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104. Yoshihisa Mizuno, Morio Ikehara, and Kyoichi A. Watanabe : Potential Antimetabolites. II.[*1] Chemical Synthesis of 6-Azacytidine.[*2]

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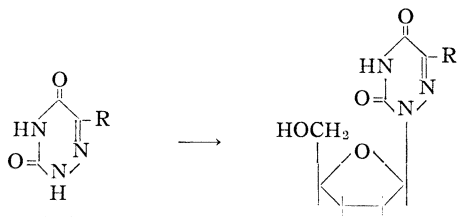
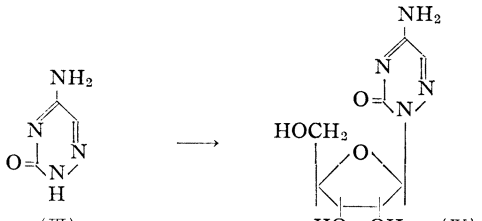
The interest which in recent years has been attached to the chemical synthesis of nucleosides of 1,2,4-triazine derivatives has increased by the remarkable progress of biochemical studies on antimetabolites. For example, Prusoff, *et al.* observed the transformation of 6-methyl-1,2,4-triazine-3,5(2*H*, 4*H*)-dione (6-azathymine) (I; R=CH_3) in bacterial systems into its deoxyriboside[1,2] (II; R=CH_3, R′=H) which, in contrast to the inertness of the free base, inhibits the biosynthesis of deoxyribonucleic acid.[3~5]



(I) (II)

Riboside of 1,2,4-triazine-3,5(2*H*, 4*H*)-dione (I; R=H) (6-azauracil), which interupts pyrimidine nucleoside formation by inhibition of orotydylic acid decarboxylase through conversion to its 5′-monophosphate[6] in some mutant of microorganisms[7,8] or mammalian tumor systems[9,10] despite the ineffectiveness of 6-azauracil, has also been prepared enzymatically.[11~13]

Accumulation of 2-(β-D-ribofuranosyl)-5-amino-1, 2, 4-triazin-3(2*H*)-one (6-azacyti-



(III) (IV)

*1 Part I. This Bulletin, **10**, 647(1962).

*2 A part of the work was reported at the 7th Hokkaido Local Meeting of the Pharmaceutical Society of Japan, held on October 8, 1960.

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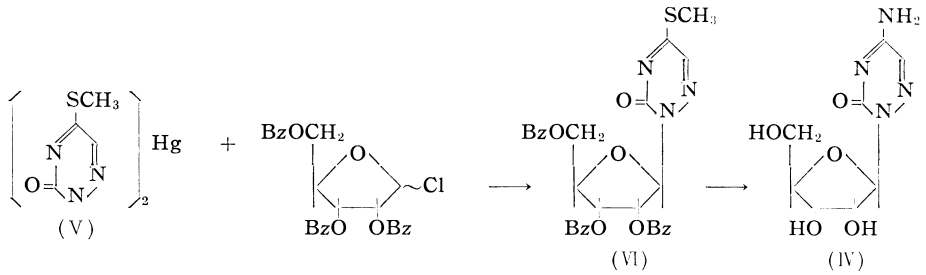
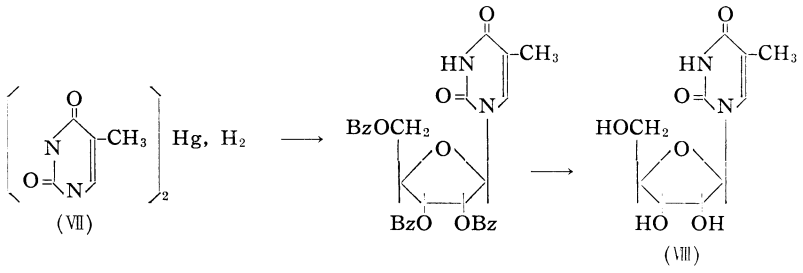
1) W. H. Prusoff, W. L. Holmes, A. D. Welch : Cancer Research, **14**, 570 (1954).
2) W. H. Prusoff : J. Biol. Chem., **215**, 809 (1955).
3) W. H. Prusoff, A. D. Welch : *Ibid.*, **218**, 929 (1956).
4) W. H. Prusoff, L. G. Lajatha, A. D. Welch : Biochim. et Biophys. Acta, **20**, 209 (1956).
5) R. H. Hall, R. Haselkorn : J. Am. Chem. Soc., **80**, 1138 (1958).
6) R. E. Handschumacher, C. A. Pasternak : Biochim. et Biophys. Acta, **30**, 451 (1958).
7) R. E. Handschumacher : *Ibid.*, **23**, 428 (1957).
8) *Idem* : J. Biol. Chem., **235**, 764 (1960).
9) R. Shindler, A. D. Welch : Science, **125**, 548 (1957).
10) C. A. Pasternak, R. E. Handschumacher : J. Biol. Chem., **234**, 2992 (1959).
11) J. Skoda, V. E. Hess, F. Šorm : Experientia, **13**, 150 (1957).
12) F. Šorm, H. Keilova : *Ibid.*, **14**, 215 (1958).
13) R. E. Handschumacher : Nature, **182**, 1090 (1958).

dine) (IV) in the medium of *Escherichia coli* B. grown in the presence of 5-amino-1,2,4-triazin-3(2*H*)-one (6-azacytosine) (III) was reported quite recently.[14]

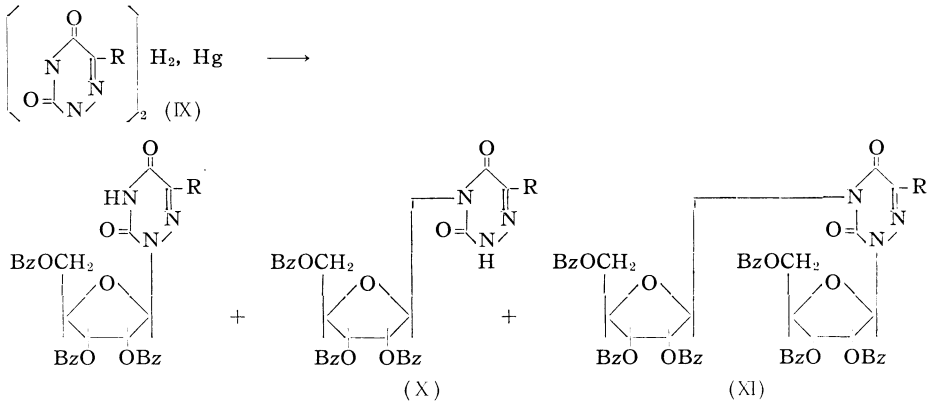
The work described in this paper was undertaken with the object of preparing 6-azacytidine by condensation of mercury salt of 5-methylthio-1,2,4-triazin-3(2*H*)-one (V) with 1-chloro-2,3,5-tri-O-benzoyl-D-ribofuranose followed by ammonolysis of the condensation product.



Although thymineriboside (VIII) was prepared[15] successfully by condensation of dithyminylmercury (VII) with halosugar in boiling xylene, according to the direction of Davoll and Lowy,[16] when the same type of mercury salt of 6-azathymine (IX; R=CH_3)[17]

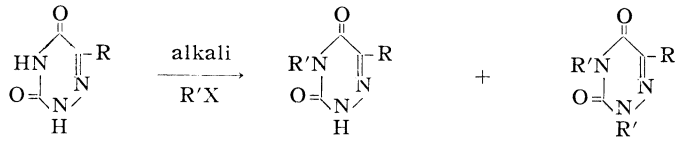


or 6-azauracil (IX; R=H)[8] is subjected to the condensation reaction under similar condition as above, the product obtained is always a mixture of three nucleosides and the isolation of the desired derivative would, therefore, be very complicated and the yield becomes poor.

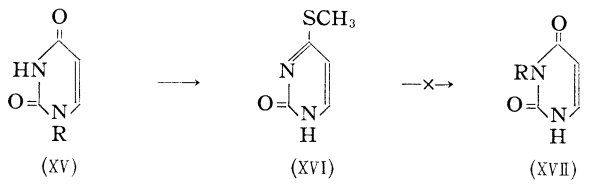


14) E. Bresnik, S. Singer, G. H. Hitchings : Biochim. et Biophys. Acta, **37**, 251 (1960).
15) J. J. Fox, N. Yung, J. Davoll, G. B. Brown : J. Am. Chem. Soc., **78**, 2117 (1956).
16) J. Davoll, B. A. Lowy : *Ibid.*, **73**, 150 (1951).
17) R. H. Hall : *Ibid.*, **80**, 1145 (1958).

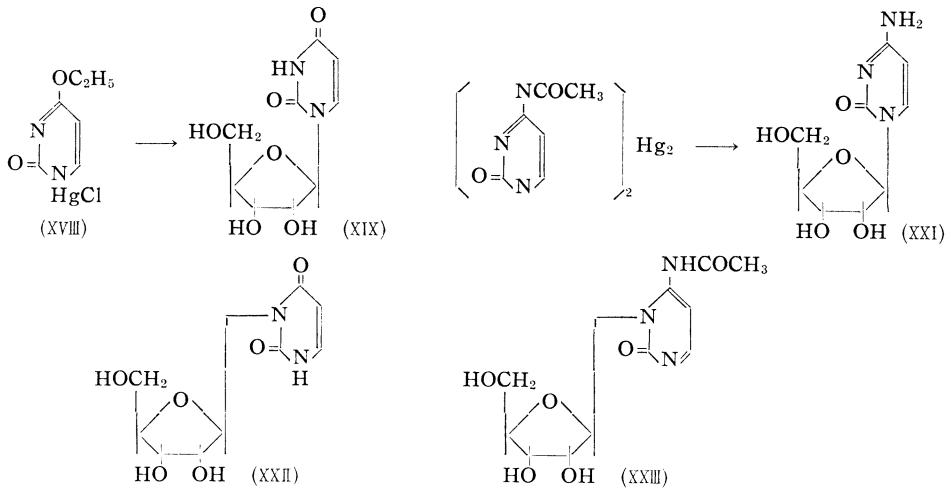
One of the main reasons for the undesirable result lies in the fact that the hydrogen atom attached to N_4 is also, or more, dissociable, and, in fact, Bougault obtained only monoalkyl derivatives accompanied with dialkyl derivatives (XIV) by treating alkali metal salts of 1,2,4-triazine-3,5(2*H*, 4*H*)-dione (XII) with alkyl halide, and the monoalkyl derivative was identified as 4-alkyl derivative (XIII).[18,19]



Consequently, if lactim-lactam tautomerism is inhibited, i.e., if π-electrons are fixed between N_4 and C_5, an improved yield of the desired nucleoside might be anticipated. Wheeler and Johnson,[20] in a series of their researches on pyrimidines, obtained 1-alkyluracil (XV) by alkylation of 4-methylthio-2(1*H*)-pyrimidinone (XVI) followed by acid hydrolysis. In this case, isomeric 3-alkyl derivative (XVII) was not detected. Later, Fox,



et al.,[21] obtained uridine (XIX) and cytidine (XXI) by treatment of mercuric chloride salt and mercury salt of 4-O-ethyluracil (XVIII) and 4-N-acetylcytosine (XX) with 2,3,5-tri-O-benzoyl-D-ribofuranosyl chloride in boiling xylene and toluene, respectively, followed by debenzoylation. In each case, also, they isolated only naturally occurring nucleosides, and the isomeric compounds, (XXII) and (XXIII), analogous to (X), were not obtained.

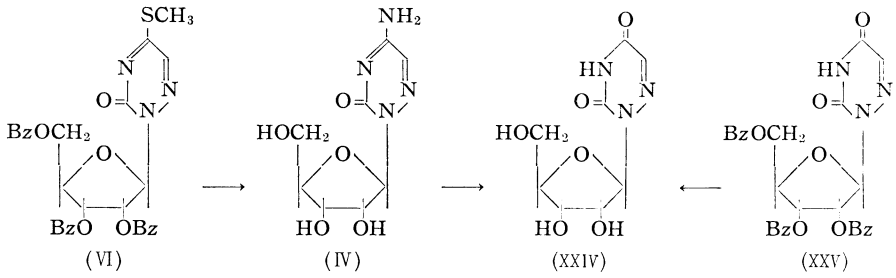


18) J. Bougault : Ann. chim., (9), **5**, 317 (1916).
19) J. Bougault : Compt. rend., **160**, 625 (1915).
20) H. L. Wheeler, T. B. Johnson : Am. Chem. J., **42**, 30 (1909).
21) J. J. Fox, N, Yung, I. Wempen, I. L. Doerr : J. Am. Chem. Soc., **79**, 5060 (1957).

As a rule it is well known that dialkyl sulfide forms a complicated complex with inorganic mercury salt and this property is useful for purifying organosulfur compounds.[22] In this laboratory, however, a successful preparation of 2′,3′,5′-tri-O-benzoyl-β-D-ribofuranoside of 2,6-dibenzylthio- or 2,6-dimethylthiopurine was effected by general mercury salt procedure.[23] Therefore, alkylthio triazine was employed as an intermediate to nucleoside. Since mercury salt of benzylthio derivative decomposed at about 91°, mercury salt of 5-methylthio-1,2,4-triazin-3(2*H*)-one (V) was employed.

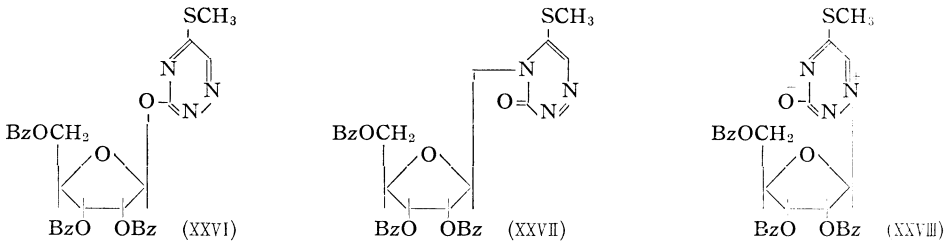
In the course of preparing this mercury compound, a curious phenomenon was observed. When an attempt was made to obtain monochloromercury derivative of the triazine using 1:1:1 molar ratio of the triazine, sodium hydroxide, and mercuric chloride, the precipitate formed contained no chlorine and its analytical data agreed with that calculated for the molar ratio of the triazine and mercury of 2:1. This salt had no definite melting point, but decomposed at about 136~145° according to the rate of heating. On the other hand, when 2:2:1 ratio of the triazine, alkali, and mercuric chloride was used, the salt obtained gave a strong test for chlorine, decomposed at about 81~91° according to the rate of heating, and its analytical data indicated the salt to be a mixture. The experiment was repeated, but the result was well reproducible.

The condensation reaction of the above mercury salt of methylthio derivative and tri-O-benzoyl-D-ribosyl chloride was carried out in boiling benzene, but the condensate was against expectation, a mixture of three nucleosides, which could easily be separated as crystalline substances by alumina chromatography. One of these nucleosides, melting sharply at 136~137°, gave a strong test for sulfur and its infrared absorption spectrum showed that it contained neither -OH- nor -NH-group. On treatment with methanolic



(VI) (IV) (XXIV) (XXV)

ammonia this compound gave almost quantitatively a free nucleoside melting at 216~217°, which, on hydrolysis with dilute hydrochloric acid, was converted into an alternate nucleoside, whose ultraviolet absorption spectrum was quite similar to the nucleoside derived from one of the other two benzoyl derivatives. The benzoyl nucleoside, melting at 186°, contained no sulfur and had a strong absorption at 3240 cm^{-1}, indicating that a hydrogen atom is attached directly to oxygen or nitrogen. The third nucleoside had

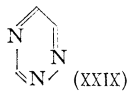


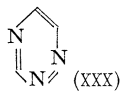
(XXVI) (XXVII) (XXVIII)

22) F. Challenger : "Aspects of the Organic Chemistry of Sulfur" 9 (1959), Butterworth Scientific Publications, London.
23) M. Ikehara, T. Ueda, S. Horikawa, A. Yamazaki : This Bulletin, **10**, 665(1962).

neither free -OH- nor -NH-group and gave a strong test for sulfur, but melted at 123～124°, and meltng point depressed to 119～120° on admixture with the above nucleoside benzoyl derivative melting at 136～137°. This compound, when treated with acid, liberated 6-azauracil, while the other two nucleosides did not afford the free base by this treatment.*4

The nucleoside of the type in which the sugar moiety is attached to N_4 (XXVII) might be unstable to acids,[17] but would be produced less than the desired N^2-nucleoside (VI) by the above procedure because of the existence of steric effect of methylthio group and according to Maccoll's calculation which indicates (XXIX) to be more stable than (XXX).[24] Another possibility for a compound is attachment of the sugar moiety at N_1 (XXVIII).


(XXIX)


(XXX)

This would mean an internally compensated quaternary compound. A positively charged linkage such as (XXVIII) would be expected to decompose easily in acid during the procedure of isolation.

Considering the above evidences, the nucleoside melting at 136～137° was assigned as the desired benzoyl derivative (VI), the one melting at 186° as the decomposed product of (VI), namely, (XXV), and the free nucleoside derived from (VI) by methanolic ammonia treatment as the desired 6-azacytidine (IV). The third nucleoside was assigned as O-glycoside (XXVI).

Experimental

Mercury Salt of 5-Methylthio-1, 2, 4-triazin-3(2*H*)-one (V)——5-Methylthio-1, 2, 4-triazin-3(2*H*)-one[25] (1.43 g., 0.01 mole) was dissolved in 25 cc. of EtOH with heating, and after cool to room temperature, freshly prepared solution of 0.2*N* NaOH (50 cc., 0.01 mole) was added. To the clear solution thus obtained, 2.72 g. of $HgCl_2$ dissolved in 12 cc. of EtOH was added with stirring and the stirring was continued for 0.5 hr. The white precipitate formed was collected by centrifugation, washed thoroughly with H_2O, EtOH, and finally with Et_2O. The salt was dried over P_2O_5 in a diminished pressure at 70～80°. White powder, decomposed at about 136～145° according to the rate of heating. This salt gave negative test for chlorine. Yield, 2.24 g., or 92.6%. *Anal.* Calcd. for $C_5H_5O_2N_6HgS_2$: C, 19.83; H, 1.63; N, 17.36. For $C_5H_5O_2N_6HgS_2 \cdot H_2O$: C, 19.12; H, 1.99; N, 16.73. Found : C, 19.12; H, 2.16; N, 17.31.

Another experiment using 2:2:1 molar ratio of the triazine (1.43 g., 0.01 mole), alkali, (50 cc. of 0.2*N* NaOH, 0.01 mole), and $HgCl_2$, (1.37 g., 0.005 mole) afforded a pale yellow precipitate (1.72 g.) which decomposed at about 81～91° according to the rate of heating. This material gave a strong positive test for chlorine. *Anal.* Calcd. for $C_5H_5O_2N_6HgS_2$: C, 19.83; H, 1.63; N, 17.36. For $C_4H_4ON_3ClHgS$: C, 12.70; H, 1.06; N, 11.1. Found : C, 14.8; H, 1.8; N, 14.1.

Condensation of the Mercury Salt (V) with 1-Chloro-2,3,5-tri-O-benzoyl-D-ribofuranose—— A solution of 10.08 g. (0.02 mole) of 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose[26,27] in 325 cc. of dehyd. Et_2O was saturated with dry HCl at 0°. After 1 week in an ice-box, Et_2O was evaporated and the residue was redissolved in 70 cc. of dehyd. benzene. Benzene was distilled off in a reduced pressure below 30° and the residue redissolved in 70 cc. of benzene to be re-evaporated. This procedure was repeated twice more, the residue was dissolved in 70 cc. of benzene, and added to a previously set up reaction as follows : the mercury salt (V), (5.26 g., 0.011 mole) decomposing at about 136～145°, was suspended in 600 cc. of dehyd. benzene in a three necked flask equipped with a stirrer and condenser. Before addition of the above sugar solution, 195 cc. of benzene was distilled off, and

*4 P. A. Levene and H. Sobotka studied the lability of pyrimidine O-glycosides in acid and base. (J. Biol. Chem., **65**, 469 (1925)).

24) A. Maccoll : J. Chem. Soc., **1946**, 670.

25) M. Barczai-Wartos : Nature, **165**, 369(1950).

26) F. Ishikawa, A. Nomura, T. Ueda, M. Ikehara, Y. Mizuno : This Bulletin, 8, 380 (1960).

27) E. F. Recondo, H. Rinderknecht : Helv. Chim. Acta, **42**, 1171 (1959).

further 30 cc. was then evaporated off. The reaction mixture was stirred for 3 hr. Pale yellow suspension gradually became brown.

The solution was filtered and the filtrate was evaporated to dryness in a diminished pressure. The residue was dissolved in 120 cc. of $CHCl_3$, and the solution was extracted twice with an equal volume of 30% KI solution twice with 5% sodium versenate solution, once with 0.1*N* HCl, and finally with H_2O. After drying over anhyd. Na_2SO_4, $CHCl_3$ was evaporated to dryness. The residue was a reddish, glassy solid, and weighed 11.6 g. This contained three nucleosides and an excess of unreacted triazine.

The mixture was separated by alumina column as follows : Merk reagent grade alumina (acid-washed, 100 g.) was used directly in preparing a column 4.5 $cm^2 \times 21.5$ cm., with benzene as the initial solvent. The mixture was dissolved in 50 cc. of benzene and passed through the column. The column was eluted at a flow rate of 100 cc. per 40 min. The first 200 cc. of benzene fraction, after evaporation to dryness, left 3.3 g. of slightly reddish syrup, which was dissloved in 12 cc. of hot EtOH, treated with animal charcoal, cooled, and yellowish syrup separated, which gradually solidified on standing below 10°. The solid was purified by repeated recrystallization from EtOH until the melting point became 123~124°. Further 300 cc. of benzene fraction gave 96 mg. of the same material. The total yield of the pure substance was 982 mg. The compound gave a strong positive test for sulfur and nitrogen and had neither -OH nor -NH-group. This was assigned as (XXVI). *Anal.* Calcd. for $C_{30}H_{25}O_8N_3S$: C, 61.33; H, 4.26; N, 7.16. Found : C, 61.38; H, 4.46; N, 7.31.

The column was then washed with 500 cc. of 10% AcOEt in benzene which eluted 4.2 g. of a substance. By a similar treatment as above, 1.2 g. of slightly yellowish nucleoside of m.p. 136~137° was obtained, and from the mother liquor, 1.8 g. of the compound, m. p. 161°, was isolated. The former gave a strong positive test for sulfur; UV $\lambda_{max}^{CHCl_3}$ mμ (ε) : 232 (4.6×10^4), 302 (1.4×10^4); $\lambda_{min}^{CHCl_3}$: 257 mμ (4.9×10^3). $[\alpha]_D^{16.0}$ −102.1° (c=5.55, $CHCl_3$). Its infrared absorption spectrum indicated that this nucleoside had no -OH or -NH-group. This was assigned as (VI). *Anal.* Calcd. for $C_{30}H_{25}O_8N_3S$: C, 61.33; H, 4.26; N, 7.16. Found : C, 61.48; H, 4.49; N, 6.76.

The compound of m.p. 161° gave a positive test for sulfur, but when the melting point rose to 183~186° after repeated recrystallization from EtOH, it gave negative test for sulfur. It had a sharp absorption band at 3240 cm^{-1} indicating the presence of -NH-group. UV : $\lambda_{max}^{CHCl_3}$ 264 mμ (8.35×10^3). This was assigned as (XXV). *Anal.* Calcd. for $C_{29}H_{23}O_9N_3$: C, 62.48; H, 4.13; N, 7.54. Found : C, 62.07; H, 4.34; N, 7.60.

Another experiment with 7.2 g. of 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose reacting for 1 hr. afforded 2.03 g. of the nucleoside of m.p. 136~137° (24.1%). Other experiments using toluene or xylene as the solvent afforded only a trace of crystalline (XXV) and not the desired derivative.

2-β-D-Ribofuranosyl-5-amino-1,2,4-triazine-3(2*H*)-one (6-Azacytidine) (IV)——This new nucleoside was obtained almost quantitatively from the pure benzoyl nucleoside (VI). A mixture of 0.615 g. (0.00105 mole) of (VI) in about 53 cc. of dehyd. MeOH was saturated with dry NH_3 below 0 and the nucleoside dissolved during the saturation of NH_3. The mixture was kept standing at room temperature for 3 days in a tightly sealed flask. When the flask was opened there was some pressure and a strong odor of CH_3SH and NH_3. The mixture was evaporated in a diminished pressure. The residue, a white powder, was washed thoroughly with $CHCl_3$ to remove BzOMe and $BzNH_2$. The yield of the crude material, melted at 215°*5 and decomposed at above 225°, was 0.245 g. (95.7%). This was purified by recrystallization from EtOH-hexane to white prisms, m.p. 216~217°. When heated above 225° it decomposed with effervescence to a brown oil. $[\alpha]_D^{16.5}$ −102.1° (c=0.595, H_2O). UV : $\lambda_{max}^{H_2O}$ 265 mμ (ε 7.2×10^3). *Anal.* Calcd. for $C_8N_{12}O_5N_4$: C, 39.3; H, 4.9; N, 23.0. Found : C, 39.3; H, 5.2; N, 23.2.

Hydrolysis of 6-Azacytidine——Above 6-azacytidine (ca. 0.02 g.) dissolved in 2*N* HCl (20 cc.) was warmed on a water bath and this deamination reaction was followed by paper chromatography. A single spot bearing both ultraviolet absorption and positive sugar reaction[28] was soon obtained. The solvent was evaporated to dryness in vacuum below 10°. The white syrup thus obtained, though it did not crystallize from EtOH, had ultraviolet absorption maximum (in H_2O) at 262 mμ (ε 6.2×10^3), which agreed well with that for 6-azauridine reported by Šorm, *et al.*[11]

Condensation of Mercury Salt of 6-Azauracil (IX; R=H) with Acetobromoglucose——A mixture of 3.7 g. (0.087 mole) of mercury salt of 6-azauracil[8] in 100 cc. of dehyd., hot xylene and of 4.1 g. (0.01 mole) of acetobromoglucose[25] was stirred under reflux. Within a few min. a clear solution resulted. After 3 hr., xylene solution was filtered and the filtrate was evaporated to dryness in a diminished pressure. The residue was dissolved in 100 cc. of $CHCl_3$ and the solution was extracted consecutively with 30% KI solution, 0.5 *M* sodium versenate solution, 0.1*N* HCl, and finally with H_2O. The $CHCl_3$ solution was dried over anhyd. Na_2SO_4. Evaporation of $CHCl_3$ left 5.1 g. of a syrup

28) M. Viscontini, O. Hoch, P. Karrer : Helv. Chim. Acta, 38, 642 (1955).

which contained three nucleosides and unreacted glucose derivative.

Separation of the Mixture on Activated Alumina——A solution of 4.0 g. of the above mixture dissolved in benzene (40 cc.) was passed through an alumina (200 g. of acid-washed, 5.65 $cm^2 \times 41$ cm.) column, with benzene as the initial solvent. The column was washed with the following solvents: 1.5 L. of benzene, then 2.2 L. of 5% AcOEt in benzene, which eluted 1.2 g. of unreacted acetyl glucose contaminated with 2,4-bistri-O-acetyl-D-glucoside (XXXIII) of the 1,2,4-triazine; 2.7 L. of 15% AcOEt in benzene, which gave 0.7 g. of (XXXIII), 0.5 L. of 20% AcOEt in benzene, which eluted 0.2 g. of a mixture of (XXXIII) and 2-glucoside (XXXI), 2 L. of 25% AcOEt in benzene, which eluted 0.3 g. of (XXXI), and 30% AcOEt in benzene, which eluted 0.4 g. of 4-glucoside (XXXII) contaminated with (XXXI). Purification by repeated recrystallization from MeOH afforded the following products: 2,4-Bis-(2′,3′,4′,6′-tetra-O-acetyl-β-D-glucopyranosyl)-1,2,4-triazine-3,5-(2*H*,4*H*)-dione (XXXIII), 170 mg., as white needles, m.p. 209°, $[\alpha]_D^{14.0}$ −41.0° (c=0.501, $CHCl_3$). *Anal.* Calcd. for $C_{31}H_{39}O_{20}N_3$: C, 48.1; H, 5.0; N, 5.4. Found: C, 48.0; H, 5.0; N, 5.3.

2-(2′,3′,4′,6′-Tetra-O-acetyl-β-D-glucopyranosyl)-1,2,4-triazine-3,5(2*H*,4*H*)-dione (XXXI), 70 mg., as white needles, m.p. 164～165°. *Anal.* Calcd. for $C_{17}H_{21}O_{11}N_3$: C, 46.1; H, 4.7; N, 9.5. Found: C, 46.0; H, 4.7; N, 9.4.

4-(2′,3′,4′,6′-Tetra-O-acetyl-β-D-glucopyranosyl)-1,2,4-triazine-3,5(2*H*,4*H*)-dione (XXXII), 70 mg., as white leaflets, m.p. 171～173°. *Anal.* Calcd. for $C_{17}H_{21}O_{11}N_3$: C, 46.1; H, 4.7; N, 9.5. Found: C, 46.2; H, 4.9; N, 9.6.

2,4-Bis-β-D-glucopyranosyl-1,2,4-triazine-3,5(2*H*,4*H*)-dione——A solution of 100 mg. of (XXXIII) in 60 cc. of NH_3-MeOH, previously saturated at 0°, was kept standing for 3 days at room temperature. The solvent was evaporated to dryness in a reduced pressure and the residue was crystallized from EtOH to white leaflets of 2,4-bis-β-D-glucopyranosyl-1,2,4-triazine-3,5(2*H*,4*H*)-dione, m.p. 165°. Yield, 38 mg. (66%). *Anal.* Calcd. for $C_{15}H_{23}O_{12}N_3 \cdot H_2O$: C, 39.6; H, 5.5; N, 9.2. Found: C, 39.3; H, 6.0; N, 9.0.

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Summary

6-Azacytidine (IV) was prepared by condensation of mercury salt of 5-methylthio-1,2,4-triazin-3(2*H*)-one with 1-chloro-2,3,5-tri-O-benzoyl-D-ribose in boiling benzene followed by ammonolysis of the condensation product. The synthesis of three isomeric N-glucosides of 6-azauracil is also described.

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