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Methylated Purines and Pyrimidines. II. Synthesis and Properties of 2,6-Diamino-5-(methylamino)-4-pyrimidinol*

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ABSTRACT: 2,6-Diamino-5-(methylamino)-4-pyrimidinol (compound VIII) was synthesized by an application of the Hinsberg reaction. Sulfonylation of 2,4,5-triamino-6-methoxypyrimidine (compound Ib) gave the 5-sulfonamidopyrimidines II and VI, which were methylated to the corresponding 5-(*N*-methylsulfonamido)pyrimidines (compounds III and VII). Removal of the sulfonyl group by reduction with sodium in liquid ammonia, and of the *O*-methyl group with HBr in acetic acid or with concentrated aqueous acid, gave compound VIII in good yields. The 5-(methylamino)pyrimidine was considerably more stable than the unmethylated compound (Ia) in hot acids, or in alkali in the presence of oxygen. Rather vigorous conditions appeared neces-

sary to cyclize the 5-(*N*-methylformamido)- and 5-(1-methylureido)pyrimidines derived from compound VIII to 7-methylguanine and 8-hydroxy-7-methylguanine, respectively. Heating of compound VIII with ethyl oxalate gave 5-methylleucopterin (compound IX). Preliminary attempts failed to convert compound VIII to 5-methylfolic acid. Unlike 2,5,6-triamino-4-pyrimidinol (compound Ia), compound VIII did not appear to react with 2,3-dibromopropanal plus *p*-aminobenzoyleglutamate. When compound VIII was treated with 2-bromo-3,3'-diethoxypropanal the reaction was very slow compared to that observed with compound Ia. There was no evidence for the formation of a dihydropteridin or a pteridin.

As discussed in the preceding paper (Borowitz *et al.*, 1965), interest in the possible role of 5-methylfolic acid in methionine formation stimulated several unsuccessful attempts to synthesize 2,6-diamino-5-(methylamino)-4-pyrimidinol (compound VIII), which it was hoped would afford 5-methylfolic acid by conventional means (Waller *et al.*, 1948). Further efforts, described in the present communication, were directed toward methylation of 5-sulfonamidopyrimidines and hydrolysis or reduction of the 5-(*N*-methylsulfonamido)pyrimi-

dines to compound VIII. It has been demonstrated conclusively by Wilson (1948) that acylation of 2,5,6-triamino-4-pyrimidinol (compound Ia) afforded the 5-acylaminopyrimidine. Sulfonylation of 2,4,5-triamino-6-methoxypyrimidine (compound Ib), therefore, appeared to be a convenient method to prepare intermediates for methylation. The 6-methoxypyrimidine (compound Ib) was chosen as starting material in order to avoid nuclear substitution, owing to anion formation on N-3, during subsequent alkylation reactions in basic medium.

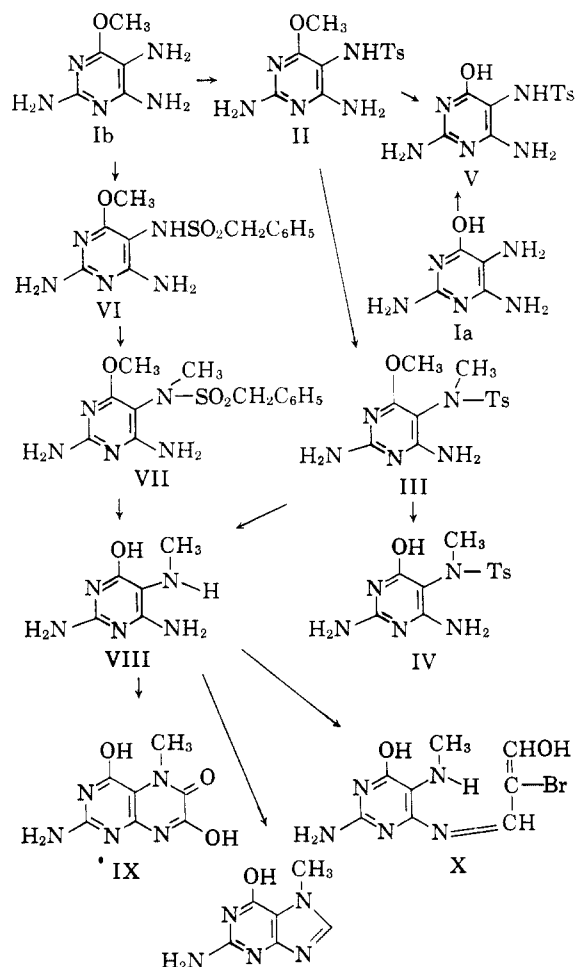
No difficulties were encountered in preparing the *p*-toluenesulfonamide II or the α -toluenesulfonamide VI by usual methods. These were methylated smoothly to the corresponding *N*-methylsulfonamides III and VII with one equivalent of methyl iodide and sodium methoxide in methanol solution, and the products were freed of unmethylated starting material by extraction

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with alkali. The *p*-toluenesulfonyl or the α -toluenesulfonyl group could not be removed by most of the available methods, such as treatment with HBr in glacial acetic acid,¹ or with HBr, HCl, or HI in aqueous solu-



tion, reduction with lithium aluminum hydride in tetrahydrofuran, and, in the case of the α -toluenesulfonyl derivative (compound VII), reduction with Raney nickel. However, reduction with sodium in liquid ammonia gave the *O*-methyl derivative of compound VIII from either III or VII if care were taken to avoid an excess of sodium at any time during the procedure. Without purifying this product the *O*-methyl group was removed with HBr in acetic acid, or with concentrated aqueous HBr, to give good yields of the desired pyrimidine VIII.

The methods developed for alkylating the 5-amino group of the pyrimidine Ib should be generally applicable to the synthesis of 5-alkylaminopyrimidines.² The

reduction of 5-formamidopyrimidines (Brown, 1955) with LiAlH_4 is severely limited by solubility requirements. Ring opening of 9-substituted 7-methylpurines to pyrimidines (Haines *et al.*, 1962; Townsend and Robins, 1963) is similarly limited by the availability of the appropriately substituted purine in which the substituent on N-9 can be removed readily. In a method recently published by Montgomery and Hewson (1961) for the synthesis of 7-methylpurines certain 6-amino-5-formamidopyrimidines were alkylated with alkyl iodides in dimethylformamide and potassium carbonate or sodium hydride, and the resulting 5-(*N*-methylformamido)pyrimidines were converted to 5-methylaminopyrimidines with methanolic HCl. In general, however, this procedure may present difficulties in dissolving the starting material and in removing the unalkylated from the alkylated pyrimidine.

The structure of compound VIII was verified by converting it to 7-methylguanine and to 8-hydroxy-7-methylguanine. Initial reaction of compound VIII with either formic acid or urea occurred under moderate conditions to yield intermediate products. Although these were not characterized completely, they were probably the 5-(*N*-methylformamido)pyrimidine and 5-(1-methylureido)pyrimidine, respectively. These intermediates appeared to require considerably more vigorous conditions for cyclization to purines than usually observed with 5,6-diaminopyrimidines. The conversion with ethyl oxalate of compound VIII to 5-methylleucopterin (compound IX) proceeded without difficulty. However, insufficient data are available for comparison with the 5,6-diaminopyrimidines. A model of compound VIII shows a reasonable amount of steric hindrance between the methyl and 4-oxo groups as ring closure is taking place and the methyl group is brought into the plane of the ring. Steric factors may therefore be responsible for the relatively more vigorous conditions required to convert compound VIII to a purine. It is also worthy of note, as documented under Experimental, that the 5-(methylamino)pyrimidine (compound VIII) is much more stable than the unmethylated analog (compound Ia) toward both acid and base.

More serious difficulties were encountered in attempts to synthesize 5-methylfolic acid from compound VIII by methods which were successful in the synthesis of folic acid from compound Ia, *p*-aminobenzoylglutamate, and a halopropionaldehyde. When compound VIII was treated with 2,3-dibromopropionaldehyde and *p*-aminobenzoylglutamate according to the method used by Waller *et al.* (1948) with compound Ia, only unchanged pyrimidine could be recovered. The 5-methylaminopyrimidine (compound VIII) was also treated with 2-bromo-3,3'-diethoxypropionaldehyde according to the initial step in the folic acid synthesis of Slettinger *et al.* (1955). Whereas in control experiments with compound Ia the water-insoluble bromoacetal reacted and disappeared rapidly, reaction with compound VIII was very slow. The only compound which could be isolated was apparently the Schiff base X resulting from a condensation of the hydrolyzed

¹ Although detosylation occurred in hot HBr-glacial acetic acid, side reactions precluded the isolation of compound VIII (see Experimental).

² After the completion of the synthesis of compound VIII described in the present communication a similar procedure was published by Brederick and Gotsmann (1962) for the synthesis of 4-amino-1,3-dimethyl-5-methylaminouracil. The preparation of compound VIII from Ib, in which the nuclear nitrogen atoms are unsubstituted, indicates the general applicability of the method in this field.

acetal and the 6-amino group of compound VIII, but this product was not characterized. A similar product was isolated by King and Spensley (1952) from reaction mixtures of 2-nitromalondialdehyde, *p*-aminobenzoylglutamate, and compound Ia. It is not clear whether steric factors, discussed in relation to hindered purine formation from compound VIII, were responsible for the apparent lack of displacement of bromine by the 5-methylamino group of compound VIII. More work is required to find the appropriate conditions for the conversion of compound VIII to a 5-methylpteroylglutamate.

The preparation of 7-methylguanine from compound VIII is more convenient than the classical method of Fischer (1897, 1898) from theobromine, via 2,6-dichloro-7-methylpurine and 6-amino-2-chloro-7-methylpurine. However, a recent procedure, depending on methylation of the readily available guanosine and acid hydrolysis of the resulting 7-methylguanosine (Jones and Robins, 1963), offers numerous advantages. On the other hand, guanosine cannot be alkylated to higher homologs of 7-methylguanosine,³ whereas the methods described later can be used to prepare the 5-(ethylamino) and 5-(butylamino) homologs of compound VIII and these can be converted to the 7-alkyl guanines.³

Experimental⁴

N-(2,4-Diamino-6-methoxy-5-pyrimidinyl)-*p*-toluenesulfonamide (Compound II). A solution of 15.2 g (56 mmoles) of 2,4,5-triamino-6-methoxypyrimidine sulfate monohydrate⁵ (compound Ib) in 360 ml of 0.3 N sodium hydroxide at 65° was treated with 11.9 g of powdered *p*-toluenesulfonyl chloride with vigorous stirring. Whenever the pH of the reaction mixture dropped to 7.5, 5 ml of 1 N NaOH was added, until a total of 70 ml had been used. During the course of these additions the temperature was slowly raised to 80° and maintained at this temperature for 15 minutes after the final addition, vigorous stirring being continued throughout. The reaction mixture was cooled and brought to pH 6 with acetic acid. The precipitate was removed by filtration, washed with water, and shaken vigorously with 600-, 300-, and 200-ml portions of 0.2 N NaOH, undissolved solid being collected each time by filtration. The residual insoluble material was discarded. The three sodium hydroxide extracts were combined and acidified to pH 5 with acetic acid, and the precipitate was collected on a filter, washed well with water, air dried, and recrystallized from methanol. The hot methanol solution was either centrifuged or filtered repeatedly to remove a red gelatinous impurity. Yield, 12.6 g (73%), mp 226–228°. The analytical sample had

mp 228.5–230°. Ultraviolet spectrum, $\lambda_{\text{max}}^{\text{H}^+}$ 283 m μ (ϵ 12,700), 233 m μ (ϵ 18,300); $\lambda_{\text{min}}^{\text{H}^+}$ 261 m μ (ϵ 6400).

Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$ (309.3): C, 46.6; H, 4.89; N, 22.6; S, 10.4. Found: C, 46.7; H, 4.92; N, 22.4; S, 10.3.

N-(2,4-Diamino-6-methoxy-5-pyrimidinyl)-*N*-methyl-*p*-toluenesulfonamide (Compound III). A warm solution of 12.6 g (40.6 mmoles) of *N*-(2,4-diamino-6-methoxy-5-pyrimidinyl)-*p*-toluenesulfonamide (compound II) and 2.19 g (40.6 mmoles) of sodium methoxide in 200 ml of dry methanol was treated with 5.77 g (40.6 mmoles) of methyl iodide and refluxed gently for 3.5 hours. It was then concentrated *in vacuo* to a small volume and treated with a large volume of water to dissolve the sodium iodide. The precipitate was removed by filtration, washed well with water, and shaken vigorously with successive 300-ml portions of 0.2 N sodium hydroxide, until the sodium hydroxide extracts no longer yielded a precipitate upon acidification to pH 5 (recovered starting material, 1.07 g). The alkali-insoluble solid was washed well with water and dried; 10.9 g (83%; 91% based on unrecovered starting material), mp 206–209°. Recrystallization from methanol yielded 9.5 g, mp 211–213°. A further 0.75 g, mp 210–213°, was obtained by concentration of the mother liquors. Analytical sample had mp 211–212.5°. Ultraviolet spectrum, $\lambda_{\text{max}}^{\text{H}^+}$ 281–282 m μ (ϵ 12,400), 237 m μ (ϵ 20,700); $\lambda_{\text{min}}^{\text{H}^+}$ 262 m μ (ϵ 6000). $\lambda_{\text{max}}^{\text{OH}^-}$ (95% ethanol) 260–264 m μ (plateau, ϵ 10,600), 235 m μ (ϵ 18,600); $\lambda_{\text{min}}^{\text{OH}^-}$ (95% ethanol) 221 m μ (ϵ 15,500).

Anal. Calcd for $\text{C}_{13}\text{H}_{17}\text{N}_5\text{O}_3\text{S}$ (323.3): C, 48.3; H, 5.30; N, 21.7; S, 9.98. Found: C, 48.4; H, 5.37; N, 21.9; S, 10.0.

N-(2,4-Diamino-6-hydroxy-5-pyrimidinyl)-*N*-methyl-*p*-toluenesulfonamide (Compound IV). To 1 liter of a saturated solution of HBr in glacial acetic acid were added 2.6 g (8.1 mmoles) of *N*-(2,4-diamino-6-methoxy-5-pyrimidinyl)-*N*-methyl-*p*-toluenesulfonamide (compound III) and 1.9 g of phenol. The mixture was stirred overnight at room temperature and concentrated *in vacuo* to a small volume. After the addition of dry ether to complete precipitation, the solid was removed by filtration and dissolved in water, and the aqueous solution was taken to dryness *in vacuo*. The residue was redissolved in water, the solution was taken to dryness, and the solid was dried *in vacuo* (oil pump) over KOH for 4 days to remove tenaciously held HBr. The product was treated with water, collected by filtration, and dried to give 2.0 g of compound IV. The water washings were concentrated to dryness and the residue was treated as described (drying over KOH, and so forth) to give an additional 0.4 g. Total yield, 95%. Recrystallization from ethanol gave 2.1 g (82%), mp 204–207° decomp. Ultraviolet absorption, $\lambda_{\text{max}}^{\text{H}^+}$ 264 m μ (ϵ 18,000), 233 m μ (ϵ 15,600); $\lambda_{\text{min}}^{\text{H}^+}$ 248 m μ (ϵ 12,600). $\lambda_{\text{max}}^{\text{OH}^-}$ 260 m μ (ϵ 7640); $\lambda_{\text{min}}^{\text{OH}^-}$ 250 m μ (ϵ 6700). $\lambda_{\text{max}}^{\text{OH}^-}$ (95% ethanol) 271 m μ (ϵ 14,200), 231 m μ (shoulder, ϵ 13,100); $\lambda_{\text{min}}^{\text{OH}^-}$ (95% ethanol) 249 m μ (ϵ 6300).

Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$ (309.3): C, 46.6; H, 4.89; N, 22.65; S, 10.4. Found: C, 46.6; H, 5.30; N, 22.6; S, 10.7.

³ Private communication from Dr. H. Plaut, Cyclo Chemical Corporation.

⁴ Ultraviolet spectra in aqueous solution were taken in 0.1 N HCl and 0.1 N NaOH.

⁵ Furnished by Cyclo Chemical Corp., Los Angeles, Calif.

N-(2,4-Diamino-6-hydroxy-5-pyrimidinyl)-*p*-toluenesulfonamide (Compound V). Using the procedure described for the preparation of compound II, 9.6 g (37 mmoles) of 2,5,6-triamino-4-pyrimidinol (compound Ia) sulfate monohydrate was treated with 7.6 g of *p*-toluenesulfonyl chloride. The product (9.1 g, 83%) was recrystallized from 1.5 liters of 70% aqueous methanol to yield 6.4 g (58%), decomp 268–270°. Ultraviolet absorption, $\lambda_{\text{max}}^{\text{H}^+}$ 265 m μ (ϵ 16,900), 229 m μ (ϵ 14,800); $\lambda_{\text{min}}^{\text{H}^+}$ 243 m μ (ϵ 9600).

Anal. Calcd for $\text{C}_{11}\text{H}_{13}\text{N}_5\text{O}_3\text{S}$ (295.3): N, 23.7; S, 10.9; Found: N, 23.9; S, 11.1.

Compound V was also obtained by treating compound II with HBr in glacial acetic acid as described for the conversion of compound III to IV.

N-(2,4-Diamino-6-methoxy-5-pyrimidinyl)- α -toluenesulfonamide (Compound VI). The procedure used for the preparation of compound II gave low yields (30–50%) when applied with α -toluenesulfonyl chloride. The following procedure gave improved yields. A well-stirred suspension of 10.1 g (37 mmoles) of 2,4,5-triamino-6-methoxypyrimidine (compound Ib) sulfate monohydrate in 200 ml of water was treated with sufficient 1 N sodium bicarbonate solution to dissolve the pyrimidine salt and bring the pH to 8. Finely powdered α -toluenesulfonyl chloride (11.4 g, 60 mmoles) was added, and the reaction mixture was stirred vigorously at room temperature for 2.5 hours, the pH being maintained at 7.8 ± 0.2 by the addition of 1 N sodium bicarbonate solution. The reaction flask was then immersed in a water bath at 65° for 10 minutes. The product was isolated and purified as described for compound II. Considerable quantities of product are recoverable from the methanol mother liquors. Total yield of recrystallized compound VI, 8.0 g (69%), mp 228–230° decomp. Ultraviolet absorption, $\lambda_{\text{max}}^{\text{H}^+}$ 282 m μ (ϵ 14,200), 238 m μ (ϵ 13,500); $\lambda_{\text{min}}^{\text{H}^+}$ 258 m μ (ϵ 4200).

Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$ (309.3): C, 46.6; H, 4.89; N, 22.6; S, 10.4. Found: C, 46.7; H, 4.97; N, 22.6; S, 10.4.

N-(2,4-Diamino-6-methoxy-5-pyrimidinyl)-*N*-methyl- α -toluenesulfonamide (Compound VII). A refluxing solution of 4.0 g (13 mmoles) of *N*-(2,4-diamino-6-methoxy-5-pyrimidinyl)- α -toluenesulfonamide (compound VI) and 0.71 g (13 mmoles) of sodium methoxide in 150 ml of 80% aqueous methanol was treated with 1.85 g (13 mmoles) of methyl iodide, and refluxing was continued for 2 hours. The solution was concentrated *in vacuo* to 20 ml, diluted with 200 ml of water, and acidified to pH 6. The precipitate was removed by filtration, washed with water, and shaken vigorously with two successive 100-ml portions of 0.2 N NaOH. Only the first extract yielded a precipitate of starting material (0.42 g) upon acidification to pH 6. The alkali-insoluble material was washed well with water and recrystallized from methanol to yield 2.6 g of colorless crystals, mp 204–206°. A further 0.2 g of equally pure product was obtained by recrystallization of material recovered from the mother liquor (yield 67%). Ultraviolet absorption, $\lambda_{\text{max}}^{\text{H}^+}$ 281 m μ (ϵ 14,300), 234 m μ (ϵ 13,400); $\lambda_{\text{min}}^{\text{H}^+}$ 256 m μ (ϵ 3000), 225 m μ

(ϵ 12,100). $\lambda_{\text{max}}^{\text{H}^+}$ (95% ethanol) 266 m μ (ϵ 8700), 239 m μ (ϵ 14,000); $\lambda_{\text{min}}^{\text{H}^+}$ (95% ethanol) 255 m μ (ϵ 7500), 227 m μ (ϵ 11,800).

Anal. Calcd for $\text{C}_{13}\text{H}_{17}\text{N}_5\text{O}_3\text{S}$ (323.3): C, 48.3; H, 5.30; N, 21.7; S, 9.98. Found: C, 48.7; H, 5.69; N, 21.9; S, 9.91.

2,6-Diamino-5-(methylamino)-4-pyrimidinol. (Compound VIII). (A) FROM *N*-(2,4-DIAMINO-6-METHOXY-5-PYRIMIDINYL)-*N*-METHYL- α -TOLUENESULFONAMIDE (COMPOUND VII). A well-stirred solution of 208 mg of compound VII in 90 ml of dry liquid ammonia under a nitrogen atmosphere was treated with small pieces of sodium, one at a time, until a blue color, stable for 20 seconds, pervaded the solution. An excess of dry ammonium chloride was added immediately to discharge the color and the ammonia was evaporated under a stream of nitrogen. The solid residue was taken up in 60 ml of 1 N HCl and diluted with 140 ml of water, and the solution was clarified by filtration and taken to dryness *in vacuo*. The dried residue ($\lambda_{\text{max}}^{\text{H}^+}$ 228 m μ , 278 m μ) was stirred for 5 hours with 100 ml of a saturated solution of HBr in acetic acid, and the mixture was taken to near dryness *in vacuo*. The residue was triturated with dry ether, collected by filtration, and dissolved in water. The solution was evaporated to dryness *in vacuo*, and the residue was redissolved in a little water and added to an excess of a saturated aqueous solution of picric acid. The picrate was collected, washed with water, and decomposed by shaking vigorously in a separatory funnel with a mixture of 2 N HCl and ether. The aqueous layer was extracted four more times with ether and evaporated to dryness *in vacuo*. Residual HCl was removed by repeated addition of absolute ethanol followed by distillation under reduced pressure. The dry residue (77 mg) was taken up in 1.1 ml of hot water, 0.2 ml of 12 N H_2SO_4 was added, and crystallization was induced by cooling and seeding. The product was collected on a filter and washed with ethanol. Yield, 94 mg (54%). Recrystallization from 1 ml of 2 N H_2SO_4 , followed by washing with 95% ethanol and drying at room temperature over P_2O_5 , gave analytically pure material. Ultraviolet spectrum, $\lambda_{\text{max}}^{\text{H}^+}$ 262 m μ (ϵ 14,800); $\lambda_{\text{max}}^{\text{OH}^-}$ 274 m μ (ϵ 13,000), 244–253 m μ (plateau, ϵ 8400). $\lambda_{\text{max}}^{\text{pH}}$ (0.1 M phosphate buffer) 276–277 m μ .

Anal. Calcd. for $\text{C}_8\text{H}_9\text{N}_5\text{O} \cdot \text{H}_2\text{SO}_4 \cdot \text{H}_2\text{O}$ (271.2): C, 22.1; H, 4.83; N, 25.8; S, 11.8; N-CH₃, 5.54. Found: C, 22.4; H, 4.66; N, 26.1; S, 12.0; N-CH₃, 6.06.

Failure was encountered in attempts to obtain compound VIII by removal of the α -toluenesulfonyl group of compound VII with Raney nickel and hydrogen under various conditions.

(B) FROM *N*-(2,4-DIAMINO-6-METHOXY-5-PYRIMIDINYL)-*N*-METHYL-*p*-TOLUENESULFONAMIDE (COMPOUND III). To a well-stirred suspension of 75 g of compound III in 2.5 liters of dry liquid ammonia under a nitrogen atmosphere were added small pieces of sodium, one at a time, each piece being allowed to react completely before the next one was added. The suspended solid gradually dissolved, and addition of sodium was continued until a blue color, stable for about 20 seconds, pervaded the solution. A little dry solid ammonium

chloride was added immediately to discharge the color. Ammonia was allowed to evaporate at room temperature, and evaporation was completed under reduced pressure. The flask containing the solid residue was immersed in an ice bath and 1 liter of cold aqueous 48% HBr containing 15 g of phenol was added carefully. A vigorous reaction took place, and the mixture was stirred at room temperature overnight. The precipitate, which contained only a slight quantity of pyrimidine and consisted principally of benzene-ligroin (1:1) soluble material and ammonium bromide, was removed by filtration and discarded. The filtrate was concentrated under vacuum (oil pump) to one-third its volume (precipitation occurred), treated with three volumes of absolute ethanol, and cooled at -20° overnight. The precipitate was removed by filtration, the filtrate was discarded, and the solid was washed (on a clean suction flask) with 1 liter of water. Undissolved solid was collected and dried, yielding 37.8 g (64%) of analytically pure compound VIII hydrobromide monohydrate (ultraviolet absorption, $\lambda_{\max}^{\text{H}^+}$ 261 μ , ϵ 14,700).

The stirred aqueous washing was treated slowly with an ethanolic solution of 60 g of picric acid and allowed to stand for 20 minutes. The precipitate was removed by filtration, washed, and added to a well-stirred mixture of 1.5 liters of benzene and 1.5 liters of 2 N HCl. When all of the solid had dissolved, the benzene layer was replaced with 1.5 liters of fresh benzene and vigorous stirring was continued for 0.5 hours. The aqueous layer was extracted continuously overnight with ether and taken to dryness under reduced pressure. The residue was dried in a vacuum over KOH and P_2O_5 and recrystallized from 2 N H_2SO_4 . The product was washed with ethanol and dried over P_2O_5 to give 14.1 g (22%) of compound VIII sulfate monohydrate (see method A).

Anal. Calcd for $\text{C}_5\text{H}_9\text{N}_5\text{O} \cdot \text{HBr} \cdot \text{H}_2\text{O}$ (254.1): C, 23.6; H, 4.76; N, 27.6; Br, 31.5. Found: C, 23.9; H, 4.31; N, 27.6; Br, 31.7.

The following reaction conditions failed to remove the tosyl group of compound III: refluxing with 25% HCl for 9 hours; heating in a sealed tube at 100° with acetic acid saturated with HCl for 4.5 hours; standing at room temperature in liquid HBr in a sealed tube for 22 hours; treating with 95% H_2SO_4 at 40° for 66 hours; and refluxing with LiAlH_4 in tetrahydrofuran. Acetic acid saturated with HBr had no effect at room temperature; heating in a sealed tube (with a little xylene to react with Br_2) at 85° for 4 hours, or at 100° for 2 hours, removed the tosyl group, but produced a mixture of products. Elemental analyses and ultraviolet absorption indicated that multiple acetylation and, in part, ring closure to 8-methylpurines had occurred. At 65° the removal of the tosyl group was extremely slow.

In addition to the methods described (HBr-acetic acid, or 48% HBr) for removing the *O*-methyl group, the following were also effective: refluxing in 6 N HCl for 15 minutes; standing in concentrated HCl at room temperature overnight.

As judged by disappearance of ultraviolet absorp-

tion, compound VIII was 35% destroyed by refluxing in 6 N HCl for 4 hours. Under the same conditions, the unmethylated pyrimidine (compound Ia) was 95% destroyed. The ultraviolet absorption of compound VIII was unchanged for over 4 hours in 0.1 N NaOH, while that of compound Ia underwent a continuous change with a shift of λ_{\max} from approximately 275 μ to 261 μ and a steady decrease of λ_{\max} .

N-(2,4-Diamino-6-hydroxy-5-pyrimidinyl)-*N*-methylformamide and 7-Methylguanine. A solution of 3.5 g (13 mmoles) of 2,6-diamino-5-(methylamino)-4-pyrimidinol sulfate monohydrate (compound VII) in 75 ml of hot water was treated with a solution of 3.3 g (13 mmoles) of barium acetate and heated for 40 minutes at 40° . The BaSO_4 was removed by filtration, and the filtrate was taken to dryness under reduced pressure. A portion (1.3 g) of the compound VIII acetate was dissolved in 70 ml of 98% formic acid, the solution was concentrated to 10 ml under reduced pressure, and a further 40 ml of formic acid was added. The solution was refluxed for 4 hours and the solvent was removed under reduced pressure. The crude (*N*-methylformamido)pyrimidine was dissolved in 50 ml of dimethylformamide and refluxed for 5 hours in an unsuccessful attempt at ring closure to 7-methylguanine. Removal of the solvent *in vacuo* and trituration of the residue with ethanol led to the recovery of 730 mg of material whose ultraviolet spectrum ($\lambda_{\max}^{\text{H}^+}$ 263 μ , $\lambda_{\max}^{\text{OH}^-}$ 262 μ) indicated it to be the essentially unchanged formamide, although about 5% of the desired purine could be obtained from it by fractional crystallization from water.

To cyclize the above-mentioned product 100 mg of the solid was heated for 15 minutes at $340\text{--}350^{\circ}$ (Woods metal bath) under a slow stream of nitrogen. The brown material was purified by dissolving in warm 3 N ammonium hydroxide, decolorizing with charcoal, and concentrating the solution under reduced pressure. The white precipitate was collected, washed with a little cold water, and dried; yield, 53 mg. It was identified as 7-methylguanine by its ultraviolet spectrum (Gulland and Story, 1938; Reiner and Zamenhoff, 1957): $\lambda_{\max}^{\text{H}^+}$ 250 μ (ϵ 13,000), 274 μ (shoulder, ϵ 7800); $\lambda_{\max}^{\text{OH}^-}$ 280 μ (ϵ 9400), and by chromatographic comparison with authentic 7-methylguanine (on Whatman No. 1 paper, ascending) using six solvent systems: (a) 1-butanol-85% formic acid-water (77:10:13, v/v); (b) 1-butanol-0.6 N ammonium hydroxide (6:1, v/v); (c) 2-propanol-water-15 N ammonium hydroxide (7:2:1, v/v); (d) 1-butanol-acetic acid-water (5:1:4, v/v, upper layer); (e) 0.05 M phosphate buffer, pH 7.2; (f) 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride buffer, pH 9.

2-Amino-6,8-dihydroxy-7-methylpurine. The free base, compound VIII, was obtained by stirring vigorously 5.05 g (20 mmoles) of compound VIII hydrobromide monohydrate with 28 ml of 1 N NaHCO_3 for 20 minutes. The fine white solid was removed by filtration, washed with water, and dried; yield, 2.71 g (17 mmoles). An intimate mixture of 0.5 g of the free base and 1 g of urea, obtained by grinding in a mortar, was heated

at 190° in an atmosphere of nitrogen until gas evolution ceased and the melt solidified. The solid was cooled, powdered, and washed well with water [$\lambda_{\text{max}}^{\text{H}^+}$ 264 m μ , 234 m μ (shoulder); $\lambda_{\text{max}}^{\text{OH}^-}$ 261 m μ , 232 m μ (shoulder)]. The insoluble portion was collected, dried *in vacuo* over P₂O₅, and reheated under nitrogen to 340–350°. At this temperature gas evolution occurred and the material turned orange. After cooling, the material was washed with 0.1 N HCl, and undissolved solid was taken up in 6 N HCl and treated with charcoal. The solution was taken to dryness *in vacuo*, and the light-yellow residue (200 mg) was dissolved in warm 3 N ammonium hydroxide. After two treatments with charcoal, the solution was concentrated *in vacuo* and the precipitate was collected by filtration (120 mg). It was recrystallized by dissolving in a minimum of hot 6 N HCl and diluting with five volumes of cold water. This material was identified as 2-amino-6,8-dihydroxy-7-methylpurine by its ultraviolet spectrum (Borowitz *et al.*, 1965): $\lambda_{\text{max}}^{\text{H}^+}$ 293 m μ (ϵ 9300), 248 m μ (ϵ 10,200); $\lambda_{\text{max}}^{\text{OH}^-}$ 286 m μ (ϵ 11,300), 255 m μ (ϵ 5200, shoulder), and by paper chromatographic comparison with an authentic sample using the six solvent systems specified previously in the identification of 7-methylguanine.

5-Methylleucopterin (2-Amino-4,7-dihydroxy-5-methyl-6-(5H)-pteridinone) (Compound IX). A suspension of 400 mg of the free base VIII in 17 ml of diethyl oxalate was heated under nitrogen at 175–180° for 1 hour. The reaction mixture was cooled and the product was collected by filtration and washed with ether; yield, 429 mg. The crude product was purified by dissolving in hot 0.1 N NaOH, treating with charcoal, and adding the hot, filtered, solution dropwise to 30 ml of boiling 2 N HCl. After cooling, the solid was collected by filtration and washed with a little cold water. The purification was repeated, yielding 207 mg. Ultraviolet spectrum, $\lambda_{\text{max}}^{\text{OH}^-}$ 241–244 m μ (ϵ 14,900), 258 m μ (ϵ 12,600, shoulder), 280 m μ (ϵ 6400, shoulder), 341–345 m μ (ϵ 8400); $\lambda_{\text{min}}^{\text{OH}^-}$ 230 m μ (ϵ 13,100), 300 m μ (ϵ 3300).

Anal. Calcd for C₇H₇N₅O₃ (209.2); C, 40.2; H, 3.37; N, 33.5. Found: C, 39.9; H, 3.55; N, 33.1.

Exploratory experiments on the condensation of compound VIII with benzil, diacetyl, and phenylglyoxal resulted either in recovery of starting material or in poor yields of highly colored products whose structures were not further investigated. Attempts to obtain 5-methylxanthopterin by the condensation of compound VIII with glyoxylic acid or its derivatives gave more encouraging results and will be the subject of further investigation.

Attempted Synthesis of 5-Methylfolic Acid. Method A (Waller *et al.*, 1948): 2,6-Diamino-5-(methylamino)-4-pyrimidinol (compound VIII) was treated with 2,3-dibromopropanal and *p*-aminobenzoylglutamate as in the method for the synthesis of folic acid from compound Ia. Only starting pyrimidine could be recovered.

Method B (Sletzinger *et al.*, 1955): To a stirred solution of 3 g of NaHCO₃ and 3 g of compound VIII·HBr·H₂O in 90 ml of water at 30°, in an atmosphere of nitrogen, was added dropwise 2.6 g of 2-bromo-3,3'-diethoxypropanal (Sletzinger *et al.*, 1955) over a

period of 25 minutes. The vigorously stirred reaction mixture was kept at 25–30° for another hour. No precipitate appeared and essentially all of the aldehyde remained undissolved. (In a control experiment with compound Ia under the same conditions the aldehyde went into solution rapidly.) After continued stirring overnight at room temperature the aldehyde went into solution, and the precipitate which appeared was removed by filtration. Recrystallization (with considerable loss) from hot 0.1 M phosphate buffer, pH 7.4, in a nitrogen atmosphere, gave 0.7 g of a light-tan product. Ultraviolet spectrum, $\lambda_{\text{max}}^{\text{H}^+}$ 272 m μ , 296 m μ , which reverted to that of compound VIII on refluxing in 0.1 N HCl for 15 minutes. Changes in ultraviolet absorption of solutions in 0.1 N NaOH indicated slow decomposition. The product had no ethoxyl groups, but contained bromine which did not react with AgNO₃ solution in the cold, and only very slowly on heating.

Anal. Calcd for C₈H₁₀N₅O₂Br (288.1); C, 33.4; H, 3.50; N, 24.3; Br, 27.7. Found: C, 34.5; H, 3.66; N, 24.2; Br, 27.3.

Unreacted pyrimidine (compound VIII) was recovered as the 5-(*N*-methylbenzamido)pyrimidine by treating the filtrate from the reaction mixture with benzoyl chloride and 1 N NaHCO₃. The product was recrystallized from 95% ethanol, mp > 300°. Ultraviolet absorption, $\lambda_{\text{max}}^{\text{H}^+}$ 267 m μ (ϵ 17,500); $\lambda_{\text{max}}^{\text{OH}^-}$ 263 m μ (ϵ 11,700).

Anal. Calcd for C₁₂H₁₃N₅O₂·H₂O (277.3); C, 52.0; H, 5.45; N, 25.3. Found: C, 52.4; H, 5.66; N, 24.5.

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A General Procedure for the Isolation of "Minor" Nucleosides from Ribonucleic Acid Hydrolysates*

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ABSTRACT: A general procedure for the isolation of minor components of ribonucleic acid (RNA) is described. Samples of RNA are hydrolyzed enzymatically to a mixture of nucleosides and the hydrolysate is resolved into major fractions corresponding to the major ribonucleosides by means of column partition chromatography, neutral solvents being used throughout. These major fractions are resolved into a number of subfractions by further column partition chromatography using different solvent systems. The minor

nucleosides contained in each of these subfractions can be readily isolated and purified by means of paper chromatography. Eighteen minor nucleosides were obtained from a sample of yeast s-RNA. Seventeen of these have been detected previously in this and in other laboratories. The eighteenth nucleoside, which had not been previously detected, appears to consist of adenine and two molecules of ribose. It has been tentatively assigned the structure 9[2'(3')-O-riboseyl- β -D-ribofuranosyl]adenine.

More than twenty minor nucleosides have been detected in the hydrolysates of samples of RNA isolated from a variety of sources. The question as to whether all these compounds are genuine constituents of RNA must be assessed on the basis of the evidence available for each compound. Many of the compounds, especially the methylated base constituents, have been isolated from several sources under different conditions. Possibly the most convincing evidence is that some of these compounds have been isolated as components of oligonucleotides obtained from partial enzymatic digests of RNA (see, for example, Staehelin, 1964). Some of the more recently described constituents have not yet been isolated as nucleotides but as will be described in this paper they and the previously known minor constituents, eighteen in all, have been isolated under mild conditions from an enzymatic digest of yeast s-RNA.

There are several classes of minor components, of which the one comprising the methylated bases contains the largest number of known members, and in fact it appears that the majority of the theoretically possible methylated derivatives of the common bases have already been found. The biological significance of the methylated bases has not been established but results

of research in several laboratories¹ have shed considerable light on their origin and possible function. These minor constituents arise by direct methylation of the intact RNA molecule and, what is most significant, the methylation appears to be highly specific. Thus, the methylated bases are not distributed randomly in an individual RNA molecule, but appear to be specifically located. These findings have led to speculation that the methylated bases may provide for a recognition mechanism and this possibility has been investigated in the case of a bacteriophage-host system (Gold *et al.*, 1964).

Essentially nothing is known about either the origin or the biological function of the other types of minor components, but subtle modifications of the physical and chemical properties conferred on individual species of RNA molecules by these compounds can be visualized. For example, Honjo *et al.* (1964) have found that 2'-O-methylribonucleoside 5'-phosphates are resistant to the action of bull semen and snake venom 5'-nucleotidases. This suggests that analogous modifications in substrate specificity toward RNA hydrolytic enzymes could be expected at points where such compounds are located.

It is doubtful that the number of minor constituents of RNA known at present even closely approaches the

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¹ Much of this work is summarized in a recent review by Srinivasan and Borek (1964).