Alkylation by 1,2:5,6-Dianhydrogalactitol of Deoxyribonucleic Acid and Guanosine

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DNA was alkylated in neutral solution at 37°C with 1,2:5,6-dianhydrogalactitol and hydrolysed to yield two principal products, identified as 7-galactitylguanine and 1,6-deoxy-1,6-di(guanin-7-yl)galactitol. The reaction products were separated by chromatography on Sephadex G-10 and Dowex 50 (H⁺ form). The two compounds were also obtained by reaction between dianhydrogalactitol and guanosine in acetic acid. The products were characterized from their u.v.-spectral data by comparison with those of the 7-alkylguanines and were also identified by mass spectrometry.

Clinical evaluation of 1,2:5,6-dianhydrogalactitol (compound NSC-1322313) has been under study in several clinics (Ahman *et al.*, 1977; Eagan *et al.*, 1977; Hoogstraten *et al.*, 1978; Espana *et al.*, 1978). This compound, like other bifunctional alkylating agents, had shown a marked cytotoxic effect in tumour-bearing animals and in cell-culture systems also (Németh *et al.*, 1972; Pályi, 1975). The cytotoxic effect of the compound was ascribed to its high affinity for tumour-cell DNA as revealed by binding studies performed *in vivo* (Institóris *et al.*, 1978). Examination of the nature of the interaction between DNA and dianhydrogalactitol by using physicochemical methods *in vitro* (Ötvös & Elekes, 1975) and at the cellular level (Institóris & Fox, 1978) showed that some interstrand cross-links could be detected in DNA. These findings prompted us to investigate the possible reactions between DNA and dianhydrogalactitol by using chemical methods. The reaction was carried out in an aqueous medium with pH maintained at 6–7, as described by Lawley & Jarman (1972) for the reaction of propylene oxide with DNA. It was expected, from previous work with various alkylating agents carried out by other researchers (Lawley & Brookes, 1963; Lawley *et al.*, 1975), that guanine would be the main target base in DNA for dianhydrogalactitol. Guanosine was therefore made to react with dianhydrogalactitol to yield appropriate alkylguanines to enable their characterization and establishment of a procedure for their separation.

Materials and Methods

Materials

1,2:5,6-Dianhydrogalactitol was obtained from Chinoin Pharmaceutical Works, Budapest, Hungary. Calf thymus DNA (highly polymerized), guanine and guanosine were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. For column chromatography the sulphonic resin Dowex 50W (X2; 200–400 mesh; H⁺ form) (Fluka, Buchs, Switzerland) and Sephadex G-10 (Pharmacia, Uppsala, Sweden) were used. For t.l.c. silica gel 60F₂₅₄ (Merck, Darmstadt, Germany) was used with the following solvents: A, methanol/aq. NH₃ (sp.gr. 0.90)/propan-2-ol (2:2:1, by vol.); B, butan-1-ol/aq. NH₃ (sp.gr. 0.90)/methanol (5:3:2, by vol.); C, ethanol/aq. NH₃ (sp.gr. 0.90) (3:2, v/v).

Mass spectrometry

The mass-spectral data were obtained with an AEI MS-902 instrument. The source was operated at a nominal ionizing energy of 70 eV and a temperature of 170°C, with the samples being introduced via a direct-inlet system. The exact mass measurements were carried out with an accuracy of ± 3 p.p.m. at a resolving power of 10000, with perfluorotributylamine and tris(perfluoroheptyl)-symthiazine as reference compounds. To avoid the thermal decomposition of samples their trimethylsilyl derivatives were examined. For silylation of the samples *NN*-bis(trimethylsilyl)trifluoroacetamide was used. The procedure was carried out as was proposed for nucleosides by Miller *et al.* (1976).

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Reaction between dianhydrogalactitol and guanosine

Guanosine (1.133 g; 4 mmol) in acetic acid (80 ml) was refluxed with dianhydrogalactitol (0.292 g; 2 mmol) for 1.5 h. The solvent was then removed by evaporation under reduced pressure. The residue was dissolved in 1 M-HCl (20 ml) and hydrolysed for 1.5 h at 100°C. The solution was then applied to a column (17 cm × 4.7 cm) of Dowex 50 (H⁺ form), which was then eluted with 1 M- and 2 M-HCl, and 13 ml fractions were collected. After 3.0 litres of 1 M-HCl had passed through the column, elution was continued with 2 M-HCl until no further u.v.-absorbing material was eluted (5.5 litres total volume). The profile of u.v. absorption showed the presence of five components.

Fractions 42–54 (I) contained a product with a u.v.-absorption spectrum that was unaffected by pH (λ_{max} , 275 nm; A_{280}/A_{260} ratio 1.75). This product could not be isolated, probably owing to destruction during evaporation. Fractions 66–88 (II) and 105–121 (III) gave identical u.v.-absorption spectra

(Table 1). After evaporation of fractions 66–88 (II) and recrystallization from ethanol/water a product was obtained (101 mg) that showed single spots in three solvent systems (Table 1). Its elementary analysis corresponded to galactitylguanine dihydrate, m.p. 264–271°C (decomp.) (Found: C, 37.7; H, 5.24; N, 19.9; C₁₁H₁₇N₅O₆·2H₂O requires C, 37.6; H, 5.98; N, 19.9%). The mass spectrum of the trimethylsilyl derivative of this compound (Fig. 1) exhibited a molecular peak at m/e 819.395 corresponding to C₃₂H₇₃N₅O₆Si₇. Fractions 105–121 (III) were pooled and evaporated under reduced pressure. The residue was recrystallized from water, giving chromatographically homogeneous material (Table 1) (106.8 mg), m.p. 264–268°C (decomp.). Its elementary analysis corresponded to an anhydrogalactitylguanine derivative (Found: C, 42.0; H, 5.39; N, 22.2; C₁₁H₁₅N₅O₅·H₂O requires C, 41.9; H, 5.39; N, 22.2%). The mass spectrum of compound (III) (Fig. 2a) showed a molecular peak at m/e 297.1071, corresponding to C₁₁H₁₅N₅O₅, whereas its trimethylsilyl derivative (for its mass

Table 1. *U.v.-absorption spectra of alkylguanines*

The products were obtained from reactions of guanosine with dianhydrogalactitol as described in the text. For the compositions of solvents A, B and C see the Materials and Methods section.

Guanine derivative	pH	λ_{max} . (nm)	$10^{-3} \times \epsilon$ (M ⁻¹ .cm ⁻¹)	λ_{min} . (nm)	A_{280}/A_{260} ratio	R_F		
						Solvent A	Solvent B	Solvent C
(II) 7-(1-Deoxygalactit-1-yl)- guanine	1	273, 250	7.2, 10.7	230	0.8			
	7	285, 245	8.0, 6.1	260	1.91	0.71	0.38	0.71
	11	280, —	7.3 —	257	1.84			
(III) 7-(1-Deoxyanhydrogalactit- 1-yl)guanine	1	273, 250	6.45, 9.6	230	0.77			
	7	285, 245	7.11, 5.6	260	1.9	0.81	0.50	0.79
	11	283, —	7.28, —	256	1.84			
(V) 1,6-Di(guanin-7-yl)-1,6- dideoxygalactitol	1	275, 250	13.7, 20.3	229	0.74			
	7	insoluble	— —	—	—	~0	~0	0.01
	11	283, —	12.91, —	256	1.74

Fig. 1. 70 eV mass spectrum of the trimethylsilyl derivative of compound (II)
For experimental details see the text.

Fig. 2. 70 eV mass spectrum of compound (III) (a) and that of its trimethylsilyl derivative (b)
For experimental details see the text.

Fig. 3. 70 eV mass spectrum of the trimethylsilyl derivative of compound (V)
For experimental details see the text.

spectrum see Fig. 2b) gave a molecular peak at m/e 657.3040, in good agreement with $C_{26}H_{33}N_5O_5Si_3$, supporting the structure assumed by elementary analysis. Fractions 160–195 contained unchanged guanine (IV), and after 3 litres of 1M-HCl had passed through the column elution was continued with 2M-HCl. The last u.v.-absorbing material (V) was obtained from fractions 420–451; this compound, however, could be directly isolated from the reaction solvent, since its guanosine derivative was precipitated out from acetic acid after standing at room temperature. The precipitate was hydrolysed in 1M-HCl at 100°C for 1 h. The hydrochloride was recrystallized from 1M-HCl (102 mg), m.p. > 300°C (Found: C, 36.4; H, 4.48; N, 26.2; Cl, 13.2; $C_{16}H_{20}N_{10}O_6 \cdot 2HCl$ requires C, 36.8; H, 4.20; N, 26.8; Cl, 13.6%). To obtain the free base the hydrochloride was dissolved in aqueous ammonia and the excess of ammonia was removed under reduced pressure (maximum temperature 40°C), and the insoluble base was precipitated (98 mg), m.p. > 300°C (Found: C, 36.9; H, 5.40; N, 26.6; $C_{16}H_{20}N_{10}O_6 \cdot 4H_2O$ requires C, 36.9; H, 5.40; N, 26.9%). The compound, like other diguaninyl derivatives (Brookes & Lawley, 1961b), had R_F approx. 0 in all solvents tested (Table 1). In the mass spectrum of the trimethylsilyl derivative of compound (V) (Fig. 3) the molecular peak appeared at m/e 1024.475, corresponding to $C_{40}H_{84}N_{10}O_6Si_3$. The three alkylated guanine derivatives were also characterized by their u.v.-absorption spectra measured at three pH values (Table 1), and the nature of their side chains was also established by mass spectrometry, as discussed in the Results and Discussion section.

Reaction between dianhydrogalactitol and DNA

Calf thymus DNA (highly polymerized) (64 mg) in 1.25M-sodium acetate buffer, pH 5.8 (20 ml), was treated with dianhydrogalactitol (409 mg; 2.8 mmol) for 6 days at 37°C. Subsequently the pH was adjusted to 1 with conc. HCl and the mixture was stirred at 70°C for 30 min. The pH of the hydrolysate was adjusted to 10.6 before chromatography on a column (2.6 cm × 32 cm) of Sephadex G-10 with 0.03M-NH₃, pH 10.6, as eluent, at a flow rate of 13 ml/h. The u.v. absorption of the fractions (6.5 ml) showed that fractions 9–19 contained pyrimidine oligonucleotides, fractions 20–22 probably 7-alkylguanines (A_{280}/A_{260} ratio 1.7), fractions 24–30 guanine (A_{280}/A_{260} ratio 1.07) and fractions 37–46 adenine (A_{280}/A_{260} ratio 0.21).

Fractions 20–22 were combined and evaporated to dryness, dissolved in 1M-HCl and rechromatographed on a column (1.2 cm × 17 cm) of Dowex 50W (H⁺ form) eluted with 1M-HCl; 6.5 ml fractions were collected. After 260 ml had passed through the column, elution was continued with 2M-

HCl (Fig. 4b). Three u.v.-absorbing peaks were obtained. The first, largest, one (fractions 12–16) was eluted in the same position as compound (II) (Figs. 4a and 4b) and its u.v.-absorption spectrum was identical with that of compound (II) (Table 1). After evaporation of fractions 12–16 and dissolution of residue in 10M-NH₃, the product gave t.l.c. R_F values exactly the same as those of compound (II) (Table 1). Fractions 19–23 also contained a product with a u.v.-absorption spectrum similar to that of 7-alkylguanine, but its behaviour on column chromatography (Fig. 4) and t.l.c. was closely similar to that of 7-anhydrogalactitylguanine (Table 1). The fractions (67–77) comprising the third peak were combined, and after addition of a large amount

Fig. 4. Comparison of chromatograms of the products from alkylation of guanosine (a) and DNA (b) with 1,2:5,6-dianhydrogalactitol

The products from alkylation of guanosine were applied to a column (1.2 cm × 17 cm) of Dowex (H⁺ form) and eluted with 1M- and 2M-HCl; 6.5 ml fractions were collected; from the point shown by the arrow, elution was continued with 2M-HCl. DNA was alkylated with dianhydrogalactitol, and after its hydrolysis the products were first separated on Sephadex G-10 and the alkylated materials were rechromatographed on Dowex (H⁺ form) with the same system as for alkylguanines. The products were identified by their spectral data and chromatographic properties. In order of elution the compounds are: 7-galactitylguanine, 7-anhydrogalactitylguanine and 1,6-di(guanin-7-yl)-1,6-dideoxygalactitol.

Fig. 5. 70 eV mass spectrum of the trimethylsilyl derivative of 7-(1-deoxygalactit-1-yl)guanine derived from alkylation of DNA by dianhydrogalactitol
For experimental details see the text.

Fig. 6. 70 eV mass spectrum of 7-(1-deoxyanhydrogalactit-1-yl)guanine derived from alkylation of DNA by dianhydrogalactitol
For experimental details see the text.

of methanol were evaporated under reduced pressure. The residue was dissolved in a small volume of 0.1 M-HCl, the clear solution was neutralized with NaOH and the resulting precipitate was collected by centrifugation. On the basis of its u.v.-absorption spectrum and chromatographic behaviour the product was shown to be identical with compound (V). All three products from DNA alkylation were also examined by mass spectrometry in the form of their trimethylsilyl derivatives, except the material from fractions 19–23, which was used in its original form for measurement (Figs. 5, 6 and 7).

Results and Discussion

Reaction of 1,2:5,6-dianhydrogalactitol with guanosine

The reaction conditions for alkylation of guanosine with dianhydrogalactitol were closely similar to those used by Brookes & Lawley (1961b) for the reaction of guanosine with 1,2:3,4-diepoxybutane in acetic acid.

In our case the reaction yielded four alkylated materials, which were separated on Dowex 50 (H⁺ form). The first eluted product (I) had a u.v.-absorption spectrum (λ_{max} , 275 nm; A_{280}/A_{260} ratio 1.75)

Fig. 7. 70 eV mass spectrum of the trimethylsilyl derivative of 1,6-di(guanin-7-yl)-1,6-dideoxygalactitol derived from alkylation of DNA by dianhydrogalactitol
For experimental details see the text.

Scheme 1. Origin of main fragment ions of compound (II) and that of the trimethylsilylated derivatives of compounds (II), (III) and (IV)

that was unaffected by any change of pH. Identification was not possible owing to decomposition of material during evaporation. Compounds (II) and (III) eluted subsequently proved to be 7-(1-deoxygalactit-1-yl) guanine and 7-(1-deoxyanhydrogalactit-1-yl)guanine, and the last one (compound V) was 1,6-di(guanin-7-yl)-1,6-dideoxygalactitol. The structures were deduced from data of the elementary analyses, from mass-spectral data and from u.v.

spectroscopy. The observed u.v.-absorption spectra of compounds (II), (III) and (V) (see Table 1) showed a close similarity to those of 7-alkylpurines reported by Brookes & Lawley (1961b) and Lawley & Jarman (1972). This fact, together with the observation that the position of alkyl substitution in the heterocyclic ring is the main determinant of the u.v.-absorption spectra and not the nature of the alkyl substituent (Lawley *et al.*, 1975), indicated that the substitution was at the N-7 position.

The molecular formulae for compound (III) and for the trimethylsilyl derivatives of compounds (II), (III) and (V) determined from exact mass measurements of molecular ions, as well as from the number of trimethylsilyl groups, are in good agreement with the assigned structures. Furthermore, the main fragment ions observed are of diagnostic character for the structures proposed. Such ions are,

for example, the fragments of common type for compounds (II), (III) and (V) (see Figs. 1, 2 and 3) shown in Scheme 1. The *a*, *b* and *c* type of ions for $R = H$ have also been observed in the spectrum of 7-(2-hydroxypropyl)guanine (Lawley & Jarman, 1972). From the appearance of fragment *c* in the case of compound (III) it follows that the anhydrogalactityl moiety should have a five- or probably four-membered anhydro ring, since the presence of an oxirane ring was ruled out by the lack of alkylating capability when tested by the method of Epstein *et al.* (1955). The abundant peaks of *b* ions observed in spectra of compounds (II) and (III) unambiguously reveal that the site of attachment of the purine ring to the galactitol should be the terminal carbon atom, i.e. the epoxide ring has opened to give 2-ol derivatives. In the case of compound (V) the appearance of ions *b* indicates that at least one of the guaninyl substitutions occurred at a terminal position of the galactitol, as for former compounds (II) and (III). At the same time the absence of *m/e* 103 ($CH_2=O^+-Me_3Si$) ions in the spectrum of the trimethylsilyl derivative of compound (V) also supports the 1,6-di(guaninyl)galactitol structure, since this fragment should appear when primary hydroxyl groups are present.

Reaction of 1,2:5,6-dianhydrogalactitol with DNA

DNA was made to react with dianhydrogalactitol in aqueous medium under the conditions established by Lawley & Jarman (1972) for the reaction of propylene oxide with DNA. The purines were liberated from the alkylated DNA by hydrolysis in 0.1 M-HCl (Lawley & Thatcher, 1970). Subsequently the alkylated bases were separated from the unchanged purines and pyrimidine oligonucleotides by column chromatography on Sephadex G-10 with dilute ammonia. All the alkylated guanine derivatives appeared in the same fractions, and they were further separated into three materials by using chromatography on Dowex (H^+ form) with 1 M- and 2 M-HCl

as eluent. They were identified by comparing their spectral and chromatographic properties with those of galactitylguanine derivatives of known structures. The first material was eluted with 1 M-HCl at the same position as compound (II) (Fig. 1), gave R_F values on t.l.c. and u.v.-spectral data characteristic of compound (II) (Table 1), and the mass spectrum of its trimethylsilyl derivative exhibited a molecular peak at *m/e* 819.395 and the same fragment peaks as those of the trimethylsilyl derivative of 7-(1-deoxygalactit-1-yl)guanine (Figs. 1 and 5). The alkylation of DNA produced a relatively small amount of 7-(1-deoxyanhydrogalactit-1-yl)guanine, identified by its chromatographic and spectral properties (Table 1 and Figs. 2*a* and 6). It is very probable that the anhydro structure was formed from the galactitylguanosine during the hydrolysis under rigorous conditions (see the Materials and Methods section); the mild conditions used for hydrolysis of DNA are not favourable for formation of the anhydro structure. The third material, eluted with 2 M-HCl, was identified on the basis of spectral data: the mass spectrum of its trimethylsilyl derivative was identical with that of compound (V) (Figs. 3 and 7), and its u.v.-spectral data were also very similar to those of the 1,6-di(guanin-7-yl)-1,6-dideoxygalactitol, confirming the 7-alkylation of guanine. This compound has no mobility in the t.l.c. systems tested (Table 1), as with other di(guanin-7-yl) derivatives (Brookes & Lawley, 1961*b*).

It is noteworthy that alkyladenine was not detectable after alkylation of DNA. In a search for galactityladenines the separation of products was simplified by the neutral hydrolysis of alkylated DNA to obtain only alkylated purines (Lawley & Jarman, 1972). With this method the hydrolysate was again free of detectable amounts of alkyladenines and contained solely the three alkylguanine derivatives.

The yields of the three alkylated products were estimated by summation of the A_{260} values for the

Table 2. *Estimated yields of products from alkylation of DNA with 1,2:5,6-dianhydrogalactitol*

DNA was treated with various concentrations of dianhydrogalactitol, and after hydrolysis the products were separated by column-chromatography systems. The yields of the alkylated guanines were estimated by the summation of the values of A_{260} for appropriate fractions, except when the highest concentration of reagent was used. Here the 7-galactitylguanines could be obtained by evaporation of the appropriate fractions, and the diguaninyl derivative was precipitated by neutralization of the eluent. N.M., Not measured.

Concn. of reagent (mmol of 1,2:5,6-dianhydro- galactitol/mmol of DNA P) ...	Extent of alkylation (mmol of alkyl derivative/mol of DNA P)		
	4.5	9.3	14
7-(1-Deoxygalactit-1-yl)guanine	8	12.5	14.0
7-(1-Deoxyanhydrogalactit-1-yl)guanine	1.07	1.4	1.9
1,6-Di(guanin-7-yl)-1,6-dideoxygalactitol	N.M.	1.45	2.3

appropriate fractions, except when a fairly high excess of dianhydrogalactitol was used (molar ratio 14:1) (Table 2); here the 7-galactitylguanine was simply obtained by evaporation of the fractions and the di(guaninyl) derivative could be precipitated by neutralization of the eluent (see the Materials and Methods section). At the highest dianhydrogalactitol concentration used in this study the molar ratio of di(guaninyl) derivative and 7-alkylguanine was found to be 1:7. The relatively low extent of alkylation could be explained by the fact that the hydrolysis rate of oxirane rings is fairly high in the dilute medium used because of the slight solubility of highly polymerized DNA.

In consequence, a considerable amount of dianhydrogalactitol reacted with acetate ion, as demonstrated by the increase in pH during the reaction.

Chemical studies of the alkylation of DNA by difunctional alkylating agents led to the proposal that N-7 of guanine in GpC sequences is the preferred site for the cross-linking formation between the twin strands of DNA (Brookes & Lawley, 1961a). A pair of these atoms are situated at a distance of 0.8 nm (8 Å). X-ray-crystallographic study of 1,2:5,6-dianhydrogalactitol revealed that the distance between the terminal carbon atoms is 0.63 nm (6.3 Å) and it is able to bind two N atoms separated not further than 0.9 nm (9 Å) (Simon & Institóris, 1979). This value suggests that the molecule can be capable of forming interstrand cross-linking in the proposed position of DNA.

On the basis of the present work it can be concluded that the interstrand cross-links demonstrated previously by physicochemical methods (Ötvös & Elekes, 1975; Institóris & Fox, 1978) proceed between guanine base residues of DNA and that the preferred alkylation site for 1,2:5,6-dianhydrogalactitol is the N-7 of guanine.

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