Nucleosides. XX. 1-α-D-Ribofuranosylthymine[1]

JIŘÍ FARKAŠ, LOUIS KAPLAN, AND JACK J. FOX

The Divisions of Nucleoprotein Chemistry and Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Sloan-Kettering Division Graduate School of Medical Sciences, Cornell University Medical College, New York 21, New York

Received October 22, 1963

"5-Methyluridine" prepared a decade ago by the Hilbert–Johnson procedure is now characterized as 1-α-D-ribofuranosylthymine (II). It is demonstrated that the Hilbert–Johnson reaction yields the known 1-β-D-ribofuranosylthymine (the true 5-methyluridine, I) as well as its α-anomer, II. A long-reported "5-methylcytidine" originating from the same procedure is most probably the α-anomer of true 5-methylcytidine, the latter prepared from I. The failure of these anomeric 1-D-ribofuranosylthymines and 5-methylcytosines to obey Hudson's Rules of isorotation shows that optical rotation cannot be used to assign configuration to anomers of pyrimidine nucleosides *in general*. The glycosyl linkage of the β-anomer (I) is cleaved by the pyrimidine phosphorylase of *Bacillus subtilis* 16; the α-anomer (II) is not cleaved by this enzyme.

5-Methyluridine (I, 1-β-D-ribofuranosylthymine) was synthesized by the Mercuri procedure[2] and shown to possess the β-configuration by its conversion to spongothymidine (1-β-D-arabinofuranosylthymine) *via* a 2,2′-anhydronucleoside intermediate.[3] Another "5-methyluridine" (II) was reported previously by Roberts and Visser[4] by use of the Hilbert–Johnson procedure.[5] Studies on both nucleosides[2] showed that II differed from I in melting point, mixture melting point, and optical rotation. Both I and II exhibited thymidine-like spectra. Both nucleosides, when treated with metaperiodate, consumed one mole of oxidant per mole rapidly without the liberation of formic acid, a behavior consistent with a furanosyl sugar moiety containing the α-*cis* glycol grouping. However, the optical rotations of the dialdehyde solution obtained from metaperiodate oxidation of I and II were different. These data suggested[2] that the Roberts–Visser "5-methyluridine" might be the α-anomer of I; But, since the rotation of II was more levorotatory than that for authentic 5-methyluridine (I) (Hudson's Rule of isorotation would require the β-anomer to be more levorotatory), the Roberts–Visser nucleoside (II) remained uncharacterized.[2,5]

On the basis of reported[6] optical rotations of several pairs of anomeric 1-(2′-deoxy-D-ribofuranosyl)pyrimidines, Fox and co-workers concluded[7,8] that assignment of configuration to pyrimidine 2′-deoxynucleosides on the basis of optical rotation *alone* was certainly unwarranted. Recent studies[9] with some of these anomeric pairs of pyrimidine 2′-deoxynucleosides with n.m.r. spectra and optical rotary dispersion measurements supported that conclusion.

If this behavior of pyrimidine 2′-deoxynucleosides is equally applicable to pyrimidine ribonucleosides, then "5-methyluridine" (II) might be the α-anomer of I. If so, it is surprising that *only* this anomer (obtained in low yield) was isolated by Roberts and Visser from their reaction, especially since Naito and Kawakami[10] recently isolated anomeric 1-D-ribopyranosylthymines in a similar Hilbert–Johnson-type condensation. Moreover, the "*trans* rule"[12,13] would require that, in the Hilbert–Johnson reaction with poly-*O*-acylglycosyl halides, the C-1,C-2 *trans* anomer should be the predominant (if not the sole) product when sugars bearing a 2-acyloxy function are employed. We therefore reinvestigated the Roberts–Visser preparation of "5-methyluridine."

Tri-*O*-acetyl-D-ribofuranosyl bromide was prepared from tetra-*O*-acetyl-β-D-ribofuranose[14] by the method of Zinner[15] and was condensed with 2,4-diethoxy-5-methylpyrimidine in anhydrous benzene at room temperature for three days. The crude condensate was subjected to acid hydrolysis. A mixture of only two nucleosides was obtained in 45% yield from which pure anomers were obtained by fractional crystallization. One of these ($[\alpha]_D$ −8°) was identical with 1-β-D-ribofuranosylthymine (I)[2] prepared by the Mercuri procedure. The other component ($[\alpha]_D$ −50°) showed properties similar to "5-methyluridine" (II)[4] and also gave elemental analyses consistent with a pentosylthymine.

The assignment of the α-configuration to the $[\alpha]_D$ −50° isomer (II) rests on the following data. II exhibits an ultraviolet absorption spectrum similar to I to show that II is an N-1-substituted pyrimidine. Both I and II consume one mole of metaperiodate per mole *rapidly* and do not liberate formic acid in this process. II is therefore a furanosyl derivative containing the α-*cis* glycol grouping. The rotation of the solution from periodate oxidation of II (−25°) differed from that resulting from I (+16°). These data exclude all 1-β-D-pentofuranosyl- and all 1-D-pentopyranosylthymines as possible structures for II. The rapid

(1) This investigation was supported in part by funds from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service (Grant No. CA 03190-07 and CA 03192-07).

(2) J. J. Fox, N. Yung, J. Davoll, and G. B. Brown, *J. Am. Chem. Soc.*, **78**, 2117 (1956).

(3) J. J. Fox, N. Yung, and A. Bendich, *ibid.*, **79**, 2775 (1957).

(4) M. Roberts and D. W. Visser, *ibid.*, **74**, 668 (1952).

(5) See J. J. Fox and I. Wempen, *Advan. Carbohydrate Chem.*, **14**, 283 (1959), for a general review of the Hilbert–Johnson and Mercuri procedures for pyrimidine nucleoside syntheses.

(6) M. Hoffer, R. Duschinsky, J. J. Fox, and N. Yung, *J. Am. Chem. Soc.*, **81**, 4112 (1959).

(7) See ref. 5, p. 340.

(8) J. J. Fox, N. C. Yung, I. Wempen, and M. Hoffer, *J. Am. Chem. Soc.*, **83**, 4066 (1961).

(9) R. U. Lemieux and M. Hoffer, *Can. J. Chem.*, **39**, 110 (1961).

(10) (a) T. Naito and T. Kawakami, *Chem. Pharm. Bull.* (Tokyo), **10**, 627 (1962). (b) These authors observed that the optical rotations of these anomers (as well as anomeric 1-D-xylopyranosylthymines prepared by an alternate route) did not obey Hudson's isorotation rules. However, their configurational assignments to those glycopyranosyl nucleoside anomers rests, in the final analysis, on the assignment of the β-configuration to the 1-D-xylopyranosylthymine prepared[2,11] by both the Mercuri and Hilbert–Johnson procedures. This latter assignment is derived from the "*trans* rule."[12,13] Though this assignment is probably correct, it is not to be considered as rigorously established.

(11) J. J. Fox and I. Goodman, *J. Am. Chem. Soc.*, **73**, 3256 (1951).

(12) B. R. Baker in Ciba Foundation Symposium, Chemistry and Biology of Purines, 1957, p. 120.

(13) For application of the *trans* rule[12] to the Hilbert–Johnson reaction, see ref. 5, p. 336.

(14) G. B. Brown, J. A. Davoll, and B. A. Lowy, *Biochem. Prep.*, **4**, 70 (1955). The authors are indebted to Dr. G. B. Brown for a generous supply of tetra-*O*-acetyl-D-ribofuranose.

(15) H. Zinner, *Chem. Ber.*, **83**, 153 (1950).

uptake of metaperiodate excludes the arabino- and xylofuranosylthymines. Thus, we are left with 1-α-D-ribofuranosyl- or 1-α-D-lyxofuranosylthymine as possibilities for the structure of II. The likelihood of II being the α-lyxo isomer[16] is indeed remote. Moreover, the paper electrophoretic behavior (borate buffer, pH 6) of I (+5.6 cm.) and II (+5.5 cm.) are almost identical. 1-β-D-Lyxofuranosylthymine[17] has a much higher mobility in this system (+7.8 cm.); a similar relationship is obtained when uridine is compared electrophoretically to its 1-β-D-lyxouracil isomer.[18] Finally, both I and II and *no other nucleosides* were found in the same reaction. It is concluded, therefore, that II is 1-α-D-ribofuranosylthymine.

A purified extract of *B. subtilis* 16 (a pyrimidine-requiring strain) containing pyrimidine nucleoside phosphorylase was incubated with either pure II or pure I. No detectable glycosyl cleavage (formation of thymine) of the α-anomer was observed, whereas the β-anomer under the same conditions underwent ~55% cleavage to form thymine. When pure samples of I and II were mixed (1:1) and treated similarly with this enzymatic extract, no inhibition of glycosyl cleavage of the β-nucleoside by the presence of the α-anomer was detected.

That the α-anomer is resistant to glycosyl cleavage by nucleoside phosphorylases is expected. Lampen[19] demonstrated a decade ago that the Roberts–Visser material (II) was resistant to nucleosidase preparations from *E. coli*, whereas 1-β-D-ribofuranosylthymine (I) was cleaved by extracts of *L. pentosus*, as well as *E. coli* B.[17]

General Considerations.—These findings indicate that, as far as the Hilbert–Johnson condensation procedure with ribofuranosyl halide is concerned, the "*trans* rule" is not wholly operative. The results of Naito and Kawakami[10a] with poly-*O*-acylribo- and -ylopyranosyl halides also show this limitation of the "*trans* rule" because they also obtained mixtures of anomeric nucleosides by the Hilbert–Johnson method.

Roberts and Visser[4] also prepared a "5-methylcytidine" by treatment of their condensate obtained from the Hilbert–Johnson reaction with ammonia. (This condensate was also used by them for the synthesis of II.) This "5-methylcytidine" differed in melting point from 5-methylcytidine[20] prepared by the thiation process from 1-β-D-ribofuranosylthymine (I). On the basis of the studies with anomeric 1-D-ribofuranosylthymines reported here, it is more than likely that the Roberts–Visser "5-methylcytidine"[4] is 1-α-D-ribofuranosyl-5-methylcytosine. These anomers also fail to obey Hudson's Rules of isorotation. The α-anomer gives an $[\alpha]^{23}D$ −78 ± 5°,[21] whereas 1-β-D-ribofuranosyl-5-methylcytosine has a specific rotation of +14°.[20,22] Therefore, on the basis of the rotations of pyrimidine ribonucleosides reported here and previous studies on pyrimidine 2′-deoxyribonucleosides,[6-9] it is obvious that Hudson's Rules of isorotation cannot be employed to assign anomeric configurations to pyrimidine nucleosides in general.

Experimental[23]

Synthesis of 1-D-Ribofuranosylthymine Anomers.—Tetra-*O*-acetyl-β-D-ribofuranose[14] (15 g., 0.047 mole) was dissolved in dry ether saturated with hydrogen bromide at 0°. The flask was tightly stoppered and the solution was allowed to remain for 45 min. at 0°. The solvent was removed *in vacuo* (bath temperature 40°), the sirupy residue was dissolved in 30 ml. of dry benzene, and the solvent was again removed *in vacuo*. This procedure was repeated twice. The sirupy residue was dissolved in 50 ml. of dry benzene and treated with 9.1 g. (0.050 mole) of 2,4-diethoxy-5-methylpyrimidine. The solution was kept in a flask protected from moisture at room temperature for 3 days, then it was heated at ~60° for 9 hr. After concentration of the solution to a sirup *in vacuo*, the residue (23 g.) was dissolved in 150 ml. of dry methanol and treated with 50 ml. of a methanolic solution previously saturated at 0° with hydrogen chloride. After 12 hr. at 0°, the methanol was removed, and the residue was dissolved in 100 ml. of water. The solution was neutralized by portionwise addition with vigorous stirring of silver carbonate. The silver salts were removed by filtration, and the colorless filtrate was concentrated *in vacuo* (bath temperature 40°) to a sirup (10 g.). This sirup was dissolved in 30 ml. of methanol and an aliquot was used for further investigation.

This solution (5 ml., containing 1.65 g. of crude material) was placed on a cellulose column with *n*-butyl alcohol–ethanol–water (40:11:19).[24] The first eluates contained free pyrimidines (negative periodate test).[25] Later fractions showed ultraviolet absorption at 266 mμ and a positive periodate test. These latter fractions were combined and concentrated to dryness. Upon trituration with a small amount of ethanol, the sirupy residue crystallized (1.02 g.) and the mother liquor was discarded. This crystalline material contained ~90% nucleosides by spectrophotometric determination. The total yield of mixed nucleosides was 45%. The crude crystalline material was dissolved in 5 ml. of hot ethanol and placed in the cold for 2 days. Needles rich in the α-anomer were collected. After three recrystallizations of these needles from ethanol, 180 mg. of 1-α-D-ribofuranosylthymine was obtained, m.p. 175–176°, lit.[4] m.p. 175–177°, λ_{max} 268.5 mμ, $[\alpha]^{23}D$ −50° (*c* 1.28, water).

Anal. Calcd. for $C_{10}H_{14}N_2O_6$: C, 46.50; H, 5.46; N, 10.85. Found: C, 46.51; H, 5.24; N, 10.50.

The mother liquors after several days in the cold, deposited tiny spherical-shaped clusters which were almost pure β-anomer. After two recrystallizations of this material from ethanol, pure 1-β-D-ribofuranosylthymine (169 mg.) was obtained, m.p. 183–184°, lit.[2] m.p. 183–185°. A mixture melting point with authentic material[2] showed no depression, $[\alpha]^{23}D$ −8° (*c* 2.3, water), lit.[2] $[\alpha]D$ −10°.

Metaperiodate Oxidation Studies.—These studies were carried out in a manner similar to those already described.[2] The consumption of metaperiodate and the rotation of the dialdehyde solutions of these anomers were as follows.

---

(16) The synthesis of 1-α-D-lyxofuranosylthymine is underway in our laboratories for use in an enzymatic study.

(17) J. J. Fox, J. F. Codington, N. C. Yung, L. Kaplan, and J. O. Lampen, Jr., *J. Am. Chem. Soc.*, **80**, 5155 (1958).

(18) J. F. Codington, R. Fecher, and J. J. Fox, *ibid.*, **82**, 2794 (1960).

(19) J. O. Lampen, "Phosphorus Metabolism," Vol. II, W. D. McElroy and B. Glass, Ed., The John Hopkins Press, Baltimore, Md., 1952, p. 368.

(20) J. J. Fox, D. Van Praag, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich, and G. B. Brown, *J. Am. Chem. Soc.*, **81**, 178 (1959).

(21) (*c* 0.05, distilled water) The authors are indebted to Dr. D. W. Visser for a sample of this compound and to Dr. David Fukushima for the determination of its optical rotation on a 1-mg. sample. Mrs. Naishun Miller kindly performed the metaperiodate oxidation of this small sample and found that it consumed one mole of oxidant per mole within three minutes. This consumption of oxidant remained constant over a two-hour period. This data supports a furanosyl structure. A 1-mg. sample of cytidine was used as a control in the metaperiodate study. The ultraviolet absorption spectrum of the Roberts-Visser "5-methylcytidine" at pH values of 1, 7, and 13 was almost identical with that reported[20] for true 5-methylcytidine, which shows that both nucleosides are 1-substituted 5-methylcytosines.

(22) (*c* 3.2, distilled water); lit.[19] $[\alpha]D$ −3° (*c* 2.5, 1.0 *N* NaOH).

(23) All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are corrected. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

(24) L. Hough and J. K. N. Jones, "Methods in Carbohydrate Chemistry," Vol. I, R. L. Whistler and M. L. Wolfrom, Ed., Academic Press, Inc., New York, N. Y., 1962, p. 21.

(25) J. A. Cifonelli and F. Smith, *Anal. Chem.*, **26**, 1132 (1954).

	Moles of IO_4^- consumed per mole		$[\alpha]_D$ of dialdehyde produced
	2 hr.	12 hr.	
β-Anomer (I)	0.94	0.97	+16°
α-Anomer (II)	0.99	1.01	−25°

Chromatographic Studies.—In the ionophoretic system[26] requiring borate buffer, pH 6.2, 4 hr., 800 volts, the following migrations were obtained: for the α-anomer, +5.5 cm.; β-anomer, +5.6 cm.; and for 1-β-D-lyxofuranosylthymine, +7.8 cm. The chromatographic system[27] (ethyl acetate–acetic acid–water, 9:2:1, thin layer chromatography on Merck Silica Gel G.) without benzeneboronic acid present gave, for the α-anomer, R_f 0.60, and, for 5-methyluridine, R_f 0.59. The same system containing 0.5% benzeneboronic acid gave these R_f values: for the α-anomer, 0.86, and, for the β-anomer, 0.75.

Enzymatic Studies.—*Bacillus subtilis* 16, a pyrimidine-requiring organism,[28] was used as a source of pyrimidine nucleoside phosphorylase, since preliminary studies had indicated that extracts of this mutant contained substantial amounts of this enzyme. The organism was grown in the Biogen[29] in a chemically defined liquid medium[30] supplemented with 0.5 μmole per ml. of uracil. The cells were harvested at the end of the exponential phase of growth in a Sharples centrifuge and resuspended in 0.05 *M* phosphate–0.02 *M* cysteine hydrochloride buffer (pH 7.08); extracts were prepared by their disruption in a 10 KC Raytheon sonic oscillator. Purification of the enzyme was carried out according to the procedure of Tono and Cohen[31] to give a preparation purified 14-fold. This preparation was used for the experiments listed in Tables I–III.

A calibration curve relating the amount of thymine formed to the amount of 1-β-D-ribofuranosylthymine initially present in the incubation mixture was obtained. The results are shown in tabular form (Table II).

TABLE I

CLEAVAGE OF 1-D-RIBOFURANOSYLTHYMINES BY PURIFIED PYRIMIDINE NUCLEOSIDE PHOSPHORYLASE DERIVED FROM *B. subtilis* 16[a]

Time, min.	% thymine formed from β-Anomer	% thymine formed from α-Anomer
30	36	0
60	51	0
90	53	0
180	56	0

[a] Each incubation mixture contained 80 μmoles of phosphate buffer, pH 6.95, 12 μmoles of mercaptoethanol, 0.2 ml. of purified enzyme preparation, and 6.4 μmoles of the β-anomer or 19.2 μmoles of α-anomer in a total volume of 2.0 ml. Incubation was carried out at 36.2°; aliquots were removed at various time intervals and pipetted into 2.8 ml. of 0.1 *N* NaOH. The thymine present as a result of enzymic action was measured by following the change in optical density at 300 mμ, pH 13.

TABLE II

CLEAVAGE OF 1-β-D-RIBOFURANOSYLTHYMINE BY PURIFIED PYRIMIDINE NUCLEOSIDE PHOSPHORYLASE DERIVED FROM *B. subtilis* 16[a]

[A] 1-β-D-Ribofuranosylthymine, μmole/ml.	[B] Thymine formed, μmole/ml.	% cleavage, [B]/[A] × 100
1.0	0.42	42
2.0	0.80	40
3.0	1.14	38
4.0	1.47	37

[a] Each incubation mixture contained 80 μmoles of phosphate buffer, pH 6.95, 12 μmoles of mercaptoethanol, 0.2 ml. of purified enzyme preparation, and the amount of β-anomer as indicated in a total volume of 2.0 ml. Incubation was carried out at 36.2°; at the end of 180 min., the reaction was stopped and the amount of thymine present was determined by the change in optical density at 300 mμ, pH 13.

It is evident that a constant percentage of thymine is formed from the β-anomer irrespective of its initial concentration. Furthermore, the addition of various levels of the α-anomer to the reaction mixture did not affect the per cent cleavage of the β-anomer. These results are shown in Table III.

TABLE III

EFFECT OF 1-α-D-RIBOFURANOSYLTHYMINE UPON CLEAVAGE OF 1-β-D-RIBOFURANOSYLTHYMINE BY PURIFIED PYRIMIDINE NUCLEOSIDE PHOSPHORYLASE[a]

Anomeric mixture [A] β-anomer, μmole/ml	Anomeric mixture [B] α-anomer, μmole/ml.	Thymine formed, μmole/ml.	% thymine formed, [B]/[A] × 100
4.0	0	1.43	36
3.6	0.4	1.34	37
3.0	1.0	1.12	37

[a] Incubation conditions similar to those presented in Table II.

Acknowledgment.—The authors express their appreciation to Drs. George B. Brown and C. Chester Stock for their warm and continued interest.

(26) M. P. Gordon, D. M. Intrieri, and G. B. Brown, *J. Am. Chem. Soc.*, **80**, 5161 (1958).

(27) E. J. Bourne, E. M. Lees, and H. Weigel, *J. Chromatog.*, **11**, 253 (1963).

(28) Obtained from Dr. R. Guthrie (Children's Hospital, Buffalo, N. Y.) who isolated and characterized this mutant with respect to its pyrimidine requirements.

(29) An instrument for the continuous cultivation of microorganisms (American Sterilizer Co.).

(30) R. E. Feeney, J. A. Garibaldi, and E. M. Humphreys, *Arch. Biochem.*, **17**, 435 (1948).

(31) H. Tono and S. S. Cohen, *J. Biol. Chem.*, **237**, 1271 (1962).

Synthesis of Higher Ketoses by Aldol Reactions. II. Unsubstituted Heptuloses

ROBERT SCHAFFER

Division of Physical Chemistry, National Bureau of Standards, Washington, D. C. 20234

Received December 10, 1963

Unsubstituted D-erythrose undergoes a mixed aldol reaction with 1,3-dihydroxy-2-propanone to give D-*allo*-, D-*altro*-, and D-*gluco*-heptuloses in an over-all yield of 37%.

Work in this laboratory has shown that substituted higher ketoses[1] as well as branched-chain higher aldoses[2] are obtained by aldol reactions of carbohydrates that (a) are unable to form intramolecular hemiacetal linkages because of blocked γ- or δ-hydroxyl groups, and (b) are precluded from isomerizing to ketoses by substitution of the α-hydroxyl group. Recently, however, the aldol self-addition reaction of an *unsubstituted ring-forming* sugar (D-erythrose to D-*gluco*-L-*glycero*-3-octulose) was discovered,[3] and this indicated that a mixed aldol reaction of unsubstituted tetroses

(1) R. Schaffer and H. S. Isbell, *J. Org. Chem.*, **27**, 3268 (1962).

(2) R. Schaffer, *J. Am. Chem. Soc.*, **81**, 542 (1959).

(3) R. Schaffer and A. Cohen, *J. Org. Chem.*, **28**, 1929 (1963).