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Synthesis of 2-Alkylthioinosine 5′-Phosphates and N^2-Methylated Guanosine 5′-Phosphates[1]

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2-Alkylthioinosine 5′-phosphates and N^2-methylated guanosine 5′-phosphates (the minor constituents of transfer RNA) were synthesized *via* 2′,3′-O-isopropylidene-2-mercaptoinosine (I) from 5-amino-1-β-D-ribofuranosyl-4-imidazolecarboxamide (AICA-riboside). The relationship between their chemical structures and flavoring activity was investigated, and 2-methylthioinosine 5′-phosphate (VII) was found to have an extremely strong flavoring activity.

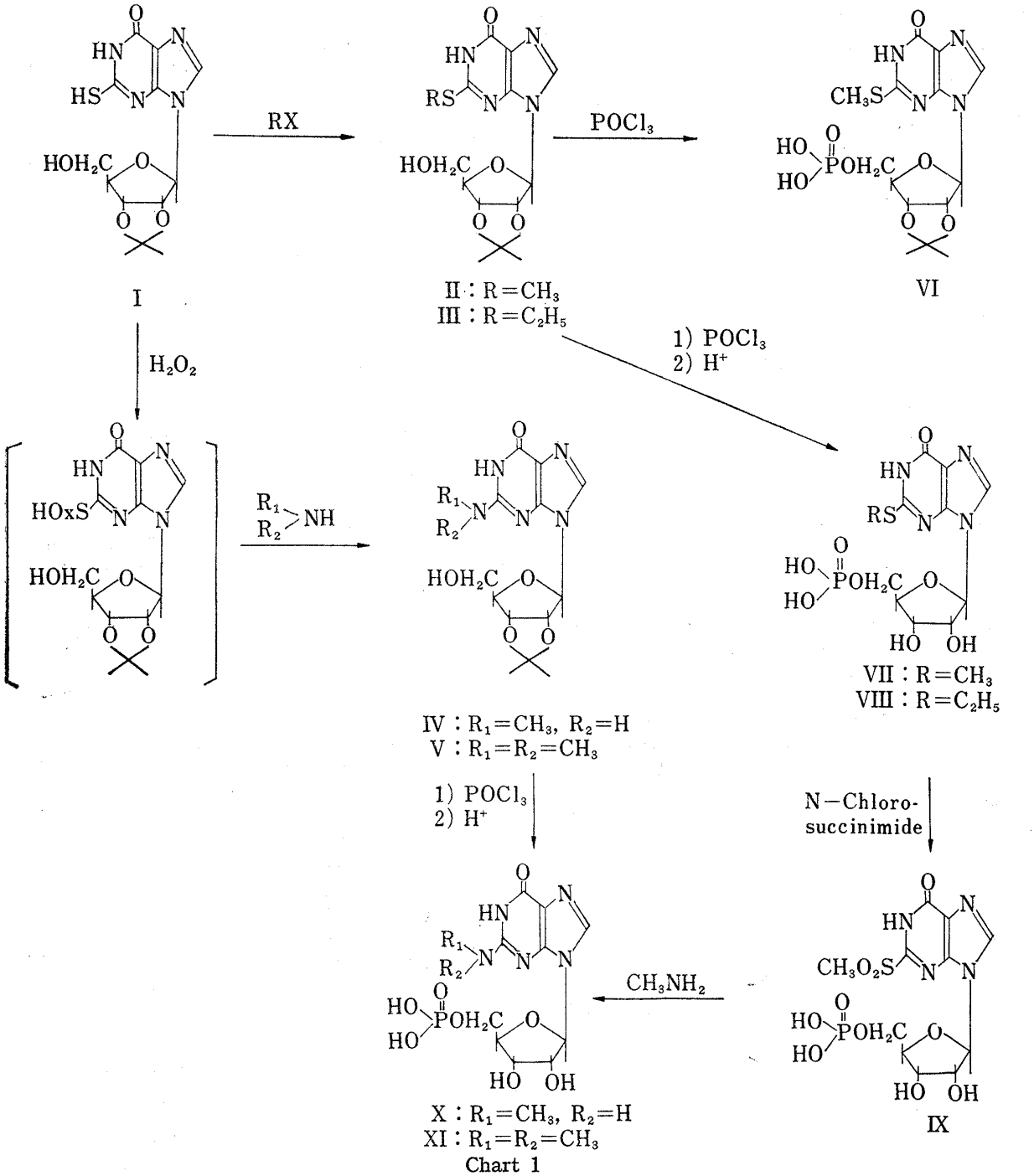
In 1960, Kuninaka[3] first investigated systematically the relationship between the flavoring activity of some naturally occurring nucleotides and their chemical structures, and proved that a structure which is essential to flavoring activity is purine 5′-mononucleotide with a hydroxy group in position 6, such as 5′-guanylic and 5′-inosinic acids. Although a number of papers have been reported on the synthesis of purine nucleotides, there are little information on the correlation of flavoring activity and chemical sturcture. Previously, the authors[4] synthesized 9-(4′-hydroxybutyl)guanine 4′-phosphate, but it had no flavoring activity. As analogs of 5′-inosinic acid, 9-β-D-glucopyranosylhypoxanthine 6′-phosphate and 4′-phosphate were prepared by Honjo, *et al.*,[5] who reported that they were ineffective in taste enhancement.

Though 5′-guanylic and 5′-inosinic acids are widely used as seasoning agents, the former has a strong flavoring activity than the latter has.[3] Obviously, this difference in the flavoring activity depends on the presence or the absence of an amino group in position 2 of purine nucleus. This fact suggests that, if a suitable substituent would be introduced to position 2 of 5′-inosinic acid, 5′-nucleotide having a stronger flavoring activity might be obtained.

In order to investigate the effect of a substituent in position 2 on flavoring activity, the authors attempted to synthesize some 2-substituted inosine 5′-phosphates.

In the previous paper,[6] we reported the versatile method for the preparation of guanosine *via* 2-mercaptoinosine from the readily available 5-amino-1-β-D-ribofuranosylimidazole (AICA-riboside),[7] and this procedure was extended to the synthesis of N^2-methyl- and N^2,-N^2-dimethylguanosines. In a similar fashion, 2′,3′-O-isopropylidene-N^2-methylguanosine (IV) and 2′,3′-O-isopropylidene-N^2,N^2-dimethylguanosine (V) were also prepared *via* 2′,3′-O-isopropylidene-2-mercaptoinsoine (I) which was obtained by treating the isopropylidene derivative[8] of AICA-riboside with sodium methylxanthate as shown in Chart 1. Further treatment of I with methyl iodide and ethyl iodide afforded the isopropylidene derivatives (II and III) of 2-methylthio- and 2-ethylthioinosines, respectively.

1) Presented at the 87th Annual Meeting of the Pharmaceutical Society of Japan, April, 1967, Kyoto, Japan.
2) Location: *Suzuki-cho, Kawasaki.*
3) A. Kuninaka, *Bull. Agr. Chem. Soc. Japan*, **34**, 489 (1960).
4) Reported at the 85th Annual Meeting of the Pharmaceutical Society of Japan, October, 1965, Tokushima (Abstract p. 293).
5) A. Nohara, K. Imai, and M. Honjo, *Chem. Pharm. Bull.* (Tokyo), **14**, 491 (1966).
6) A. Yamazaki, I. Kumashiro, and T. Takenishi, *J. Org. Chem.*, **32**, 3032 (1967).
7) T. Shiro, A. Yamanoi, S. Konishi, S. Okumura, and M. Takahashi, *Agr. Biol. Chem.*, **26**, 785 (1962).
8) Ajinomoto Co., Inc. Neth. Patent 6409142 (1965) (*C.A.* **63**, 5731 (1965)).



I

II : R=CH_3
III : R=C_2H_5

VI

VII : R=CH_3
VIII : R=C_2H_5

IV : R_1=CH_3, R_2=H
V : R_1=R_2=CH_3

X : R_1=CH_3, R_2=H
XI : R_1=R_2=CH_3

IX

Chart 1

The synthesis of 5′-nucleotides was achieved by a method developed in our labolatories,[9] in which the above isopropylidene derivatives were phosphorylated in good yields. Compound II was treated with 3 equiv. of phosphoryl chloride in trimethyl phosphate at −5° for 3 hr. The phosphorylation was proved to proceed quantitatively by the fact that an aliquot from the reaction mixture showed a single spot of 2-methylthioinosine 5′-phosphate (VII) after acidic treatment on the paper chromatogram. After the resulting nucleotide was absorbed on the decolorizing resin[10] and eluted with 0.5N ammonium hydroxide, the eluate was

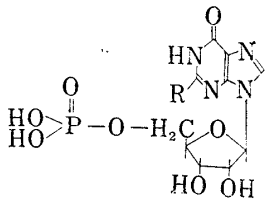
9) Reported by M. Yoshikawa, K. Kusashio, T. Kato, and T. Takenishi at the 19th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1966 (Abstract IV, p. 52).

10) This decolorizing resin was prepared in our Laboratories by co-polymerization of metaphenylenediamine, resorcin and formalin.

concentrated *in vacuo* to give the ammonium salt of VII, which was converted to the disodium salt. The yield was found to be 64%. Characterization of this compound was carried out by elemental analysis and spectral properties. Similarly, 2-ethylthioinosine 5'-phosphate (VIII) was prepared from III in 48% yield.

Phosphorylation of IV and V followed by acidic treatment afforded N^2-methylguanosine 5'-phosphate (X) and N^2,N^2-dimethylguanosine 5'-phosphate (XI), which were of interest

TABLE I.



Compound No.	R	mp (°C)	$[\alpha]_D$ (°C)	*Rf* values in solvent[a] A	B	C
III	C_2H_5S (Isopropylidene derivative)	208—208.5		0.76	0.80	
VI	CH_3S (Isopropylidene derivative)	120—122 (decomp.)	$[\alpha]^{27}$ −6.6 (c=0.92, water)	0.22	0.07	0.21
VII	CH_3S	195—210 (decomp.)	$[\alpha]^{23}$ −16.4 (c=2.92, water)	0.10	0.30	0.39
VIII	C_2H_5S	195—205 (decomp.)	$[\alpha]^{28}$ −14.0 (c=1, water)	0.10	0.32	0.42
IX	CH_3SO_2	250—260 (decomp.)	$[\alpha]^{26}$ −43.0 (c=1, water)	0.02	0.08	0.58
X	CH_3NH	>240	$[\alpha]^{28}$ −22.5 (c=1, water)	0.10	0.25	0.42
XI	$(CH_3)_2N$	179—180 (decomp.)	$[\alpha]^{28}$ −20.0 (c=1, water)	0.10	0.28	0.44

	Formula	Analysis (%) Calcd. C	H	N	P	Found C	H	N	P
III	$C_{15}H_{20}O_5N_4S$	48.90	5.47	15.21		49.24	5.52	15.23	
VI	$C_{14}H_{17}O_8N_4SPNa_2 \cdot {}^3/_2H_2O$	33.27	3.97	11.09	6.14	33.86	4.60	10.46	6.33
VII	$C_{11}H_{13}O_8N_4SPNa_2 \cdot 2H_2O$	27.85	3.59	11.81	6.54	28.11	3.49	12.15	6.64
VIII	$C_{12}H_{15}O_8N_4SPNa_2 \cdot 2H_2O$	29.51	3.89	11.48	6.35	28.99	3.62	11.51	6.23
IX	$C_{11}H_{13}O_{10}N_4SPBa \cdot 4H_2O$	20.65	3.03	8.35	4.89	20.86	3.32	8.85	4.52
X	$C_{11}H_{14}O_8N_5PNa_2 \cdot H_2O$	30.07	3.65	15.95	7.06	30.03	4.11	15.88	6.56
XI	$C_{12}H_{16}O_8N_5PNa_2 \cdot 2.5H_2O$	30.00	4.38	14.58	6.46	29.61	4.59	14.63	6.16

a) Paper chromatography was carried out on Toyo Filter Paper No. 51 by the ascending method. Solvent systems: A, *n*-butyl alcohol–acetic acid–water, 4:1:1 (v./v.); B, *n*-propyl alcohol–ammonia (28%)–water, 20:12:3 (v./v.); C, iso-propyl alcohol–sat. ammonium sulfate–water, 2:79:19 (v./v.).

as the minor constituents[11,12] of transfer RNA, in 58 and 51% yields, respectively. Their ultraviolet absorption spectra closely resemble those of 5′-guanylic acid, and when the spectra of XI were compared with those of X at various pH ranges, a little bathochromic shift was observed. The nuclear magnetic resonance spectra of X in deuterium oxide exhibited a singlet at 2.82 ppm due to methyl group, while XI did a singlet at 3.09 ppm due to dimethyl group.

Previously, Dunn, *et al.*[11] separated N^2-methylated guanosine 2′- and 3′-phosphates from alkaline hydrolyzate of ribonucleic acid, but there is no report on the isolation of 5′-phosphates, X and XI. Although the biological significance of minor constituents is unknown, it is expected that the synthesis of X and XI could contribute to the studies of the biological, chemical and physical properties of transfer RNA, since these compounds can now be readily prepared from AICA-riboside in good yields.

Oxidation[13] of VII with N-chlorosuccinimide in aqueous solution afforded 2-methylsulfonylinosine 5′-phosphate (IX), which was isolated as barium salt in 59% yield. This compound showed infrared absorption band at 1300 cm^{-1} due to sulfonyl group, and ultraviolet absorption maxima at 255 and 285 mμ in 0.1 N hydrochloric acid.

Compound X was also prepared by reaction of IX with methylamine although in low yield. When dimethylamine was employed, however, dephosphorylation preceded the amination to yield N^2,N^2-dimethylguanosine. Physical properties and analytical data of these nucleotides are summarized in Table I and II.

Table II. Ultraviolet Absorption Spectra

Compound	λ_{max} mμ (ε)		
	pH 1.1	pH 6.6	pH 12.8
VI	268 (10, 400)	261 (9, 500) 281 (infl.)	270 (10, 000)
VII	271 (15, 700)	265 (13, 600) 284 (infl.)	274 (13, 900)
VIII	270 (18, 500)	264 (17, 500) 285 (infl.)	273 (18, 100)
IX	255 (8, 300) 286 (4, 100)	257 (8, 500) 284 (5, 500)	258 (7, 300) 285 (5, 800)
X	261 (13, 400) 288 (infl.)	256 (13, 500) 285 (infl.)	262 (10, 500) 280 (infl.)
XI	267 (15, 800) 295 (infl.)	262 (15, 700) 288 (infl.)	264 (12, 000) 284 (infl.)

It is well known[3,14] that 5′-inosinic acid synergistically enhances the flavoring activity of monosodium L-glutamate (MSG). The above nucleotides themselves were also found to have a flavoring activity and moreover, showed a synergistic effect with MSG. Then, the flavoring intensity of an aqueous solution containing 0.05% of a mixture of the nucleotide and MSG in the ratio of 1 to 100 was measured by taste panels and compared with that of the standard sample of 5′-inosinic acid and MSG.[15] The results, in which the flavoring intensity of 5′-inosinic acid is shown as 1.0 are given in Table I. From the fact that X and XI possess almost the same intensity as that of 5′-guanylic acid, it is obvious that the introduction of one or two methyl group to the amino group of 5′-guanylic acid does not affect substantially its original activity.

11) J.D. Smith and D.B. Dunn, *Biochem. J.*, **72**, 294 (1959).
12) P.L. Bergquist and R.E.F. Matthews, *Biochem. J.*, **85**, 305 (1962).
13) M. Ikehara, A. Yamazaki, and T. Fujieda, *Chem. Pharm. Bull.* (Tokyo), **10**, 1075 (1962).
14) S. Ikeda, H. Furukawa, and S. Yamaguchi, *Statistical Quality Control* (Japan), **13**, 76 (1962).
15) Reported by T. Ninomiya, S. Ikeda, S. Yamaguchi, and T. Yoshikawa of our Laboratories at the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April 1, 1967 (Abstract p. 187).

Table III. Flavoring Intensity of 5′-Nucleotides in Synergistic Effect with MSG

Nucleotide	Relative intensity
5′-Inosinic acid·2Na·7.5H_2O	1.0
5′-Guanylic acid·2Na·7H_2O	2.3[16]
VII·2Na·6H_2O	8.0
VIII·2Na·2H_2O	7.5
X·2Na·5.5H_2O	2.3
XI·2Na·2.5H_2O	2.4

On the other hand, VII had an extremely strong flavoring activity and exhibited far higher synergistic flavoring intensity than 5′-guanylic acid, whereas VIII was slightly less effective than VII. Thus it is of interest to note that the methylthio group in position 2 has large influence on the flavoring activity of 5′-inosinic acid. However, 2′,3′-O-isopropylidene-2-methylthioinosine 5′-phosphate (VI) prepared from II was found to be tasteless. Since 2′-deoxyinosine 5′-phosphate[17] and 2′-deoxyguanosine 5′-phosphate have a flavoring activity, the 3′-hydroxy group in the ribose moiety might be essential to the flavoring activity of 5′-purine nucleotide. When VII was converted to IX, the activity decreased considerably. The details of measurement of the flavoring intensity will be reported by Dr. Ninomiya, *et al.* in our laboratories elsewhere.

Further investigation along this line is now in progress.

Experimental[18]

2′,3′-O-Isopropylidene-2-ethylthioinosine (III)——To a solution of the ammonium salt of 2′,3′-O-isopropylidene-2-mercaptoinosine[6] (I, 15 g, 42 mmoles) in 150 ml of water, ethyl iodide (7.8 g, 50 mmoles) was added portionwise. The mixture was stirred vigorously at room temperature for 2 hr. The resulting crystals were filtered and recrystallized from aqueous ethanol to give 12 g (78%) of a pure product.

2-Methylthioinosine 5′-Phosphate (VII)——Phosphoryl chloride (2.8 ml, 30.5 mmoles) was mixed with 20 ml of trimethyl phosphate cooled at −10° in a four-necked flask equipped with a thermometer and a silica gel drying tube. To this solution was added 2′,3′-O-isopropylidene-2-methylthioinosine[6] (II, 5.31 g, 15 mmoles) with stirring while maintaining the temperature below −5°, and the mixture was stirred at −5° for 3 hr. Within 30 minutes, it became clear and turned viscous. Then, the solution was poured into 500 ml of ice water to decompose the excess of phosphoryl chloride and the pH of the resulting solution was adjusted to 1.5 with 4N sodium hydroxide. Paper chromatographical examination exhibited a single spot of VII. The above solution was heated at 70° for 40 minutes with stirring to remove the isopropylidene group. After cooling, the pH was adjusted to 2 with alkaline solution and the solution was passed through a column (3×60 cm) of 200 ml of decolorizing resin.[10] The column was washed with 1 liter of water, and the nucleotide was eluted with 0.5N ammonium hydroxide until eluate became free from ultraviolet absorbing material. After the eluate(1.5 liter) was concentrated to *ca.* 100 ml, one volume of ethanol was added to precipitate the ammonium salt of VII, which was collected by filtration and dissolved in 100 ml of water. The pH of the solution was then adjusted to 7.8 with 1N sodium hydroxide and the solution was heated at 100° for 10 minutes. This procedure was repeated several times to convert completely the ammonium salt of VII to the disodium salt, and two volume of ethanol was added. After being kept for 2 hr at 0°, the precipitate was collected by filtration, recrystallized from aqueous ethanol, and dried at 80° (5 mm) for 5 hr to give

16) M. Ohara, T. Ninomiya, S. Ikeda, S. Yamaguchi, and T. Yoshikawa, *J. Agr. Chem. Soc. Japan*, **40**, 169 (1966).

17) Y. Nakao and K. Ogata reported at the Kanto Branch Meeting of the Agricultural Chemical Society of Japan, Tokyo, November, 1960.

18) All melting points are uncorrected. Ultraviolet absorption spectra were taken with a Hitachi Type EPS-2 automatic recording spectrophotometer, and infrared absorption spectra were measured with a Jasco Model IR-S spectrophotometer. The NMR spectra were measured with a Varian A-60 using tetramethylsilane as internal standard. The pK_a values were determined potentiometrically.

4.3 g (64%) of pure samples[19]; pK_{a_1} 3.05, pK_{a_2} 6.58. The moving distance in paper electrophoresis (10% acetic acid buffer, 800 V, 2 hr) was 10.0 cm. This compound showed infrared absorption bands at 1675 (C=O), 1222 (P=O), 1080 (C–O–C), and 976 (P–O–C) cm^{-1}.

2-Ethylthioinosine 5′-Phosphate (VIII)——2′,3′-O-Isopropylidene-2-ethylthioinosine (III, 5.5 g, 15 mmoles) was treated with phosphoryl chloride (4.1 ml, 45 mmoles) in a manner similar to that described for VII to yield 3.5 g (48%) of a pure product; pK_{a_1} 3.07, pK_{a_2} 6.50.

2′,3′-O-Isopropylidene-2-methylthioinosine 5′-Phosphate (VI)——The compound II (5.3 g, 15 mmoles) was treated with phosphoryl chloride (2.8 ml, 30 mmoles) in 20 ml of trimethyl phosphate in the same manner as that described for VII. After the reaction, the solution was poured into 300 ml of ice water, the pH was adjusted to 6 with alkaline solution, and the solution was passed through a column of the decolorizing resin (300 ml). In this case, the column had been thoroughly washed with ice water of pH 6 to avoid the hydrolysis of isopropylidene group. The column was washed with 2 liter of ice water, and then the nucleotide was eluted with 0.5N ammonium hydroxide. The eluate was concentrated *in vacuo* to afford a gummy product. Trituration of the gum with aqueous ethanol yielded white crystals, which were treated with aqueous sodium hydroxide to give the disodium salt of VI. A pure product was obtained by recrystallization from 50% aqueous ethanol. Yield 1.2 g (16%).

2-Methylsulfonylinosine 5′-Phosphate (IX)——To a solution of the disodium salt of VII (2 g, 4.6 mmoles) in 100 ml of water was added N-chlorosuccinimide (2.4 g, 18 mmoles), and the mixture was stirred at 50° for 2 hr. Paper chromatogram of this solution showed a single spot in solvent C. The solution was made acidic pH 2 by addition of 1N hydrochloric acid and applied to a column of the above resin (80 ml). The ultraviolet absorbing eluate, which was obtained upon eluting the column with 0.5N ammonium hydroxide, was concentrated *in vacuo* below 40°. The resulting residue was dissolved in water, and a solution of barium acetate (1.12 g, 4.1 mmoles) in 15 ml of water was added with stirring after the pH of the solution was adjusted to 8.5 with 1N sodium hydroxide. Addition of a half volume of ethanol gave precipitate, which was washed with ethanol and dried *in vacuo* at 60° for 4 hr. Yield 1.5 g (59%).

N^2-Methylguanosine 5′-Phosphate (X)[20]——Method A: To a solution of phosphoryl chloride (2.8 ml, 30.5 mmoles)in 20 ml of trimethyl phosphate cooled to −10°, 2′,3′-O-isopropylidene-N^2-methylguanosine[6] (IV, 5.4 g, 14 mmoles) was added portionwise, and the mixture was stirred for 3 hr. Within 1 hr, it became clear. Then, the solution was poured into 750 ml of ice water, the pH was adjusted to 1.5 with 4N sodium hydroxide, and the resulting solution was heated at 70° for 40 minutes to remove the isopropylidene group. An aliquot from the solution exhibited two spots on paper chromatogram. The major spot was that of X, and the other (minor) identical with that of N^2-methylguanosine. After the pH being adjusted to 2, the solution was passed through a column of the above resin to absorb X. The column was eluted with 2 liter of 0.5N ammonium hydroxide. Concentration of the eluate to a small volume afforded a gummy product, which was converted to the disodium salt as usual. Although this compound was chromatographically homogeneous, it is difficult to isolate it as crystals. After the above ammonium salt was dissolved in 200 ml of water, the pH of the solution was adjusted to 8.5, and to this, a solution of barium acetate (100 mg) in water was added with stirring. The resulting precipitate, mainly consisted of barium phosphate, was removed by centrifugation. Further addition of a solution of barium acetate (4 g, 14 mmoles) in 30 ml of water gave a precipitate of barium salt, which was collected by centrifugation and washed with water, ethanol, and then ether. A small second crop of product was obtained by adding one volume of ethanol to the filtrate. The combined products, dried *in vacuo*, weighed 6.5 g and analyzed as follows. *Anal.* Calcd. for $C_{11}H_{14}O_8N_5$-PBa·$2H_2O$: C, 24.09; H, 3.29; N, 12.77. Found: C, 24.60; H, 3.81; N, 12.53. The barium salt was suspended in 200 ml of water, 45 ml of Amberlite IR-120 (Na^+ form) resin was added, and the mixture was stirred until all the barium salt dissolved . After the resin filtered off, the filtrate was evaporated under reduced pressure to *ca.* 20 ml. The pH of this solution was 7.7. Addition of a small volume of ethanol afforded the disodium salt as an amorphous powder, which was dried to give 4.1 g (58%) of a pure product; the moving distance in paper electrophoresis (10% acetic acid buffer, 800 V, 2 hr): 5.5 cm. The infrared absorption spectra showed absorption bands at 1680 (C=O), 1100 (C–O–C), and 975 (P–O–C) cm^{-1}.

Method B: To a solution of the disodium salt of VII (1g, 2.1 mmoles) in 40 ml of water was added N-chlorosuccinimide (1.22 g, 9.1 mmoles). After being stirred at 50° for 2 hr, the solution was saturated with gaseous methylamine at 0° and heated in an autoclave at 140° for 2 hr. The solvent was removed *in vacuo*, and the residue was dissolved in water. After being adjusted to pH 2, the solution was passed through a column of the above resin to absorb IX and worked up as usual. The crude product obtained was dissolved in water, the pH was adjusted to 8.5, and a solution of barium acetate (1.1 g, 4.1 mmoles) in 15 ml of water was added. The barium salt, precipitated by addition of ethanol, was collected by centrifugation, washed

19) It was found by Mr. N. Muramatsu, Y. Miyasaka, and K. Kusashio in our Laboratories that the disodium salt hexahydrate of VII was most stable in an ordinary atmosphere. This sample was used in the measurement of flavoring intensity.

20) In the measurement of flavoring intensity, X·Na_2·$5.5H_2O$ was used.

with ethanol, and dried *in vacuo* at 110° for 4 hr. Yield 0.3 g (26%). This compound had an identical infrared spectrum as that obtained by method A.

N^2,N^2-Dimethylguanosine 5'-Phosphate (XI)——2',3'-O-Isopropylidene-N^2,N^2-dimethylguanosine[6] (V, 5 g, 14 mmoles) was phosphorylated with 6.5 g (42 mmoles) of phosphoryl chloride in trimethyl phosphate. The mixture was worked up in the same manner as that described for VII. After phosphorylation followed by acidic hydrolysis, paper chromatogram of the solution revealed a single spot of XI. Recrystallization of crude disodium salt from 50% aqueous ethanol gave colorless crystals, 3.5 g (51%); the moving distance in paper electrophoresis (10% acetic acid buffer, 800 V, 2 hr): 5.5 cm.

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