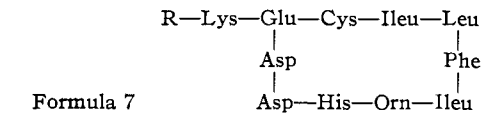
that the isoleucine connected to the cysteine is covered with a DNP group in tri-DNP-bacitracin A. Only a poor yield of DNP-isoleucine results from complete hydrolysis. Apparently the linkages around the sulfur do not present sufficient stability to strong acid to give good yields of degradation products.

A short time ago Porath[18] suggested a very tentative sequence for the amino acids present in bacitracin A which is given in formula 7. A tripeptide containing Phe, Ileu and Orn such as peptide A could arise from the sequence of formula 7. Indeed peptides clearly showing the sequence Orn → Ileu → Phe were reported in a previous paper.[1]

(18) J. Porath, *Nature*, **172**, 871 (1953).



Formula 7

However, peptide D containing only Lys, Asp, Orn and Ileu could not arise from formula 7 since it lacks a glutamic acid and a histidine residue. Nor could the pentapeptide E with two N-terminal residues, glutamic and aspartic acids, arise from the sequence in formula 7.

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Growth Inhibition of *Escherichia coli* by New Thymidine Analogs

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The preparation of 3-methylthymidine, 5-hydroxydeoxyuridine, and 5-bromodeoxyuridine and an improved method for the deamination of deoxycytidine are described. 5-Hydroxydeoxyuridine has been shown to suppress completely the growth of *Escherichia coli* K-12 at 18 hours at a level of 20 μg. per ml. 5-Bromodeoxyuridine is about $^1/_{30}$th as effective, while 3-methylthymidine, thymidine, deoxycytidine and deoxyuridine produce no inhibition. It is of interest that 5-substituted deoxyribosylpyrimidines are the only substituted nucleosides which inhibit growth of microörganisms which require neither purine nor pyrimidine compounds for growth.

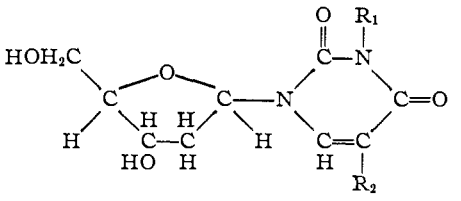
Studies with totally-labeled cytidine[1] have indicated that the incorporation of this nucleoside into DNA and RNA pyrimidine nucleosides takes place without prior cleavage of the ribosyl bond, suggesting the existence of a mechanism for the conversion of ribose to deoxyribose without disruption of the glycosyl linkage. That the reverse of such a reaction does not take place is indicated by the observation of Reichard and Estborn[2] that N^{15}-labeled deoxycytidine is incorporated to a small but significant extent only into DNA cytosine and thymine, while N^{15}-labeled thymidine functions exclusively as a precursor of polynucleotide thymine. In neither case was the isotope detected in the RNA pyrimidines.

The synthesis and testing of a series of substituted deoxyribosylpyrimidines was undertaken in view of the foregoing findings with the possibility in mind that the nucleoside derivatives might block DNA biosynthesis specifically. A further incentive for synthesis of substituted deoxyribosylpyrimidines was based on the previous findings that various ribosylpyrimidine derivatives inhibit growth of microörganisms, *Neurospora*, and Theilers GD VII virus *in vitro*, and that the inhibitory effects are overcome by the addition of selected hydrolytic derivatives of RNA.[3-7] It seemed reasonable that the methods reported for the synthesis of substituted ribosylpyrimidines[3,7] might be adapted to the synthesis of substituted deoxyribosylpyrimidines, and that the latter compounds, should they prove to be inhibitory, could then be utilized by the technique of inhibition analysis[8] as a means of gaining information concerning intermediary reactions, particularly those concerning DNA biosynthesis about which relatively little is known. Furthermore, because of the unique properties ascribed to DNA, antimetabolites capable of blocking DNA biosynthesis specifically might be of value in chemotherapy.

Deoxyuridine was chosen as the nucleoside to be substituted since deoxyuridine derivatives may be considered as analogs of thymidine, a specific precursor of DNA thymine, and because methods for substitution of the uracil moiety of nucleosides are available.[3,7] The commercial scarcity of deoxyuridine and the relatively low yield of this nucleoside obtained by isolation from a DNA hydrolysate by the method of Anderson, *et al.*,[9] made it necessary to undertake the development of a method for the deamination of deoxycytidine. Modifications of the procedures described for the synthesis of uridine derivatives[3,7] were required for the synthesis of the analogous deoxyribosylpyrimidines because the latter are relatively more unstable under the conditions employed. Considerable difficulty was encountered in initiating crystallization of 5-bromodeoxyuridine and 5-hydroxydeoxyuridine. Allowing the solutions to stand uncovered

(1) I. A. Rose and B. S. Schweigert, *J. Biol. Chem.*, **202**, 635 (1953).
(2) P. Reichard and B. Estborn, *ibid.*, **188**, 839 (1951).
(3) T. Kay Fukuhara and D. W. Visser, *ibid.*, **190**, 95 (1951).
(4) M. Roberts and D. W. Visser, *ibid.*, **194**, 695 (1952).
(5) I. J. Slotnick, D. W. Visser and S. C. Rittenberg, *ibid.*, **203**, 647 (1953).
(6) D. W. Visser, D. L. Lagerborg and H. E. Pearson, *Proc. Soc. Exptl. Biol. Med.*, **76**, 689 (1951).
(7) M. Roberts and D. W. Visser, THIS JOURNAL, **74**, 668 (1952).
(8) W. Shive, *Ann. N. Y. Acad. Sci.*, **52**, 1212 (1950).
(9) W. Anderson, C. A. Dekker and A. R. Todd, *J. Chem. Soc.*, 2721 (1952).

at room temperature for long periods of time gave the best results. Recrystallization of both compounds, however, was readily carried out with water or absolute ethanol as the solvent.



No.	R_1	R_2	Name
I	H	OH	5-Hydroxydeoxyuridine
II	H	Br	5-Bromodeoxyuridine
III	CH_3	CH_3	3-Methylthymidine

The microbiological data indicate that I is a potent inhibitor of *E. coli* K-12, completely suppressing growth up to 18 hours at a level of 20 μg. per ml. II is less effective, producing comparable inhibition of growth at thirty- to forty-fold higher concentrations, while III stimulates the growth rate slightly. At the end of 48 hours, growth in tubes containing I approached or exceeded maximal control growth, whereas growth in tubes containing II remained partially inhibited. Preliminary determination of oxygen utilization by resting-cell suspensions of *E. coli* K-12 in the presence of I and II indicated that the spontaneous reversal of inhibition is due to destruction of the deoxynucleoside derivatives,[10] perhaps in a manner analogous to that reported by Slotnick, *et al.*,[11] for the degradation of a number of substituted ribosylpyrimidines.

Deoxyuridine and deoxycytidine at a level of 1,600 μg. per ml. neither stimulate nor inhibit growth, while thymidine at the same level accelerates somewhat the appearance of maximal growth.

I and II are of particular interest since they are the only substituted nucleosides known to inhibit microörganisms which do not require purine or pyrimidine compounds for growth. Preliminary studies[10] have shown that I inhibits the growth of a purine-requiring *E. coli* mutant (M45B4) in the same concentration range as that required for comparable inhibition of *E. coli* K-12. 5-Hydroxyuridine, which has no effect on *E. coli* K-12, has been reported to suppress completely the growth of this mutant at a concentration of 2 μg. per ml.[5]

Experimental[12]

Deamination of Deoxycytidine.—The method of Dekker and Elmore[13] for deamination of 5-methylcytidine was adapted to the deamination of deoxycytidine in gram quantities. Twenty ml. of glacial acetic acid was added dropwise to a mechanically-stirred solution of deoxycytidine hydrochloride (3.3 g.. 0.0121 mole) and sodium nitrite (15.3 g., 0.222 mole) in 45 ml. of distilled water. Stirring was continued for 1 hour after the addition of acetic acid was complete. The mixture was allowed to remain at room temperature for 24 hours, whereupon it was evaporated to dryness at 20° *in vacuo*. The residue was taken up in 100 ml. of distilled water and stirred with increments of Amberlite IR-120 cation-exchange resin until bubbling could no longer be detected in the solution. The mixture was then placed on a column of Amberlite IR-120 (120 ml. wet resin) and eluted with distilled water until the effluent no longer exhibited appreciable ultraviolet light absorption as determined with a Beckman spectrophotometer at 270 mμ. The total effluent volume was reduced by evaporation with an air stream to 75 ml.. and the concentrated solution was lyophilized. The product was crystallized from absolute ethanol. A second crop of crystals was obtained from the mother liquor. The yield was 2.57 g. (90%) of deoxyuridine melting at 159–162°.

5-Bromodeoxyuridine.—Bromine water saturated at 5° was added with stirring to deoxyuridine (1 g., 0.0044 mole) until the nucleoside dissolved and a permanent red color appeared. Air was then bubbled through the solution to remove excess bromine, and the resulting colorless solution was mixed with 150 ml. of glacial acetic acid and lyophilized nearly to dryness. Rapid decomposition of the product results if the lyophilization is carried too far. The addition of glacial acetic acid, followed by partial lyophilization, was repeated several times with progressively smaller portions of acetic acid until most of the water had been removed. Finally, the nearly-dry residue was taken up in 250 ml. of absolute ethanol and boiled for 12 minutes in a hot water-bath. Absolute ethanol was added from time to time to maintain the original volume. The solution was then concentrated to a thick sirup at reduced pressure at room temperature. To the sirupy residue was added 10 ml. of absolute ethanol. The sirup dissolved readily upon warming. Ethyl ether was added to opalescence and the solution was allowed to remain about 2 days at room temperature. The product crystallized as white needles. The mother liquor was concentrated, treated with methanol–ethyl acetate, and allowed to stand, whereupon additional crystalline product was obtained. The yield was 0.88 g. (65%). The product recrystallized from absolute ethanol melted at 187–189°. *Anal.* Calcd. for $C_9H_{11}N_2O_5Br$: C, 35.20; H, 3.61; N, 9.12. Found: C, 35.17; H, 3.68; N, 8.85.

5-Hydroxydeoxyuridine.—Deoxyuridine (2.25 g., 0.0099 mole) was brominated and freed of excess bromine by the procedure described for the synthesis of 5-bromodeoxyuridine. Finely-powdered lead oxide (PbO, 4.1 g.) was added to the colorless solution, and the mixture was mechanically stirred for 15 minutes at room temperature and for 30 minutes in a boiling water-bath. The mixture was then chilled just to the freezing point, and the precipitated lead bromide and lead oxide were removed by filtration and washed twice with small portions of cold distilled water. The filtrate was treated with increments of Amberlite IR-120 ion-exchange resin until a negative test for lead was obtained with sodium sulfide. The resin was removed by filtration and the filtrate was reduced to $^1/_5$th of its original volume by lyophilization. It was found that further reduction of the volume resulted in rapid decomposition of the product. Glacial acetic acid (150 ml.) was added to the concentrated solution and the mixture was lyophilized nearly to dryness. The addition of glacial acetic acid followed by partial lyophilization was repeated several times to remove most of the remaining water and acid. Finally, the nearly-dry white residue was taken up in 300 ml. of absolute ethanol and placed in a constant temperature bath at 50°. Air was bubbled through the solution for 4 hours, and the volume was subsequently reduced to 30 ml. by evaporation at reduced pressure at room temperature. The concentrated solution was allowed to stand at room temperature. Crystallization ensued as the volume was slowly reduced to about 15 ml. The crystals were removed by filtration. and the mother liquor was evaporated to a thick sirup which was treated with methanol–ethyl acetate, stirred and allowed to stand overnight. The crystals which formed were isolated and combined with the first crop. The yield was 0.71 g. (30%) of white crystals melting at 209–211°. *Anal.* Calcd. for $C_9H_{12}N_2O_6$: C, 44.26: H, 4.95; N, 11.47. Found: C, 44.53; H, 5.23; N, 11.55.

3',5'-Diacetylthymidine.—Dry thymidine (10 g., 0.0413 mole) was suspended in 500 ml. of freshly-distilled acetic anhydride in a 2-liter round bottom flask fitted with a ground-glass stopper. The mixture was shaken while warming intermittently under the tap. Small portions of anhydrous pyridine were added at intervals until the thymidine had dissolved completely. The solution was allowed to

(10) I. J. Slotnick, personal communication.

(11) I. J. Slotnick, D. W. Visser and S. C. Rittenberg, *J. Biol. Chem.*, **208**, 217 (1954).

(12) All melting points were taken on a Fisher–Johns melting point apparatus and are uncorrected.

(13) C. A. Dekker and D. T. Elmore, *J. Chem. Soc.*, 2864 (1951).

stand at room temperature for 24 hours and was then slowly concentrated to an amber sirup at 20° *in vacuo*. The sirup was taken up in a small amount of methanol and the solution was again evaporated to dryness *in vacuo*. This was repeated twice more to ensure complete removal of pyridine and other volatile products. The sticky residue was taken up in 50 ml. of absolute ethanol. Crystallization ensued as the solution was slowly concentrated at reduced pressure and room temperature. A second crop of crystals was obtained from the mother liquor in similar fashion. The combined product was recrystallized from water. The yield was 10.6 g. (80%) of a white crystalline compound melting at 123–125°. *Anal.* Calcd. for $C_{14}H_{18}N_2O_7$: C, 51.53; H, 5.56; N, 8.58. Found: C, 51.54; H, 5.69; N, 8.28.

3-Methylthymidine.—The method was adapted from the procedure described by Visser, *et al.*,[14] for the synthesis of 3-methyluridine. 3′,5′-Diacetylthymidine (6.67 g., 0.0204 mole) was dissolved in a minimum amount of anhydrous ethylene dichloride. The solution was cooled to 0° in an ice-bath and cold diazomethane (3 g., 0.074 mole) in ether was added slowly with shaking. The solution was a deep yellow color after the addition was complete. The solution, in a flask fitted with a stopper containing a drying tube, was allowed to remain at room temperature for 24 hours. The yellow solution was subsequently evaporated to dryness at reduced pressure and room temperature. The residue was taken up 2 times in 50 ml. of anhydrous methanol and evaporated to dryness after each addition. The residue was dissolved in 74 ml. of anhydrous methanol, and 26 ml. of a solution of dry HCl in anhydrous methanol (25% HCl by weight) was added. The solution was allowed to remain 3 days at room temperature in a stoppered flask. The mixture was then neutralized by dropwise addition of alcoholic sodium hydroxide. Sodium chloride precipitated and was removed by filtration. and the filtrate was evaporated to dryness with an air stream. The residue was easily crystallized from water, yielding 1.7 g. (33%) of white crystals which melted at 129–131° after drying in a vacuum desiccator first at room temperature overnight, then at 100° *in vacuo* for 2 hours. *Anal.* Calcd. for $C_{11}H_{16}N_2O_5$: C, 51.55; H, 6.30; N, 10.93. Found: C, 51.10; H, 6.50; N, 10.92.

Spectrophotometric Data.—The ultraviolet absorption maxima and molar extinction coefficients of the thymidine analogs are shown in Table I. Each compound was dissolved in 0.1 *N* HCl and in 0.1 *N* NaOH at a concentration of 20 μg. per ml., and measured in a Beckman spectrophotometer, model DU.

TABLE I

ULTRAVIOLET ABSORPTION OF THYMIDINE ANALOGS

	Spectra in acid		Spectra in alkali	
	$\lambda_{max.}$ mμ	ϵ_{max} × 10^{-3}	$\lambda_{max.}$ mμ	ϵ_{max} × 10^{-3}
5-Hydroxydeoxyuridine	281	8.5	303	6.8
5-Bromodeoxyuridine	280	9.9	277	7.2
3-Methylthymidine	267	9.6	267	9.6

Microbiological.—*E. coli* K-12 was cultured on a glucose-salts-agar medium.[15] Inoculated slants were incubated 24 hours at 37° and stored at 5°. The cultures were transferred biweekly.

The inoculum was prepared by transferring a loopful of the organism to 5 ml. of liquid medium having the following composition: 0.25 g. of glucose, 0.005 g. of $MgSO_4 \cdot 7H_2O$, 0.05 g. of NH_4NO_3, 0.5 g. of K_2HPO_4 and 0.2 g. of KH_2PO_4 per 100 ml. of medium. After incubating 24 hours at 37°, a loopful of the cell suspension was transferred to another identical broth tube and the incubation was repeated. Finally, 0.2 ml. of the resulting cell suspension was transferred to 50 ml. of sterile saline and each assay tube was inoculated with 0.1 ml. of this suspension.

Each nucleoside tested was dissolved in a double-strength salts solution (see "liquid medium" above) and aliquots of the solution containing varying amounts of the nucleoside were added to the assay tubes. The volume in each tube was adjusted to 2.5 ml. with double-strength salts solution and the tubes were autoclaved at 15 pounds pressure for 15 minutes. Sterile glucose (2.5 ml., 0.5% glucose weight-volume) was added to each tube prior to inoculation. The tubes were incubated at 37°. Growth was measured as turbidity in a Klett-Summerson photoelectric colorimeter (Filter No. 66). Maximum growth was attained in the control tubes at 18 hours. The results are shown in Table II.

TABLE II

GROWTH OF *E. Coli* K-12 IN THE PRESENCE OF PYRIMIDINE DEOXYNUCLEOSIDES

Compound	Concn., μg./ml.	Time and Klett units 15 hr.	18 hr.
(Control)[a]	..	36 ± 1	72 ± 3
5-Hydroxydeoxyuridine	6	23	65
	10	14	58
	12	5	37
	14	0	23
	20	0	0
	40	0	0
5-Bromodeoxyuridine	40	26	66
	120	23	43
	200	20	36
	400	10	16
	600	5	9
	800	0	
3-Methylthymidine	100	39	61
	400	45	64
	800	59	73
Deoxycytidine	1600	42	72
Deoxyuridine	1600	38	70
Thymidine	1600	47	73

[a] Control values constitute the average of triplicate tubes. Growth at 18 hours in the controls was maximal.

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We are indebted to Dr. Irving J. Slotnick and Mr. R. J. Martinez for preliminary data concerning oxygen utilization by resting-cell suspensions of *E. coli* K-12 in the presence of 5-bromo-, and 5-hydroxydeoxyuridine. We wish to thank Dr. Slotnick for supplying the *E. coli* K-12 culture.

LOS ANGELES, CALIF.

(14) D. W. Visser, G. Barron and R. Beltz, THIS JOURNAL, **75**, 2017 (1953).

(15) B. D. Davis and E. S. Mingioli, *J. Bact.*, **60**, 17 (1950).