Synthesis and Quantification of DNA Adducts of 4,4′-Methylenedianiline

Dietrich Schütze,[†] Peter Sagelsdorff,[‡] Ovnair Sepai,[§] and Gabriele Sabbioni*[,†]

Institut für Pharmakologie und Toxikologie, Universität Würzburg, Versbacherstrasse 9, D-97078 Würzburg, Germany, Ciba-Geigy AG, Cell Biology Unit, CH-4001 Basel, Switzerland, and Department of Environmental and Occupational Medicine, The Medical School, University of Newcastle upon Tyne, Newcastle NE2 4HH, U.K.

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4,4′-Methylenedianiline (MDA) is used as a hardener in the manufacture of plastics and polyurethanes. MDA has been classified as a carcinogen in animals and is a suspected human carcinogen. Assuming that MDA would yield similar DNA adducts to other arylamines, we synthesized the following C-8 guanine adducts: *N*′-acetyl-*N*-(deoxyguanosin-8-yl)-MDA, *N*-(deoxyguanosin-8-yl)-MDA, *N*-(deoxyguanosin-8-yl)-4MA, and their corresponding 3′-monophosphate derivatives. We developed methods to identify these adducts of MDA in liver DNA using ^{32}P-postlabeling, HPLC, and GC-MS techniques. Liver DNA was obtained from rats treated with radiolabeled MDA (1.11 and 116.5 μmol/kg body weight). The total radioactivity bound to the DNA corresponded to 0.06 and 2.7 adducts per 10^7 nucleotides [covalent binding index (CBI = (μmol of adduct per mol of nucleotide)/(mmol of compound per kg body weight)) of 1.05 and 2.3]. This DNA-binding potency is in the range of weakly genotoxic compounds. The liver DNA was analyzed for the presence of the synthesized adducts by the following methods: (I) HPLC analysis of nucleotides and purines after enzymatic and acid hydrolysis, and (II) ^{32}P-postlabeling after enzymatic hydrolysis. The major adducts found *in vivo* did not correspond to the synthesized standards. Further work was carried out to determine the structure of the unidentified adducts. It was possible to release MDA and MDA-d_4 from DNA of rats dosed with MDA and/or MDA-d_4 and from the synthesized adducts using strong base hydrolysis. Liver of two female Wistar rats given 500 μmol/kg MDA·2HCl was hydrolyzed in 0.1 M NaOH overnight at 110 °C. GC-MS analysis of the heptafluorobutyric anhydride derivatized dichloromethane extracts detected 428 ± 40 fmol of MDA/mg of DNA. In the control animals no MDA was found. The experiment was repeated with livers from animals dosed 500 μmol/kg MDA-d_4·2DCl. In these rats 488 ± 19 fmol MDA-d_4 was found to be bound at liver DNA. Taking into account a 68% yield of the method, the CBI found in these cases was 0.82 and 1.0, respectively.

Introduction

4,4′-Methylenedianiline (MDA)[1] is used as a hardener in the manufacture of plastics and the production of polyurethanes. The compound has a low mammalian toxicity with an oral LD_{50} in mice, rats, guinea pigs, and rabbits which range between 200 and 800 mg/kg (*1*). It has been reported to be hepatotoxic in rats (*2*), dogs (*3*), and man (*4*). It was mutagenic in the Ames test (*5*) in the presence of an S9 metabolizing system and carcinogenic in rats and mice (*2*). MDA has been classified as a carcinogen in animals and as a suspected carcinogen in humans (*6*).

Exposure to MDA has been monitored by several researchers by measuring MDA or *N*′-acetyl-4,4′-methylenedianiline (AcMDA) in urine (*7*−*9*) and as hemoglobin adducts (*8*, *10*). Most arylamines react with C-8 of guanine (*11*, *12*). In rats dosed with 4,4′-methylenebis-(2-chloroaniline) (MOCA), two major adducts of monocyclic arylamines with adenine have been isolated: *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol and *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorotoluene (*13*, *14*). No adducts with guanine were found in this case. The authors postulate that the selective reaction of *N*-hydroxy-MOCA with DNA-adenine and the formation of the single arylamine ring adducts is a consequence of the strong inductive effect of the ortho-chlorine (*13*). Assuming that MDA would yield similar DNA adducts like most arylamines (*11*, *12*), we synthesized the following C-8 guanine adducts of MDA: *N*′-acetyl-*N*-(deoxyguanosin-8-yl)-MDA (dG-AcMDA), *N*-(deoxyguanosin-8-yl)-MDA (dG-MDA), and their corresponding 3′-monophosphate derivatives. The C-8 adduct of 4-methyl-

* Corresponding author. Tel/FAX: (0931) 201 2281; email: Gabriele.Sabbioni@t-online.de.
[†] Universität Würzburg.
[‡] Ciba-Geigy AG.
[§] University of Newcastle upon Tyne.
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[1] Abbreviations: 4,4′-methylenedianiline (MDA), covalent binding index (CBI), *N*′-acetyl-4,4′-methylenedianiline (AcMDA), 4,4′-methylenebis(2-chloroaniline) (MOCA), 4-methylaniline (4MA), *N*′-acetyl-*N*-(deoxyguanosin-8-yl)-MDA (dG-AcMDA), *N*-(deoxyguanosin-8-yl)-MDA (dG-MDA), *N*-(deoxyguanosin-8-yl)-4MA (dG-4MA), 2′-deoxyguanosine monohydrate (dG), 2′-deoxyguanosine 3′-monophosphate (dGp), T4 polynucleotide kinase (PNK), direct insertion probe (DIP), desorption chemical ionization (DCI), field desorption (FD), electrospray ionization mass spectrometry (ESI-MS), *N*′-acetyl-4′-amino-4-nitrodiphenylmethane (nitro-AcMDA), *N*-acetoxy-*N*′-acetyl-MDA (OAc-AcMDA), ammonium formate (AF), 3-chloroperbenzoic acid (mCPBA), *N*′-acetyl-*N*-(3′-monophospho-2′-deoxyguanosin-8-yl)-MDA (dGp-AcMDA), *N*-(3′-monophosphate-2′-deoxyguanosin-8-yl)-MDA (dGp-MDA), *N*-(3′-monophosphate-2′-deoxyguanosin-8-yl)-4MA (dGp-4MA), 250 mM mannitol, 70 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4 (HM buffer), heptafluorobutyric anhydride (HFBA), 2′-deoxyadenosine (dA), N^2-(2′-deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-PhiP), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), nuclease P1 (NP1), tetrabutylammonium chloride (TBA), units (U), 2′-deoxycytidine 3′-phosphate (dCp), 2′-deoxythymidine 3′-phosphate (dTp), and 2′-deoxyadenosine 3′-phosphate (dAp).

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aniline (4MA) was synthesized in case the *N*-(deoxyguanosin-8-yl)-4MA (dG-4MA) would be formed from an unstable dG-MDA adduct as seen for the adenine adducts of MOCA. These DNA adducts were used to develop methods of adduct quantification by the ^{32}P-postlabeling technique and by GC-MS following the procedure recently published by Lin *et al.* (*15*) and Friesen *et al.* (*16*).

Experimental Procedures

Chemicals. Nuclease P1 (NP1) (from *Penicillium citrinium*, 236225), RNase A (from bovine pancreas, 109142), and proteinase K (from *Tritirachium album*, 745723) were from Boehringer (Mannheim, Germany); 2′-deoxyguanosine monohydrate (dG), pyruvonitrile, heptafluorobutyric anhydride (HFBA), and MDA were purchased from Fluka (Neu-Ulm, Germany); PEI-cellulose thin-layer plates were from Macherey-Nagel (Düren, Germany); water (chromatography grade, 15333) and anhydrous sodium sulfate (p.a. 6639) were from Merck (Darmstadt, Germany); T4 polynucleotide kinase (PNK) was from Pharmacia (Freiburg, Germany); ethanol (Rotipuran >99.8%, 9062.2) and Roti-Phenol (=redistilled phenol, equilibrated in and covered with 10 mM Tris, 1 mM EDTA-Na_2 (pH 7.5−8.0)) were from Roth (Karlsruhe, Germany); 2′-deoxyguanosine 3′-monophosphate (dGp) and dichloromethane (Pestanal) were from Riedel de Haen (Seelze, Germany); alkaline phosphatase from calf intestine (P-4252), apyrase (A6132), calf thymus DNA, micrococcal endonuclease (N-3755), spleen phosphodiesterase (P-6897), and pig liver esterase (E-3128) were acquired from Sigma (Deisenhofen, Germany); MDA-d_4 was synthesized as described by Schütze *et al.* (*8*). All the other chemicals were from Aldrich (Steinheim, Germany). [^{14}C]MDA (6.84 mCi/mmol) was obtained from Amersham (Little Chalfont, U.K.). The radioactive purity (>98%) was checked by TLC (toluene/acetone = 1:1).

Syntheses. Caution: *N-Arylhydroxylamines and their O-acyl derivatives are potentially carcinogenic. They should be handled with protective clothing in a well-ventilated fume hood.*

Instrumentation. HPLC was performed with a quaternary HPLC pump of the 1050 series by Hewlett Packard (Waldbronn, Germany) equipped with a photodiode array detector LKB 2140 (Pharmacia, Freiburg, Germany). The following HPLC columns were used: LiChrospher RP 18 (250 × 4 mm, 5 μm) and LiChrospher RP Select B (250 × 4 mm, 5 μm) from Merck (Darmstadt, Germany); Nucleosil C18 (250 × 12.5 mm, 7 μm) and Nucleosil C18 (250 × 4.6 mm, 10 μm) from Macherey-Nagel (Düren, Germany). NMR spectra were recorded on a Bruker AC 250 and Bruker WM 400 spectrometer. The ^{13}C signals were distinguished by a DEPT (distortionless enhancement by polarization transfer) experiment. Internal standard was tetramethylsilane or DMSO. Mass spectra of volatile compounds were measured on a mass spectrometer 5989A with a GC-MS interface or with a direct insertion probe (DIP). Mass spectra were measured on a Finnigan MAT 90 using the field desorption (FD) technique or on a Finnigan MAT 8200 with isobutane (0.3 mbar) or ammonia (0.3 mbar) as reactant gas using the desorption chemical ionization (DCI) technique (Dr. Gerda Lange, Department of Organic Chemistry of the University of Würzburg). The electrospray ionization mass spectra (ESI-MS) were provided by Dr. Peter Farmer (MRC Toxicology, Leicester). The experiments were conducted on a Fisons-Quattro mass spectrometer using cone-induced fragmentation. Samples were introduced via loop injection in water/acetonitrile. UV spectra were recorded on a Kontron spectrophotometer Uvikon 860. IR spectra were registered on a Perkin-Elmer 1420 recording spectrophotometer. TLC was carried out with TLC-plates Alugram SIL G/UV254 with fluorescence indicator (Macherey-Nagel, Düren, Germany).

***N′*-Acetyl-4′-amino-4-nitrodiphenylmethane (Nitro-AcMDA).** AcMDA (5.8 g, 24.1 mmol) in dichloromethane (200 mL) was added dropwise over 2 h to 50% 3-chloroperbenzoic acid (mCPBA) (25 g, 72.4 mmol) in dichloromethane (300 mL). After a further 1 h stirring at room temperature, the reaction solution was washed with 1 M NaOH (5 × 100 mL) and 1 M HCl (1 x 150 mL). The organic phase was dried with magnesium sulfate, and the solvent was evaporated *in vacuo*. The yellow solid (6.2 g) obtained was purified on a silica gel column with ethyl acetate as solvent. The collected fraction was evaporated *in vacuo*, yielding 5.2 g (80%) of yellowish needles of nitro-AcMDA, which melt at 164-165 °C (lit. (*17*) 160−161 °C). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 2.02 (s, 3 H), 4.02 (s, 2 H), 7.17 (d, J = 8.5 Hz, 2 H), 7.47 (d, J = 8.8 Hz, 2 H), 7.51 (d, J = 8.5 Hz, 2 H), 8.14 (d, J = 8.8 Hz, 2 H), 9.90 (s, 1 H). MS (70eV): m/z (%) = 271 (9), 270 (46) [M$^+$], 229 (15), 228 (100), 227 (34), 182 (35), 181 (17), 180 (11), 107 (10), 106 (66).

***N*,*N′*-Diacetyl-*N*-hydroxy-MDA.** Hydrazine hydrate (206 mg, 4.12 mmol) was added to nitro-AcMDA (270 mg, 1 mmol) and 5% palladium on charcoal (Pd/C) (50 mg) in THF (25 mL), keeping the temperature below 5 °C. After 1 h, further hydrazine hydrate (103 mg, 2.06 mmol) was added. After a total of 2 h, no starting material was detectable by TLC (silica gel, acetone/hexane 1:1). Triethylamine (219 mg, 2.16 mmol) was added. Acetyl chloride (1.65 mg, 21.0 mmol) in THF (5 mL) was added very slowly, keeping the reaction temperature below 10 °C, and the reaction mixture was kept stirring at room temperature for a further 30 min. The solution was filtered, and the solid residue was washed with diethyl ether. The combined organic phases were vigorously stirred with saturated sodium bicarbonate solution and then washed with 1 M NaOH (6 × 25 mL). The pH of the aqueous solution was adjusted to between pH 1 and 2 with HCl and then extracted with ethyl acetate (5 × 25 mL). After the organic phases were dried with magnesium sulfate and evaporated down *in vacuo*, the orange brown oil was then taken up in 0.1 M NaOH (30 mL) and stored overnight after addition of 37% HCl (15 mL). The precipitate was filtered, washed with water, and dried in a desiccator. Colorless short needles of *N*,*N′*-diacetyl-*N*-hydroxy-MDA (202 mg, 68%) with a melting point of 75−77 °C (lit. (*18*) 138-139 °C) were obtained. ^{1}H NMR (250 MHz, DMSO-d_6): δ = 2.02 (s, 3 H), 2.19 (s, 3 H), 3.86 (s, 2 H), 7.13 (d, J = 8.3 Hz, 2 H), 7.19 (d, J = 8.4 Hz, 2 H), 7.50 (d, J = 8.3 Hz, 2 H), 7.52 (d, J = 8.4 Hz, 2 H), 9.89 (s, 1 H), 10.56 (br s, 1 H). ^{13}C NMR (63 MHz, DMSO-d_6): δ = 22.7 (q), the other signals are listed in Table 2. MS (70eV): m/z (%) = 299 (3), 298 (12) [M$^+$], 283 (15), 282 (78), 256 (47), 241 (11), 240 (59), 239 (26), 199 (10), 198 (65), 197 (100), 195 (11), 183 (12), 182 (41), 181 (19), 180 (24), 148 (10), 136 (19), 121 (29), 106 (76), 93 (15).

***N′*-Acetyl-*N*-hydroxy-MDA.** Hydrazine hydrate (5.16 g, 103 mmol) was added over 15 min at 5 °C to a suspension of nitro-AcMDA (6.76 g, 25 mmol) in THF (400 mL) and 5% Pd/C (1 g). After 1.5 h no more starting material was present according to TLC analysis (silica gel, acetone/hexane = 1:1). The reaction mixture was dried over anhydrous sodium sulfate and filtered through Celite. The solid on the filter was washed with THF. The combined organic phases were evaporated *in vacuo* to about 50 mL, covered with hexane (400 mL), and stored overnight at −20 °C. After filtration and drying with a vacuum pump, yellowish crystals were obtained with a yield of 95% (6.09 g). AcMDA (15%) was present as a byproduct. For further purification, the hydroxylamine was dissolved in a minimum quantity of dichloromethane/THF 2:3, filtered, and covered with a 4−5-fold volume of diethyl ether and stored at −20 °C. The precipitate was filtered and washed twice with diethyl ether and dried at the vacuum pump. The slightly yellow needles decompose at 115 °C (lit. (*18*) ca. 110 °C). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 2.02 (s, 3 H), 3.76 (s, 2 H), 6.77 (d, J = 8.4 Hz, 2 H), 7.00 (d, J = 8.4 Hz, 2 H), 7.10 (d, J = 8.4 Hz, 2 H), 7.48 (d, J = 8.4 Hz, 2 H), 8.13 (br s, 1 H), 9.86 (s, 1 H). MS (70eV): m/z (%) = 256 (3) [M$^+$], 255 (7), 254 (38), 241 (18), 240 (100), 212 (21), 198 (45), 197 (95), 183 (19), 182 (64), 181 (22), 180 (31), 165 (16), 106 (97), 104 (10), 93 (14), 89 (12), 77 (13).

***N*-Acetoxy-*N′*-acetyl-MDA (OAc-AcMDA).** Pyruvonitrile (1.07 mL, 15 mmol) in THF (25 mL) was added slowly to *N′*-acetyl-*N*-hydroxy-MDA (3.84 g, 15.0 mmol) and triethylamine (2.36 mL, 17 mmol) in THF at −50 to −40 °C over an hour. After stirring the solution for 1.5 h at −20 °C, the solvents were evaporated *in vacuo* at 0 °C. The yellow-orange oil was

dissolved in THF and used without further purification for the reactions with dG and dGp.

***N′*-Acetyl-*N*-(2′-deoxyguanosin-8-yl)-MDA (dG-AcMDA).** A cold solution (0 °C) of OAc-AcMDA (ca. 5 mmol) in THF (8 mL) was added to dG (200 mg, 701 μmol) and triethylamine (607 mg, 6 mmol) in water (8 mL) at 37 °C. After 1.5 h at room temperature, water (400 mL) was added and the pH was adjusted to 5 with formic acid. Extraction with diethyl ether was followed by evaporation of the water phase *in vacuo*. The residue was taken up in water/methanol 8:2 (10 mL) and purified by HPLC on a 250 × 12.5 mm RP18 column with 50 mM ammonium formate (AF) buffer, pH 7.4/methanol 50:50 and a flow of 3 mL/min. The fraction at 17 min was collected, evaporated, and repurified by HPLC with the same conditions but with water instead of the AF buffer. After evaporation, the residue was taken up in DMSO (1.5 mL) and purified by HPLC (250 × 4 mm, RP select B column, THF gradient in water: 10−40% in 15 min, then 5 min at 80%, flow of 1 mL/min). The peak at 14.1 min was collected (the main byproduct, *N,N′*-diacetyl-*N*-hydroxy-MDA eluted at 15.7 min). Evaporation *in vacuo* of THF was followed by freeze-drying. Colorless, fine needles of dG-AcMDA were obtained. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.99 (dd, J = 6.6 Hz, 2 H), 2.00 (s, 3 H), 3.73 (m, 2 H), 3.81 (s, 2 H), 3.91 (d, J = 1.9 Hz, 1 H), 4.40 (m, 1 H), 5.32 (d, J = 3.6 Hz, 1 H), 5.87 (t, J = 4.8 Hz, 1 H), 6.31 (dd, J = 5.8 Hz, J = 9.8 Hz, 1 H), 6.33 (s, 2 H), 7.09 (d, J = 8.6 Hz, 2 H), 7.11 (d, J = 8.5 Hz, 2 H), 7.46 (d, J = 8.5 Hz, 2 H), 7.61 (d, J = 8.6 Hz, 2 H), 8.56 (s, 1 H), 9.84 (s, 1 H), 10.51 (br s, 1 H). The signal for 2′-H$_a$[dG] is covered by the DMSO-d_6-signal. UV (methanol): λ_{max} (log ϵ) = 204 nm (4.787), 246 (4.386), 284 (4.533). MS (DCI/isobutane, 70 eV): m/z (%) = 432 (2), 428 (1), 391 (8), 390 (31) [M$^+$ − D-ribose], 117 (100), 116 (16), 113 (16). MS (FD): m/z (%) = 528 (77) [M$^+$ + Na], 507 (58), 506 (41), 505 (100) [M$^+$].

***N′*-Acetyl-*N*-(3′-monophosphate-2′-deoxyguanosin-8-yl)-MDA (dGp-AcMDA).** The cold solution (0 °C) of OAc-AcMDA (ca. 0.7 mmol) in THF (1.8 mL) was added to dGp (22.5 mg, 60.4 μmol) and triethylamine (63 μL, 454 μmol) in water (8 mL) at 0 °C under nitrogen. After 1.5 h at 37 °C the THF was evaporated with a stream of nitrogen and AF buffer, pH 5.1 (10 mL), was added. dGp-AcMDA was purified by HPLC [250 × 4 mm, RP18 column, methanol gradient in AF buffer (pH 5.1): 5−65% in 20 min, then 65−85% in 1 min, flow 1 mL/min]. The peak at 20 min was collected, evaporated *in vacuo*, taken up in 2 mL of AF buffer (pH 5.1)/methanol 4:1, and rechromatographed [250 × 4 mm, RP select B column, THF gradient in AF buffer (pH 5.1): 0−30% in 15 min, then 5 min 80%, flow 1 mL/min]. The peak at 13.5 min was collected. After evaporation of the organic solvents *in vacuo* and freeze-drying, colorless crystals (2.02 μmol, 3.3%, see below) of dGp-AcMDA were obtained. For further analysis, the crystals were dissolved in water/methanol 1:4 and transferred to a 1 mL volumetric flask. The purity determined by HPLC at the wavelengths 245 and 280 nm was 99% and 99.5% [250 × 4 mm, RP18 column, methanol gradient in AF buffer (pH 5.1): 10−80% in 30 min, flow 1 mL/min, t_R = 21.6 min]. Structural elucidation and quantitation of dGp-AcMDA: dGp-AcMDA solution (10 μL) in 30 mM sodium acetate buffer, pH 7.2 (500 μL), and 10 mM zinc sulfate (20 μL) was incubated at 37 °C with a solution of NP1 (20 μL, ca. 20 U) in 30 mM sodium acetate buffer (pH 5.3). After 20 h the whole reaction mixture was analyzed by HPLC with the same conditions described above. The peak areas at 245 and 305 nm were quantified against a calibration curve obtained under the same HPLC conditions with 12.1 −36.2 nmol of dG-AcMDA. A concentration of 2.02 nmol/μL was calculated for dGp-AcMDA. The collected peak was then reanalyzed by HPLC [250 × 4 mm, RP select B column, THF gradient in AF buffer (pH 5.1): 0−30% in 20 min, then 5 min at 80%, flow of 1 mL/min]. The retention time (19.7 min) and the UV spectra were the same as the standard solution of dG-AcMDA.

***N*-(2′-Deoxyguanosin-8-yl)-MDA (dG-MDA).** dG-AcMDA (5.00 mg, 9.90 μmol) was dissolved in DMSO (1 mL) and diluted with 50 mM sodium phosphate buffer, pH 7.7 (20 mL). After addition of carboxylesterase suspension (500 μL, 1150 U) in 3.2 M ammonium sulfate, the reaction mixture was kept at 37 °C. After 36 h, the pH was adjusted to 7.7 with 1 M NaOH, and further carboxylesterase (100 μL, 230 U) was added. HPLC analysis [250 × 4 mm, RP18 column, methanol gradient in AF buffer (pH 5.1): 30−60% in 15 min, 60−80% in 1 min, flow 1 mL/min] showed that all dG-AcMDA was used up after further 12 h reaction time. The reaction mixture was then evaporated *in vacuo* and taken up in water (1 mL) and purified by HPLC with the same conditions described above. The peak eluting at 15.3 min was collected, evaporated, taken up in a minimum quantity of DMSO, and rechromatographed [250 × 4 mm, RP select B column, THF gradient in water: 10−40% in 15 min, 40−80% in 1 min, flow 1 mL/min]. The peak eluting at 14.6 min was collected. THF was evaporated *in vacuo*. Fine colorless needles (3.4 mg, 7.34 μmol, 74%) were obtained after freeze-drying.

^{1}H NMR (250 MHz, DMSO-d_6): δ = 1.99 (dd, J = 13.2 Hz, J = 5.6 Hz, 2 H), 3.68 (s, 2 H), 3.73 (m, 2 H), 3.91 (d, J = 1.8 Hz, 1 H), 4.40 (m, 1 H), 4.84 (br s, 2 H), 5.32 (d, J = 3.2 Hz, 1H), 5.87 (t, J = 4.5 Hz, 1 H), 6.31 (dd, J = 5.6 Hz, J = 9.6 Hz, 1 H), 6.34 (s, 2 H), 6.47 (d, J = 8.4 Hz, 2 H), 6.84 (d, J = 8.4 Hz, 2 H), 7.05 (d, J = 8.6 Hz, 2 H), 7.60 (d, J = 8.6 Hz, 2 H), 8.54 (s, 1 H), 10.50 (br s, 1 H). The signal for 2′-H$_a$[dG] is covered by the DMSO-d_6 signal. UV (methanol): λ_{max} (log ϵ) = 204 nm (4.734), 236 (4.208), 285 (4.472). MS (DCI/isobutane, 70 eV): m/z (%) = 349 (2), 348 (10) [M$^+$ − D-ribose], 197 (9), 169 (12), 142 (19), 141 (100), 140 (24), 129 (13), 127 (13), 125 (11), 118 (23), 117 (100), 116 (29), 113 (16).

***N*-(3′-Monophosphate-2′-deoxyguanosin-8-yl)-MDA (dGp-MDA).** dGp-AcMDA solution in water/methanol 4:1 (500 μL, 1.01 μmol) was evaporated to dryness with a stream of nitrogen. The residue was taken up in 10 mL of 50 mM phosphate buffer and incubated with a carboxylesterase suspension (500 μL, ca. 1150 U) in 3.2 M ammonium sulfate solution (pH 8) at 37 °C under nitrogen. An HPLC run of the reaction mixture after 24 h [250 × 4 mm, RP18 column, methanol gradient in AF buffer (pH 5.1): 10−80% in 30 min, flow 1 mL/min, t_R (dGp-MDA) = 20.2 min, t_R (dGp-AcMDA) = 21.5 min] showed that dGp-AcMDA was still present. The pH of the reaction solution was readjusted to 7.7 with NaOH, and further carboxylesterase (125 μL, ca. 300 U) was added. After a total of 40 h reaction time, dGp-MDA was obtained after HPLC purification [250 × 4 mm, RP18 column, THF gradient in AF buffer (pH 5.1): 10−30% in 15 min and 30−80% in 1 min, flow 1 mL/min, t_R = 10.1 min]. Evaporation of THF *in vacuo* was followed by freeze-drying. The colorless crystals of dGp-MDA were transferred to a 1 mL volumetric flask and dissolved in water/methanol 4:1 for further analysis. Structural elucidation and quantitation of dGp-MDA: dGp-MDA solution (10 μL) in 30 mM sodium acetate buffer, pH 7.2 (500 μL), and 10 mM zinc sulfate (20 μL) was incubated at 37 °C with a solution of NP1 (20 μL, ca. 20 U) in 30 mM sodium acetate buffer (pH 5.3) for 36 h. The whole reaction solution was analyzed with the same HPLC conditions used for the control of the reaction after 24 h described above. The peak area at 27.6 min was quantified against a calibration curve obtained with dG-AcMDA (5.9−23.5 nmol) at three wavelengths: 230, 280, and 310 nm. The concentration of the dGp-MDA solution was 710 nmol/mL. The yield of the deacetylation was therefore 70%. The collected peak was then reanalyzed by HPLC [250 × 4 mm, RP select B column, THF gradient in AF buffer (pH 5.1): 0−45% in 20 min, then 5 min at 80%, flow 1 mL/min]. The retention time (23.7 min) and the UV spectra were the same as the standard solution of dG-MDA.

***N*-Hydroxy-4MA.** Hydrazine hydrate (20.6 g, 412 mmol) was added slowly to 4-nitrotoluene (13.7 g, 100 mmol) and 5% Pd/C (1 g) in THF (500 mL) at 0−5 °C. After 2.5 and 3.5 h, a further 5% Pd/C (500 mg) was added. The reaction was stopped after 5 h. The reaction suspension was dried over sodium sulfate and filtered through Celite. The filtrate was evaporated to 50 mL, then poured to cold hexane (400 mL), and stored overnight at −20 °C. The precipitate was filtered, washed with cold hexane, and dried at a vacuum pump. Slightly yellow and shiny leaves of *N*-hydroxy-4MA (8.95 g, 73%) were obtained,

which melt at 92−93 °C (lit. (*19*) 93−94 °C). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 2.20 (s, 3 H), 6.76 (d, J = 8.4 Hz, 2 H), 6.98 (d, J = 8.4 Hz, 2 H), 8.05 (br s, 1 H). ^{13}C-NMR (63 MHz, DMSO-d_6): δ = 20.6 (q, -CH_3), 113.7 (d, C-2,6), 128.2 (s, C-4), 129.2 (d, C-3,5), 150.1 (s, C-1). MS (70eV): m/z (%) = 124 (5), 123 (56) [M^+], 121 (10), 107 (38), 106 (100), 91 (22), 79 (43), 78 (19), 77 (48), 65 (20), 63 (13), 61 (13), 53 (12), 52 (24), 51 (29).

***N*-(2′-Deoxyguanosin-8-yl)-4-methylaniline (dG-4MA).** Pyruvonitrile (1.73 g, 25.0 mmol) dissolved in diethyl ether (20 mL) was added to *N*-hydroxy-4MA (3.08 g, 25.0 mmol) and triethylamine (2.73 g , 27.0 mmol) in diethyl ether (50 mL) under nitrogen in 20 min at −40 °C. The temperature was allowed to rise slowly to −15 to −10 °C. After 2 h, the reaction mixture was dried over sodium sulfate and filtered at −20 °C. The solvents were evaporated at 0 °C. The obtained yellow oil of *N*-acetoxy-4MA was dissolved at 0 °C in THF (110 mL). Then a suspension of 2′-deoxyguanosine monohydrate (1.14 g, 4.00 mmol) in water (40 mL) was added under vigorous stirring. The mixture was warmed up in about 30 min to room temperature and stirred for another 1.5 h. The solvents were then evaporated *in vacuo*. The dark residue was taken up in water (400 mL) and extracted with diethyl ether (5 × 100 mL) and 1-butanol (5 × 100 mL). The 1-butanol phase was evaporated. The orange residue was taken up in water/methanol 2:8 (10 mL) and purified by semipreparative HPLC [250 × 12.5 mm, RP18 column, methanol gradient in water: 30−90% in 13 min, flow 3 mL/min]. The peak eluting at 10.5 min was collected. After evaporation of the methanol and freeze-drying, pale yellow needles of dG-4MA (70 mg) were obtained in 4.7% yield. Purity by HPLC with UV detection at 254 nm was >95%. ^{1}H NMR (250 MHz, DMSO-d_6): δ = 1.99 (dd, J = 12.8 Hz, J = 5.7 Hz, 1 H), 2.24 (s, 3 H), 3.74 (m, 2 H), 3.92 (d, J = 1.6 Hz, 1 H), 4.41 (m, 1 H), 5.33 (d, J = 3.4 Hz, 1 H), 5.90 (t, J = 4.5 Hz, 1 H), 6.32 (dd, J = 5.7 Hz, 1 H), 6.34 (s, 2 H), 7.16 (d, J = 8.4 Hz, 2 H), 7.48 (d, J = 8.4 Hz, 2 H), 8.54 (s, 1 H), 10.51 (br s, 1 H). The signal of 2′-H_a[dG] was covered by the DMSO-d_6 signal. ^{13}C NMR (63 MHz, DMSO-d_6): δ = 20.7 (q, PhCH_3), 38.5 (t, C-2′-[dG]), 61.6 (t, C-5′[dG]), 71.6 (d, C-3′[dG]), 83.2 (d, C-1′[dG]), 87.5 (d, C-4′[dG]), 112.5 (s, C-5[dG]), 117.8 (d, C-2,6), 129.2 (d, C-3,5), 129.6 (s, C-4), 138.7 (s, C-1), 143.9 (s, C-8[dG]), 149.8 (s, C-4[dG]), 153.1 (s, C-2[dG]), 156.0 (s, C-6[dG]). UV (methanol): λ_{max} (log ϵ) = 202 nm (4.492), 282 (4.398). MS (DCI/isobutane, 70 eV): m/z (%) = 373 (7) [M^+ + H], 257 (12) [M^+ − D-ribose], 124 (13), 117 (100), 116 (17), 114 (11), 113 (41), 112 (16).

***N*-(3′-Monophosphate-2′-deoxyguanosin-8-yl)-4MA (dGp-4MA).** A cold solution of *N*-acetoxy-4MA (1.5 mmol) in THF (2 mL) was added to dGp (14.8 mg, 42.6 μmol) and triethylamine (152 mg, 1.50 mmol) in water (2 mL) under nitrogen at 37 °C. After 1 h, the reaction solution was poured into 50 mM AF buffer, pH 5.1 (200 mL), and extracted with diethyl ether (5 × 50 mL). Then the water phase was evaporated *in vacuo*. The brown residue was taken up in 50 mM AF buffer (pH 5.1)/methanol 3:1 (8.0 mL) and purified by HPLC [250 × 4 mm, RP18 column, methanol gradient in AF buffer (pH 5.1): 10−55% in 15 min, 55−85% in 1 min, flow 1 mL/min]. The peak eluting at 15.5 min was collected. After evaporation of the solvents *in vacuo*, the residue was rechromatographed [250 × 4 mm, RP select B column, THF/acetonitrile = 9:1 gradient in water: 0−30% in 15 min, then 5 min 80%, flow 1 mL/min]. The peak eluting at 13.5 min was collected. After evaporation of the organic solvents and freeze-drying, slightly yellow crystals of dGp-4-MA were obtained and dissolved in methanol/water 2:8 for storage. The purity of the product was checked by HPLC and UV detection [250 × 4 mm, RP18 column, methanol gradient in AF buffer (pH 5.1): 10−80% in 30 min, flow of 1 mL/min, t_R = 17.5 min]. The purity at wavelengths 245 and 280 nm was 97% and 99%, respectively. Structural elucidation and quantitation of the yield: dGp-4MA solution (10 μL) in 30 mM sodium acetate buffer, pH 7.2 (500 μL), and 10 mM zinc sulfate (20 μL) was incubated at 37 °C with a solution of NP1 (20 μL, ca. 20 U) in 30 mM sodium acetate buffer (pH 5.3). After 20 h the whole reaction mixture was analyzed by HPLC with the same conditions described for the purity check (t_R = 20.2 min). The peak areas at 240 and 310 nm were quantified against a calibration curve obtained with the same HPLC conditions with 29.3−58.5 nmol of dG-4MA. A concentration of 6.43 nmol/μL was calculated for dGp-4MA**.** This corresponds to a total yield of 15% (6.43 μmol). The collected peak was then reanalyzed by HPLC [250 × 4 mm, RP select B column, THF gradient in AF buffer (pH 5.1): 0−30% in 20 min, then 5 min at 80%, flow 1 mL/min]. The retention time (17.3 min) and the UV spectra were the same as the standard solution of dG-4MA.

Animal Experiments. Female Wistar rats (200−225 g) were obtained from the Zentralinstitut für Versuchstierkunde (Hannover, FRG). They had free access to feed (Altromin 1324) and water. MDA·2HCl and MDA-d_4·2DCl were given in H_2O and D_2O by gavage to groups of two animals. Two control animals received only H_2O. After 24 h the animals were anesthetized with diethyl ether, and blood (4−6 mL) was taken by heart puncture. The livers were perfused with 0.9% saline solution. Livers of male Wistar rats (190−235 g) given 5.6 μmol/kg (0.3 mCi/kg) and 116.5 μmol/kg (3 mCi/kg) [^{14}C]MDA by ip were kindly provided by Dr. Eric Bailey (MRC Toxicology, Leicester). The rats were sacrificed after 24 h.

Isolation of Rat Liver DNA. Liver tissue (1 g) in 5 mL HM buffer (250 mM mannitol, 70 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid, pH 7.4) was homogenized in a teflon-potter-glass for 10 s at medium speed. DNA was isolated as described by Gupta (*20*, *21*) and dissolved in water (Merck, HPLC water) for the GC-MS analysis and in nucleotide buffer (8 mM calcium chloride, 20 mM sodium succinate, pH 6.0) for the postlabeling analysis. An aliquot of the solution (25 μL) was added to 1 mL of water and the DNA determined by UV [DNA (mg/mL) = extinction $E_{260\ nm}$/20]. About 1 mg of DNA was obtained from 1 g of liver by this method.

DNA Binding Assay. Rat liver DNA was isolated from the liver chromatin fraction and purified to constant specific radioactivity by lysis, extraction with phenol/chloroform/isoamyl alcohol, chromatography on hydroxylapatite, dialysis and precipitation with ethanol according to Sagelsdorff *et al.* (*22*, *23*). Using this method, about 10 mg of DNA could be isolated from a rat liver. The specific activity of the DNA was determined by scintillation counting of 1−5 mg of the isolated DNA. [^{14}C]-MDA−DNA adducts were assessed by HPLC analysis of digested DNA using the following methods:

(i) Rat liver DNA (1 mg) in nucleotide buffer (1 mL) was hydrolyzed with micrococcal endonuclease (5 U) and spleen phosphodiesterase (0.1 U) for 16 h at 37 °C and was analyzed with method A [Nucleosil C18 column (250 × 4.6 mm, 10 μm), 3% methanol in 50 mM AF buffer (pH 4.5) for 10 min, followed by a linear gradient to 100% methanol in 35 min, flow 1.5 mL/min]. The retention times for the unmodified deoxyribonucleotides: 2′-deoxycytidine 3′-phosphate (dCp), dGp, 2′-deoxythymidine 3′-phosphate (dTp), and 2′-deoxyadenosine 3′-phosphate (dAp) were 3.5, 5.8, 6.9, and 12.4 min, respectively. Two minute fractions were collected and assessed for radioactivity by scintillation counting (60 min). Completeness of the hydrolysis was checked by analyzing 10 μL of the hydrolysate with method A. The amount of liberated nucleotides was calculated according to the respective peak areas.

(ii) Alternatively, the DNA hydrolysates were analyzed after the addition of the synthetic standards dGp-4MA, dGp-MDA, and dGp-AcMDA with method B [Nucleosil C18 column (250 × 4.6 mm, 5 μm), flow of 1.5 mL/min, 40 min gradient of 20−80% methanol in 50 mM AF buffer, pH 4.5]. The synthesized adducts dGp-4MA, dGp-MDA, and dGp-AcMDA eluted at 14.6, 18.9, and 20.4 min, respectively. Two minute fractions were collected and assessed for radioactivity by scintillation counting (60 min).

(iii) The purines were liberated from the rat liver DNA (1 mg in 1 mL of nucleotide buffer) by the addition of 0.1 volume of 1 M HCl and hydrolysis at 90 °C for 1h. The hydrolysate was neutralized with NaOH and loaded on a Bondelut SAX cartridge, which was previously treated with methanol (1 mL) and water (2 × 1 mL). The column was then washed with 100 μL of water. The eluates containing the purines were combined.

The apurinic DNA was eluted with 0.48 M sodium phosphate, pH 3.2 (1 mL). The radioactivity of all collected fractions was determined by scintillation counting. The purines were analyzed after the addition of the purine adducts G-4MA, G-MDA, and G-AcMDA and the corresponding nucleotide adducts by HPLC with method C [Nucleosil C18 column (250 × 4.6 mm, 5 μm), 35 min gradient 20−80% MeOH in 50 mM AF buffer (pH 4.5), flow of 1.5 mL/min]. G-4MA, G-MDA, and G-AcMDA eluted at 16.4, 18.4, and 20.4 min, respectively. Fractions of 2 min were collected and assessed for radioactivity by scintillation counting. The samples were counted for 60 min.

^{32}P-Postlabeling Analysis. Rat liver DNA (10 μg in 12.5 μL) was hydrolyzed with micrococcal endonuclease (30 mU/μL) and spleen phosphodiesterase (6 mU/μL). The deoxynucleotide adducts were enriched by extraction with water saturated double distilled 1-butanol in the presence of 3 mM tetrabutylammonium chloride (*21*, *24*). After evaporation at reduced pressure, the residue was dissolved in water (10 μL) and ^{32}P-labeled with 100 μCi [^{32}P]ATP (60 pmol) and T4 polynucleotide kinase (7.5 U) in a final volume of 15 μL, for 2 h at room temperature. Excess [^{32}P]ATP was digested with apyrase (20 mU). The adducts were separated by multidirectional TLC on PEI cellulose with the following solvents: D1: 2.3 M NaH_2PO_4/NaOH, pH 5.77 (overnight onto a 10 cm wick); D3: 1 M NaH_2PO_4/NaOH, 3.8 M urea, pH 3.5; and D4: 1 M NaH_2PO_4, 5 M urea, pH 7.6. For the determination of the amount of nucleotides, an aliquot of the diluted DNA hydrolysate was ^{32}P-labeled (see above) and analyzed by TLC on PEI cellulose sheets with 120 mM NaH_2PO_4 (pH 6.8) as eluent.

Hydrolysis of Liver DNA and Analysis by GC-MS. DNA (1 mg) isolated as described above was taken up in 1 mL of water (Merck, HPLC water) and transferred to Teflon centrifuge tubes (Nalgene). The solution was made basic with NaOH (final concentration 0.1 M) and extracted twice with dichloromethane. The internal standard (5 pmol), MDA-d_4 for MDA dosed rats, MDA for MDA-d_4 dosed rats, and 4MA (24 μg) as carrier were added, and the samples were hydrolyzed at 110 °C overnight. The hydrolysate was extracted with dichloromethane (5 mL). The organic phase was passed through a Pasteur pipet with anhydrous sodium sulfate. HFBA (5 μL) was added. After 15 min, a methanolic solution of 4MA (10 μL = 24 μg) was added. After evaporation in a speed evaporator, the residue was taken up in ethyl acetate (2 × 50 μL), transferred to a microinsert for autosampler vials, and evaporated again to dryness. The residue was dissolved in ethyl acetate (15 μL) and analyzed by GC-MS with the conditions published recently (*8*).

Results and Discussion

Synthesis of Deoxyguanosine Adducts. DNA adducts of MDA (Scheme 1) were synthesized following a method Boche used for the preparation of DNA adducts of other arylamines (*25*−*30*). The intermediate nitro-AcMDA was obtained after oxidation of AcMDA with mCPBA. The *N*-hydroxyarylamine of AcMDA was obtained after reduction of nitro-AcMDA with hydrazine hydrate on 5% Pd/C. Addition of pyruvonitrile to the *N*-hydroxyarylamine yielded OAc-AcMDA, which was added to a solution of dG or dGp. The dG adduct dG-AcMDA was isolated with 1.1% yield. dG-MDA was obtained by deacetylation of AcMDA with a pig liver carboxylesterase following a method described by Martin *et al.* (*31*) for the deacetylation of *N*′-acetyl-*N*-(2′-deoxyguanosin-8-yl)benzidine. The dG adduct of 4MA was synthesized according to the procedure by Meier and Boche (*25*). The corresponding dGp-4MA, dGp-AcMDA, and dGp-MDA were synthesized following the method used for the synthesis of the dG adducts. These products were identified by comparing the retention times and the UV spectra with the corresponding dG adducts after dephosphorylation with NP1. The yields of dGp-AcMDA and dGp-4MA were 3 times higher than for the corresponding nucleoside adduct syntheses.

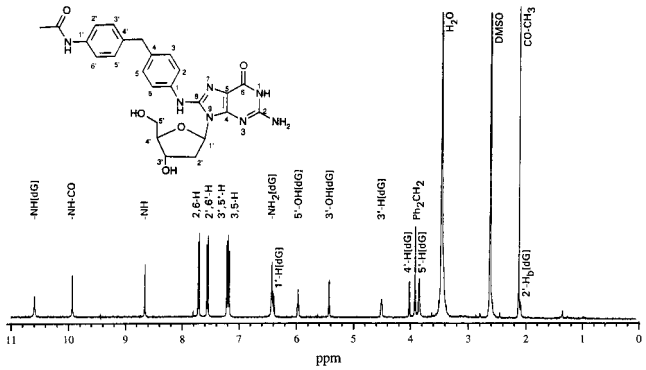
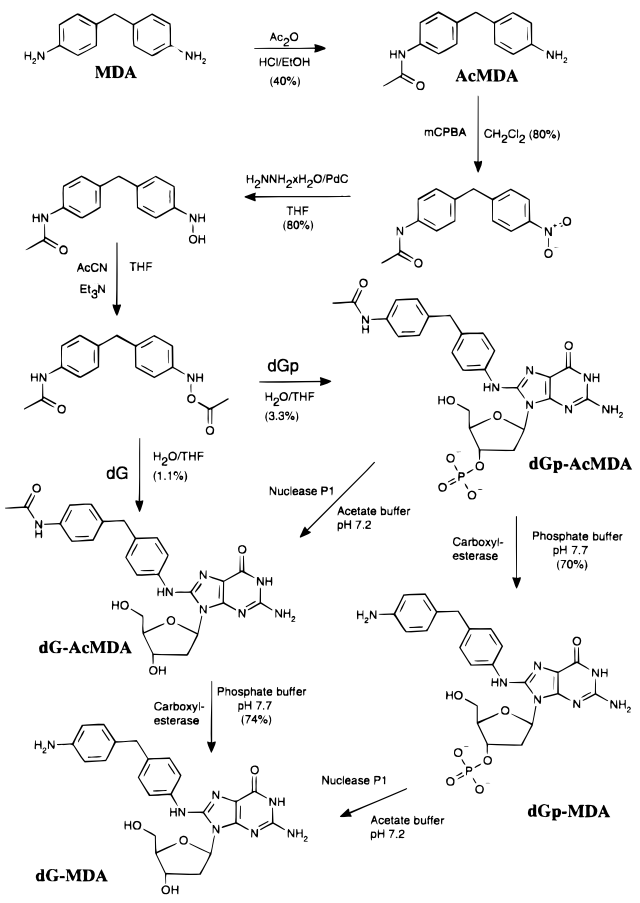


Figure 1. ^{1}H NMR (400 MHz, DMSO-d_6) of the synthesized dG-AcMDA.

Scheme 1. Synthesis of dG and dGp Adducts of MDA and AcMDA



Our attempts to synthesize the deoxyadenosine (dA) adduct with OAc-AcMDA, according to the method described for the synthesis of dG-AcMDA, failed. The synthesis may be successful, following the method published recently for *N*-(3′-monophosphate-2′-deoxyadenosin-8-yl)-4-aminobiphenyl (*32*, *33*). Another approach is the reaction of the OAc-AcMDA with calf thymus DNA following the method for the synthesis of the dA adducts of MOCA (*13*, *14*). However, it should be noted that the yields for dG-AcMDA (*34*) and *N*-(deoxyguanosin-8-yl)-aniline (*29*) are 20 times lower using DNA instead of dG as reactant. This might be also the case for dA adducts.

Spectroscopic Properties. All synthesized compounds were characterized by NMR and MS (Figures 1−3). The spectra of known derivatives of MDA have

Table 1. Comparison of ^{1}H NMR Data (DMSO-d_6) of MDA Derivatives and dG-MDA

compds[a] (MDA derivatives)	ring with changing functional groups				ring with *N*-acetyl group			
	2,6-H	3,5-H	NH_x	Ph_{2CH2}	2′,6′-H	3′,5′-H	NH-Ac	Ac
MDA	6.47	6.82	4.82	3.56				
AcMDA	6.47	6.84	4.87	3.67	7.45	7.07	9.86	2.02
hydroxyarylamine[a]	6.77	7.00	8.13	3.76	7.48	7.10	9.86	2.02
hydroxamic acid[a]	7.52	7.19		3.86	7.50	7.13	9.89	2.02
nitroarene[a]	8.14	7.47		4.02	7.51	7.17	9.90	2.02
dG-AcMDA[b]	7.61	7.09	8.56	3.81	7.46	7.11	9.84	2.00
dG-MDA[b,c]	7.60	7.05	8.54	3.68				
	6.47	6.84	4.84					

[a] Hydroxyarylamine = *N*′-acetyl-*N*-hydroxy-MDA, hydroxamic acid = *N*,*N*′-diacetyl-*N*-hydroxy-MDA, nitroarene = *N*′-acetyl-4-amino-4-nitrodiphenylmethane. [b] The signals of the nucleoside protons are not listed in this table. [c] The signals of the aromatic ring with nitrogen attached to C-8 of guanine are listed in the first row. The signals of the other aromatic protons are listed in the second row.

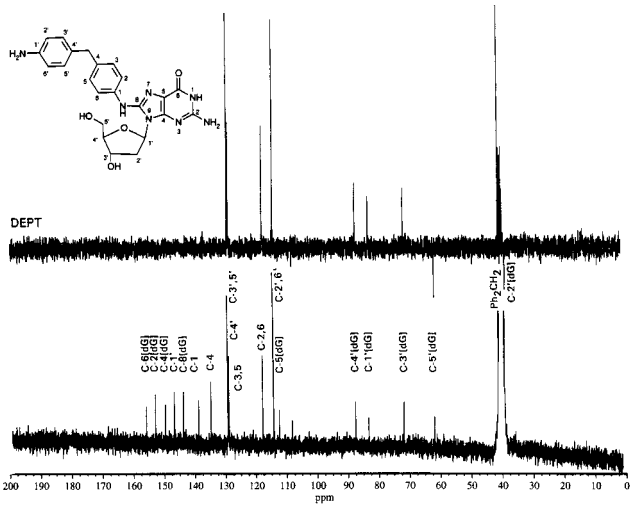


Figure 2. ^{13}C NMR (63 MHz, DMSO-d_6) of dG-MDA.

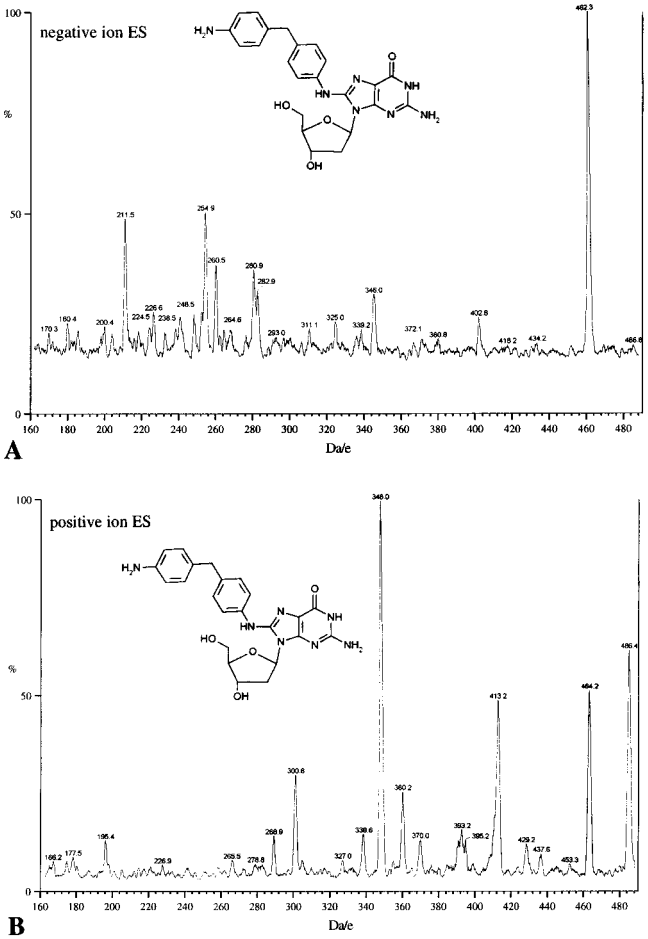


Figure 3. MS of dG-MDA using (A) negative and (B) positive electrospray ionization on a Quattro-BQ mass spectrometer.

also been included in the tables, since NMR data were not available from the literature. For the aromatic protons, spectra of the AA′BB′ type are expected. However, in general the spectra were of the AB type and were interpreted accordingly. The change of the proton shifts due to different functional groups are shown in Table 1. The acetylation of the amino group in MDA shifts the proton in the *meta* and *ortho* position 0.23 and 0.98 ppm downfield. Oxidation of MDA to the *N*-hydroxyarylamine shifts the same protons 0.18 and 0.30 ppm downfield. With the introduction of an acetyl and a hydroxy group as in the hydroxamic acid, the protons shift 0.37 and 1.05 ppm downfield. A nitro group instead of an amino group as in nitro-AcMDA shifts the *meta* and *ortho* protons 0.65 and 1.67 ppm downfield. Binding of the nitrogen at the C-8 position of guanine shifts the protons 0.23 and 1.13 ppm downfield. These shifts are comparable to those seen in the hydroxamic acid. Similar shifts have been registered for the 4MA derivatives as shown in the Experimental Procedures. The downfield shift of the proton on the nitrogen of the aromatic amine by 4 ppm in comparison to the parent aromatic amine was characteristic for the dG adducts. The signals for the dG protons correspond well with the C-8-dG adducts of 4-methoxyaniline (*25*, *30*), 4-chloroaniline (*25*), aniline (*29*), 4-aminoazobenzene (*35*), 4-aminobiphenyl (*36*), 3,2′-dimethylaminobiphenyl (*37*), benzidine (*31*), 2-naphthylamine (*38*), 2-aminofluorene (*39*), 2-aminophenanthrene (*40*), 2,4-dimethylaniline (*41*, *42*), and 2,6-dimethylaniline (*41*). ^{1}H NMR data have been used to elucidate the conformation of dG adducts of arylamines (*30*, *42*−*44*). The comparison of the chemical shifts shows that the conformation of the new adducts correspond to the structures as elaborated by Meier and Boche for dG-4MA (*30*, *43*).

The ^{13}C NMR spectra of the synthesized adducts were interpreted according to the C-8 guanine adducts of 4MA (*42*), 4-methoxyaniline (*30*), 4-chloroaniline (*30*), aniline (*29*), and 4-aminobiphenyl (*28*). The carbons of the dG unit are very similar for all compounds except for N^2-(2′-deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (dG-PhiP) (*44*). The multiplicity of the carbons was determined with DEPT experiments. For the ^{13}C NMR data (Table 2) of the aryl carbons of all MDA derivatives, the largest differences are seen for the carbons attached to the nitrogen (±5.90 ppm) and for the carbon C4 in the *para* position (±8.2 ppm). The chemical shifts of the carbons in the *meta* position change only slightly (±0.6 ppm). The ^{13}C chemical shifts of the carbon in the *ortho* and *para* position shift downfield with the

Table 2. Comparison of the ^{13}C NMR Data (DMSO-d_6) MDA Derivatives and dG Adducts of MDA and AcMDA

compds (MDA derivatives)	ring with changing functional groups					ring with the *N*-acetyl group					
	C-1	C-2,6	C-3,5	C-4	CH_2	C-1′	C-2′,6′	C-3′,5′	C-4′	C=O	CH_3
MDA	146.8	114.3	129.4	129.9	40.1						
AcMDA	147.0	114.4	129.4	128.8	40.1	137.5	119.4	129.0	137.4	168.4	24.3
hydroxyarylamine[a]	150.5	113.6	129.1	132.6	40.2	137.5	119.5	129.0	140.0	168.4	24.3
hydroxamic acid[a]	138.3[b]	121.1	129.2	140.1	40.2	137.8	119.5	128.9	136.1	168.4	24.3
nitroarene[a]	150.1	123.9	130.1	146.2	40.5	138.1	119.7	129.4	134.6	168.5	24.3
dG-AcMDA[c]	139.1	117.9	129.0	133.9	40.1	137.5	119.5	129.0	136.8	168.4	24.3
dG-MDA[c,d]	138.9	117.8	128.9	134.9	40.2						
	146.9	114.3	129.3	129.2							

[a] Hydroxyarylamine = *N*′-acetyl-*N*-hydroxy-MDA, hydroxamic acid = *N*,*N*′-diacetyl-*N*-hydroxy-MDA, nitroarene = *N*′-acetyl-4′-amino-4-nitro-diphenylmethane. [b] Broad signal. [c] The signals of the nucleoside carbons are not listed in this table. [d] The signals of the aromatic ring with nitrogen attached to C-8 of guanine are listed in the first row. The signals of the other aryl carbons are listed in the second row.

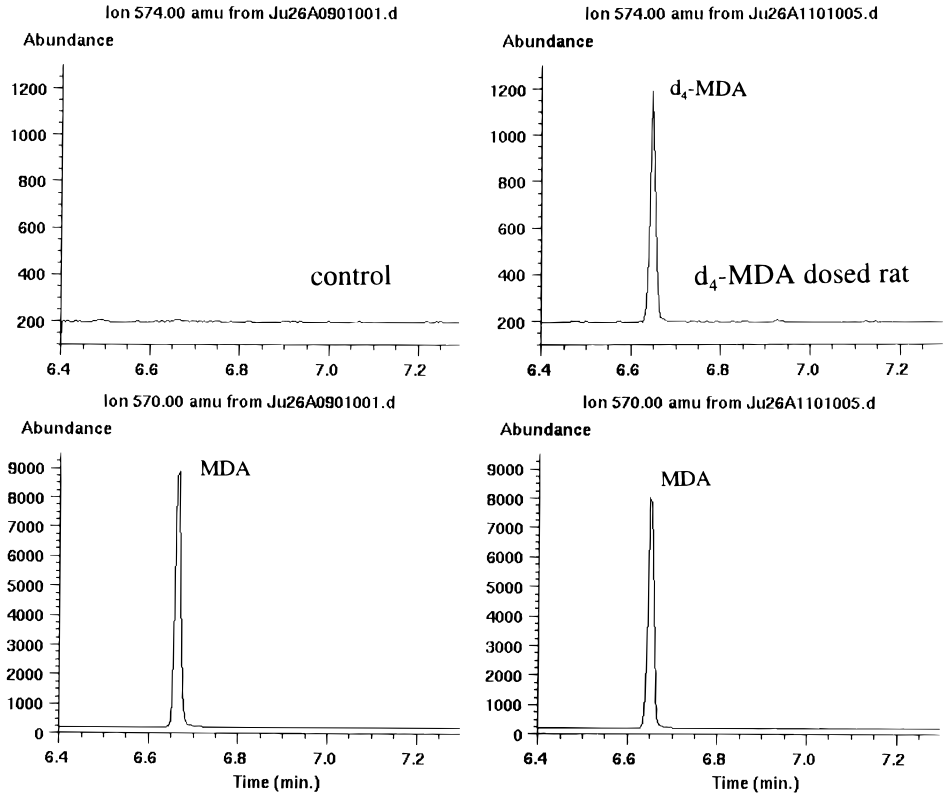


Figure 4. GC-MS with negative chemical ionization of MDA-d_4 released from liver DNA of control rats and of rats dosed with MDA-d_4. The dichloromethane extracts were derivatized with HFBA. The fragment ion 574 [570] corresponds to the molecular ion minus HF of the di-HFBA derivative of MDA-d_4 [MDA].

increasing electron withdrawing properties of the substituent (NO_2 > NHAcOH > NAc > NHOH). The dG substituent shifts the *ortho* carbon 3.5 ppm and the *para* carbon of the arylamine 5.0 ppm downfield. These downfield shifts are similar to those caused by an acetyl group on nitrogen. Similar effects were seen for the DNA adducts of 4MA (Experimental Procedures). The position of the nitrogen binding on guanine was seen by the disappearance of the H-8 of guanine at 7.93 ppm and a 8 ppm downfield shift of the C-8.

The mass spectra were acquired using different methods. The molecular ion of dG-4MA could be detected using the DCI technique; however, for dG-MDA and dG-AcMDA only the fragments resulting from the loss of the deoxyribose unit could be recorded. Mass spectra were also obtained by electrospray ionization. The molecular ion was the major ion in the negative ion spectra. For the positive ion spectra M^+ − deoxyribose was the major peak (Figure 3).

The UV spectra of the adducts shift by about 30 nm to longer wavelengths compared to the spectrum of dG. This suggests an extension of the conjugation system of guanine with MDA.

Hydrolysis of dG Adduct Standards. Recently the parent aromatic amines have been released by base hydrolysis from DNA of animals treated with 4-aminobiphenyl (*15*) and with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (*16*). This method was applied to the quantification of synthetic dG adducts of MDA. dG-MDA (0.6, 2.4, and 4.8 pmol) was hydrolyzed in Teflon tubes overnight with 0.1 M NaOH in the presence of DNA (1 mg) of control rats and 4MA (24 μg) as carrier. The released MDA was extracted in CH_2Cl_2 and analyzed by GC-MS. The release of MDA was linear over a range of 0−5 pmol; MDA (pmol) = 0.02 + 0.68 × dG-MDA (pmol), $r^2 = 1.0$. The yield for released MDA was 68 ± 5%. For dG-AcMDA treated under the same conditions, MDA was found with a yield of 79 ± 7%.

^{32}P-Postlabeling of dG Adduct Standards. The postlabeling method was optimized for the quantitation of dGp-4MA, dGp-AcMDA, and dGp-MDA. The synthesized 3′-monophosphate-dG adducts were incubated with

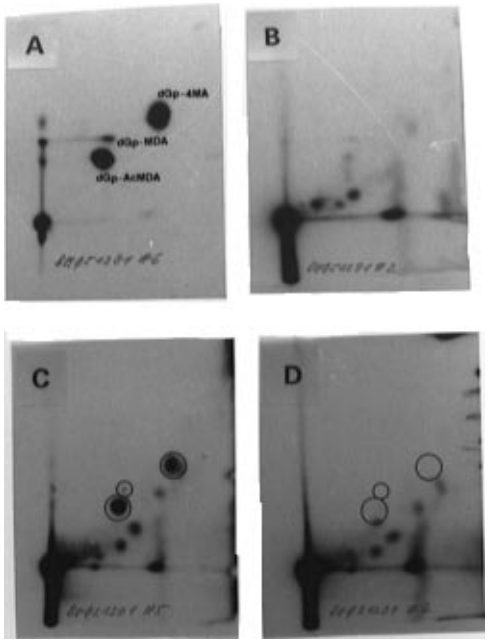


Figure 5. ^{32}P-Postlabeling analysis of (A) standard DNA adducts: dG-MDA, dG-AcMDA, and dG-4MA; (B) control DNA; (C) DNA of control rats spiked with adduct standards resulting in a RAL of 1×10^{-6}; and (D) DNA (10 nmol) from an MDA treated rat (500 μmol/kg).

T4 polynucleotide kinase (PNK) (0.2−0.5 U/μL) and [^{32}P]-ATP (1−5 pmol/μL) for various time periods (1−3 h) to check the labeling efficiency. The products were analyzed by multidirectional TLC on PEI cellulose. After the labeling reaction, a multidirectional chromatography on PEI coated cellulose TLC sheets was chosen to resolve the adducted nucleotides (Figure 5). The first development (D1) with 2.3 M NaH_2PO_4/NaOH (pH 5.77) as eluent was used to elute impurities and contaminating normal nucleotides, while dGp-AcMDA and dGp-MDA remained at the origin. The dGp-4MA slowly migrated under this condition to 1−2 cm above the origin. The development in the D3 and D4 solvent system resulted in sufficient separation of the adducts (Figure 5A−D). The best labeling efficiencies were obtained after an incubation time of 2 h with 0.5 U/μL PNK and 4 μM [^{32}P]-ATP: ca. 70% for dGp-4MA and 60% for dGp-AcMDA and 20% for dGp-MDA. The methods of adduct enrichment—1-butanol extraction and NP1 digestion—were tested with the dGp adduct standards. Using NP1 digestion, the recoveries for the adducts were much less than 1%. This indicates that the respective dGp adducts were dephosphorylated by NP1 as well as the normal nucleotides. This confirms the results obtained with other dGp adducts of aromatic amines (*45*). Using 1-butanol extraction, the recoveries for the adducts improved with increasing concentrations of TBA. In the presence of 3 mM TBA, dGp-4MA, dGp-AcMDA, and dGp-MDA were recovered with 56%, 71%, and 23%, respectively. Therefore, the overall procedure with 1-butanol extraction in the presence of 3 mM TBA and labeling for 2 h with 0.5 U PNK/μL and 4 μM [^{32}P]ATP should result in recoveries of about 40%, 40%, and 4% for dGp-4MA, dGp-AcMDA, and dGp-MDA, respectively. This is in agreement with the experimentally found recoveries of 32%, 30%, and 2%. These recoveries were much lower (3%, 6%, and 0.8% for dGp-4MA, dGp-AcMDA, and dGp-MDA, respectively) when the adducts were incubated with micrococcal nuclease (30 mU/μL) and phosphodiesterase (6 mU/μL), prior to the enrichment and labeling procedure. This indicates that the synthetic adducts are not stable under the conditions used for DNA hydrolysis. Therefore, different hydrolysis conditions were tested with calf thymus DNA spiked with the synthetic adducts to increase the recoveries. DNA (5−10 μg) was spiked with the synthetic adduct standards (20−600 fmol) and was hydrolyzed to the nucleotides by incubation with different amounts of micrococcal nuclease (10−75 mU/μL) and spleen phosphodiesterase (0.6−6 mU/μL) in a final volume of 12 μL of nucleotide buffer for 3 h at 37 °C. The hydrolysate was extracted with 1-butanol and ^{32}P-postlabeled as described above. The adduct recoveries were increased by a factor of 2, using 15 mU/μL micrococcal nuclease and 1 mU/μL phosphodiesterase. The DNA was not completely digested with lower amounts of enzymes. The limit of detection of the assay under the present conditions allows the detection of about 1 fmol of adduct in 30 nmol of normal nucleotides. This corresponds to an adduct level of 1 adduct in 3×10^7 normal nucleotides (RAL = 3.3×10^{-8}).

Liver DNA Adducts *in Vivo*. Three approaches were used for the analysis and quantification of liver−DNA adducts: (i) HPLC analysis of DNA isolated from animals dosed with radiolabeled MDA, (ii) analysis of the DNA by ^{32}P-postlabeling, and (iii) base hydrolysis of the DNA and determination of the released MDA by GC-MS. In rats given ip ^{14}C-labeled MDA (5.6 μmol/kg, 0.3 mCi/kg; 116.5 μmol/kg, 3 mCi/kg), radioactivity was associated with the purified liver DNA. Specific activities of 2.4 and 50.4 dpm/mg of DNA were found with the DNA isolated from the low and high dosed animals. Repurification of the DNA sample from the high dosed animal did not result in a reduction of the specific DNA radioactivity. This indicates that, during the DNA isolation process, the DNA adducts formed with DNA were stable and all noncovalently bound radiolabeled metabolites of MDA were removed. For the animals dosed with 5.6 and 116.5 μmol [^{14}C]MDA/kg body weight, the chemical binding index (CBI) [(μmol of chemical bound/mol of DNA)/(mmol of chemical applied/kg body weight)] was 1.05 and 2.3, respectively. This classifies MDA as a weak genotoxic carcinogen (*46*). DNA was hydrolyzed to the nucleotides and analyzed by HPLC with method A. The corresponding radioactivity profile (Figure 6A) showed that no radioactivity coeluted with the normal nucleotides. The major peak of the recovered radioactivity (78%) eluted distant from the nucleotides, in the region known to contain the more lipophilic DNA adducts. This indicates covalent adduct formation of MDA. To investigate the type of MDA adducts, DNA hydrolysate was analyzed by HPLC with method B in the presence of the synthetic adducts (Figure 6B). Additionally, the DNA spiked with the synthetic adducts was chemically hydrolyzed to the purines and analyzed by HPLC with method C (Figure 6C). The recoveries of the radioactivity for the two analyses were 79% and 70%, respectively. The results clearly showed that the radioactivity did not coelute with one of the synthesized standards. The same DNA was used for the analysis of DNA adducts by the optimized postlabeling technique. No adducts corresponding to dGp-4MA, dGp-MDA, or dGp-AcMDA could be detected. The same result was obtained with DNA of female Wistar rats dosed with 500 μmol/kg MDA·2HCl by gavage. No adducts corresponding to the synthetic standards could be detected (Figure 5D), although the adduct level (see

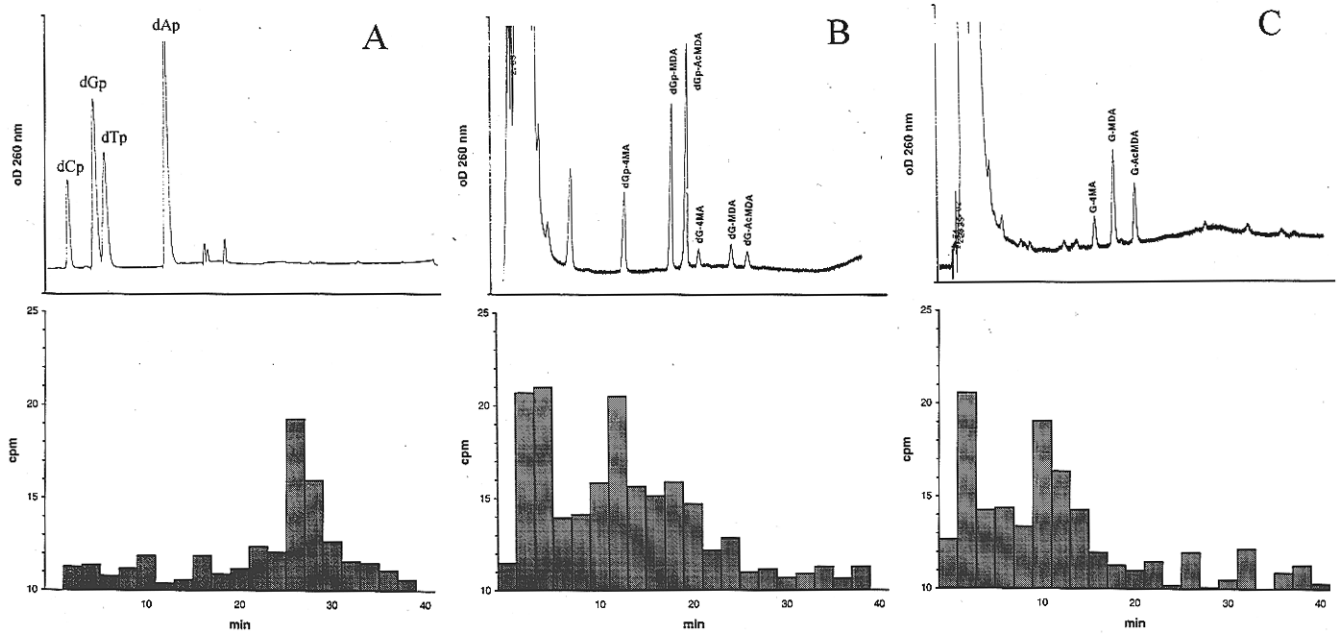


Figure 6. Analysis of liver DNA isolated from an animal after treatment with radiolabeled MDA. The radioactive background levels (11 cpm) obtained from experiments with control DNA were not subtracted. DNA was digested with spleen phosphodiesterase and micrococcal nuclease and analyzed by HPLC (A) with method A. (B) The DNA hydrolysate was spiked with the adducts dGp-4MA, dGp-MDA, and dGp-AcMDA and analyzed by HPLC with method B. (C) DNA spiked with the adducts dGp-4MA, dGp-MDA, and dGp-AcMDA was chemically hydrolyzed and analyzed by HPLC with method C.

GC-MS analysis) is higher than the determination limit found for the postlabeling method of the synthetic standards spiked to control DNA (Figure 5C).

Livers of female Wistar rats treated with MDA·2HCl and/or MDA-d_4·2DCl were analyzed by GC-MS following the third approach (Figure 4). DNA was isolated as described for the postlabeling analyses. Livers from animals dosed the same day with only the vehicle were used as controls, in order to account for background levels of MDA. Liver DNA of two female Wistar rats given 500 μmol/kg MDA·2HCl was hydrolyzed in 0.1 M NaOH overnight at 110 °C in the presence of the recovery standard MDA-d_4. GC-MS analysis of the HFBA derivatized dichloromethane extracts detected 428 ± 40 fmol of MDA/mg of DNA. In the control animals no MDA was found. The experiment was repeated with livers from animals dosed with 500 μmol/kg MDA-d_4. In these rats 488 ± 19 fmol of MDA-d_4/mg of DNA was found to be bound. Taking into account a 68% yield of the method, the CBI found in these cases was 0.82 and 1.0, respectively. Chemical release of the parent compound from protein or DNA adducts always raises the question about the covalent character of the binding. DNA from rats given MDA-d_4 was thoroughly tested for the presence of noncovalently bound MDA-d_4. DNA was dissolved in 0.1 M NaOH, spiked with the internal standard 3,3′-methylenedianiline, and extracted with dichloromethane. No MDA-d_4 could be found in this first extract; but MDA-d_4 was released after hydrolysis overnight (see above). In addition, the workup procedure was tested with regard to the detection of noncovalently bound MDA. DNA (1 mg) in 0.1 M NaOH was spiked with 2 pmol of MDA. After incubation for 1 h at room temperature, the amines were extracted twice with dichloromethane. The water phase was then spiked with MDA-d_4 (5 pmol) as internal standard and 4MA (24 μg) as carrier, hydrolyzed overnight at 110 °C, and extracted with dichloromethane. MDA was quantified against MDA-d_4. No MDA could be detected in the final extract. Therefore, extraction of the DNA solution with dichloromethane prior to hydrolysis overnight eliminates noncovalently bound MDA.

The structure of the MDA−DNA adduct found *in vivo* is still unknown. At the moment it is unclear if only C-8 adducts of guanine are cleavable with base. It is conceivable that also the C-8 adducts with deoxyadenosine (dA) release MDA after base treatment. The release of MDA with base suggests that a MDA bond with the C-8 of a purine base is present.

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