Synthesis, Characterization, and Quantitation of the Major Adducts Formed between Sulfur Mustard and DNA of Calf Thymus and Human Blood

Alex Fidder,† Gerrit W. H. Moes,† Annehieke G. Scheffer,‡ Govert P. van der Schans,‡ Robert A. Baan,‡ Leo P. A. de Jong,† and Hendrik P. Benschop*,†

TNO Prins Maurits Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands, and TNO Medical Biological Laboratory, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands

Received April 20, 1993®

As part of a program to develop methods for verification of alleged exposure to sulfur mustard, we synthesized and characterized the adducts most likely formed by alkylation of DNA with sulfur mustard: *N*7-[2-[(2-hydroxyethyl)thio]ethyl]guanine (**1**), bis[2-(guanin-7-yl)ethyl] sulfide (**2**), *N*3-[2-[(2-hydroxyethyl)thio]ethyl]adenine (**3**), and O^6-[2-[(2-hydroxyethyl)thio]ethyl]-guanine and its 2′-deoxyguanosine derivative. Incubation of double-stranded calf thymus DNA and human blood with [^{35}S]sulfur mustard in vitro followed by enzymatic degradation of the DNA and mild depurination afforded three major radioactive peaks upon HPLC analysis. These peaks were identified as **1–3** by coelution with the synthetic markers and mass spectrometric and electronic spectra. Compound **1** appeared to be the most abundant adduct, which is in agreement with previous investigations on DNA alkylation with sulfur mustard.

Introduction

Reports on treatment of the casualties from chemical warfare during the First Gulf War (1980–1988) in hospitals all over Europe have shown the need for reliable methods to establish the nature of the agent to which the casualties were exposed (*1*). In addition, unequivocal methods to verify alleged exposure of victims to chemical warfare agents are needed in order to sustain the credibility of the recently signed treaty to ban chemical weapons (*2*). As a first effort in this field, we focused our attention toward the development of such methods for the chemical warfare agent sulfur mustard. Due to the strong alkylating properties of sulfur mustard, adducts formed with DNA and proteins from various biopsies may serve to monitor exposure to the agent, e.g., by immunochemical detection of the adducts.

The alkylation of DNA by sulfur mustard was investigated in the 1960s by Brookes and Lawley (*3–11*). They suggested that *N*7-[2-[(2-hydroxyethyl)thio]ethyl]guanine (**1**; see Chart 1), the interstrand and intrastrand diadduct bis[2-(guanin-7-yl)ethyl] sulfide (**2**), and *N*3-[2-[(2-hydroxyethyl)thio]ethyl]adenine (**3**) are formed upon in vitro alkylation of DNA by sulfur mustard. More recently, Ludlum et al. (*12, 13*) reported that O^6-[2-(ethylthio)-ethyl]guanine was formed upon reaction of DNA with the sulfur mustard analogue 2-chloroethyl ethyl sulfide.

Up to now, the characterization of the reference compounds for identification of the adducts formed after exposure of DNA to sulfur mustard was based only on similarity of their UV spectra with those of analogous alkyl-substituted purines. Moreover, **1** was the only reference compound for which the isolation has been described explicitly (*3, 4*). In this paper, we report the resynthesis of **1–3** and a new route for the synthesis of O^6-[2-[(2-hydroxyethyl)thio]ethyl]guanine (**4**) and O^6-[2-[(2-hydroxyethyl)thio]ethyl]-2′-deoxyguanosine (**5**) and provide unequivocal spectroscopic evidence for the structure of the adducts. The adducts formed upon in vitro exposure of double-stranded calf thymus DNA and of DNA in human white blood cells to [^{35}S]sulfur mustard were identified and quantitated using the synthesized adducts as reference compounds.

Experimental Procedures

Chemicals. Caution: *Sulfur mustard [2,2′-bis(2-chloroethyl) sulfide] is a carcinogenic, vesicant, and cytotoxic agent.*

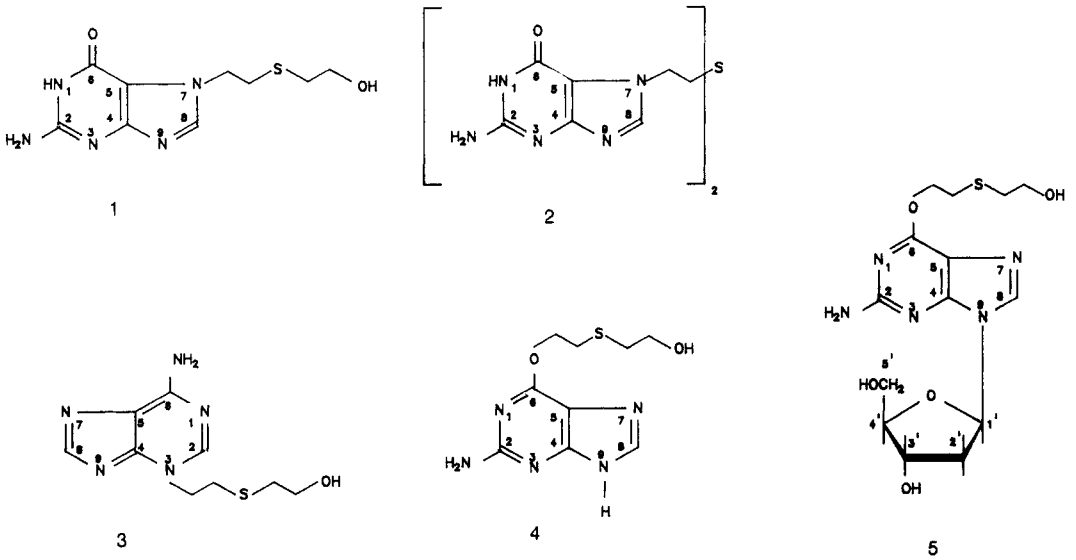
Adenosine, alkaline phosphatase type III, double-stranded calf thymus DNA, 2′-deoxynucleosides, deoxyribonuclease I, GMP, and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease P1 and RNase T1 were purchased from Boehringer (Mannheim, Germany), and proteinase K was obtained from Merck (Darmstadt, Germany). O^6-Ethylguanine was procured from Chemsyn Science Laboratories (Lenexa, KS). Technical-grade sulfur mustard was distilled before use to a gas chromatographic purity exceeding 99.5%. [^{35}S]Hydrogen sulfide (8.4 GBq/mmol) was purchased from Amersham International (Houten, The Netherlands). Human blood was obtained from volunteers in our laboratories.

Instrumentation. HPLC analyses described in the synthetic part of this paper were carried out on a Lichrosorb reverse-phase C18 column (250 × 5 mm) using a Waters model 510 HPLC pump (Bedford, MA); the eluent (1 mL/min) was 30% methanol in water (v/v; system A). Preparative column chromatography was performed using a VZE pump (at pressures ≤0.6 MPa; Verder, The Netherlands), an Applied Biosystems 757 detector (San Ramon, CA) or a Waters 990 array detector, and a Lobar column (440 × 37 mm, Merck) filled with Lichrosorb reverse-phase C18 material (particle size 40–63 μm); the eluent (4–6 mL/min) was 25 mM NH_4HCO_3 in methanol/water (1/3 v/v; system B). HPLC analyses of DNA hydrolysates were performed on a reverse-phase C18 column (250 × 4.6 mm, 5-μm particles ODS Ultrasphere; Beckman, Fullerton, CA); the eluent (1 mL/min) was 25 mM NH_4HCO_3 (pH 8) and a linear gradient to 25 mM NH_4HCO_3 in

* Correspondence should be addressed to this author at the TNO Prins Maurits Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands.
† TNO Prins Maurits Laboratory.
‡ TNO Medical Biological Laboratory.
® Abstract published in *Advance ACS Abstracts*, February 1, 1994.

0893-228x/94/2707-0199$04.50/0

Chart 1. Structures and Ring Numbering of Sulfur Mustard–DNA Base Adducts



16% methanol over 20 min, followed by a linear gradient to 25 mM NH_4HCO_3 in 48% methanol over 20 min (system C). TLC was performed on Merck HPTLC plates (60F 254; 5 × 10 cm) or on Merck RP-18 plates (5 × 20 cm) using methanol/dichloromethane (8/92 v/v) or acetone/chloroform (45/55 v/v) as the mobile phase. Gel filtration on Sephadex G-10 (Pharmacia, Uppsala, Sweden) and anion-exchange chromatography on Q-Sepharose (Pharmacia) were performed with a P-1 pump, GP-250 gradient programmer, Frac-100 fraction collector, UV-1 optical unit (254 nm), and UV-1 control unit (Pharmacia). GLC was performed on a Chrompack (Bergen op Zoom, The Netherlands) 483A gas chromatograph, equipped with a flame-ionization detector and a wide-bore glass capillary column (0.7 mm × 50 m) coated with SE-30 (1-μm film thickness). Thermospray LC/MS spectra were recorded on a Nermag (Paris, France) R10-10C quadrupole instrument operated in the positive mode and equipped with a thermospray ion source (Nermag) coupled via a Vestec interface (Vestec Co., Houston, TX). The LC system comprised a reverse-phase C18 column (Lichrosorb 5-μm particles) with 0.1 M aqueous ammonium acetate and methanol in varying ratios as eluent. ^1H and ^{13}C NMR spectra were recorded on a Varian (Palo Alto, CA) VXR 400S spectrometer operating at 400.0 and 100.6 MHz, respectively. Chemical shifts are given in ppm relative to TMS. The solvent signals at 2.525 ppm (residual Me_2SO-d_5 in Me_2SO-d_6) served as a reference for ^1H NMR spectroscopy, whereas the solvent signals at 39.6 ppm (Me_2SO-d_6), 77.1 ppm ($CDCl_3$), or 115.7 and 162.9 ppm [$CF_3C(O)OD$] were used as a reference for ^{13}C NMR spectroscopy. UV spectra were recorded on a Beckman UV-7 spectrophotometer (Beckman Instruments, Irvine, CA). Radioactivity counts were performed on a Packard Tri-Carb series Minaxi (Downers Grove, IL) or a Packard Mark III liquid scintillation spectrometer with Picofluor 30 (Packard) as a scintillation cocktail. Thin-layer chromatograms of radioactive products were scanned using a Berthold LB 2723 DC scanner (Wildbad, Germany) equipped with a windowless proportional counting tube.

Synthesis. [^{35}S]Sulfur mustard was prepared on a 1-mmol scale by reaction of [^{35}S]hydrogen sulfide with ethylene oxide followed by chlorination of the formed [^{35}S]thiodiglycol with thionyl chloride, as described by Boursnell et al. (*14*): yield 24%; radiochemical purity (TLC) 98%; chemical purity (GC) 95%; specific activity 850 MBq/mmol.

2-Acetoxyethyl 2′-Hydroxyethyl Sulfide. Thiodiglycol (122 g, 1 mol) and dry pyridine (7.9 g, 0.1 mol) were dissolved in dichloromethane (1 L). A solution of acetyl chloride (7.85 g, 0.1 mol) in dichloromethane (100 mL) was added dropwise with stirring at room temperature over 1.5 h. After stirring for another 2 h, the reaction mixture was poured into water (250 mL). The organic layer was washed with water (3 × 100 mL). Distillation of the crude compound gave 8.7 g (49%) of the desired compound as a thin, colorless oil: bp 131–135 °C/266 Pa; purity ca. 97% (GC; HPLC, system A); ^1H NMR ($CDCl_3$) δ 4.24 (t, J = 6.7 Hz, 2H, $AcOCH_2$), 3.76 (q, J = 6.0 Hz, 2H, CH_2OH), 2.78 (t, J = 6.0 Hz, 2H, $HOCCH_2S$), 2.78 (t, J = 6.7 Hz, 2H, SCH_2COAc), 2.10 (t, J = 6.0 Hz, 1H, OH), 2.08 (s, 3H, CH_3).

***N*7-[2-[(2-Hydroxyethyl)thio]ethyl]guanine (1).** A solution of GMP (407 mg, 1 mmol) in water (10 mL) was adjusted to pH 4.5 with 1 M HCl. Sulfur mustard (0.25 mL, 2 mmol) was added, and the pH was maintained at 4.5 with 0.1 M NaOH. After 6 h, HPLC analysis (system A) showed one main product peak (ca. 40%) in addition to starting material and thiodiglycol. The reaction mixture was neutralized with 0.1 M NaOH, extracted with dichloromethane (3 × 10 mL), and purified on a Q-Sepharose column (2 × 30 cm) in portions of 3 mL. The reaction product and thiodiglycol were eluted (3 mL/min) using water as an eluent (90 mL), after which the starting material was washed from the column with 1 M NaCl (90 mL) and with water (120 mL). The appropriate fractions were evaporated to dryness under reduced pressure and dissolved in 25 mL of 1 M HCl. Depurination of *N*7-alkylated GMP was achieved by boiling this solution for 1 h. HPLC analysis (system A) showed the presence of thiodiglycol and the supposed 1. The mixture was evaporated to a small volume (5 mL) under reduced pressure and neutralized with ammonia. An insoluble light-yellow material appeared, which was washed with water and then dissolved in 5 mL of 1 M HCl. This solution was desalted on a Sephadex G-10 column (2 × 100 cm) which was eluted with water (15 mL/h). UV-positive fractions were pooled and lyophilized to give 40 mg (15% yield) of a white solid material: mp >280 °C dec [lit.: >280 °C dec (*3*)]; ^1H NMR (Me_2SO-d_6) δ 10.8 (bs, 1H, NH), 7.94 (s, 1H, H-8), 6.17 (s, 2H, NH_2), 4.78 (t, J = 5.5 Hz, 1H, OH), 4.35 (t, J = 6.7 Hz, 2H, NCH_2), 3.54 (dt, J = 5.4 and 6.7 Hz, 2H, CH_2OH), 2.97 (t, J = 6.7 Hz, 2H, $NCCH_2S$), 2.59 (t, J = 6.7 Hz, 2H, SCH_2COH); ^{13}C NMR (Me_2SO-d_6) δ 160.2 (C-4), 154.6 (C-6), 152.8 (C-2), 143.5 (C-8), 107.9 (C-5), 60.8 (CH_2OH), 46.0 (NCH_2), 33.8 (SCH_2COH), 32.2 ($NCCH_2S$); thermospray LC/MS m/z 256 [MH^+]; UV (values in parentheses from ref 3) pH 1, UV_{max} 249 nm (250 nm); pH 7, UV_{max} 284 nm (284 nm); pH 12, UV_{max} 280 nm (281 nm).

Bis[2-(guanin-7-yl)ethyl] Sulfide (2). The compound was synthesized starting from equimolar amounts of GMP and sulfur mustard (4.2 mmol), according to Brookes and Lawley (*4*). Extractions of the crude product with 0.01 M HCl (3 × 10 mL) left a product containing 86% of 2 which could be crystallized from boiling in 0.01 M HCl (25 mL): yield 115 mg (6.9%); purity 97% (HPLC, system A); mp >230 °C dec; ^1H NMR [$CF_3C(O)$-

OD] δ 8.95 (s, 2H, 2H-8), 4.86 (bt, J = ca. 5 Hz, 4H, $2NCH_2$), 3.27 (bt, J = ca. 5 Hz, 4H, $2CH_2S$); ^{13}C NMR [$CF_3C(O)OD$] δ 154.6 (C-6), 154.0 (C-2), 145.9 (C-4), 140.7 (C-8), 109.5 (C-5), 50.0 (NCH_2), 32.1 (CH_2S); thermospray LC/MS m/z 389 [M^+H^+], 411 [MNa^+]; UV (values in parentheses from ref 3) pH 1, UV_{max} 249 nm (250 nm); pH 7, UV_{max} 284 nm (284 nm); pH 14, UV_{max} 280 nm (281 nm).

N3-[2-[(2-Hydroxyethyl)thio]ethyl]adenine (3). The compound was synthesized from adenosine (18.7 mmol) analogously to the procedure described by Brookes and Lawley (*3*) for the synthesis of 1. The desired product was isolated by low-pressure liquid chromatography (system B) of 10 portions of 0.5 mL each of the crude product dissolved in water. The UV-positive fractions (254 nm) were pooled and lyophilized to give a pale-brown solid (51 mg, 1.1%): purity 98% (HPLC, system A); mp >188 °C dec; ^1H NMR (Me_2SO-d_6) δ 8.36 (s, 1H, H-2), 7.87 (b, 2H, NH_2), 7.78 (s, 1H, H-8), 4.87 (bt, J = ca. 5 Hz, 1H, C-OH) 4.49 (t, J = 6.7 Hz, 2H, NCH_2), 3.56 (bdt, J = ca. 5 and ca. 7 Hz, 2H, CH_2OH), 3.12 (t, J = 6.7 Hz, 2H, $NCCH_2S$), 2.64 (t, J = 6.7 Hz, 2H, SCH_2COH); ^{13}C NMR (Me_2SO-d_6) δ 155.2 (C-6), 152.4 (C-8), 149.5 (C-4), 143.8 (C-2), 120.3 (C-5), 60.8 (CH_2OH), 49.0 (NCH_2), 33.9 (SCH_2COH), 30.3 (SCH_2CN); thermospray LC/MS m/z 240 [MH^+]; UV (values in parentheses from ref 15) pH 1, UV_{max} 274 nm (274.5 nm); pH 7, UV_{max} 273 nm; pH 12, UV_{max} 272 nm (274 nm).

3',5',N²-Triacetyl-2'-deoxyguanosine (6). This compound was prepared from 2'-deoxyguanosine (5 mmol) according to Gaffney et al. (*16*): yield 1.4 g (70%); mp 203–206 °C [lit.: 190 °C (softening), >225 °C dec (*16*)]. The ^1H and ^{13}C NMR spectra (Me_2SO-d_6) were in accordance with the structure. Thermospray LC/MS m/z 394 [MH^+], 416 [MNa^+], 432 [MK^+]; UV in methanol (values in parentheses from ref 16) UV_{max} 256.5 nm (255 nm); UV_{min} 224.5 nm (224 nm); UV_{sh} 227.5 nm (278 nm).

O⁶-[2-[(2-Hydroxyethyl)thio]ethyl]-2'-deoxyguanosine (5). Compound 5 was prepared analogously to the procedure of Li and Swann (*17*). Briefly, 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl;[1] 1.2 g, 4 mmol), triethylamine (1.2 mL, 8 mmol), and 4-(dimethylamino)pyridine (15 mg) were added to 6 (0.8 g, 2 mmol) dissolved in anhydrous dichloromethane (8 mL). After 30 min, the formed O^6-[(2,4,6-triisopropylphenyl)sulfonyl]-3',5',N^2-triacetyl-2'-deoxyguanosine (7) was purified by flash chromatography on a silica gel column (2 × 8 cm), using dichloromethane as an eluent. The product dissolved in dry dichloromethane (1 mL) was cooled to 0 °C, and N-methylpyrrolidine (NMP; 0.93 g, 11 mmol) was added to give 6-(N-methylpyrrolidiniumyl)-3',5',N^2-triacetyl-6,2'-dideoxyguanosine (8). After stirring for 10 min, 2-acetoxyethyl 2'-hydroxyethyl sulfide (3.3 g, 20 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 0.23 g, 1.5 mmol) were added to the solution. The mixture was allowed to warm to room temperature and stirred for another 2 h to give O^6-[2-[(2-acetoxyethyl)thio]ethyl]-3',5',N^2-triacetyl-2'-deoxyguanosine (9). The solution was diluted with dichloromethane (50 mL) and washed with a saturated aqueous solution of NH_4Cl (3 × 10 mL). Dichloromethane was evaporated in vacuo. The residue was purified on a silica gel column (3 × 30 cm) using a gradient of petroleum ether (boiling range 40–60 °C)/dichloromethane (1/1 v/v) to dichloromethane/methanol (9/1 v/v). Removal of the acetyl groups was accomplished using 25% aqueous ammonia/dioxane (50 mL, 1/1 v/v) at 50 °C for 20 h to give crude 5. The latter product was isolated using a Sephadex G-10 column (100 × 2 cm) which was eluted with water (15 mL/h): yield 90 mg (12%); mp 230 °C dec; ^1H NMR (Me_2SO-d_6) δ 8.10 (s, 1H, H-8), 6.42 (bs, 2H, NH_2), 6.24 (dd, J = 6.1 and 7.7 Hz, 1H, H-1'), 5.27 (d, J = 4.0 Hz, 1H, C-3'-OH), 4.99 (t, J = 5.6 Hz, 1H, OH), 4.83 (t, J = 5.5 Hz, 1H, OH), 4.56 (t, J = 7.0 Hz, 2H, O^6-CH_2), 4.38 (m, 1H, H-3'), 3.85 (dt, J = 2.8 and 4.6 Hz, 1H, H-4'), 3.64–3.49 (m, 4H, $2CH_2OH$), 2.96 (t, J = 7.0 Hz, 2H, O^6-CCH_2S), 2.71 (t, J = 6.7 Hz, 2H, SCH_2COH), 2.60 and 2.24 (ddd, 2H, H-2' and H-2''); ^{13}C NMR (D_2O) δ 159.9 (C-6), 159.6 (C-2), 153.9 (C-4), 137.8 (C-8), 113.8 (C-5), 87.6 (C-4'), 82.8 (C-1'), 70.8 (C-3'), 65.2 (O^6-CH_2CS), 61.8 (C-5'), 61.0 (CH_2OH), 39.1 (C-2'), 34.3 (SCH_2COH), 30.1 (O^6-CCH_2S); thermospray LC/MS m/z 372 [MH^+], 256 [MH^+ of 4], 152 [MH^+ of Gua]; UV: pH 1, UV_{max} 288.5, 243 nm, UV_{min} 261, 232 nm; pH 7, UV_{max} 281, 247.5 nm, UV_{min} 262, 228 nm; pH 13, UV_{max} 281, 247.5 nm, UV_{min} 227, 262 nm.

O⁶-[2-[(2-Hydroxyethyl)thio]ethyl]guanine (4). Compound 5 (30 mg, 0.08 mmol) was stirred with 0.1 M HCl (25 mL) for 10 min. The reaction mixture was neutralized with ammonia and concentrated to a small volume under reduced pressure at 25 °C. The residue was applied to a Sephadex G-10 column (100 × 2 cm) which was eluted with water (15 mL/h). UV-positive fractions (254 nm) were collected and lyophilized: yield 8 mg (0.03 mmol, 39%); purity (^1H NMR) >95%; ^1H NMR (Me_2SO-d_6) δ 12.4 (b, 1H, NH), 7.84 (s, 1H, H-8), 6.20 (s, 2H, NH_2), 4.56 (t, J = 6.9 Hz, 2H, O^6-CH_2), 3.61 (t, J = 6.8 Hz, 2H, CH_2OH), 2.96 (t, J = 6.9 Hz, 2H, O^6-CCH_2S), 2.72 (t, = 6.8 Hz, 2H, SCH_2COH); ^{13}C NMR (Me_2SO-d_6) δ 159.7 and 159.6 (C-2 and C-6), 156.0 (b, C-4), 138.3 (C-8), 113.0 (b, C-5), 65.1 (O^6-CH_2), 61.0 ($SCCH_2OH$), 34.3 (SCH_2COH), 30.1 (O^6-CCH_2S); thermospray LC/MS m/z 256 [MH^+], 278 [MNa^+], 152 [MH^+ of Gua]; UV pH 1, UV_{max} 287.5 nm, UV_{min} 253; pH 7, UV_{max} 281.5, 239.5 nm, UV_{min} 258, 228 nm; pH 13, UV_{max} 284, UV_{min} 258 nm, UV_{sh} 245 nm.

Exposure of Double-Stranded Calf Thymus DNA and Human Blood to Sulfur Mustard. A solution of double-stranded calf thymus DNA in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, and 15 mM KH_2PO_4, pH 7.4; 1 mg/mL) or human blood (10 mL) was treated with [^{35}S]sulfur mustard in acetone (0.1 and 1 mM; final acetone concentration 1%) for 30–60 min at 37 °C.

Isolation of DNA from White Blood Cells, Enzymatic Hydrolysis of Isolated DNA, and Analysis of Degraded DNA. The isolation of white blood cells and of DNA from the cells and the breakdown of DNA into nucleosides were performed according to standard techniques used in our laboratory (*18*, *19*). Next, nucleosides alkylated at a position which resulted in relatively instable adducts were depurinated by heating the nucleoside mixture at 100 °C for 5 min.

Results and Discussion

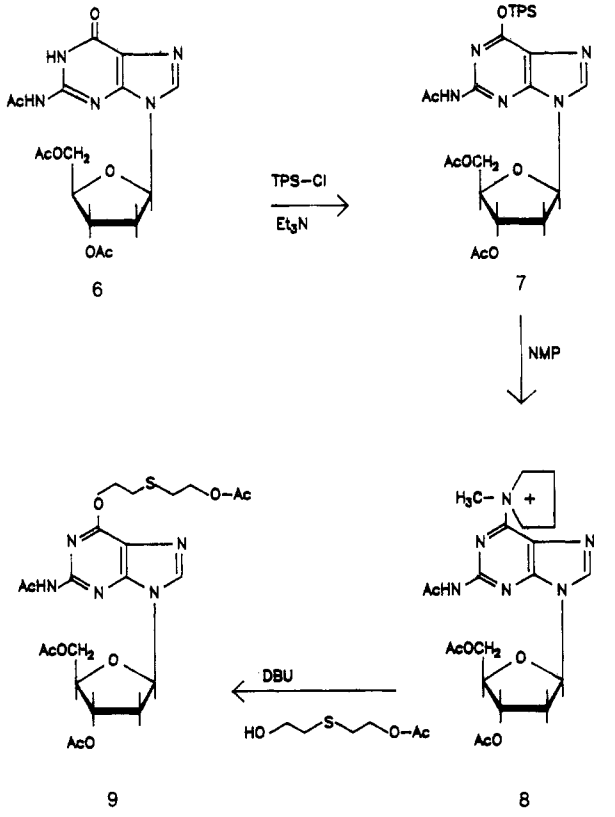
Synthesis and Characterization of the Adducts. The pH was maintained at 4.5 during alkylation of GMP, the starting material for the synthesis of 1 and 2, in order to avoid alkylation of the phosphate moiety (*20*). The presence of the phosphate moiety allowed a rapid isolation of $N7$-alkylated GMP by means of anion-exchange chromatography. Subsequent depurination gave 1 in 15% overall yield. The diadduct 2 could easily be obtained in analytically pure state by crystallization from dilute aqueous HCl after the depurination step, taking advantage of its extremely low solubility in water. We found no evidence that the $N1$-adduct is formed simultaneously with the $N3$-adduct 3 upon alkylation of Ade in glacial acetic acid, while this product has been isolated from reaction of 2'-deoxyguanosine 5'-phosphate (*10*) and of Ade (*15*) with semi-sulfur mustard in aqueous solution.

The method for synthesis of 5 starting from 6-chloro-3',5'-di-O-acetyl-2'-deoxyguanosine and the monosodium salt of thiodiglycol as reported by Ludlum (*12*, *13*) proved to be unsatisfactory in our hands. We pursued a recent and convenient route (Scheme 1) for the synthesis of this compound, starting from easily accessible 8 (*16*, *17*).

The m/z values of the molecular ions of 1–3 in their thermospray LC/MS spectra correspond with the proposed structures. UV spectra of the adducts were identical with those reported earlier (*3*, *4*, *15*). Final proof for the

[1] Abbreviations: DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; NMP, N-methylpyrrolidine; PBS, phosphate-buffered saline; TPS-Cl, 2,4,6-triisopropylbenzenesulfonyl chloride.

Scheme 1. Reaction Pathway for the O^6 Alkylation of 6



supposed structures was obtained from ^1H and ^{13}C NMR spectroscopy by assignment of the carbon signals from single-frequency decoupling. The long-range couplings found between the N-CH_2 protons of the 2-[(2-hydroxyethyl)thio]ethyl moiety and C5/C8 of the Gua ring (3 and 4 Hz, respectively) and between these protons and C2/C4 of the Ade ring (5 and 3 Hz, respectively) showed that this moiety was attached to the $N7$-position of Gua and to the $N3$-position of Ade, respectively.

The structure of **5** followed from the route of synthesis. Results of UV, thermospray LC/MS, and ^1H and ^{13}C NMR analysis are consistent with the proposed structure. Furthermore, the shift of the carbon of 2-[(2-hydroxyethyl)thio]ethyl moiety directly attached to O^6 of the Gua ring in the ^{13}C NMR spectrum of the compound is relatively high (65.2 ppm), as compared with the shift of the same carbon in **1** (46 ppm), which is in accordance with binding of this moiety of **5** to oxygen rather than to nitrogen.

Compound **4** was obtained after depurination of **5** in 0.1 M aqueous HCl for 10 min at room temperature. This treatment led only to partial dealkylation, which was unexpected in view of the high lability of O^6-[2-[(2-hydroxyethyl)thio]ethyl] and O^6-[2-(ethylthio)ethyl] bonds in acidic medium for the O^6-substituted Gua nucleosides as reported by Ludlum et al. (*12*). The reasonable stability of **4** in acidic solution found in our work was confirmed by the half-lifetime for dealkylation at pH 0.5 and 25 °C (57 min), as determined from the increase of absorbance at 248 nm in duplicate experiments. However, dealkylation proceeded more than 2 orders of magnitude slower when the 2-[(2-hydroxyethyl)thio]ethyl moiety was replaced by an ethyl group ($t_{1/2}$ = 260 h). It is suggested that anchimeric assistance by bivalent sulfur with intermediate formation of an episulfonium ion provides the extra driving force for the accelerated hydrolysis of **4**.

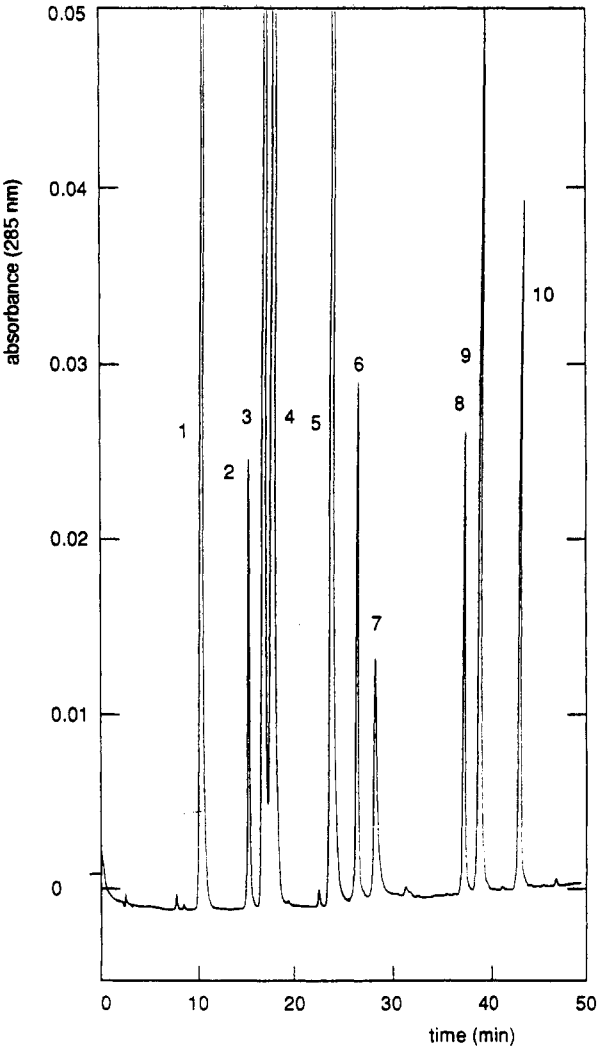


Figure 1. HPLC chromatogram of DNA nucleosides and synthetic sulfur mustard–DNA adducts. The products were analyzed on a reverse-phase C18 column (ODS Ultrasphere) with UV monitoring (285 nm). The column was eluted using system C. Peaks: 1, 2′-deoxycytidine; 2, 2′-deoxy-5-methylcytidine; 3, 2′-deoxyguanosine; 4, dThd; 5, 2′-deoxyadenosine; 6, 1; 7, 3; 8, 2; 9, 4; 10, 5.

Identification of the Adducts in DNA. The adducts formed with DNA were identified in double-stranded calf thymus DNA and in human blood exposed to sulfur mustard. Alkylation of sulfur mustard at the $N7$-position of 2′-deoxyguanosine or at the $N3$-position of 2′-deoxyadenosine results in adducts which are unstable due to the presence of a positive charge in the purine moiety. Therefore, selective release of the modified purines formed upon exposure of DNA to sulfur mustard was brought about by mild, enzymatic hydrolysis of the isolated DNA into nucleosides, followed by a short heating step (5 min, 100 °C). The heating step, however, would not depurinate **5**. This was confirmed by HPLC analysis (system A) and thermospray LC/MS analysis of a heat-treated solution of **5** (PBS, pH 7.4).

An HPLC system was developed which separates the four major nucleosides, 2′-deoxy-5-methylcytidine, and the five synthesized adducts (Figure 1). HPLC analysis (system C) of double-stranded calf thymus DNA treated with 1 or 0.1 mM [^{35}S]sulfur mustard and subsequently hydrolyzed and depurinated revealed five main peaks in the UV absorbance profiles, which could be attributed to the expected four major nucleosides and to 2′-deoxy-5-

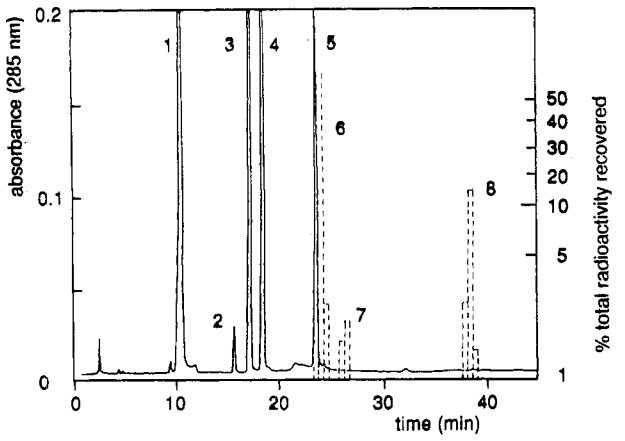


Figure 2. HPLC chromatogram of hydrolyzed DNA from human blood exposed to 1 mM [^{35}S]sulfur mustard (30 min, 37 °C). The DNA hydrolysate was analyzed on a reverse-phase C18 column (ODS Ultrasphere); UV absorbance (285 nm) and radioactivity of 0.5-min fractions were monitored. The column was eluted using system C. Peaks: 1, 2′-deoxycytidine; 2, 2′-deoxy-5-methylcytidine; 3, 2′-deoxyguanosine; 4, dThd; 5, 2′-deoxyadenosine; 6, 1; 7, 3; 8, 2.

methylcytidine from comparison with HPLC analysis of nontreated DNA and from coelution with the commercially available nucleosides. Upon spiking with the synthesized adducts, three radioactive peaks were assigned to 1–3. The three major adducts were also identified by UV spectroscopy using a diode array detector as well as by thermospray LC/MS.

HPLC analyses of the DNA hydrolysates showed no significant amount of radioactivity at the positions of **4** and **5**. The latter product was coeluted to make sure that depurination of **5** had not occurred in any way during workup of DNA. At most, a very low degree of alkylation at this position by sulfur mustard should be expected on the basis of the high *s* value (0.95) of the episulfonium ion in the Swain–Scott equation (*21*, *22*).

DNA which was isolated from human blood after treatment with [^{35}S]sulfur mustard and degraded gave similar results as were obtained with double-stranded calf thymus DNA (Figure 2). Although three radioactive peaks were found which coeluted with the synthesized adducts, the amount of adducts were below the detection limits for UV and thermospray LC/MS detection.

Quantitation of the Adducts in DNA. The three major adducts were quantitated in two experiments. The amount of [^{35}S]sulfur mustard bound to DNA after exposure of human blood and double-stranded calf thymus DNA to 1 and 0.1 mM [^{35}S]sulfur mustard is shown in Table 1. Much more radioactivity had reacted per gram of DNA with double-stranded calf thymus DNA than with DNA in the white blood cells of human blood. This suggests that components of blood interfere with the reaction of sulfur mustard with nuclear DNA.

Treatment with a 10-fold higher concentration of [^{35}S]-sulfur mustard resulted in an approximately 7-fold increased level of alkylation. Evidently, the concentration of sulfur mustard is not the only rate-determining factor in the formation of DNA adducts.

The distribution of bound radioactivity over the three major adducts as found after HPLC separation is also given in Table 1. These adducts are responsible for most of the radioactivity in the HPLC eluate. The proportion of **3** in double-stranded calf thymus DNA was higher than in DNA from white blood cells, whereas in one experiment

Table 1. Degree of Alkylation of DNA[a] upon Treatment of Double-Stranded Calf Thymus DNA (1 mg/mL PBS) and of Human Blood (20-25 μg of DNA/mL) with [^{35}S]Sulfur Mustard, and Molar Ratios of Individual Purines Alkylated in DNA[a]

sample	concn of [^{35}S]sulfur mustard (mM)	amt of [^{35}S]sulfur mustard bound to DNA (μmol of [^{35}S]sulfur mustard/g of DNA)	molar ratio of alkylated purines (% of total radioactivity)[b] 1	2	3
calf thymus DNA	1	110	66	14	10
	0.1	16	55	15	11
blood	1	7	61	15	5
	0.1	0.9	61	20	7

[a] Averages of two experiments. [b] The percentages were corrected for the amount of thiodiglycol recovered.

with human blood **3** was not found at all. Higher concentrations of sulfur mustard resulted in relatively more **1** and less **2**.[2]

The results obtained after treatment of DNA from both sources with 0.1 mM sulfur mustard are roughly comparable with the ratios for the principal adducts reported in previous studies on [^{35}S]sulfur mustard alkylation of DNA from bacteriophage T2 (*6*), T7 (*11*), salmon sperm (*9*), and yeast (*24*); i.e., 1.6–2.0-fold higher amounts of **1** are formed than of the two other adducts together. Somewhat more **3** than **2** was found in bacteriophages and yeast, whereas more diadduct was found in salmon sperm DNA and in our studies.

Confirming the results of Lawley and co-workers (*3–11*), we conclude that exposure of double-stranded calf thymus DNA to sulfur mustard results mainly in the formation of **1**, together with minor amounts of **2** and **3**. Similar results were obtained in human blood. Adduct **5** was not formed in measurable amounts in our experiments, which is not in accordance with the results of Ludlum et al. (*12*, *13*).

Since **1** appears to be the major adduct formed in all cases, a suitable derivative of this compound will be used as a hapten for the development of an immunochemical detection method in order to verify exposure to sulfur mustard.[3] In forthcoming papers, detection of protein adducts of sulfur mustard will be described, which are in general longer lived than DNA adducts.

Acknowledgment. This work was supported in part by Grant DAMD17-88-Z-8022 from the U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD, and in part by the Directorate of Military Medical Services of the Ministry of Defense, The Netherlands. The authors are grateful to Simon H. van Krimpen and P. Eugène J. Verwiel for performing NMR spectroscopy, to Albert G. Hulst and Eric R. J. Wils for performing thermospray LC/MS, to Ad L. de Jong for performing diode array spectroscopy, to Richard H. Bode

[2] In a separate experiment where single-stranded calf thymus DNA was used instead of double-stranded calf thymus DNA only a slightly lower proportion of **2** was formed. This suggests that the majority of these adducts do result from intrastrand cross-links, which is in accordance with the findings of Lawley et al. (*11*) for reaction of bacteriophage T7 with sulfur mustard that one-fourth or less of the diadducts formed cross-links between the two DNA chains. In most cases, **2** appears to be formed via reaction of one molecule of sulfur mustard with two adjacent Gua bases in the same DNA strand (*23*).

[3] G. P. van der Schans, A. G. Scheffer, R. H. Mars-Groenendijk, A. Fidder, H. P. Benschop, and R. A. Baan, unpublished results.

for technical assistance, and to Dr. Daan Noort for critically reading the manuscript.

References

(1) *Report of the specialists appointed by the Secretary-General to investigate allegations by the Islamic Republic of Iran concerning the use of chemical weapons.* UN document S/16, 433, 26 March, 1984.

(2) *Draft convention on the prohibition of the development, production, stockpiling and use of chemical weapons and on their destruction.* Report of the Conference on Disarmament to the General Assembly of the United Nations, CD/1173, 3 September, 1992.

(3) Brookes, P., and Lawley, P. D. (1960) The reaction of mustard gas with nucleic acids *in vitro* and *in vivo*. *Biochem. J.* **77**, 478–484.

(4) Brookes, P., and Lawley, P. D. (1961) The alkylation of guanosine and guanylic acid. *J. Chem. Soc.*, 3923–3928.

(5) Brookes, P., and Lawley, P. D. (1961) The reaction of mono- and di-functional alkylating agents with nucleic acids. *Biochem. J.* **80**, 496–503.

(6) Brookes, P., and Lawley, P. D. (1963) Effects of alkylating agents on T2 and T4 bacteriophages. *Biochem. J.* **89**, 138–144.

(7) Brookes, P., and Lawley, P. D. (1963) Evidence for the action of alkylating agents on deoxyribonucleic acid. *Exp. Cell Res. Suppl.* **9**, 521–524.

(8) Lawley, P. D., and Brookes, P. (1963) Further studies on the alkylation of nucleic acids and their constituent nucleotides. *Biochem. J.* **89**, 127–138.

(9) Lawley, P. D., and Brookes, P. (1967) Interstrand cross-linking of DNA by difunctional alkylating agents. *J. Mol. Biol.* **25**, 143–160.

(10) Lawley, P. D., and Brookes, P. (1968) Cytotoxicity of alkylating agents towards sensitive and resistant strains of *Escherichia coli* in relation to extent and mode of alkylation of cellular macromolecules and repair of alkylation lesions in deoxyribonucleic acids. *Biochem. J.* **109**, 433–447.

(11) Lawley, P. D., Lethbridge, J. H., Edwards, P. A., and Shooter, K. V. (1969) Inactivation of bacteriophage T7 by mono- and difunctional sulphur mustards in relation to cross-linking and depurination of bacteriophage DNA. *J. Mol. Biol.* **39**, 181–198.

(12) Ludlum, D. B., Tong, W. P., Metha, J. R., Kirk, M. C., and Papirmeister, B. (1984) Formation of O^6-ethylthioethyldeoxyguanosine from the reaction of chloroethyl ethyl sulfide with deoxyguanosine. *Cancer Res.* **44**, 5698–5701.

(13) Ludlum, D. B., Kent, S., and Metha, J. R. (1986) Formation of O^6-ethylthioethylguanine in DNA by reaction with the sulfur mustard, chloroethyl sulfide, and its apparent lack of repair by O^6-alkylguanine–DNA alkyltransferase. *Carcinogenesis* **7**, 1203–1206.

(14) Boursnell, J. C., Francis, G. E., and Wormall, A. (1946) Studies on mustard gas and some related compounds. III. The preparation and use of mustard gas containing a) radioactive sulphur and b) deuterium. *Biochem. J.* **40**, 743–745.

(15) Shooter, K. V., Edwards, P. A., and Lawley, P. D. (1971) The action of mono- and di-functional sulphur mustards on the ribonucleic acid-containing bacteriophage $\mu 2$. *Biochem. J.* **125**, 829–840.

(16) Gaffney, B. L., Marky, L. A., and Jones, R. A. (1984) Synthesis and characterization of a set of four dodecadeoxyribonucleoside undecaphosphates containing O^6-methylguanine opposite adenine, cytosine, guanine, and thymine. *Biochemistry* **23**, 5686–5691.

(17) Li, B. F. L., and Swann, P. F. (1989) Synthesis and characterization of oligodeoxynucleotides containing O^6-methyl-, O^6-ethyl-, and O^6-isopropylguanine. *Biochemistry* **28**, 5779–5786.

(18) Fichtinger-Schepman, A. M. J., Van der Veer, J. L., Den Hartog, J. H. J., and Lohman, P. H. M. (1985) Adducts of the antitumor drug *cis*-diamminedichloroplatinum(II) with DNA: formation, identification and quantitation. *Biochemistry* **24**, 707–713.

(19) Fichtinger-Schepman, A. M. J., Van Oosterom, A. T., Lohman, P. H. M., and Berends, F. (1987) Cis-Diamminedichloroplatinum(II)-induced DNA adducts in peripheral leukocytes from seven cancer patients: quantitative immunochemical detection of the adduct induction and removal after a single dose of cis-diamminedichloroplatinum(II). *Cancer Res.* **47**, 3000–3004.

(20) Griffin, B. E., and Reese, C. B. (1963) The synthesis of $N1$- and N^6-methyladenosine 5′-pyrophosphates. Possible substrates for polynucleotide phosphorylase. *Biochim. Biophys. Acta* **68**, 183–192.

(21) Swain, C. G., and Scott, C. B. (1953) Quantitative correlation of relative rates. Comparison of hydroxide ion with other nucleophilic reagents toward alkyl halides, esters, epoxides and acyl halides. *J. Am. Chem. Soc.* **75**, 141–147.

(22) Lawley, P. D. (1974) Alkylation of nucleic acids and mutagenesis. In *Molecular and environmental aspects of mutagenesis* (Prakash, L., Sherman, F., Miller, M. W., Lawrence, C. W., and Taber, H. W., Eds.) pp 17–31, C. C. Thomas, Springfield, IL.

(23) Edwards, P. A., and Shooter, K. V. (1971) Sedimentation characteristics of DNA multiply crosslinked by a difunctional alkylating agent, mustard gas. *Biopolymers* **10**, 2079–2082.

(24) Kircher, M., and Brendel, M. (1983) DNA alkylation by mustard gas in yeast strains of different repair capacity. *Chem.-Biol. Interact.* **44**, 27–39.