on palladium catalyst gave 4-aminophenyl ester *IX*, chromatographically homogeneous, giving a positive reaction both for the P=S bond and also with ninhydrin.



VII

VIII, R = NO_2
IX, R = NH_2

SCHEME 2

The compounds prepared in this study were bound to affinity supports and compared with the materials obtained from corresponding derivatives of inosine 2′(3′)-phosphates and 2′(3′)-phosphorothioates. The results of this study are published elsewhere[15].

EXPERIMENTAL

Unless stated otherwise the solutions were evaporated at 40°C/2 kPa and the compounds dried at 13 Pa. Paper chromatography was carried out on paper Whatman No 3 MM in 2-propanol–conc. aq. ammonia–water (7 : 1 : 2), paper electrophoresis on the same paper at 20 V/cm in 0·1M triethylammonium hydrogen carbonate at pH 7·5. The separation on DEAE cellulose (80 × 4 cm, Cellex D) was carried out at elution rate of 3 ml per min, the elution course was monitored with a Uvicord instrument (LKB, Sweden). The UV spectra were measured in aqueous solutions on a Specord UV–VIS spectrophotometer, and quantitative determinations on a Beckmann DU apparatus.

Inosine 5'-Phosphorothioate (*IIc*)

2',3'-O-Isopropylideneinosine (*VII*) (see[16]) was codistilled with pyridine at 40°C/13 Pa, dissolved in 10 ml of dimethylformamide and mixed with 20 ml of 1M solution of freshly prepared tri-n-butylammonium salt of thiophosphoric acid[17]. The mixture was concentrated at 40°C/13 Pa, heated at 70°C and under exclusion of moisture for 12 h, refluxed with 100 ml of 80% acetic acid for 1 h, evaporated in a vacuum, codistilled 3 times with water (50 ml each time), dissolved in 50 ml of water, alkalized with triethylamine and put on a column of DEAE cellulose (see above). The column was washed with water until UV-absorption decreased and then a linear gradient of 0—0·2M triethylammonium bicarbonate of pH 7·5 (2 l each) was applied. The fraction of the product was evaporated *in vacuo*, the residue codistilled three times with ethanol (50 ml portions), dissolved in a minimum amount of water and introduced onto a column of 100 ml of Dowex 50X8 (Li^+-form). The neutral aqueous eluate absorbing in UV light was evaporated in a vacuum, codistilled with three 50 ml portions of ethanol and precipitated from methanol (10 ml) with ether (100 ml). The product was filtered off, washed with ether and dried in a vacuum. Yield of the lithium salt of compound *IIc*, 40%, content (determined spectrophotometrically): 80%. Repeated precipitation increased the content to 90%. The product was chromatographically and electrophoretically homogeneous, the reaction to P=S was positive. According to high-performance liquid chromatography it does not contain inosine 5'-phosphate.

Phosphorylation of Uridine (*Ia*) with Thiophosphoryl Chloride in Triethyl Phosphate

Thiophosphoryl chloride[18] (0·3 ml, *i.e.* 3 mmol, or 0·5 ml, *i.e.* 5 mmol) was added to a suspension of 0·5 mmol of uridine (*Ia*) in 2·5 ml of triethyl phosphate and the mixture was stirred at room temperature. Samples of the mixture (0·1 ml) were pipetted into 1 ml of 1M triethylammonium hydrogen carbonate (pH 7·5) and aliquots were analysed by paper electrophoresis and paper chromatography in system S1. The results are shown in Table I.

Uridine 5'-Phosphorothioate (*IIa*)

A mixture of 1·22 g (5 mmol) of uridine, 10 ml of ethyl orthoformate, 10 ml of dimethylformamide and 1 ml of 6M hydrogen chloride in dimethylformamide was stirred until complete dissolution and then allowed to stand at room temperature overnight. After alkalization with triethylamine the mixture was filtered under suction and the filtrate evaporated at 40°C/13 Pa. The residue was allowed to stand with 20 ml of 50% aqueous ethanol for 1 h, evaporated again in a vacuum and codistilled 3 times with 50 ml portions of pyridine in a vacuum. The residue was dissolved in 35 ml of dioxane, pyridine (1·2 ml, 15 mmol) and thiophosphoryl chloride (4·1 ml, 15 mmol) were added consecutively, and the mixture was allowed to stand at room temperature overnight.

After filtration through Celite the filtrate was evaporated *in vacuo*. The residue was heated with 25 ml of 50% acetic acid at 50°C, for 1 h evaporated at 40°C/13 Pa, and the residue codistilled with three 30 ml portions of ethanol. The residue was dissolved in 50 ml of water and alkalized with ammonia and worked up as above (for *IIc*). Yield, 0·38 g (21·6%) of lithium salt of compound *IIa*, content (spectrophotometrically): 92%. The product was chromatographically and electrophoretically homogeneous.

Adenosine 5'-Phosphorothioate (*IIb*)

This was prepared in the same manner as compound *IIa* from 3 mmol of adenosine. Yield of the lithium salt of *IIb*, 0·225 g (20·0%), content, 94%. The product was chromatographically and electrophoretically homogeneous.

Reaction of 2',3'-O-Ethoxymethylene Derivatives of Ribonucleosides *III* with Thiophosphoric Acid Triimidazolide

Imidazole (6·1 g, 90 mmol) was added to a solution of 2·54 g (15 mmol) of thiophosphoryl-chloride in 50 ml of acetonitrile and the mixture was stirred at room temperature for 1 h. 2',3'-O-Ethoxymethyleneuridine (10 mmol) (*IIIa*, prepared according to the procedure mentioned above under *IIa*) in 50 ml of acetonitrile was then added and the mixture stirred at room temperature for 3 days. After filtration under suction the material on the filter was washed with acetonitrile and the filtrate evaporated in a vacuum. The residue was heated in 50 ml of 50% acetic acid at 50°C for 1 h, evaporated at 40°C/13 Pa and the residue analysed by paper chromatography and electrophoresis. The mixture contained 70% of compound *IIa*. The residue was dissolved in 50 ml of water and acidified to pH 4·0 with hydrochloric acid, then treated gradually with charcoal (washed with hydrochloric acid and water) until A_{260} dropped to 1·0. The mixture was centrifuged, washed with three 50 ml portions of water and suspended in 50 ml of a mixture of methanol, conc. ammonia and water (3 : 1 : 6). After 30 min stirring the mixture was centrifuged, washed three times with 25 ml of the same mixture and the supernatants were evaporated. According to chromatographic analysis in S1 and HPLC the mixture contained only uridine and uridine 5'-phosphate.

The reaction with 2',3'-O-ethoxymethylene-N^6-dimethylaminomethyleneadenosine (see[19]) was carried out in a similar manner. The conversion to compound *IIb* was 53% after 3 days. After desalting on active charcoal the mixture contained only adenosine and adenosine 5'-phosphate.

Stability of Compound *IIc*

A 10^{-3} M solution of compound *IIc* in 0·1M-TRIS buffer of pH 1—8 (in units of pH) was incubated at 37°C and aliquots of the mixtures were analysed by high-performance liquid chromatography.

A solution of 0·1 mmol of compound *IIc* in 6 ml of water was stirred at room temperature and 190 mg (0·5 mmol) of *p*-toluenesulfonate of N-cyclohexyl-N'-(3-trimethylammoniumpropyl)-carbodiimide[20] were added to it and pH was kept at 6·0 with 0·1M hydrochloric acid in a pH-stat. According to electrophoresis quantitative desulfuration of compound *IIc* can be observed already after 1 h on the basis of the detection for the P=S bond.

A solution of 0·5 mmol of compound *IIc* (lithium salt) in 3 ml of water was stirred with 0·87 ml of tri-n-butylamine and 0·25 ml of ethyl chloroformate for 1 h. The mixture was evaporated *in vacuo*, the residue dissolved in 0·1M-TRIS buffer of pH 8·5 and the sample analysed by paper chromatography. The mixture contained compound *IIc* exclusively.

2',3'-O-2-(2-Carboxyethyl)ethylideneinosine 5'-Phosphate (*Vb*)

A mixture of 100 mg (0·21 mmol) of sodium salt of inosine 5'-phosphate, 1 ml of dimethylformamide, 0·1 ml of ethyl 4-oxopentanoate, 0·1 ml of ethyl orthoformate and 0·1 ml of 6M hydrogen chloride in dimethylformamide was stirred at room temperature for two days. The mixture was alkalized with triethylamine, evaporated at 40°C/13 Pa and the residue chromatographed on a plate with silica gel (40 × 16 × 0·3 cm) in methanol–chloroform (1 : 1) and the zone of the product was eluted with a mixture of 500 ml of methanol and 200 ml of 50% methanol. The residue of compound *Va* was dissolved in 2 ml of 0·5M sodium hydroxide and allowed to stand at room temperature overnight, then neutralized with Dowex 50 X 8 (H^+-form) to pH 7·2, filtered under suction, washed with water and the filtrate evaporated; the residue was dissolved in 2 ml of methanol and precipitated with 50 ml of ether. The yield of the chromatographically and electrophoretically homogeneous trisodium salt of compound *Vb* was 54 mg (50%), the content, determined spectrophotometrically was 88%.

2',3'-O-2-(2-Carboxyethyl)ethylideneinosine 5'-Phosphorothioate (*VIb*)

Ethyl 4-oxopentanoate (0·9 ml; 6 mmol), ethyl orthoformate (1 ml) and 0·5 ml of 6M hydrogen chloride in dimethylformamide were added to a suspension of 0·378 g (1 mmol) of dilithium salt of inosine 5'-phophorothioate (*IIc*) in 5 ml dimethylformamide. The clear mixture was allowed to stand at room temperature for two days, then alkalized with triethylamine, evaporated at 40°C/13 Pa and the residue chromatographed on a silica gel plate (see compound *Vb*) in chloroform–methanol (4 : 1). The band of the product was eluted with methanol (500 ml), evaporated and the residue of compound *VIa* was allowed to stand in a mixture of 10 ml of ethanol and 10 ml of 0·5M sodium hydroxide for one hour, when the reaction was complete according to electrophoresis. The mixture was neutralized with Dowex 50X8 (H^+-form) to pH 7·2, evaporated *in vacuo* and the residue precipitated from methanol (2 ml) with ether (50 ml). The yield of the chromatographically and electrophoretically homogeneous trisodium salt of compound *VIb* was 10%, the content (spectrophotometrically determined) was 93%. The product contained a P=S bond.

O-(3-Aminophenyl)inosine 5'-Phosphorothioate (*IX*)

Imidazole (3·0 g; 44·1 mmol) was added to a solution of 2·8 g (10·3 mmol) of 4-nitrophenylthiophosphoryl dichloride[21] in 70 ml of acetonitrile and the mixture stirred at room temperature for 20 min. 2',3'-O-Isopropylideneinosine (3·1 g; 10 mmol) in 30 ml of acetonitrile was then added and the mixture stirred at room temperature for 2 days. The mixture was filtered, the filter washed with 20 ml of acetonitrile and the filtrate evaporated in a vacuum. The residue was refluxed with 100 ml of 80% acetic acid, evaporated in a vacuum, codistilled with three 50 ml portions of water, diluted with 300 ml of water, alkalized with triethylamine to pH 9, extracted twice with 50 ml of ether and the aqueous phase concentrated under reduced pressure to 50 ml. This solution was applied onto a column of DEAE cellulose (see above), this was washed with water until the UV absorption decreased and the product was eluted with a linear gradient of buffer (0—0·2M triethylammonium hydrogen carbonate of pH 7·5, 2 l of each). The fraction of the product was evaporated *in vacuo* and the residue purified on 2 silica gel plates (see above) in chloroform–methanol (4 : 1). The zones with the product were eluted with methanol (300 ml) and the eluate evaporated in a vacuum. The residue (*VIII*) was then dissolved in 100 ml of water, 1 g of 10% palladium on charcoal and 1 ml of 28% palladium chloride were added, and the mixture was hydrogenated at normal pressure until the consumption of hydrogen ceased (160 ml). The

suspension was filtered through Celite and the neutral filtrate was evaporated *in vacuo*. The residue was converted to lithium salt (see *IIa*) and precipitated from methanol (2 ml) with ether (100 ml). The yield of the chromatographically and electrophoretically homogeneous lithium salt of compound *IX* was 820 mg (50%). The product reacted positively with the reagent for the P=S bond and with ninhydrin.

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