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Gentiobiose, a Constituent of Deoxyribonucleic Acid from Coliphage T6*

SIGERU KUNO† AND I. R. LEHMAN

From The Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California

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The deoxyribonucleic acid of the T-even coliphages contains glucosylated and nonglucosylated 5-hydroxymethylcytosine in place of cytosine (1–6). In the case of deoxyribonucleic acid from phage T6, 72% of the 5-hydroxymethylcytosine residues are diglucosylated. In T2 deoxyribonucleic acid, 70% of the 5-hydroxymethylcytosine residues are monoglucosylated, but 5% bear diglucosyl units. A recent investigation of the configuration of the disaccharides bound to the 5-hydroxymethylcytosine nucleotides of deoxyribonucleic acid from T2 and T6 showed the glucose units to be β linked to each other and joined to the hydroxymethyl group of the 5-hydroxymethylcytosine by an α linkage (6). In the present study, the disaccharide from T6 deoxyribonucleic acid was identified as gentiobiose, 6-*O*-β-D-glucopyranosyl-D-glucose. This disaccharide is unique to deoxyribonucleic acid from phage T6 and possibly T2 and has been observed only in such glycosides as amygdalin and crocin, and as part of the trisaccharide, gentianose.

EXPERIMENTAL PROCEDURE

Sugars and Enzymes—Maltose and cellobiose were purchased from Pfanstiehl Laboratries, Inc. Gentiobiose and amygdalin were products of the Nutritional Biochemicals Corporation. Sophorose was kindly supplied by Drs. M. Wolfrom and H. G. Fletcher; laminaribiose was the gift of Dr. E. Neufeld; β-methylmaltoside was the gift of Dr. D. S. Hogness. Glucose 6-phosphate dehydrogenase and hexokinase were purchased from the Sigma Chemical Company. α- and β-Glucosidases were purified as described previously (6). Crystalline tetrahydrofolate formylase (7) and tetrahydrofolic acid were gifts from Dr. J. C. Rabinowitz. The *Escherichia coli* phosphodiesterase was purified as described previously (8).

Isolation of DNA—Phages T2 and T6 were grown and purified as described previously (6). DNA was isolated from phage T6 by osmotic shock followed by treatment with chloroform-octanol (6, 9). DNA was isolated from phage T2 by extraction with sodium dodecyl sulfate in the following way. To 285 ml of a phage suspension (6×10^{12} per ml) were added, with vigorous stirring, 500 ml of 1% sodium dodecyl sulfate. Stirring was continued for 2 hours at room temperature. The solution was cooled to 4° and then adjusted to pH 6 with 4 N HCl. To this solution were added, with stirring, 215 ml of 4.5 M NaCl. The precipitate which formed was removed from solution by centrifugation at 10,000 × *g* for 30 minutes, leaving most of the DNA in the supernatant fluid. DNA remaining in the precipitate was recovered by extraction with 150 ml of 1 M NaCl, followed by centrifugation at 10,000 × *g* for 30 minutes. The extraction procedure was repeated three times. The supernatant fluid and the extracts were combined, and the DNA was precipitated by the addition of 2 volumes of 95% ethanol. The precipitated DNA was dissolved in 700 ml of 1 M NaCl, and residual protein was removed by treatment with chloroform-octanol (9). The DNA was again precipitated, washed with 95% ethanol, and dried at 20–25°; it was then dissolved in 0.02 M NaCl.

Isolation of 5-Hydroxymethylcytosine Nucleotides—The DNA from phages T2 and T6 was degraded enzymatically to 5′-mononucleotides, and the HMC[1] nucleotides were isolated by chromatography on Dowex 1 columns as described previously (6). dHMP was purified by crystallization according to the method of Koerner and Varadarajan (10). Mono- and diglucosylated dHMP were purified by dissolving them in a minimal amount of water and then precipitating them from solution by the addition of 9 volumes of absolute ethanol. The precipitates were washed twice with 95% ethanol. The three HMC nucleotides have identical ultraviolet absorption spectra at pH 1, λ_{max} = 284 mμ, $a_M = 12.5 \times 10^3$ (based on phosphate estimation), A_{280} to A_{260} = 2.75, and A_{290} to A_{270} = 1.36. The molar extinction value of 12.5×10^3 is somewhat lower than the value of 13.2×10^3 reported earlier (6). There is no immediately apparent reason for this discrepancy.

Conversion of HMC Nucleotides to 5-Hydroxymethyluracil Derivatives—The diglucosylated 5-hydroxymethyluracil nucleotides were prepared by deamination of the corresponding HMC nucleotides according to the procedure of Wyatt and Cohen (1). Diglucosylated dHMP (5 μmoles) was mixed with 0.04 ml of glacial acetic acid and 0.20 ml of 2 M $NaNO_2$. The solution was kept at 20–25° for 40 hours with occasional stirring and was then evaporated to dryness under a stream of air at 37°. The dried material was dissolved in 1.0 ml of water and passed through a Dowex 50-W column (2 × 1 cm), H^+ form. The column was washed four times with 1-ml portions of water. The unadsorbed effluent fluid and washings were combined and taken to dryness by lyophilization.

The deamination reaction was essentially complete, and neither unchanged material nor any other byproducts could be detected by paper chromatography of the deaminated nucleotide in the

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† Rockefeller Foundation Fellow. Present address, Department of Medical Chemistry, University of Kyoto Faculty of Medicine, Kyoto, Japan.

[1] The abbreviations used are: HMC, 5-hydroxymethylcytosine; dHMP, 5-hydroxymethyl deoxycytidine 5′-phosphate; hydroxymethyl-dUMP, 5-hydroxymethyl deoxyuridine 5′-phosphate.

isobutyric acid-ammonia solvent. The diglucosylated hydroxymethyl-dUMP has an absorption maximum (pH 2) at 264 mμ, $a_M = 10.2 \times 10^3$ (based on phosphate estimation), A_{280} to $A_{260} = 0.56$, and A_{250} to $A_{260} = 0.69$.

The non- and monoglucosylated 5-hydroxymethyluracil nucleotides were prepared in the same way from the corresponding non- and monoglucosylated HMC nucleotides. The ultraviolet absorption spectra of these compounds are identical with that of the diglucosylated nucleotide at pH 2.

Periodate Oxidation of Glucosylated Nucleotides—The oxidation by periodate of the glucosylated nucleotides was followed by the spectrophotometric method of Dixon and Lipkin (11) as modified by Rammler and Rabinowitz.[2] The reaction mixture (1.2 ml) contained 500 μmoles of sodium acetate buffer, pH 4.3, and 0.2 to 0.4 μmole of nucleotide. The reaction was started by the addition of 6.0 μmoles of sodium metaperiodate which had previously been standardized with sodium arsenate (12), and the decrease in absorbancy at 305 mμ was followed for 30 hours at room temperature. A decrease of 0.093 in the absorbancy at 305 mμ corresponds to the consumption of 1.0 μmole of periodate. The blank cuvette contained all of the components except substrate. The change of the optical density in the blank cuvette during the reaction was usually less than 0.02.

The amount of formate produced at the completion of the periodate oxidation reaction was measured enzymatically by means of the tetrahydrofolate formylase reaction (7) as modified by Rammler and Rabinowitz.[2]

Enzymatic Hydrolysis of Disaccharides—The hydrolysis of disaccharides by β-glucosidase was measured in a coupled reaction in which the liberation of glucose by the glucosidase was determined by its conversion to glucose 6-phosphate and then to 6-phosphogluconic acid by the sequential action of hexokinase and glucose 6-phosphate dehydrogenase (13). The reaction mixture (1.0 ml) contained 50 μmoles of glycylglycine buffer, pH 7.0, 0.5 μmole of ATP, 5.0 μmoles of $MgCl_2$, 0.3 μmole of TPN, 0.03 to 0.20 μmole of substrate, 1.2 units of glucose 6-phosphate dehydrogenase (13), and 15 units of hexokinase (14). The reaction was started by the addition of β-glucosidase and was followed by measuring the increase in optical density at 340 mμ at 1-minute intervals. After a 1- to 3-minute lag period, the optical density increased linearly. After the lag, the reaction rate was a linear function of β-glucosidase concentration in the range from 800 to 6000 units (6). The Michaelis-Menten constant, K_m, and the maximal velocity, V_{max}, were calculated from the Lineweaver-Burk plots (15).

Paper Chromatography and Paper Electrophoresis—Descending paper chromatography of sugars was carried out on Whatman No. 1 paper with *n*-propanol-ethyl acetate-water (7:1:2) as a solvent (16). After development of the chromatogram for 48 hours, the paper was dried and reducing sugars were located by means of the aniline oxalate reagent (17). The $R_{glucose}$ values for the β linked disaccharides were as follows: laminaribiose, 0.62; sophorose, 0.54; cellobiose, 0.45; and gentiobiose, 0.37. Paper chromatographic separation of the glucosylated nucleotides was carried out by means of the isobutyric acid-ammonia solvent (18).

Paper electrophoresis was carried out by the procedure of Foster (19), which was modified slightly by the use of Whatman No. 1 paper, 12.5 × 56 cm, 0.1 M borate buffer, pH 10, and 600 volts for 2 to 3 hours at room temperature. Reducing sugars were located with the aniline oxalate reagent. The $M_{glucose}$ values (electrophoretic mobility as compared with glucose) of the β linked disaccharides were as follows: gentiobiose, 0.73; laminaribiose, 0.68; cellobiose, 0.33; and sophorose, 0.33.

Glucose bound to DNA or nucleotide or in disaccharides, was determined by the anthrone procedure (20). Free glucose was also measured by this procedure or by the coupled hexokinase-glucose 6-phosphate dehydrogenase reaction (13). Protein was determined by the method of Lowry *et al.* (21). The procedure of Chen, Toribara, and Warner (22) as modified by Ames and Dubin (23) was used to estimate phosphate.

RESULTS

Periodate Oxidation of Diglucosylated dHMP from T6 DNA—The four isomeric β linked glucosyl glucoside derivatives of dHMP can be partially distinguished by measurements of periodate consumption and formate release after treatment of these compounds with periodate (24) (Table I). The values of 4.4 moles of periodate consumed and 2.3 moles of formate released per mole of diglucosylated dHMP from T6 DNA are in good agreement with those expected for a gentiobioside, (β 1 → 6 glucoside) (Table II). The somewhat elevated values are probably the result of "overoxidation" (24). Thus, with amygdalin (mandelonitrile-β-gentiobioside), the values for periodate consumption and formate liberation are also higher than theo-

TABLE I

Theoretical values of periodate consumption and formate release after periodate oxidation of glucosyl glucosides

Glucosyl-glucoside	Periodate consumed per mole of substrate oxidized	Formate liberated per mole of substrate oxidized
	moles	*moles*
Sophoroside, β (1 → 2)	3	1
Laminaribioside, β (1 → 3) (nigeroside, α (1 → 3))	2	1
Cellobioside, β (1 → 4) (maltoside, α (1 → 4))	3	1
Gentiobioside, β (1 → 6) (isomaltoside, α (1 → 6))	4	2

TABLE II

Periodate oxidation of glucosyl glucosides

Substrate	Amount of substrate added	Periodate consumed per mole of substrate		Formate released per mole of substrate	
		Observed	Theoretical	Observed	Theoretical
	μmoles	*moles*	*moles*	*moles*	*moles*
Diglucosylated dHMP (T6)	0.394	4.47		2.36	
Diglucosylated dHMP (T6)	0.788	4.40		2.17	
Amygdalin	0.420	4.41	4.0	2.36	2.0
Amygdalin	0.840	4.30	4.0	2.51	2.0
β-Methyl maltoside	0.950	3.14	3.0	1.25	1.0
dHMP	0.680	0.45	0	0	0

[2] We are grateful to Dr. Rabinowitz for supplying us with the details of this method before its publication.

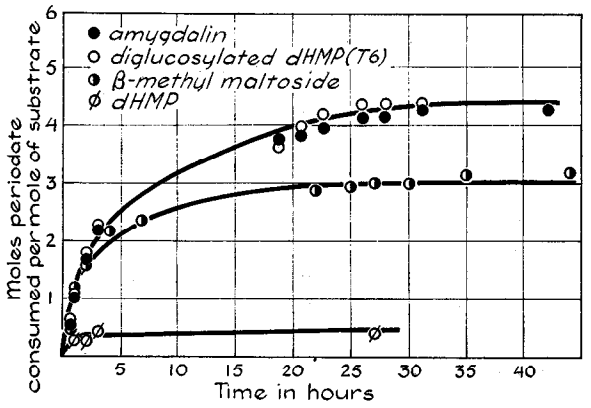

FIG. 1. Time course of periodate consumption by glucosyl glucosides. The conditions used were those described under "Experimental Procedure."

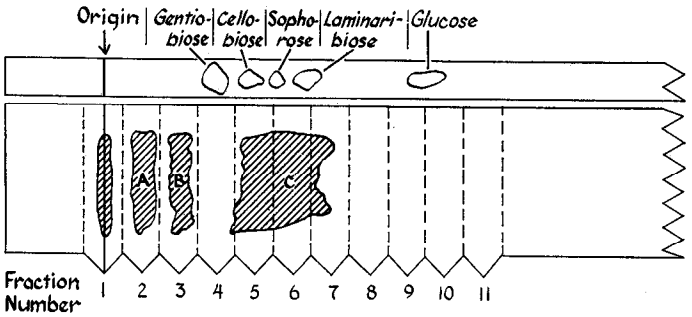

FIG. 2. Paper chromatogram of acid hydrolysate of diglucosylated hydroxymethyl-dUMP from T6 DNA. Hydrolysis of the diglucosylated hydroxymethyl-dUMP was carried out in the following way: 4.9 μmoles of nucleotide were dissolved in 2.5 ml of 0.04 N HCl and heated for 90 minutes in a boiling water bath (sealed tube). After cooling, the solution was taken to dryness under a stream of air at 37°. The dried residue was dissolved in water and transferred quantitatively to Whatman No. 1 paper that had been washed thoroughly with distilled water. The chromatogram was developed with the *n*-propanol-ethyl acetate-water solvent for 40 hours. The paper was dried, and the nucleotides were located under ultraviolet light and marked. The paper was then cut as shown, and the fractions were eluted with water for approximately 10 hours. The eluates (approximately 1.5 ml) were taken to dryness under a stream of air at 37°, and the dried residues were each dissolved in 1.0 ml of water. An aliquot from each fraction was removed and analyzed for its ultraviolet absorption and glucose and phosphate contents (Table III). The *shaded areas* represent ultraviolet-absorbing compounds.

retical; they are, however, identical with those obtained with the diglucosylated dHMP. As a further check on the validity of this method, the values observed with β-methyl maltoside (3.14 moles of periodate consumed; 1.25 moles of formate released) are in agreement with those expected for the 1,4-glucoside and significantly different from the 1,6 isomer. The time course of the periodate oxidation of the various glucosyl glucosides is shown in Fig. 1.

Identification of Isolated Disaccharide—Attempts to isolate the intact disaccharide by acid treatment of the diglucosylated dHMP resulted in extensive hydrolysis of the disaccharide to free glucose. The small amount of disaccharide recovered was tentatively identified as gentiobiose on the basis of its paper chromatographic and paper electrophoretic behavior. However, diglucosylated hydroxymethyl-dUMP formed by deamination of diglucosylated dHMP from T6 DNA yielded approximately 30% of the total glucose in the form of disaccharide after mild acid treatment of the nucleotide.

The products formed by acid hydrolysis of diglucosylated hydroxymethyl-dUMP were separated by paper chromatography in the *n*-propanol-ethyl acetate-water system (Fig. 2), and a series of 11 fractions was obtained (Table III). The material recovered in Fraction 1 (approximately 7% of the nucleotide initially hydrolyzed) had an ultraviolet absorption spectrum and glucose and phosphate content consistent with diglucosylated hydroxymethyl-dUMP, but showed significantly different chromatographic properties; it was not further identified. The other three ultraviolet-absorbing compounds, A (Fraction 2), B (Fraction 3), and C (Fractions 4, 5, 6, and 7) were identified as the di-, mono-, and nonglucosylated 5-hydroxymethyluracil nucleotides, respectively, on the basis of their ultraviolet absorption spectra, their contents of nucleotide-bound glucose and phosphate, and paper chromatography in the isobutyric acid-ammonia solvent. Fractions 4 and 5, which contained approximately 30% of the total sugar of the hydrolysate (measured as glucose) were freed from residual hydroxymethyl-dUMP by passing them through Dowex 1 columns (3 × 1 cm, OH^- form). The sugar was recovered quantitatively in the unadsorbed effluent fluid. It was identified as gentiobiose on the basis of the following paper chromatographic, paper electrophoretic, and enzymatic evidence. On chromatography in the *n*-propanol-ethyl acetate-water solvent, only one spot was detected which migrated at the same rate as gentiobiose ($R_{glucose}$ = 0.36). Similarly, a single component with the same mobility as gentiobiose ($M_{glucose}$ = 0.75) was observed after paper electrophoresis. On treatment of 0.18 μmole of the sugar (expressed as glucose equivalents) with 1200 units of β-glucosidase for 1 hour, approximately 90% was converted to free glucose. Less than 1% hydrolysis could be detected with α-glucosidase.

Although all of the β linked glucosyl glucosides are susceptible to β-glucosidase, their K_m and V_{max} values differ markedly from each other. The K_m and V_{max} values for the disaccharide

TABLE III

Analysis of acid hydrolysate of diglucosylated hydroxymethyl-dUMP (T6)

	Nucleotide*	Glucose	Phosphate	Glucose to nucleotide ratio
	μmoles	μmoles	μmoles	μmoles
Fraction 1	0.35	0.62	0.34	1.8
Fraction 2	1.74	3.47	1.73	2.0
Fraction 3	0.53	0.72	0.53	1.35
Fraction 4	0.18	1.88	0.15	10.4
Fraction 5	0.64	1.24	0.72	1.95
Fraction 6	0.71	0.08	0.78	0.11
Fraction 7	0.21	0.04	0.23	0.02
Fraction 8	<0.006	0.02	0.05	
Fraction 9	<0.006	0.94	0.04	
Fraction 10	<0.006	0.40	0.04	
Fraction 11	<0.006	0.06	0.04	
Total	4.36	9.47	4.66	
Percentage recovered	89	96	95	

* Calculated with a value of 10.2×10^3 as the molar extinction at 264 mμ of hydroxymethyl-dUMP.

in Fractions 4 and 5 were very close to those of gentiobiose, and different from those of the other β-glucosides (Table IV).

The remainder of the sugar in the hydrolysate was found in Fractions 9 and 10. It was identified as free glucose by chromatography in the *n*-propanol-ethyl acetate-water solvent. This identification was verified by means of the hexokinase-glucose 6-phosphate dehydrogenase reaction. Thus, Fractions 9 and 10, when combined, contained 1.34 μmoles of glucose as measured by the anthrone reaction and 1.37 μmoles of glucose as determined by enzymatic assay.

Since approximately 48% of the diglucosylated hydroxymethyl-dUMP was recovered unchanged after treatment with acid (Table V), the possibility existed that there are two forms of the glucosylated nucleotide with differing stabilities toward acid hydrolysis. This possibility was ruled out by the following experiment. Unhydrolyzed diglucosylated hydroxymethyl-dUMP (0.88 μmole) obtained from Fraction 2 of the acid hydrolysate (Fig. 2) was dissolved in 0.45 ml of 0.04 N HCl and heated at 100° for 90 minutes in a sealed tube. The hydrolysate was chromatographed and analyzed as before. The products were identified as di-, mono-, and nonglucosylated hydroxymethyl-dUMP, gentiobiose, and glucose, and the relative distribution of products was very close to that of the first hydrolysate (Table V). These results indicate that the structure of the disaccharide moiety of the diglucosylated HMC from phage T6 is gentiobiose, 6-*O*-β-D-glucopyranosyl-D-glucose, and that the diglucosylated HMC is therefore HMC-α-gentiobioside (Fig. 3).

TABLE IV

Kinetic data for hydrolysis of β linked disaccharides by β-glucosidase

Substrate	K_m	V_{max} *
	$M \times 10^4$	
Sophorose	10.0	0.578
Laminaribiose	5.6	1.45
Cellobiose	7.7	0.249
Gentiobiose	4.6	0.079
Disaccharide from diglucosylated hydroxymethyl-dUMP (T6)	4.4	0.070

* Micromoles of glucose released per minute per mg of protein.

TABLE V

Distribution of products after acid hydrolysis of diglucosylated hydroxymethyl-dUMP

Compound hydrolyzed	Products formed				
	Diglucosylated hydroxymethyl-dUMP*	Monoglucosylated hydroxymethyl-dUMP*	Hydroxymethyl-dUMP*	Gentiobiose†	Glucose†
	%	%	%	%	%
Gentiobiose				83.7	16.3
Diglucosylated hydroxymethyl-dUMP (T6)	47.8	12.2	40.0	33.0	14.2
Diglucosylated hydroxymethyl-dUMP (T6)‡	39.0	14.8	46.2	34.7	14.9
Diglucosylated hydroxymethyl-dUMP (T2)	40.6	16.9	42.5	32.1	20.7

* Percentage of total nucleotide recovered (calculated from the absorbancy at 264 mμ). The values for diglucosylated hydroxymethyl-dUMP include the unidentified nucleotide found at the origin of the paper chromatogram (see Fraction 1, Fig. 2 and Table III).

† Percentage of total glucose recovered (calculated as glucose equivalents).

‡ Reisolated from the initial acid hydrolysate (Fraction 2, Fig. 2).

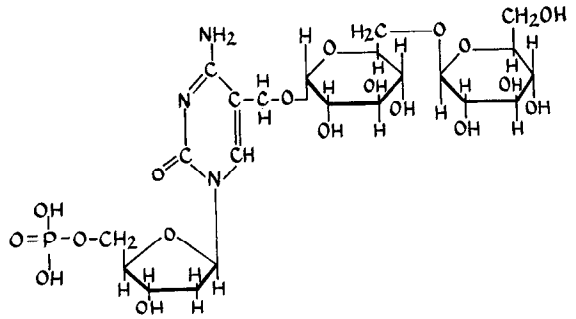

FIG. 3. Postulated structure of diglucosylated dHMP from T6 DNA.

In the case of the diglucosylated dHMP from T2 DNA, extensive characterization of the disaccharide was not possible because of the relatively small amounts of the nucleotide available. In one experiment, the diglucosylated dHMP from T2 was converted to the deoxyuridylate derivative and subjected to acid hydrolysis, and the products were analyzed. The results were almost identical with those obtained with the diglucosylated hydroxymethyl-dUMP from T6 (Table V), suggesting that the diglucosylated HMC nucleotides from T2 and T6 have the same structures.

DISCUSSION

Identification of the diglucosylated HMC from T6 DNA as HMC-α-gentiobioside essentially completes the analysis of the glucosylated constituents of the DNA from the T-even coliphages (6). The results of this analysis may be summarized as follows. T4 contains HMC-α-glucoside (70% of the total HMC) and HMC-β-glucoside (30%); T2 DNA contains HMC-α-glucoside (70%); T6 DNA contains HMC-α-glucoside (3%) and HMC-α-gentiobioside (72%). Although it seems probable that T2 DNA also contains a small amount of the HMC-α-gentiobioside, more work will be needed to prove this point rigorously. Approximately 25% of the HMC residues in T2 and T6 DNA are not glucosylated.

Since the glucose appears to be an integral component of the DNA, *i.e.* not separated from the DNA during replication (25), identification of these sugars should permit a more definitive genetic evaluation of the T-even coliphages. Similarly, it should permit a more positive identification of mutant and hybrid phages derived from them whose over-all glucose contents appear to differ from the wild type (26–28).

Although the evidence presented here is entirely compatible with the assignment of the β (1 → 6) linkage to the glucosyl glucoside moiety of DNA from phage T6, it should be emphasized that the methods used are not sufficiently sensitive to

detect up to 10% of another disaccharide; therefore, the presence to this extent of other sugar components in this DNA cannot be ruled out.

SUMMARY

The disaccharide bound to the 5-hydroxymethylcytosine residues of deoxyribonucleic acid from phage T6 was identified by (*a*) measurement of periodate consumption and formate liberation after periodate oxidation of the diglucosylated hydroxymethyldeoxycytidylate and (*b*) chromatographic, electrophoretic, and enzymatic examination of the isolated disaccharide. All of the evidence obtained indicates that the disaccharide is gentiobiose, 6-*O*-β-D-glucopyranosyl-D-glucose, and that it is distinct from the other known isomeric β linked glucosyl glucosides. Since the gentiobiosyl unit is bound to the hydroxymethyl group of the 5-hydroxymethylcytosine in an α linkage, diglucosylated 5-hydroxymethylcytosine in T6 deoxyribonucleic acid is 5-hydroxymethylcytosine-α-gentiobioside.

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