SYNTHESIS OF 2′(3′)-PHOSPHATES AND 2′(3′)-PHOSPHOROTHIOATES OF 5′-O-CARBOXYMETHYLINOSINE AND RELATED COMPOUNDS

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Received February 14th, 1980

5′-O-Carboxymethylinosine (*IX*) was prepared from disodium salt of 2′,3′-O-isopropylideneinosine (*VIII*) by treatment with sodium chloroacetate, followed by acid hydrolysis. 5′-O-(2-Aminoethylamidocarbonylmethyl)inosine (*XIII*) was obtained by reaction of *IX* with *p*-nitrophenol and subsequent treatment with ethylenediamine. Action of triethyl phosphite on the compounds *IX* and *XIII* afforded the corresponding 5′-O-substituted 2′(3′)-phosphites *X* and *XIV* which on reaction with trimethylsilyl chloride and sulfur gave the 2′(3′)-phosphorothioates *XI* and *XV*. The compound *IX* was transformed by phosphorus oxychloride in 5′-O-carboxymethylinosine 2′(3′)-phosphate (*XII*). Uridine, adenosine and inosine 2′(3′)-phosphorothioates (*Ia—Ic*) were obtained from 2′,3′-O-di-n-butylstannylene derivatives of the nucleosides *IV* by treatment with thiophosphoryl chloride followed by alkaline hydrolysis; inosine and guanosine 2′(3′)-phosphorothioates (*Ic*, *Id*) were prepared by reaction of the corresponding 2′(3′)-phosphites *VIc*, *VId* with trimethylsilyl chloride and sulfur. Cyclisation of *Ic* and *Id* with ethyl chloroformate in the presence of tri-n-butylamine afforded inosine 2′,3′-O,O-cyclophosphorothioate (*VIIc*) and the corresponding guanosine derivative *VIId*. Compounds *VIIc* and *VIId* are not cleaved by *Streptomyces aureofaciens* ribonuclease.

Affinity chromatography finds a still wider application as a method for purification of enzymes. It was also employed in purification of ribonuclease from beef[1] and hog[2] pancreas as well as in purification of the snake venom exonuclease[3] and uridine-specific cyclising ribonucleotide-2′-transferase from rape seedlings[4]. In all these cases either aromatic ester of uridine-5′-phosphate, 3′,5′-diphosphate, or aromatic diester of thiophosphoric acid, was used as the active ligand.

In the course of our investigations we were interested in the use of affinity chromatography for purification of guanyl-specific ribonuclease from *Streptomyces aureofaciens*[5,6]. Since this enzyme cleaves not only the ester bonds of guanosine 3′-phosphate but also the analogous bonds of inosine 3′-phosphate, we turned our attention to ligands derived from inosine. It can be expected that both inosine 2′-phosphate and 3′-phosphate, and even more the corresponding isomeric esters of thiophosphoric acid, will have an inhibitory action on the mentioned enzyme. Naturally, a ligand, suitable for this purpose, must contain also a group capable of a covalent binding to the carrier without disturbing the characteristic structure of the inhibitor. In our previous

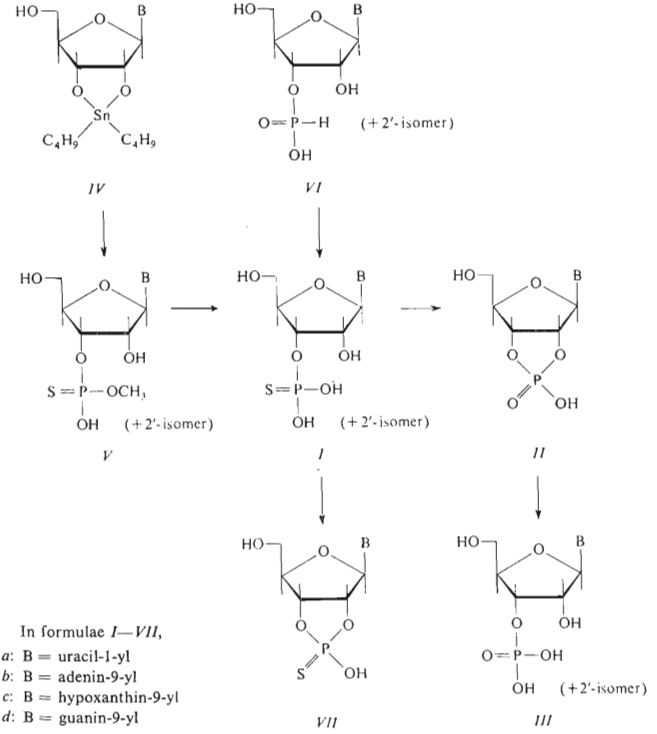
investigations on bonding to proteins, we obtained good results with 5′-O-carboxymethyl derivatives of ribo- and 2′-deoxyribonucleosides[7–10]. In these compounds, the carboxymethyl group is covalently bonded, does not affect the fundamental properties of the nucleoside skeleton, and can easily be activated by large variety of reagents for the binding to an activated carrier. This paper concerns the preparation of 5′-O-carboxymethylinosine, its 2′(3′)-phosphate, 2′(3′)-phosphorothioate and 2′,3′-cyclic phosphodiesters, as well as analogous derivatives with modified carboxymethyl moiety.

We checked several methods of preparation of nucleoside 2′(3′)-phosphorothioates *I* with respect to their possible application to synthesis of 5′-O-carboxymethylinosine derivatives, to small-scale preparations and to contamination of the desired product with phosphoric acid derivatives. Thiophosphorylation of 5′-O-protected ribonucleosides by reaction with thiophosphoric acid triimidazolide[11,12] has been studied with 5′-O-(4,4′-dimethoxytrityl)-N^6-dimethylaminomethyleneadenosine as model compound. Although the conversion to the 2′(3′)-phosphorothioate *Ib* was sufficiently high, the work-up of the reaction mixture proved to be difficult owing to a high content of salts; adsorption on charcoal, usually employed for demineralisation, in the case of the phosphorothioate *Ib* led to its quantitative desulfuration. Obviously, this desulfuration is of general character and was observed also with 5′-phosphorothioates[13]; it proceeds probably *via* an S-activated intermediate because the main product proved to be adenosine 2′,3′-cyclic phosphate (*IIb*), arising from such an intermediate by an intramolecular attack by the neighbouring 2′(3′)-hydroxy group. This product was characterized unequivocally by comparison with an authentic material and by quantitative cleavage with T2-ribonuclease to adenosine 3′-phosphate (*IIIb*).

In order to avoid the above complication, we elaborated a new synthesis of ribonucleoside 2′(3′)-phosphorothioates *I* from unprotected ribonucleosides, analogous to the reaction employed in the selective phosphorylation of *cis*-diol grouping in ribonucleosides *via* their 2′,3′-O-di-n-butylstannylene derivatives[14]. Compounds *IV* are formed in an *in situ* reaction of ribonucleosides with di-n-butyltin oxide in methanol and react then with thiophosphoryl chloride in the presence of tri-n-butylamine in the same solvent to give methyl esters of ribonucleoside 2′(3′)-phosphorothioates *V*. These intermediates can be either isolated as lithium salts or directly transformed in an alkaline medium to the corresponding ribonucleoside 2′(3′)-phosphorothioates *I*. In the relatively strongly alkaline medium the P=S bond is sufficiently stable and therefore the product *I* is contaminated only with less than 3–5% of the corresponding nucleotide *III*. In order to check the general applicability of this method, we prepared in this manner the phosphorothioates *Ia–Ic*, derived from uridine, adenosine and inosine.

As a third alternative preparation of nucleoside 2′(3′)-phosphorothioates *I* we made use of an already described[15] method, consisting in sulfuration of trimethylsilyl

derivatives of nucleoside 2′(3′)-phosphites *VI* with elemental sulfur. This method is preparatively suitable because the starting phosphites *VI* can easily be prepared by acid catalysed reesterification of free ribonucleosides with triethyl phosphite[16], even in small scale preparations. The reaction proceeds with high yield and the product *VI* is transformed by an excess of trimethylsilyl chloride in the presence of triethylamine directly into the pertrimethylsilyl derivative with protected sugar hydroxy groups as well as the acidic function on the heterocyclic ring. Reaction with elemental



IV *VI* *V* *I* *II* *VII* *III*

In formulae *I*—*VII*,
a: B = uracil-1-yl
b: B = adenin-9-yl
c: B = hypoxanthin-9-yl
d: B = guanin-9-yl

SCHEME 1

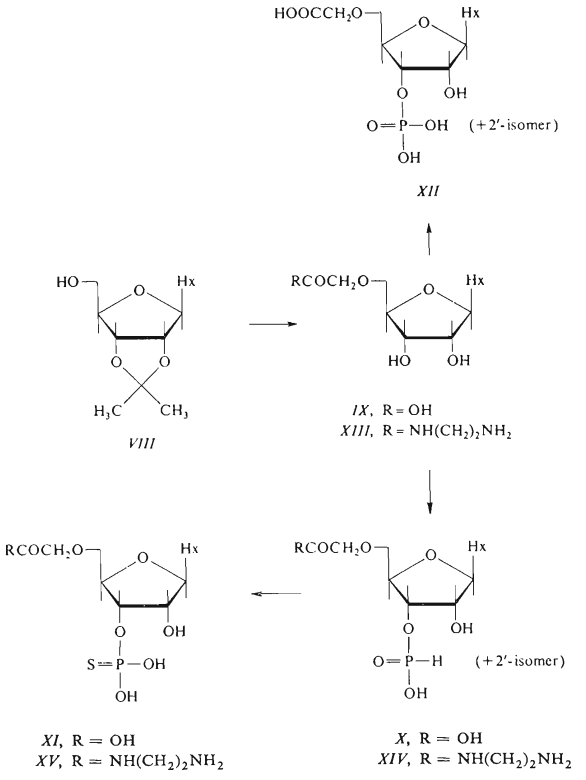
sulfur affords then practically quantitatively the compound *I*. This method was used in the preparation of inosine and guanosine derivatives (*Ic, d*) which contained less than 2% of the corresponding nucleotide *III*.

Ribonucleoside 2′, 3′-O,O-cyclophosphorothioates *VII* were used several times for the study of the stereochemistry of the hydrolytic step of some ribonucleases[17,18]. We investigated the preparation of these compounds as possible substrates for the ribonuclease of *S. aureofaciens*. Their preparation by cyclisation of compounds *I* requires activation of the phosphorothioate which involves the danger of desulfuration. The published data on the possibility of cyclisation of alkali metal salts of compounds *I* by treatment with 2,4,6-triisopropylbenzenesulfonyl chloride in dimethylformamide[11] have not been confirmed in our case. Under these conditions the inosine derivative *Ic* afforded a mixture of cyclic diesters, containing a substantial amount of the 2′,3′-cyclic phosphate *IIc* (Scheme 1). Reaction of the compound *Ic* with N,N′-dicyclohexylcarbodiimide in pyridine or N-cyclohexyl-N′-trimethylammoniumpropylcarbodiimide *p*-toluenesulfonate in an aqueous medium led to the 2′,3′-cyclophosphate *IIc* as the sole product (with the latter reagent the reaction was complete during several hours at room temperature). All the three reagents mentioned react thus preferentially at the sulfur atom under formation of activated derivatives of the O=P.SR type. On the other hand, the classical activation of carboxylic and phosphoryl groups by formation of mixed anhydrides with esters of chloroformic acid[19,20] transforms the compounds *I* rapidly into practically pure O,O-cyclophosphorothioates *VII*. Their purity was checked by high pressure liquid chromatography, electrophoresis and by their hydrolysis back to the compounds *I* without any contamination with the nucleotide. This method was applied to preparation of the cyclic diesters of the thiophosphoric acid derived from inosine (*VIIc*) and guanosine (*VIId*) which were isolated as lithium salts.

The compounds *VIIc* and *VIId* were studied as substrates for the ribonuclease of *S. aureofaciens*. They are completely resistant towards the enzyme under conditions of degradation of the corresponding 2′,3′-cyclophosphates *IIc* and *IId* to the nucleotides *IIIc*, *IIId*. Thus, the ribonuclease of *S. aureofaciens* differs in this respect from the guanyloribonuclease T1 (*Aspergillus oryzae*) which is capable of splitting one of the diastereoisomers of the compound *VIId* (see[18]).

The above-mentioned method was then applied to inosine derivatives, containing the bonding group in the position 5′: the starting 5′-O-carboxymethylinosine *IX* was prepared by reaction of 2′,3′-O-isopropylideneinosine (*VIII*) with an equimolecular amount of sodium chloroacetate in the presence of two equivalents of sodium hydride (the monosodium salt of *VIII* afforded mainly the N^1-carboxymethyl derivative). Similarly as in the case of pyrimidine nucleosides[7,8], dimethyl sulfoxide proved to be the solvent of choice. After removal of the protecting group in an acidic medium, the product was obtained in an analytically pure state by chromatography

on a strongly basic anex. Its electrophoretical mobility corresponded to the presence of an acidic function in the molecule and the UV spectrum confirmed the presence of an NH group in the hypoxanthine moiety.



In formulae *VIII*—*XV*,
Hx = hypoxanthin-9-yl residue

SCHEME 2

The compound *IX* was transformed by reaction with triethyl phosphite into the 2′(3′)-phosphite *X* which, as its per-trimethylsilyl derivative, was treated with elemental sulfur according to ref.[15], affording the desired 5′-O-carboxymethylinosine 2′(3′)-phosphorothioate *XI*. Structure of the compound *XI* was confirmed by its electrophoretical properties, positive reaction for the P=S bond (using the reagent described in ref.[21]) and its UV spectrum. Phosphorylation of the compound *IX* with phosphorus oxychloride in triethyl phosphate, followed by alkaline hydrolysis, afforded 5′-O-carboxymethylinosine 2′(3′)-phosphate (*XII*) (Scheme 2). Contrary to nucleosides with free primary hydroxyl[22], this compound reacts very slowly and an excess of the reagent must be employed.

In addition to the 5′-O-carboxymethylinosine derivatives *XI* and *XII* which can be bonded *e.g.* to a polymeric carrier, containing aliphatic amino groups, we prepared also the corresponding compounds, derived from 5′-O-carboxymethylinosine 2-aminoethylamide (*XIII*). This type could be bound to other types of carrier, *e.g.* to cyanogene bromide activated Sepharose (*i.e.* without any external activation reagent); the inserted 2-aminoethylamide group can serve as a spacer. The compound *XIII* was prepared from *IX* by treatment with *p*-nitrophenol in the presence of N,N′-dicyclohexylcarbodiimide and subsequent reaction of this activated ester with an excess of 1,2-diaminoethane. The product *XIII* was separated from a small amount of the N,N′-disubstituted derivative by chromatography on a strongly acidic catex which retained only the compound *XIII*. The 2′(3′)-phosphorothioate *XV* was then prepared by the usual reaction sequence (Scheme 2) *via* the 2′(3′)-phosphite *XIV*. The obtained compounds were chromatographically homogeneous and their electrophoretic and UV-spectral properties corresponded to their structure.

The methods described in this paper were thus applied to synthesis of inosine 2′(3′)-phosphate and 2′(3′)-phosphorothioate, carrying in the position 5 carboxymethyl or 2-aminoethylamidocarbonylmethyl groups, suitable for various kinds of bonds to affinity polymeric carriers. The bonding reaction of these compounds, as well as the application of the obtained affinity carriers, is described elsewhere[23].

EXPERIMENTAL

The melting points were determined on a Kofler block and are uncorrected. Unless otherwise stated, the solutions were taken down at 40°C/2 kPa and the compounds were dried over phosphorus pentoxide at 13 Pa. Paper chromatography was carried out on a Whatman No 1 paper (preparative separation on Whatman No 3 MM, 50 mg of the nucleotidic material per sheet) in the system S1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2), paper electrophoresis was performed on the same paper in 0·1M triethylammonium hydrogen carbonate, pH 7·5 (20 Vcm^{-1}, 1 h). The compounds were detected under UV-light (Chromatolight), the P=S bonds were detected by the reagent, prepared according to ref.[21]. The UV absorption spectra were measured in aqueous solutions on a Specord UV-VIS instrument (Carl Zeiss, Jena, GDR); for quantitative determination we used the values from ref.[24]. Chromatography on DEAE cellulose (Cellex D, 80 × 4 cm; 3 ml/min) was followed on a Uvicord instrument (LKB, Sweden).

Reaction of 5'-O-(4,4'-Dimethoxytrityl)-N^6-dimethylaminomethyleneadenosine with Thiophosphoric Acid Triimidazolide

Imidazole (4·1 g; 60 mmol) was added to a stirred solution of thiophosphoryl chloride[25] (1·7 g; 10 mmol) in acetonitrile (40 ml). After stirring for 1 h, a solution of the nucleoside derivative (4·15 g; 6·7 mmol; see ref.[26]) in acetonitrile (40 ml) was added and the mixture was stirred for 3 days in a stoppered flask. The suspension was filtered, washed with acetonitrile (20 ml) and the filtrate taken down *in vacuo*. The residue was dissolved in 50% acetic acid (50 ml), the solution left aside for 2 h at room temperature, taken down *in vacuo* and acetic acid removed by codistillation with ethanol. According to chromatography in the system S1, the mixture contained, in addition to adenosine, 55% of the compound *Ib*, identical with the authentic material.

The residue was dissolved in water (50 ml), acidified with hydrochloric acid to pH 4·0, treated with charcoal under stirring (the charcoal was deactivated by boiling with hydrochloric acid and washed with water until neutral) till the absorbance at 260 nm dropped to 1·0. The mixture was centrifuged, washed three times with water and resuspended in a methanol–aqueous ammonia–water (3 : 1 : 6) mixture (50 ml). After stirring for 1 h, the mixture was again centrifuged, washed with the same solvent system (20 ml) and the supernatant was taken down *in vacuo*. Analysis of the mixture in the system S1 and electrophoresis showed the absence of the compound *Ib*; the mixture contained only adenosine, adenosine 2',3'-cyclophosphate *IIb* and adenosine 2'(3')-phosphate (*IIIb*), the latter two components in the ratio 3 : 1. Cleavage of *IIb* with T2 ribonuclease (50 EU per 2 μmol of *IIb* in 0·1 ml of TRIS buffer, pH 7·0, 37°C, 6 h) afforded quantitatively adenosine 3'-phosphate (*IIIb*) (according to chromatography in S1, electrophoresis).

Uridine 2'(3')-Phosphorothioate (*Ia*)

A mixture of uridine (1·22 g; 5 mmol), di-n-butyltin oxide (1·20 g; 6 mmol) and methanol (100 ml) was refluxed for 2 h and taken down *in vacuo*. The residue was dissolved in methanol (70 ml) and treated with tri-n-butylamine (7 ml), followed with thiophosphoryl chloride (2·1 ml), under cooling with ice. The mixture was set aside for 1 h and evaporated *in vacuo*. The residue was dissolved in water (200 ml), made alkaline with 10% lithium hydroxide (pH 9), extracted with ether (3 . 50 ml), the aqueous layer concentrated *in vacuo* to about 20 ml and treated with 10% lithium hydroxide (20 ml). After standing overnight at room temperature, the pH of the mixture was adjusted (pH-meter) by addition of Dowex 50X8 (H^+-form) to 7·1, the mixture was filtered, the solid on the filter washed with water and the filtrate taken down *in vacuo*. The residue was applied on a column of DEAE-cellulose (*vide supra*), the neutral fraction was eluted with water and the product obtained by gradient elution with triethylammonium hydrogen carbonate (0—0·2M), pH 7·5 (à 2 l). The product fractions were combined, taken down *in vacuo*, the residue codistilled three times with ethanol and applied to a column (50 ml) of Dowex 50X8 (Li^+-cycle). The UV-absorbing fraction, obtained by elution with water, was taken down *in vacuo*, the residue codistilled with water and ethanol and precipitated from methanol (10 ml) by addition of ether (100 ml). The product was filtered, washed with ether and dried *in vacuo*, affording in 40% yield the chromatographically (S1) and electrophoretically pure product *Ia* (lithium salt) in purity higher than 90% (determined spectrophotometrically).

Adenosine 2'(3')-Phosphorothioate (*Ib*)

This compound was prepared from adenosine (5 mmol) in the same manner as *Ia*; yield of chromatographically pure lithium salt of compound *Ib* 56%, purity 96% (determined spectrophotometrically).

Inosine 2′(3′)-Phosphorothioate (*Ic*)

A) A mixture of inosine (5 mmol), di-n-butyltin oxide (1·20 g; 6 mmol), methanol (250 ml) and triethylamine (7 ml) was refluxed for 2 h, taken down *in vacuo*, the residue suspended in methanol (150 ml) and treated with thiophosphoryl chloride (2·1 ml, 20 mmol). After the end of the exothermic reaction, the mixture was set aside for 2 h and taken down *in vacuo*. The residue was taken up in water (200 ml) and the pH of this solution was adjusted with lithium hydroxide to 10·7. The mixture was extracted with ether (2 . 50 ml) and the aqueous layer neutralized with Dowex 50X8 (H^+-form) to pH 7·1. The mixture was filtered, the filtrate concentrated *in vacuo* and chromatographed on a column (100 × 4 cm) of Sephadex G—10 (coarse) in 0·02M triethylammonium hydrogen carbonate, pH 7·5 (40 ml h^{-1}). The product fraction (contaminated with a small amount of inosine) was taken down and separated on DEAE cellulose under conditions described for *Ia*. The product fraction was evaporated *in vacuo* and transformed into the lithium salt (see compound *Ia*) which, after drying, was precipitated from methanol (5 ml) with ether, affording 1·40 g (74%) of the chromatographically pure lithium salt of *Vc*. The product was stable in 50% acetic acid (100°C, 1 h).

The compound *Vc* (1·4 g; 3·7 mmol) in 5% lithium hydroxide (20 ml) was set aside overnight at room temperature, the mixture was neutralised with Dowex 50X8 (H^+-form) (pH 7·0), filtered, the filtrate was taken down *in vacuo*, the residue codistilled with ethanol (2 . 50 ml) and precipitated from methanol (5 ml) with ether, yielding 1·40 g (100%) of chromatographically pure *Ic* (lithium salt) of 97% purity (determined spectrophotometrically). According to HPLC analysis, the compound contained less than 4% of inosine 2′(3′)-phosphate (*IIIc*).

B) To a solution of triethylammonium salt of inosine 2′(3′)-phosphite (*VIc*; 2·83 g; 6·52 mmol; prepared according to ref.[15]) in pyridine (50 ml) was added triethylamine (5·6 ml; 40 mmol) and then with stirring trimethylsilyl chloride (5·1 ml; 40 mmol). After stirring for 10 min, ground crystalline sulfur (419 mg; 13 mgat) was added and the mixture was stirred for 24 h at room temperature in a stoppered flask. The mixture was filtered, the solid washed with pyridine (5 ml), the filtrate diluted with water (100 ml) and taken down *in vacuo*. An aqueous solution of the residue (100 ml) was extracted with chloroform (3 . 50 ml) and ether (2 × 50 ml). The aqueous layer was evaporated and the residue transformed into the lithium salt *Ic* (1·75 g; 72%), using the procedure described for *Ia*. The product was chromatographically and electrophoretically homogeneous and identical with the compound prepared by the procedure *A*); purity 94% (determined spectrophotometrically). According to HPLC analysis, the product did not contain any detectable amount of inosine 2′(3′)-phosphate (*IIIc*).

Guanosine 2′(3′)-Phosphorothioate (*Id*)

Guanosine (20 mmol) was transformed into the 2′(3′)-phosphite *VId* using the method described in ref.[15]. The thus-obtained triethylammonium salt of the compound *VId* was dried *in vacuo* and the residue treated with pyridine (150 ml), triethylamine (17 ml) and then, with stirring, with trimethylsilyl chloride (15·5 ml). After 10 min, crystalline sulfur (1·26 g; 39·4 mgat) was added. The mixture was stirred for 24 h in a stoppered bottle, filtered, the solid washed with pyridine (20 ml), the filtrate diluted with water (200 ml) and extracted with chloroform (3 . 25 ml) and ether (25 ml). The aqueous layer was concentrated *in vacuo* and the product transformed to the lithium salt (as described for *Ia*) which, after evaporation, was precipitated from ethanol (50 ml) on addition of acetone (300 ml). The suspension was filtered, washed successively with acetone (200 ml) and ether (200 ml) and dried *in vacuo*, yielding 5·6 g (62·3% based on guanosine) of the chromatographically pure lithium salt *Id*, free of guanosine 2′(3′)-phosphate (*IIId*); purity 87% (spectrophotometrically).

Inosine 2′(3′)-Phosphate (*IIIc*)

n-Butyl nitrite (1·5 ml) was added to a solution of adenosine 2′(3′)-phosphate (*IIIb* free acid; 1·0 g; 2·88 mmol) in 50% acetic acid (10 ml), the mixture was stirred at 0°C for 3 h and then set aside at room temperature for 48 h. After evaporation *in vacuo*, the residue was codistilled with water (3 . 20 ml), taken up in water (20 ml), and after adjusting the pH to 3 by addition of formic acid, applied on a column of Dowex 50X8 (H^+-form; 100 ml). After elution with water, the UV-absorbing eluate was taken down, the residue coevaporated with ethanol (3 . 20 ml), taken up in water (50 ml), neutralized with lithium hydroxide (pH 7·1) and evaporated *in vacuo*. Precipitation with ethanol–ether (see *IVa*) afforded 0·69 g (50%) of the chromatographically homogeneous lithium salt of compound *IIIc* (content 87%).

Reaction of Inosine 2′(3′)-Phosphorothioate (*Ic*) with Activating Agents

A) *With* N-*cyclohexyl*-N′-(3-*trimethylammonium*-1-*propyl*)*carbodiimide*: The title reagent[27] (190 mg; 0·5 mmol) was added to a solution of the lithium salt of *Ic* (0·1 mmol) in water (6 ml) and the mixture was kept at pH 6·0 (pH-state) by addition of 0·2M hydrochloric acid. Aliquotes of the mixture were analyzed by paper chromatography and electrophoresis, the P=S bonds being detected by the above-described method. After 1 h, the reaction product consisted solely of inosine 2′,3′-cyclic phosphate (*IIc*).

B) *With* 2,4,6-*triisopropylbenzenesulfonyl chloride*: The title reagent (1 g; 4 mmol) was added to a solution of lithium salt of *Ic* (1 mmol) in pyridine (5 ml) and the mixture was stirred at room temperature. After 1 h, the mixture contained no starting *Ic* and consisted of the 2′,3′-cyclic phosphate *IIc* and the 2′,3′-O,O-cyclophosphorothioate *VIIc* in the ratio 9 : 1 (determined spectrophotometrically).

Inosine 2′,3′-O,O-Cyclophosphorothioate (*VIIc*)

Tri-n-butylamine (1·75 ml), followed by ethyl chloroformate (0·5 ml) was added to a solution of lithium salt of compound *Ic* (1 mmol) in water (6 ml). After stirring for 1 h at room temperature, the mixture was applied to 3 sheets of paper Whatman No 3 MM and chromatographed in the system S1. The product bands were eluted with dilute ammonia (pH 9), the eluate was taken down *in vacuo* and the residue transformed into the lithium salt (see *Ia*). Yield of chromatographically and electrophoretically homogeneous product *VIIc* 96%, purity 98% (determined spectrophotometrically).

The compound *VIIc* (1 mg) on heating in 50% acetic acid (50 µl) to 50°C for 2 h afforded quantitatively the chromatographically homogeneous compound *Ic* which, according to HPLC, contained only traces of the nucleotide *IIIc*.

Guanosine 2′,3′-O,O-Cyclophosphorothioate (*VIId*)

This compound was prepared in 86% yield from lithium salt of *Id* (1 mmol) analogously as described for *VIIc*; purity 95%. Cleavage with acetic acid (*VIIc*) afforded pure starting *Id*.

Cleavage of Compounds *VIIc*, *VIId* and *IId* with Ribonuclease of *S. aureofaciens*

The incubation mixture was 7 mM in the substrate, dissolved in 0·05M-TRIS-buffer (pH 7·2), and contained 10000 EU of the enzyme per 1 ml. After incubation for 15 h at 37°C, the mixture

was analyzed by HPLC (Table I). Under these conditions, 45—48% of compound *IId* were cleaved; compounds *VIIc* and *VIId* were completely resistant.

5'-O-Carboxymethylinosine (*IX*)

Sodium hydride (0·33 g; 13·6 mmol) was added to a solution of 2',3'-O-isopropylideneinosine (*VIII*; see ref.[28]; 2·1 g; 6·8 mmol) in dimethyl sulfoxide (35 ml) and the mixture was stirred for 15 min with exclusion of moisture. Sodium chloroacetate (0·79g; 6·8 mmol), dried over phosphorus pentoxide at 60°C/13 Pa, was added and the mixture was stirred for 4 days in a stoppered flask. After addition of ethanol (5 ml) and acetic acid (1 ml) the mixture was taken down at 60°C/13 Pa and the residue refluxed with 80% acetic acid (50 ml) for 2 h. The mixture was taken down *in vacuo*, the residue coevaporated with water (3 . 50 ml), diluted with water (50 ml) and the pH was adjusted to 8·0. The mixture was applied on a column of Dowex 1X2 (200 ml, 100—200 mesh, acetate form). The column was washed with water until the eluate showed no UV-absorption, then with dilute acetic acid (gradient 0—1M, à 1 l; gradient 1—2M, à 0·5 l). The product was eluted when the concentration of acetic acid was 1·5M. The product-containing fractions were combined, taken down *in vacuo*, coevaporated with water (3 . 50 ml) and ethanol (2 . 50 ml) and the residue was precipitated from methanol (5 ml) with ether (100 ml). The obtained product, m.p. 150°C, (1·05 g; 47%) was chromatographically and electrophoretically homogeneous, purity 94% (determined spectrophotometrically). For $C_{14}H_{14}N_4O_7$ (326·3) calculated: 44·17% C, 4·32% H, 17·18% N; found: 44·28% C, 4·53% H, 16·79% N.

TABLE I

Paper Chromatography and Electrophoresis

Compound	R_F [a]	E_{Up} [b]	Compound	R_F [a]	E_{Up} [b]
Ia	0·16	0·96	*Va*	0·45	0·65
Ib	0·23	0·84	*Vb*	0·50	0·70
Ic	0·10	0·98	*Vc*	0·42	0·56
Id	0·06	0·88	*Vd*	0·38	0·46
IIa	0·45	0·67	*VIIc*	0·44	0·66
IIb	0·52	0·70	*VIId*	0·36	0·54
IIc	0·40	0·60	*IX*	0·32	0·57
IId	0·37	0·50	*X*	0·10	0·93
IIIa	0·20	1·00	*XI*	0·03	1·27
IIIb	0·27	0·87	*XII*	0·06	1·16
IIIc	0·15	1·05	*XIV*	0·31	0·25
IIId	0·10	0·92	*XV*	0·18	0·54

[a] In S1; [b] related to uridine 3'-phosphate (*IIIa*).

5′-O-Carboxymethylinosine 2′(3′)-Phosphorothioate (*XI*)

A mixture of the compound *IX* (980 mg; 3 mmol), dimethylformamide (20 ml) and triethyl phosphite (10 ml) was acidified with 6M-HCl in dimethylformamide and set aside for 3 days in a stoppered flask. Water (20 ml) was added and pH was adjusted to 8—9 by addition of ammonia. After evaporation *in vacuo*, the residue was dissolved in water (20 ml) and applied on a column of DEAE cellulose (*vide supra*). The column was washed with water until the eluate exhibited no UV-absorption and then with triethylammonium hydrogen carbonate solution pH 7·5 (linear gradient 0—0·3M à 2 l). The product-containing fraction was taken down, the residue codistilled with ethanol (3 . 50 ml) and dried *in vacuo*, affording the chromatographically homogeneous product X (0·88 g; 50%).

Trimethylsilyl chloride (1·3 ml; 10 mmol) was added to a mixture of *X* (0·67 g; 1·14 mmol), pyridine (15 ml) and triethylamine (1·4 ml; 10 mmol) and the mixture was stirred for 10 min. Crystalline sulfur (96 mg; 3 mgat) was added and the mixture was stirred in a stoppered flask for 24 h at room temperature. The suspension was filtered, the solid on the filter washed with pyridine (5 ml), the filtrate diluted with water (100 ml) and extracted with chloroform (3 . 25 ml) and ether (25 ml). The aqueous phase was taken down *in vacuo*, the residue applied on 4 sheets of paper Whatman No 3 MM and chromatographed in the system S1. The bands of the product were eluted with dilute ammonia (pH 9), the eluate was taken down and the residue transformed into the lithium salt. Yield of the chromatographically homogeneous product *XI* (lithium salt) was 210 mg (42%), purity 90% (determined spectrophotometrically); it shows positive reaction for P=S.

5′-O-Carboxymethylinosine 2′(3′)-Phosphate (*XII*)

A mixture of the compound *IX* (47·7 mg; 0·146 mmol), triethyl phosphate (1 ml) and phosphorus oxychloride (50 µl, 83·5 mg; 0·545 mmol) in a stoppered flask was set aside for 4 days at room temperature, poured into water (20 ml) and the pH was maintained at 7·5 for 2 h by means of 10% lithium hydroxide (pH-state). The mixture was concentrated *in vacuo* and the residue chromatographed on 1 sheet of paper Whatman No 3 MM in the system S1. The band of the product was eluted with dilute ammonia (pH 9), the eluate taken down and transformed into the lithium salt of the compound *XII* (see *Ia*) which was chromatographically and electrophoretically homogeneous; yield 24·6 mg (40%), purity 80% (determined spectrophotometrically).

5′-O-(2-Aminoethylamidocarbonylmethyl)inosine (*XIII*)

N,N′-Dicyclohexylcarbodiimide (0·45 g; 2·2 mmol) was added to a solution of the compound *IX* (0·66 g; 2 mmol) and *p*-nitrophenol (0·31 g; 2·2 mmol) in dimethylformamide (8 ml) and the mixture was stirred overnight in a stoppered flask. Dioxane (8 ml) was added, the mixture was filtered and the precipitate on the filter washed with dioxane (5 ml). The filtrate was taken down *in vacuo* and the residue chromatographed on a loose layer (40 × 16 × 0 3 cm) of silica gel, containing a fluorescence indicator, in a methanol–chloroform mixture (1 : 1). The product band was eluted with methanol (500 ml) and the eluate taken down *in vacuo*. The obtained product (2 mmol) was dissolved in dioxane (20 ml), mixed with 1,2-diaminoethane (3 ml) and the solution stirred overnight. The mixture was taken down *in vacuo*, the residue coevaporated with toluene (25 ml) and applied on a column of Dowex 50X8 (H^+-form; 50 ml). After washing with water till the eluate exhibited no UV-absorption, the product was eluted with dilute (1 : 10) ammonia. The UV-absorbing eluate was taken down *in vacuo* and after drying used in the further preparation of the compound *XIV*. The product showed a positive ninhydrine reaction. Yield (determined spectrophotometrically) 1·7 mmol (85%).

5'-O-(2-Aminoethylamidocarbonylmethyl)inosine 2'(3')-Phosphorothioate (*XV*)

The product *XIII* (1·7 mmol; see the preceding preparation) was stirred with a mixture of dimethylformamide (3 ml), 6M hydrogen chloride in dimethylformamide and triethyl phosphite (1 ml) till the mixture became homogeneous, then set aside overnight and worked up in the same manner as described for the compound *X*. Yield of the chromatographically homogeneous ammonium salt *XIV* was 1 mmol (60%), as determined spectrophotometrically. To a solution of ammonium salt of the compound *XIV* (1 mmol) in pyridine (10 ml) was added triethylamine (1·4 ml; 10 mmol) and trimethylsilyl chloride (1·27 ml; 10 mmol), and after stirring for 10 min crystalline sulfur (64 mg; 2 mgat). The mixture was then stirred for 3 days at room temperature, filtered, the filtrate was taken down *in vacuo*, the residue dissolved in water (50 ml), the solution washed with chloroform (3 . 20 ml) and ether (20 ml) and the aqueous phase taken down *in vacuo*. Chromatography of the residue on two sheets of paper Whatman No 3 MM in the system S1 and and elution of the product-containing bands with dilute ammonia (pH 9), followed by transformation of the product into the lithium salt as decsribed for *Ia*, afforded the chromatographically and electrophoretically homogeneous lithium salt of *XV* in 65% yield. Purity 95% (spectrophotometrically), the product gives a positive P=S test and ninhydrine reaction.

The authors are indebted to Dr K. Šebesta, Director of the Institute of Organic Chemistry and Biochemistry, Prague, for enabling a stay of one of the authors (P. K.) at this Institute.

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Translated by M. Tichý.