Studies of Phosphorylation. V.[1] The Synthesis of Inosine-5′-thiophosphates

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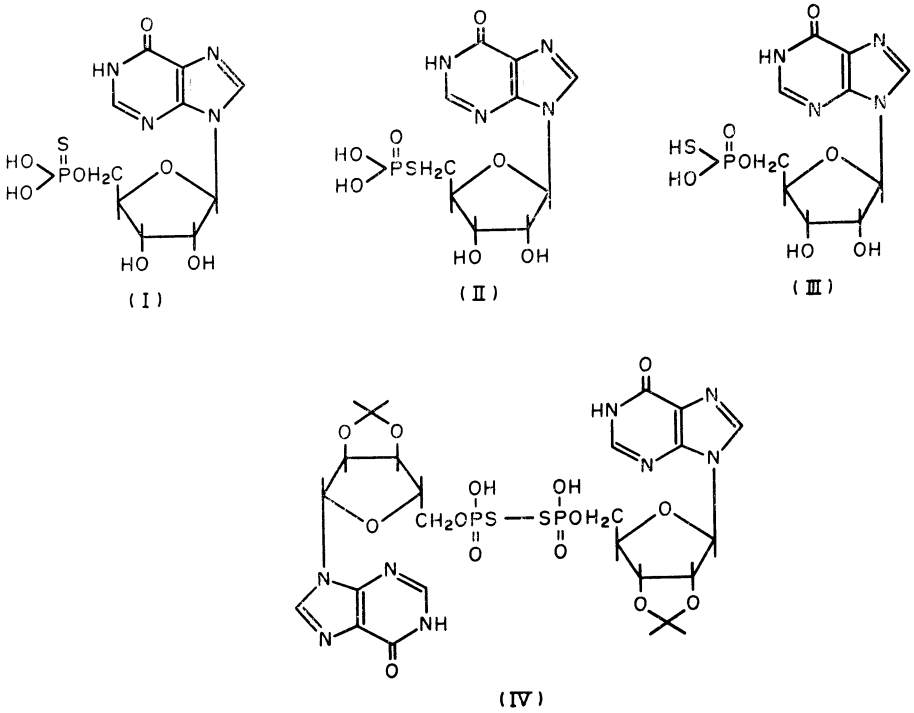
Two isomers of inosine-5′-thiophosphates, inosine-5′-phosphorothionate and 5′-deoxy-5′-thioinosine-5′-phosphorothioate, were synthesized by the direct phosphorylation of inosine with phosphorus thiochloride, and by the treatment of 5′-deoxy-5′-iodoinosine with trisodium phosphorothioate, respectively. The structure of the thiophosphate residues was identified by means of ^{31}P NMR. The flavoring activities of these thiophosphates were measured as the synergistic strength with monosodium glutamate; it was found that the strength of each compound was lower than that of inosine-5′-phosphate.

The nucleoside-5′-thiphosphates are of interest in connection with the relationship of the flavoring activity of the 5′-nucleotide to the structure of the phosphate residues. The present authors have attempted to prepare inosine-5′-thiophosphates and have obtained two isomers: inosine-5′-phosphorothionate (I) and 5′-deoxy-5′-thioinosine-5′-phosphorothioate (II). The other possible isomer, inosine-5′-phosphorothiolate (III), was not detected in the product. The synergistic flavoring strengths[2] of the nucleoside thiophosphates with monosodium glutamate were compared with that of inosine-5′-phosphate; the relative strengths of I and II were shown to be one-half and one-fortieth respectively. This paper will deal with the synthesis of the two isomers of inosine-5′-thiophosphates and with their structural identification by ^{31}P NMR.[3]

Results and Discussion

Synthesis of Inosine-5′-thiophosphates. The pyrimidine nucleoside phosphorothionates have been synthesized by the treatment of 3′-*O*-acetylthymidine and 2′,3′-*O*-dimethoxybenzylidene uridine with triimidazoyl-1-phosphinsulfide in only 25—30% yields.[4] In our previous report,[5] it has been shown that nucleosides were phosphorylated with phosphoryl chloride in trialkyl phosphate to give, selectively, the 5′-phosphates in good yields. This method was successfully applied to the phosphorylation of inosine with phosphorus thiochloride; inosine-5′-phosphorothionate (I) was obtained after the hydrolysis of the phosphorylation mixture. The product (I) was isolated by column chromatography and was identified by elemental analysis and by ^{31}P NMR spectroscopy, as will be



(I) (II) (III) (IV)

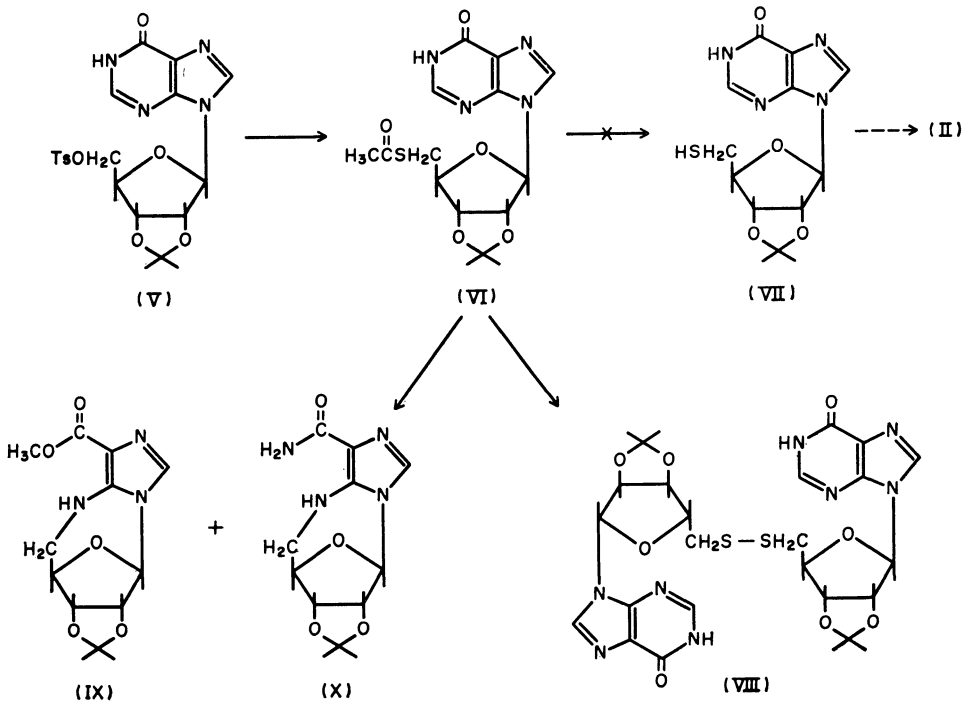
1) Part IV: M. Yoshikawa, M. Sakuraba, and K. Kusashio, This Bulletin, **43**, 456 (1970).

2) M. Ohara, T. Ninomiya, S. Ikeda, S. Yamaguchi, and T. Yoshikawa, *J. Agr. Chem. Soc. Jap.*, **40**, 69 (1966).

3) The synthesis of nucleoside-5′-thiophosphates in similar manners was reported by A. Hampton *et al.* (*Biochemistry*, **8**, 2303 (1969)) and by A. W. Murray *et al.* (*Biochemistry*, **7**, 4023 (1968)) while we were making our structural investigation.

4) F. Eckstein, *J. Amer. Chem. Soc.*, **88**, 4292 (1966).

5) M. Yoshikawa, T. Kato, and T. Takenishi, This Bulletin, **42**, 3505 (1969).



described later. The phosphorothionate was completely desulfurized to give inosine-5′-phosphate by being left for a few hours in an aqueous solution of pH 1—5, but it was stable in a neutral or mildly alkaline medium. When the phosphorothionate (I) was treated with active charcoal in order to purify it, the I was observed by paper chromatography to be entirely transformed into a different compound. The product was positive in sulfur analysis and was reduced quantitatively to I by the treatment of sodium in liquid ammonia. It can be concluded, from these results and from those of the ^{31}P NMR, that the oxidized compound was P^1,P^2-bis(inosine-5′-phosphoryl) disulfide (IV).

For the preparation of 5′-deoxy-5′-thioinosine-5′-phosphorothioate (II), we attempted to prepare 5′-deoxy-5′-thio-2′,3′-*O*-isopropylideneinosine (VII) as an intermediate. The acetylthio derivative of inosine (VI) was prepared by heating 2′,3′-*O*-isopropylidene-5′-*O*-(*p*-toluenesulfonyl)inosine (V) with sodium thioacetate using a modification of the method reported by Baddiley and Jamieson.[6] However, the treatment of the product with methanolic ammonia for deacetylation produced 5′,5′-bis(5′-deoxy-2′,3′-*O*-isopropylideneinosyl) disulfide (VIII), contrary to our expectations. The structure of disulfide was confirmed by its positive test with a Grote reagent[7] only after the treatment with potassium cyanate. The interesting result was observed, in the course of the preparative investigation of the acetylthio derivative, that two kinds of cyclonucleosides were produced by the self-exothermic degradation when the reaction has carried out without cooling. These products were isolated by alumina column chromatography. The PMR spectrum of one of the products has two peaks at 8.65 τ due to the isopropylidene group, a single peak at 6.19 τ due to the methyl ester, and a single peak at 3.91 τ due to the C_1'-proton, the latter of which is a typical signal of a cyclonucleoside group. The spectral results and the results of the elemental analysis prove that the product is 5-amino-4-methoxycarbonyl-N^5,5′-cyclo-1-β-D-(2′,3′-*O*-isopropylideneribofuranosyl)imidazole (IX). The other product was confirmed to be 5-amino-4-carbamoyl-N^5,5′-cyclo-1-β-D-(2′,3′-*O*-isopropylideneribofuranosyl)imidazole (X) by a comparison with an authentic sample.[8]

Some *S*-phosphoric esters have been synthesized by the treatment of halo-compounds with trisodium phosphorothioate.[9] When the 5′-deoxy-5′-iodoinosine was treated with trisodium phosphorothioate in water at room temperature, 5′-deoxy-5′-thioinosine-5′-phosphorothioate (II) was produced quantitatively; it was then isolated from the reaction mixture by means of cellulose-column chromatography. The phosphorothioate was extremely labile in acidic media and was completely hydrolyzed, in less than one minute, to produce 5′-deoxy-5′-thioinosine and orthophosphoric acid under the acidic conditions of pH 3 at 60°C. The compound, however, was relatively stable in neutral or alkaline media.

^{31}P NMR Spectra of Inosine-5′-thiophosphates. In order to clarify the ambiguities of inosine-5′-thiophosphates, the ^{31}P NMR spectra were determined at 40.5 MHz; the results are summarized in Table 1. The ^{31}P signal of I in aqueous ammonia appeared as a rather broad triplet (caused by the spin-coupling with the two protons at *C*-5′) at 2812 Hz upfield from the locked P_4O_6. This chemical shift is approximately equal to that of −43.1 ppm downfield from 85%

6) J. Baddiley and G. A. Jamieson, *J. Chem. Soc.*, **1955**, 1085.
7) J. W. Grote, *J. Biol. Chem.*, **93**, 25 (1931).
8) K. Kusashio and M. Yoshikawa, This Bulletin, **41**, 142 (1968).
9) S. Akerfeldt, *Acta Chem. Scand.*, **13**, 1479, 1897 (1959).

TABLE 1. PARAMETERS OF THE ^{31}P NMR SPECTRA[a)]

Compound	Solvent	Chemical shifts[b)] (multiplicity; J[c)])
I	NH_4OH aq.	−43.1 (broad triplet; $J \simeq 4.5$ Hz)
II	H_2O	−16.1 (triplet; $J \simeq 10$ Hz)
IV	H_2O	−17.3 (broad singlet)
5'-IMP 2Na	H_2O	−3.9 (triplet; $J \simeq 4.5$ Hz)
$S=P(OEt)_2$ | ONa (XI)	—	−56 −59
$O=P(OEt)_2$[d)] | SH (XII)	—	−24
$O=P(OEt)_2$ | ONa (XIII)	—	−3.8

a) All spectra were measured at room temperature (34°C).
b) Chemical shifts (ppm) are represented from 85% H_3PO_4 signal which is assumed to appear 4556 Hz upfield from P_4O_6.
c) Multiplicities and coupling constants are estimated based on the first order approximations.
d) "^{31}P Nuclear Magnetic Resonance," M. M. Crutchfield, C. H. Dungen, J. H. Letcher, V. Mark, and J. R. Van Wazer, Interscience Publishers, New York (1967).

H_3PO_4 which shows the phosphorothionate type (P=S), as is also supposed from the ^{31}P NMR spectrum of sodium diethyl phosphorothionate (XII). On the other hand, the ^{31}P NMR spectra of II and IV showed well-resolved triplets (J 10 Hz) at −16.1 and −17.3 ppm from 85% H_3PO_4 respectively. By comparing the chemical-shift data with those of the acyclic analogues, diethyl phosphorothiolate (XII), II and IV can be easily identified as thiophosphates which have a P–S single bond. The considerable differences encountered are attributable to the differences in the monoesters and diesters. Disodium inosine-5'-phosphate showed a triplet at −3.9 ppm downfield from 85% H_3PO_4, which is quite similar to that of sodium diethyl phosphate (XIII).[10)]

Experimental

Measurement of the ^{31}P NMR Spectra of Thiophosphates. The 100 MHz PMR spectra were recorded on a Varian HA-100 spectrometer by the frequency-sweep mode. The 40.5 MHz phosphorus-31 NMR spectra were also measured by the frequency-swept HA-mode, using a Varian HA-100 apparatus. A moltein P_4O_6 (supplied by Gallard-Schlesinger Co.) in a co-axial capillary held in the usual 5 mm sample tube offered a stable lock signal for the field/frequency control. A modulation frequency for the lock signal was generated by a Hewlett-Puckard 4204A audio-oscillator, and the chemical shifts from the lock signal were determined by a Hewlett-Puckard 5512A digital counter.

Paper Chromatographies of the Thio-compounds. The paper chromatographies were carried out by the ascending technique on Toyo Roshi No. 51 paper (40×40 cm), using the following solvent systems: Solv. A, *n*-propanol - concentrated ammonium hydroxide - water, 20 : 12 : 3; Solv. B, *n*-butanol - acetic acid - water, 4 : 1 : 1; Solv. C, isopropanol-saturated ammonium sulfate - water, 2 : 79 : 19. The R_f values of the thio-compounds are listed in Table 2.

TABLE 2. R_f VALUES OF THE THIO-COMPOUNDS

Compound	R_f Value		
	A	B	C
5'-Deoxy-5'-thionosine	0.43	0.38	0.30
5',5'-Bis(5'-deoxyinosyl)disulfide	0.39	0.08	0.01
5',5'-Bis(5'-deoxy-2',3'-*O*-isopropylideneinosyl)disulfide (VIII)	0.86	0.88	0.01
Inosine-5'-phosphorothionate (I)	0.30	0.02	0.54
5'-deoxy-5'-thioinosine-5'-phosphorothioate (II)	0.25	0.02	0.58
5'-acetylthio-5'-deoxy-2',3'-*O*-isopropylideneinosine (VI)	0.83	0.74	0.01
P',P^2-Bis(inosine-5'phosphoryl)-disulfide (IV)	0.32	0.02	0.29

Disodium Salt of Inosine 5'-Phosphorothionate (I). To a solution of phosphorus thiochloride (24 ml, 0.12 mol) in cold trimethyl phosphate (100 ml), inosine (10 g, 0.04 mol) was added, and then the solution was stirred for 8—9 hr at 0°C. The reaction mixture was poured into ice water, and the aqueous solution was adjusted to pH 3 with sodium hydroxide. The acidic solution was passed through a column of decolorizing resin (Resinous Adsorbent of Hokuetsu Tanso Kogyo Co.). The column was then washed well with water and eluted with a large volume of 0.5 N ammonium hydroxide. The eluate was concentrated and adjusted to pH 9—10 with concentrated ammonium hydroxide. The solution was then passed through a column of Dowex 1×2 ($HCOO^-$ form). After having been washed with water, the phosphorylated product was eluted with a large volume of 2—4 N formic acid. The eluate was evaporated to dryness *in vacuo*. Pure inosine 5'-phosphorothionate was obtained by means of cellulose (Toyo Roshi) column chromatography, which was developed in *n*-propanol-concentrated ammonium hydroxide-water (20 : 12 : 3). Ammonium salt of inosine 5'-phosphorothionate was converted to sodium salt and then recrystallized from water-methanol to give 9.5 g of the product, mp 205°C (dec.).

Found: C, 29.94; H, 3.56; N, 13.46%. Calcd for $C_{10}H_{11}N_4O_7SPNa_2$: C, 29.42; H, 2.72; N, 13.72%.

Disodium Salt of 5'-Deoxy-5'-thioinosine-5'-phosphorothioate (II). Trisodium phosphorothioate (7 g, 40 mmol) and 5'-deoxy-5'-iodoinosine (3.78 g, 10 mmol) were dissolved in water (100 ml), and then the solution was stirred for 7 hr at 40°C. The reaction mixture was evaporated *in vacuo* below 30°C, and the residual oil was dissolved in concentrated ammonium hydroxide (20 ml). After standing for 30 min, the precipitate was filtered off; the filtrate was then passed through a cellulose column (Toyo Roshi) which was developed in *n*-propanol-concentrated ammonium hydroxide-water (20 : 12 : 3). The fraction of II was evaporated to dryness *in vacuo* at 30°C, and the residue was dissolved in water. After filtration, ethanol was added to the filtrate and the resulting precipitate was recrystallized from water-ethanol to yield 3.5 g of the product, mp 208°C (dec).

Found: C, 27.22; H, 3.18; N, 12.68%. Calcd for $C_{10}H_{11}N_4O_7SPNa_2 \cdot 1.5\,H_2O$: C, 27.59; H, 3.24; N, 12.87%.

5'-Acetylthio-5'-deoxy-2',3'-O-isopropylideneinosine (VI). To a solution of sodium (2.9 g, 0.13 mol) in anhydrous methanol (75 ml), thioacetic acid (8.8 ml, 0.13 mol) was added with

10) Detailed accounts of the ^{31}P NMR spectra of nucleotides will be published elsewhere (M. Kainosho, A. Nakamura, and M. Tsuboi).

cooling on an ice bath; acetone (75 ml) and 2',3'-*O*-isopropylidene-5'-*O*-(*p*-toluenesulfonyl)inosine (V) (11.5 g, 0.025 mol) were then added. The reaction mixture was refluxed for 3 hr and evaporated to dryness at 40°C. The residue was dissolved in chloroform (150—200 ml), and the insoluble material was filtered off. The filtrate was evaporated to dryness, and the residue was dissolved in methanol (100 ml). After standing at room temperature for a few hours, the precipitate was separated and recrystallized from methanol to yield 8.5 g of the product, mp 241°C.

Found: C, 48.96; H, 4.69; N, 15.53%. Calcd for $C_{15}H_{18}N_4O_5S$: C, 49.17; H, 4.96; N, 15.29%.

5-Amino-4-methoxycarbonyl-N^5*,5'-cyclo-1-*β*-*D*-(2',3'-O-isopropylideneribofuranosyl)imidazole (IX).* V was treated without cooling in the same manner as in the preceding paragraph. The reaction products soluble in chloroform were dissolved in a small amount of methanol and then passed through a column of alumina. The column was eluted with 5% methanolic-ether. The eluate was concentrated and ether was added to the residue to crystallize IX, mp 155°C.

Found: C, 52.37; H, 5.65; N, 13.98%. Calcd for $C_{13}H_{17}N_3O_5S$: C, 52.87; H, 5.80; N, 14.23%.

X was isolated by eluting the column with 50% methanolic ether.

*5',5'-Bis-(5'-deoxy-2',3'-*O*-isopropylideneinosyl)Disulfide (VIII).* 5'-Acetylthio-5'-deoxy-2',3'-*O*-isopropylideneinosine (VI) was dissolved in methanol, and the solution was saturated with anhydrous ammonia at 0°C. After standing overnight, the solution was concentrated and methanol was added to the residue. The resulting precipitate was recrystallized from water to give 5.5 g of the product, mp 235°C.

Found: C, 47.76; H, 5.04; N, 17.26; S, 9.89%. Calcd for $C_{26}H_{30}O_8N_8S_2$: C, 48.28; H, 4.67; N, 17.33; S, 9.92%.

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