Synthesis and analysis of DNA adducts of arylamines

Armin Beyerbach, Peter B. Farmer and Gabriele Sabbioni

Arylamines and nitroarenes are very important environmental and occupational pollutants. Genotoxic effects of arylamines are believed to be initiated by the formation of DNA adducts. DNA adducts of arylamines have been found in experimental animals and in exposed humans, and are predominantly formed with the carbon 8 of 2′-deoxyguanosine. Reference standards are necessary to develop methods for the quantification of DNA-adducts. Therefore, we have synthesized the 2′-deoxyguanosin-8-yl adducts of 2-methylaniline, 2-chloroaniline, 4-chloroaniline, 2,4-dimethylaniline, and 2,6-dimethylaniline. The products were characterized by ^{1}H-NMR, ^{13}C-NMR, MS and UV. The corresponding 2′-deoxyguanosine-3′-monophosphate adducts were synthesized for the quantification of DNA adducts by the ^{32}P-postlabelling technique. A GC–MS method was developed for the analysis of the new adducts as an alternative to the ^{32}P-postlabelling. DNA was spiked with the synthesized adducts and treated with 0.3 M NaOH overnight at 110 °C in the presence of a deuterated internal standard. We observed up to 80% recovery from about 1 adduct in 10^{8} to 1 in 10^{5} nucleotides.

Keywords: *DNA adducts, arylamines, GC–MS.*

Introduction

Aromatic amines and nitroarenes are very important industrial intermediates. *N*-Oxidation is a key step in the metabolism of aromatic amines and aromatic amides to toxic products. Aromatic amines are metabolized to highly reactive *N*-hydroxyarylamines (Kadlubar and Beland 1985, Beland and Kadlubar 1990) by mixed function mono-oxygenases. *N*-hydroxyarylamines can be further metabolized to *N*-sulphonyloxy-arylamines, *N*-acetoxyarylamines or *N*-hydroxyarylamine-*N*-glucuronides. Aromatic amides might be oxidized to *N*-hydroxyarylamides which are metabolized further to *N*-sulphonyloxyarylamides and *N*-acetoxyarylamines. These highly reactive intermediates are responsible for the genotoxic effects of this class of compounds (Kadlubar and Beland 1985, Beland and Kadlubar 1990). DNA adducts of arylamines have been found in several organs of exposed experimental animals. Advances in analytical techniques have allowed the quantification of DNA adducts of arylamines in human tissue. Adducts of 4-aminobiphenyl (4ABP) have been found in biopsies of urinary bladder, and exfoliated urothelial bladder cells (Talaska *et al.* 1991, 1994), in placenta and in peripheral lung tissues (Lin *et al.* 1994). Adducts of 4,4′-methylenebis(2-chloroaniline) (Kaderlik *et al.* 1993) have been detected in exfoliated bladder urothelial cells.

Reference standards of DNA adducts have been synthesized (i) as UV markers for chromatography-based molecular dosimetry (Kriek 1972), (ii) as haptens for the development of immunoassays (Poirier *et al.* 1990), (iii) as reference compounds for the ^{32}P-postlabelling technique (Beach and Gupta 1992) and (iv) for mass spectral analyses (Annan *et al.* 1990). Several methods have been used for the generation of DNA adducts of arylamines. *N*-(Guanosin-8-yl)-aniline was synthesized by nucleophilic attack of aniline at the C-8 of 8-bromo-2′,3′,5′-tri-*O*-acetylguanosine (Jacobsen *et al.* 1988). Recently C-8-guanine adducts were prepared by the reaction of 6-hydroxy-2,4,5-triaminopyrimidine with arylisothiocyanates or arylamine dithiocarbamates and subsequent thermal cyclization (Swenson *et al.* 1993). However, most adducts have been synthesized according to the *in vivo* situation by electrophilic attack of the activated arylamine at the DNA base (Kadlubar 1994).

Quantitative analysis of modified DNA bases is assuming increasing importance as a marker of exposure to carcinogens (Farmer *et al.* 1993). To date, the ^{32}P-postlabelling technique has been the method of choice for the quantification of DNA adducts (Talaska *et al.* 1992, Kadlubar, 1994, Marques and Beland 1994). With advances in mass spectrometry, methods for quantification of modified DNA bases have been developed using this technique (Blair 1993). Recently DNA-adducts of 2-aminofluorene (Bakthavachalam 1991), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Friesen *et al.* 1995) and 4ABP (Lin *et al.* 1994) have been quantified by the chemical release of the parent arylamine after treatment with hydrazine or sodium hydroxide.

The arylamines investigated for the present study are used to produce pesticides, dyes, antioxidants, pharmaceuticals and other products (IARC 1993). Humans are exposed for example to 2-methylaniline (2MA) at the workplace (Ward *et al.* 1991), to 2,6-dimethylaniline (26DMA) from the pharmaceutical lidocaine (Bryant *et al.* 1994) and to methyl- and dimethylanilines from cigarette smoke (Bryant *et al.* 1988, Luceri *et al.* 1993). 2MA, 26DMA and 4-chloroaniline (4CA) are classified in group 2B as possibly carcinogenic in humans (IARC 1987, 1993).

We synthesized DNA adducts and developed methods for the low level detection of DNA adduct of arylamines. The DNA adducts were prepared by the reaction of *N*-acetoxyarylamines with 2′-deoxyguanosine (dG), 2′-deoxyguanosine-5′-monophosphate (dG5′P) and 2′-deoxyguanosine-3′-monophosphate (dG3′P). The structure of the adducts was determined by ^{1}H- and ^{13}C-NMR-spectroscopy and/or mass spectrometry. The synthesized adducts were used as reference standards for alkaline DNA hydrolysis and mass spectral analysis.

Armin Beyerbach, Gabriele Sabbioni *(author for correspondence) are at the Institut für Pharmakologie und Toxikologie, Universität Würzburg, Versbacher Str. 9, D-97078 Würzburg, Germany; and* **Peter Farmer** *is at the MRC Toxicology Unit, University of Leicester, Lancaster Road, Leicester LE1 9HN, UK.*

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MATERIALS AND METHODS

Chemicals

2′-Deoxyguanosine-monohydrate (31070), disodium-2′-deoxyguanosine-5′-monophosphate (31080), pyruvonitrile (16000), and anhydrous sodium sulphate (71959) were purchased from Fluka (Neu-Ulm, Germany), 2′-deoxyguanosine-3′-monophosphate (D4147), and calf thymus DNA (D1051) from Sigma (Deisenhofen, Germany), *n*-hexane (34484) from Riedel-de Haën (Seelze, Germany), pentafluoropropionic anhydride (65193) from Pierce (Oud-Beijerland, The Netherlands). Alkaline phosphatase from calf intestine (EC 3.1.3.1., P-4252) was acquired from Sigma (Deisenhofen, Germany) and nuclease P1 from *Penicillium citrinum* (EC 3.1.30.1, 236225) from Boehringer (Mannheim, Germany).

Instrumentation

HPLC was performed with a quaternary HPLC pump series 1050 (Hewlett Packard) or LKB HPLC pump 2249 with a photodiode array detector LKB 2240 (Pharmacia) or a UV detector (Hewlett Packard). The HPLC runs were performed with the following methods:

Method A: LiChrospher 100 RP18 (125 × 4 mm, 5 µm, Merck) eluting with a 30 min methanol (MeOH) 10–100% gradient in water and a flow of 1 ml min^{-1}.

Method B: Nucleosil C_{18} (250 × 25 mm, 7 µm, Macherey-Nagel) with a 30 min MeOH 10–100% gradient in water, then 10 min MeOH 100% and flow of 5 ml min^{-1}.

Method C: Nucleosil C_{18} (250 × 25 mm, 7 µm, Macherey-Nagel) with a 30 min 25–100% MeOH gradient in water and a flow of 5 ml min^{-1}.

Method D: Nucleosil C_{18} (250 × 13 mm, 7 µm, Macherey-Nagel), 50 mM ammonium formate buffer pH 5.1, MeOH gradient 10–55% in 15 min, 55–85% in 1 min, isocratic 85% to 30 min, flow 2.5 ml min^{-1}.

Method E: LiChrospher 100 RP18 (125 × 4 mm, 5 µm, Merck), 50 mM ammonium formate buffer pH 5.1, MeOH gradient 10–55% in 15 min, 55–85% in 1 min, isocratic 85% to 30 min, flow 1 ml min^{-1}.

NMR spectra were recorded on a Bruker AC 250 spectrometer. The ^{13}C-NMR signals were distinguished by a DEPT (Distortionless Enhancement by Polarization Transfer) experiment. Internal standard was tetramethylsilane (TMS) or the deuterated solvent. The UV spectra were recorded on a Kontron spectral photometer Uvikon 860. Analytical thin layer chromatography (TLC) was carried out with TLC-plates Alugram SIL G/UV_{254} with fluorescence indicator (Macherey-Nagel, Düren, Germany).

Fast atom bombardment mass spectrometry (FAB-MS) was conducted on a Fisons 70-SEQ mass spectrometer by xenon atoms at 8 keV acceleration. For positive FAB the samples were introduced in a *p*-toluene sulphonic acid/glycerol matrix, for negative FAB in an aminopropane diol matrix. Electrospray ionization mass spectrometry was conducted on a Fisons Quattro-BQ mass spectrometer. Experiments were performed using cone-induced fragmentation. Samples were introduced via loop injection in water/acetonitrile.

Synthesis of the N-hydroxyarylamines

All *N*-hydroxyarylamines were prepared from the corresponding nitro compounds following published protocols (hydrazine and palladium on charcoal (Westra 1981), and zinc dust in aqueous ammonium chloride (Bamberger and Rising 1901)). All experiments with *N*-hydroxyarylamines were carried out in a dry nitrogen atmosphere.

Synthesis of DNA adducts

N-(2′-deoxyguanosin-8-yl)-2-methylaniline (dG-2MA)

N-(2-Methylphenyl)hydroxylamine (2MA-NHOH) (739 mg, 6 mmol) and triethylamine (NEt_3) (830 µl, 6 mmol) were dissolved in tetrahydrofuran (THF) (15 ml) at –50 °C. Pyruvonitrile (430 µl, 6 mmol) was added while stirring. The reaction was monitored by TLC (silica gel, $CHCl_3$; R_f: 2MA-NHOH 0.13, *N*-acetoxy-2-methylaniline 0.69). After stirring for 40 min at –50 °C the reaction was complete. This cold solution was added to dG*H_2O (713 mg, 2.5 mmol) and NEt_3 (350 µl, 2.5 mmol) in H_2O (30 ml). The reaction was monitored by HPLC with method A. With a flow rate of 1 ml min^{-1} dG-2MA elutes at 16.3 min. After stirring for 21 h at room temperature, the reaction mixture was evaporated and the dark residue was redissolved in H_2O (60 ml). The solution was extracted with diethyl ether (Et_2O) (6 × 40 ml), ethyl acetate (EtOAc) (6 × 40 ml), and with *n*-butanol (*n*-BuOH) (6 × 40 ml).The butanol extracts were concentrated *in vacuo*. The residue was redissolved in MeOH and was purified by preparative HPLC with method B [retention time (t_R) (dG-2MA) = 14.72 min]. After evaporation of the eluents 18.0 mg (48 µmol, 1.9%) of colourless crystals of dG-2MA were obtained.

UV (MeOH : H_2O = 3 : 2): λ_{max} = 276 nm, λ_{min} = 234 nm.

^1H-NMR (250 MHz, [D_6]DMSO); δ [ppm] = 2.05 (dd, $J_{2'a,2'b}$ = 11.6, $J_{1',2'b}$ = 6.1 Hz, 1 H), 2.18 (s, 3 H, CH_3), 2.71 (ddd, $J_{2'b,2'a}$ = 11.6, $J_{1',2'a}$ = 9.5, $J_{3',2'a}$ = 9.5, $J_{3',2'a}$ = 6.2 Hz, 1 H), 3.67 (m, 2 H), 3.88 (d, $J_{5',4'}$ = 2.4 Hz, 1 H), 4.38 (m, 1 H), 5.31 (dd, 1 H, 5′-OH[dG]), 5.51 (d, 1 H, 3′-OH[dG]), 6.27 (dd, $J_{2'a,1'}$ = 9.5, $J_{2'b,1'}$ = 6.1 Hz, 1 H), 6.34 (br. s, 2 H), 6.99 (ddd, $J_{3,4}$ = $J_{5,4}$ = 7.3, 1 H), 7.12 (dd, $J_{4,5}$ = $J_{6,5}$ = 7.3 Hz, 1 H), 7.17 (d, $J_{4,3}$ = 7.3 Hz, 1 H), 7.32 (d, $J_{5,6}$ = 7.3 Hz, 1 H), 7.93 (s, 1 H), 10.59 (br. s, 1 H).

^{13}C-NMR (63 MHz, [D_6]DMSO): δ [ppm] = 18.07 (q, CH_3). All other signals are listed in Tables 1 and 2.

FAB-MS: *m/z* (%) = 373 (33, $[M + H]^+$), 257 (100, $[M–C_5H_7O_3]^+$), 177 (74), 169 (37).

N-(2′-Deoxyguanosin-3′-monophosphate-8-yl)-2-methylaniline (dG3′P-2MA)

2MA-NHOH (51 mg, 414 µmol) and NEt_3 (58 µl, 420 µmol) were dissolved in THF (1.1 ml). Pyruvonitrile (30 µl, 420 µmol) was added while stirring at –40 °C. This cold solution was added to dG3′P (10 mg, 26.8 µmol) and NEt_3 (10 µl) in H_2O (0.8 ml) at 40 °C. The reaction was monitored by HPLC with method A [t_R (dG3′P-2MA) = 4.25 min]. The addition of the *N*-acetoxy-solution was repeated twice in an interval of 1 h. After stirring for 12 h at 25 °C the reaction mixture was concentrated in vacuo, dissolved in H_2O (20 ml) and extracted with Et_2O. The aqueous layer was evaporated to dryness and the residue redissolved in 2 ml of 50 mM ammonium formate buffer pH 5.3.

A prepurification with a preparative C_{18}-column was performed [40 × 15 mm C-135 B Bondpak C_{18}/Porasil B (Waters Association), 50 ml of 50 mM ammonium formate buffer pH 5.3, then 50 ml of 50 mM ammonium formate buffer pH 5.3: MeOH = 4 : 1, then 50 ml of 50 mM ammonium formate buffer pH 5.3: MeOH = 45 : 5]. The middle fraction was evaporated and lyophilized. A sequential purification of the residue by HPLC with method C, method D and method E yielded 32.3 µg (86.6 nmol, 0.3%) of dG3′P-2MA after lyophilization.

UV (MeOH : H_2O = 1 : 4): λ_{max} = 276 nm, λ_{min} = 234 nm.

ESI-MS (cone-induced fragmentation) *m/z* (%) : 451 (20, $[M–H]^-$), 255 (16, $[M–C_5H_{10}O_6P]^-$), 212 (39), 199 (20), 195 (74, $[C_5H_8O_6P]^-$), 183 (11), 171 (22), 157 (13), 143 (38), 141 (15), 127 (24), 122 (25), 113 (13).

N-(2′-Deoxyguanosin-8-yl)-2,4-dimethylaniline (dG-24DMA)

N-(2,4-Dimethylphenyl)hydroxylamine (24DMA-NHOH) (823 mg, 6 mmol) and NEt_3 (830 µl, 6 mmol) were dissolved in THF (25 ml). Pyruvonitrile (430 µl, 6 mmol) was added dropwise over 5 min at –50 °C. Then the solution was stirred for 20 min at –50 °C. Complete acetylation was monitored by TLC on silica gel with petroleum ether : EtOAc = 5 : 3. An aliquot of this reaction solution was added to dG*H_2O (668 mg, 2.34 mmol) and NEt_3 (340 µl, 2.5 mmol) in H_2O (25 ml) at 40 °C. The reaction was monitored by HPLC with method A [t_R (dG-24DMA) = 16.0 min]. After stirring for 3 h at 40 °C and 13 h at 25 °C the solvents were distilled *in vacuo* and the residue was dissolved in H_2O (50 ml). The reaction mixture was extracted first with Et_2O then with EtOAc till the organic layers were colourless. After evaporation of the H_2O the residue was suspended in MeOH

Compound	C-6	C-2	C-4	C-8	C-5	C-1′	C-2′	C-3′	C-4′	C-5′
dG	157.05	153.85	151.20	135.68	116.89	82.89	39.70	71.04	87.86	62.00
dG-2MA	155.98	152.84	150.06	145.33	112.57	83.12	38.23	71.25	87.43	61.46
dG-24DMA	156.15	152.91	150.34	146.08	112.86	83.37	38.52	71.58	87.68	61.74
dG-26DMA	155.74	152.62	150.08	146.06	112.54	82.96	38.05	71.65	87.39	61.50
dG-2CA		no data								
dG-4CA[a]	155.91	153.16	149.68	143.11	112.27	83.09	38.70	71.50	87.42	61.51
dG-4MA[a,b]	155.81	152.88	149.46	143.51	112.11	82.77	38.43	71.23	87.14	61.26
dG-4OMeA[a,c]	155.68	153.60	149.52	144.01	112.02	82.81	38.10	71.29	87.17	61.27
dG-A[d,e]	155.9	153.0	149.5	143.1	112.1	82.7	38.4	71.1	87.1	61.2
dG-4ABP[f,g]	155.9	153.0	149.5	143.1	112.1	82.8	38.4	71.2	87.2	61.2

Table 1. ^{13}C-NMR data of the guanine and sugar (2′-deoxyribose) moiety of dG and dG adducts in [D$_6$]DMSO. Chemical shifts are given in ppm.

Key: [a] Meier and Boche (1990); [b] dG-4MA: *N*-(2′-deoxyguanosin-8-yl)-4-methylaniline; [c] dG-4OMeA: *N*-(2′-deoxyguanosin-8-yl)-4-methoxyaniline; [d] dG-A: *N*-(2′-deoxyguanosin-8-yl)-aniline; [e] Famulok *et al.* (1989); [f] dG-4ABP: *N*-(2′-deoxyguanosin-8-yl)-4-aminobiphenyl; [g] Famulok and Boche (1989).

Compound	C-1	C-2	C-3	C-4	C-5	C-6
dG-2MA	139.39	130.55	130.55	123.46	126.25	123.33
NAc-2MA[a]	137.22	132.24	130.93	125.69	126.56	125.69
2MA	146.69	121.21	130.09	116.30	126.63	114.17
dG-24DMA	136.97	131.37	131.30	132.94	126.98	124.29
NAc-24DMA[b]	134.70	132.24	131.43	134.64	127.05	125.78
24DMA	144.14	121.28	130.70	124.47	129.97	114.32
dG-26DMA	137.51	135.20	128.01	125.58	128.01	135.20
NAc-26DMA[c]	135.61	135.34	127.77	126.47	127.77	128.38
26DMA	144.30	120.72	127.93	115.99	127.93	120.72
dG-2CA		no data				
NAc-2CA[d]	135.26	126.48	129.59	126.33	127.50	126.33
2CA	145.33	117.93	129.68	117.60	128.34	116.19
dG-4CA[e]	139.93	118.99	128.48	124.15	128.48	118.99
NAc-4CA[f,g]	138.40	121.30	129.00	127.40	129.00	121.30
4CA	148.40	115.89	129.20	119.41	129.20	115.89
dG-4MA[e]	138.7	117.8	129.2	129.6	129.2	117.8
NAc-4MA[h]	137.20	119.40	129.40	132.20	129.40	119.40
4MA	146.26	114.25	129.43	124.14	129.43	114.25
dG-A[i]	140.77	117.21	128.39	120.44	128.39	117.21
NAc-A[j,k]	139.50	119.30	128.70	123.20	128.70	119.30
A	149.30	114.62	129.53	116.41	129.53	114.62

Table 2. ^{13}C-NMR data of the aryl-carbons of arylamines, *N*-acetylarylamines and dG-adducts of arylamines. Chemical shifts are given in ppm.

Key: [a] NAc-2MA: *N*-acetyl-2-methylaniline, [b] NAc-24DMA: *N*-acetyl-2,4-dimethylaniline, [c] NAc-26DMA: *N*-acetyl-2,6-dimethylaniline; [d] NAc-2CA: *N*-acetyl-2-chloroaniline; [e] Meier and Boche (1990), [f] NAc-4CA: *N*-acetyl-4-chloroaniline; [g] Patra and Mukhopadhyay (1981); [h] NAc-4MA: *N*-acetyl-4-methylaniline, [i] Famulok *et al.* (1989), [j] NAc-A: *N*-acetyl-aniline; [k] Llinares *et al.* (1980).

(3 × 25 ml). The suspension was centrifuged to remove the undissolved residue (dG). The combined MeOH fractions were evaporated. The resulting solid was dissolved in H_2O : MeOH = 9 : 1 and purified with preparative HPLC with method C yielding 23.0 mg (59 µmol, 2.5%) dG-24DMA [t_R (dG-24DMA) = 22.0 min].

UV (MeOH : H_2O = 3 : 2): λ_{max} = 272 nm, λ_{min} = 233 nm.

^{1}H-NMR (250 MHz, [D$_6$]DMSO) : δ [ppm] = 2.04 (dd, $J_{2'a,2'b}$ = 12.2 Hz, $J_{3',2'b}$ = 6.6 Hz, 1 H), 2.13 (s, 3 H, CH_3), 2.24 (s, 3 H, CH_3), 2.69 (dd, 1 H), 3.67 (d, 2 H), 3.88 (ddd, *J* = 2.3 Hz, 1 H) 4.41 (ddd, $J_{2'a,3'}$ = 5.6 Hz, 1 H), 5.31 (m, 1 H, OH[dG]), 5.51 (m, 1 H, OH[dG]), 6.27 (dd, 1 H), 6.33 (s, 2 H), 6.94 (d, $J_{6,5}$ = 8.0 Hz, 1 H), 7.00 (s, 1 H), 7.19 (d, $J_{5,6}$ = 8.0 Hz, 1 H), 7.87 (s, 1 H), 10.59 (s, 1 H).

^{13}C-NMR (63 MHz, [D$_6$]DMSO: δ [ppm] = 18.09 (q, CH_3), 20.69 (q, CH_3). All other signals are listed in Tables 1 and 2.

FAB-MS: *m/z* (%) = 388 (7), 387 (26) $[M+H]^+$, 271 (100) $[M-C_5H_8O_3]^+$, 270 (18), 269 (20), 177 (7), 85 (42).

N-(2′-Deoxyguanosin-3′-monophosphate-8-yl)-2,4-dimethylaniline (dG3′P-24DMA)

24DMA-NHOH (100 mg, 729 µmol) and NEt_3 (102 µl, 729 µmol) were dissolved in THF (3.1 ml) at –50 °C. Pyruvonitrile (52 µl, 729 µmol) was added while stirring. Half of the cold solution was added to dG3′P*H_2O (10 mg, 26.8 µmol) and NEt_3 (4 µl, 29 µmol) in H_2O (0.5 ml) at 40 °C. The rest of the cold solution was added after 20 min of stirring. Stirring was continued for 4 h at 40 °C and overnight at 25 °C. The reaction was monitored by HPLC with method A [t_R (dG3′P-24DMA) = 7.8 min]. The reaction solution was extracted with Et_2O and concentrated *in vacuo*. The residue was purified by HPLC with method C, method D and method E and lyophilized, yielding 0.148 mg (315.6 nmol, 1.18%) of dG3′P-24DMA.

UV (MeOH : H_2O = 1 : 4): λ_{max} = 272 nm, λ_{min} = 233 nm.

ESI-MS (cone-induced fragmentation) *m/z* (%): 466 (6), 465 (26, $[M-H]^-$), 269 (13, $[M-C_5H_{10}O_6P]^-$), 268 (8), 212 (12), 195 (100, $[C_5H_8O_6P]^-$).

N-(2´-Deoxyguanosin-5´-monophosphate-8-yl)-2,4-dimethylaniline (dG5´P-24DMA)

24DMA-NHOH (179 mg, 1.31 mmol) and NEt_3 (182 µl, 1.31 mmol) were dissolved in THF (5.7 ml) and treated with pyruvonitrile (94 µl, 1.31 mmol) at –50 °C. After stirring for 15 min the *O*-acetylation was complete. Half of this solution was added to dG5´P*H_2O (100 mg, 255 µmol) and NEt_3 (40 µl, 0.288 mmol) in H_2O (2 ml) at 40 °C. The remaining solution was added after 20 min of stirring. The reaction was monitored by HPLC with method A. With a flow rate of 1 ml min^{-1} dG5´P-24DMA elutes at 6.1 min. After 12 h stirring at 25 °C the reaction solution was evaporated, the residue was redissolved in H_2O (6 ml) and extracted with portions of Et_2O, till the organic layer remained colourless. Then the aqueous layer was evaporated to dryness, redissolved in H_2O (620 µl) and purified by HPLC with method C [t_R (dG5´P-24DMA) = 17.5 min]. The resulting residue was lyophilized, yielding 15.0 mg (32 µmol, 11%) of dG5´P-24DMA.

UV (MeOH : H_2O = 1 : 4): λ_{max} = 271 nm, λ_{min} = 232 nm.

N-(2´-Deoxyguanosin-8-yl)-2,6-dimethylaniline (dG-26DMA)

N-(2,6-Dimethylphenyl)hydroxylamine (26DMA-NHOH) (823 mg, 6 mmol) and NEt_3 (830 µl, 6 mmol) were dissolved in THF (25 ml) at –50 °C. Over a period of 5 min pyruvonitrile (430 µl, 6 mmol) was added. The solution was stirred for 20 min at –50 °C. The completion of the reaction was determined by TLC (silica gel, petroleum ether : ethyl acetate = 5 : 3). An aliquot of the cold solution (22 ml) was added at 40 °C to dG*H_2O (668 mg, 2.34 mmol) and NEt_3 (350 µl, 2.5 mmol) in H_2O (25 ml). The reaction was monitored by HPLC with method A. dG-26DMA elutes at 14.5 min. After stirring for 3 h at 40 °C and 13 h at 25 °C the solution was concentrated and the residue was dissolved in H_2O (40 ml). The reaction mixture was extracted with Et_2O and then with EtOAc, till the organic layers were colourless. The aqueous layer was extracted with *n*-BuOH till the organic layer remained colourless. The *n*-BuOH layer was evaporated to dryness and the resulting solid was washed twice with 10 ml MeOH. The insoluble dG was removed by centrifugation. The extract was evaporated and the resulting solid was redissolved in H_2O : MeOH = 9 : 1. The crude product was purified by preparative HPLC with method C. The peak eluting at 23.5 min was purified again on a Nucleosil C_{18} (250 × 13 mm, 7 µm, Macherey-Nagel) with a 30 min 55–80% MeOH gradient in H_2O with a flow rate of 2.5 ml min^{-1}, yielding 15.0 mg (38.7 µmol, 1.7%) of dG-26DMA.

UV (MeOH : H_2O = 3 : 2): λ_{max} = 268 nm, λ_{min} = 232 nm.

^{1}H-NMR (250 MHz, [D_6]DMSO): δ [ppm] = 2.04 (dd, $J_{2'a,2'b}$ = 12.0 Hz, $J_{3',2'b}$ = 6.5 Hz, 1 H), 2.12 (s, 6 H, 2 CH_3), 2.73 (dd, 1 H), 3.67 (d, 2 H), 3.92 (m, *J* = 1.8 Hz, 1 H), 4.42 (ddd, *J* = 5.3 Hz, 1 H), 5.52 (m, 1 H, OH[dG]), 5.79 (m, 1 H, OH[dG]), 6.32 (s, 2 H), 6.34 (dd, *J* = 5.6 Hz, 1 H), 7.04 (m, 3 H), 8.00 (s, 1 H), 10.52 (s, 1 H).

^{13}C-NMR (63 MHz, [D_6]DMSO): δ [ppm] = 18.06 (q, CH_3). All other signals are listed in Tables 1 and 2.

ESI-MS (cone-induced fragmentation): *m/z* (%) = 387 (33, $[M+H]^+$), 297 (7), 271 (100, $[M-C_5H_7O_3]^+$), 253 (10), 210 (13), 196 (5), 188 (18), 168 (6).

N-(2´-Deoxyguanosin-3´-monophosphate-8-yl)-2,6-dimethylaniline (dG3´P-26DMA)

26DMA-NHOH (37 mg, 270 µmol) and NEt_3 (38 µl, 270 µmol) were dissolved in THF (1.2 ml) at –50 °C. Pyruvonitrile (25 µl, 337 µmol) was added while stirring. Half of the cold solution was added to dG3´P*H_2O (10 mg, 26.8 µmol) and NEt_3 (4 µl, 29 µmol) in H_2O (0.5 ml) at 40 °C. The remaining solution was added after 20 min of stirring. The reaction was stirred for 10 h at 40 °C and overnight at 25 °C. The reaction was monitored by HPLC with method A [t_R (dG3´P-26DMA) = 6.3 min]. The organic solvents were removed *in vacuo* and the remaining aqueous solution was extracted with Et_2O. HPLC purification was performed with method C [t_R (dG3´P-26DMA): 14.6 min] and method D [t_R (dG3´P-26DMA) = 19.5 min]. The resulting residue was lyophilized, yielding 0.238 mg (508.3 nmol, 1.9%) dG3´P-26DMA.

UV (MeOH : H_2O = 1 : 4): λ_{max} = 269 nm, λ_{min} = 232 nm.

ESI-MS (cone-induced fragmentation) *m/z* (%): 466 (6, $[M]^-$), 465 (33, $[M-H]^-$), 269 (14, $[M-C_5H_{10}O_6P]^-$), 268 (9), 212 (16), 207 (11), 195 (100, $[C_5H_8O_6P]^-$), 143 (7), 141 (6), 113 (7).

N-(2´-Deoxyguanosin-5´-monophosphate-8-yl)-2,6-dimethylaniline (dG5´P-26DMA)

26DMA-NHOH (180 mg, 1.31 mmol) and NEt_3 (182 µl, 1.31 mmol) were dissolved in THF (5.7 ml). Pyruvonitrile (94 µl, 1.31 mmol) was added at –50 °C. Half of the cold solution was added to dG5´P*H_2O (100 mg, 0.255 mmol) and NEt_3 (40 µl, 0.288 mmol) in H_2O (2 ml) at 40 °C. The remaining solution was added after 20 min of stirring. The reaction was monitored by HPLC with method A [t_R (dG5´P-26DMA) = 4.4 min]. After 12 h of stirring at 25 °C the reaction solution was concentrated *in vacuo*. The residue was redissolved in H_2O (3 ml) and extracted with Et_2O, till the organic layer remained colourless. The aqueous layer was evaporated to dryness, the residue was dissolved in H_2O (1 ml) and purified by HPLC with method C [t_R (dG5´P-26DMA) = 21.1 min].

UV (MeOH : H_2O = 1 : 4) : λ_{max} = 267 nm, λ_{min} = 232 nm

N-(2´-Deoxyguanosin-8-yl)-2-chloraniline (dG-2CA)

N-(2-Chlorophenyl)hydroxylamine (2CA-NHOH) (344 mg, 2.4 mmol) and NEt_3 (330 µl, 2.4 mmol) in THF (4 ml) were treated with pyruvonitrile (170 µl, 2.4 mmol) at –40 °C. The reaction was monitored by TLC (silica gel, $CHCl_3$). After 20 min the *O*-acetylation was complete. The cold solution was added to dG*H_2O (571 mg, 2 mmol) and NEt_3 (275 µl, 2 mmol) in H_2O (34 ml) at 40 °C. Stirring was continued overnight at 40 °C. The reaction was monitored by HPLC with method A [t_R (dG-2CA) = 17.2 min]. The aqueous solution was evaporated to dryness, redissolved in H_2O (50 ml) and extracted with Et_2O (6 × 250 ml). The residue was suspended in MeOH (5 ml) filtered over a G-3-frit and washed with MeOH (4 × 5 ml). The filtrate was concentrated *in vacuo*, resuspended in MeOH and filtered again. HPLC runs showed that the solid residue was pure dG and the filtrate was enriched with the dG-2CA adduct. Preparative HPLC with method C [t_R (dG-2CA) = 25.0 min] yielded 8.0 mg (20 µmol, 1.0%) of dG-2CA as light brown crystals.

UV (MeOH): λ_{max} = 284, 242 nm, λ_{min} = 261, 229 nm. UV (MeOH : H_2O = 3 : 2): λ_{max} = 279 nm, λ_{min} = 234 nm.

^{1}H-NMR (250 MHz, [D_6]DMSO): δ [ppm] = 2.08 (m, 1 H), 2.72 (m, 1 H), 3.65 (m, 2 H), 3.89 (m, 1 H), 4.36 (m, 1 H), 5.28 (br. s. 2 H, 3´-OH[dG], 5´-OH[dG]), 6.23 (dd, $J_{2'a,1'}$ = 8.9 Hz, $J_{2'b,1'}$ = 5.9 Hz, 1 H), 6.40 (s, 2 H), 7.03 (dd, $J_{3,4}$ = $J_{5,4}$ = 7.7 Hz, 1 H), 7.30 (dd, $J_{4,5}$ = $J_{6,5}$ = 7.7 Hz, 1 H), 7.44 (d, $J_{5,6}$ = 7.7 Hz, 1 H), 7.75 (d, $J_{4,3}$ = 8.0 Hz, 1 H), 8.11 (s, 1 H), 10.60 (br. s, 1 H).

N-(2´-Deoxyguanosin-8-yl)-4-chloroaniline (dG-4CA)

N-(4-Chlorophenyl)hydroxylamine (4CA-NHOH) (861 mg, 6 mmol) and NEt_3 (830 µl, 6 mmol) were dissolved in THF (30 ml). Pyruvonitrile (430 µl, 6 mmol) was added while stirring at –40 °C. The reaction was monitored by TLC (silica gel, $CHCl_3$). The cold solution was added to dG*H_2O (668 mg, 2.34 mmol) and NEt_3 (340 µl, 2.5 mmol) in $CHCl_3$: EtOH : H_2O = 3 : 7 : 4 (42 ml) at 37 °C. The reaction was monitored by HPLC with method A [t_R (dG-4CA) = 17.9 min]. After stirring for 2 h at 37 °C the mixture was concentrated *in vacuo* and redissolved in H_2O (100 ml). After extraction with Et_2O (6 × 50 ml) the aqueous layer was re-extracted with *n*-BuOH (5 × 30 ml). The organic layer was evaporated to dryness and the residue was purified by preparative HPLC with method B [t_R (dG-4CA) = 31.4 min]. After lyophilization 13 mg (28 µmol, 1.2%) of a light brown solid was obtained.

UV (MeOH : H_2O = 3 : 2): λ_{max} = 285 nm, λ_{min} = 243 nm.

The NMR data are in agreement with the literature (Meier and Boche 1990).

FAB-MS: *m/z* (%) = 395 (11), 394 (8), 393 (24, $[M+H]^+$), 279 (37), 278 (26), 277 (100, $[M-C_5H_7O_3]^+$), 276 (17), 185 (26), 149 (29), 131 (18), 117 (42), 115 (40), 103 (18).

N-(2′-Deoxyguanosin-3′-monophosphate-8-yl)-4-chloroaniline (dG3′P-4CA)

4CA-NHOH (45.5 mg, 264 µmol) and NEt_3 (37 µl, 264 µmol) were dissolved in THF (1.1 ml) at –50 °C. Pyruvonitrile (25 µl, 351 µmol) was added dropwise. Half of the cold solution was added to dG3′P*H_2O (10 mg, 26.8 µmol) and NEt_3 (4 µl, 29 µmol) in H_2O (0.5 ml) at 40 °C. The remaining solution was added after 20 min of stirring. The reaction was monitored by HPLC with method A [t_R (dG3′P-4CA) = 5.9 min]. After 2 h of stirring at 40 °C and 12 h at 25 °C again a cold *N*-acetoxy solution (as shown above) was added in two portions. The solution was stirred for 2 h at 40 °C and 12 h at 25 °C. The organic solvents were removed *in vacuo* and the aqueous solution was extracted with diethyl ether. The crude product was purified with HPLC with method C. The combined adduct fractions were purified with method D and method E. The resulting residue was lyophilized, yielding 179 µg (383.8 nmol, 1.4%) of dG3′P-4CA.

UV (MeOH : H_2O = 1 : 4): λ_{max} = 284 nm, λ_{min} = 242 nm.

ESI-MS (cone-induced fragmentation) *m/z* (%): 473 (14), 471 (41, $[M-H]^-$), 277 (20), 275 (64, $[M-C_5H_{10}O_6P]^-$), 274 (16), 212 (30), 195 (100, $[C_5H_8O_6P]^-$), 143 (13), 141 (12), 127 (25).

N-(2′-Deoxyguanosin-5′-monophosphate-8-yl)-2-chloroaniline (dG5′P-2CA) and N-(2′-Deoxyguanosin-5′-monophosphate-8-yl)-4-chloroaniline (dG5′P-4CA)

2CA-NHOH or 4CA-NHOH (186 mg, 1.3 mmol) and NEt_3 (180 µl, 1.3 mmol) were dissolved in THF (5.4 ml) at –40 °C. Pyruvonitrile (100 µl, 1.4 mmol) was added while stirring. The reaction was monitored by TLC (silica gel, $CHCl_3$, R_f : 2CA-NHOH 0.28, 4CA-NHOH 0.40). The *O*-acetylation was complete within 20 min. Half of the cold solution was added to dG5′P*H_2O (100 mg, 255 µmol) and NEt_3 (40 µl, 288 µmol) in H_2O (3.7 ml) at 40 °C. Stirring was continued for 2 h at 40 °C. The reaction was monitored by HPLC method A [t_R (dG5′P-2CA) = 5.0 min; (dG5′P-4CA) = 4.8 min]. The reaction solution was concentrated *in vacuo*, dissolved in H_2O (5 ml) and extracted with Et_2O (7 × 1 ml). NEt_3 (40 µl, 288 µmol) and the rest of the *N*-acetoxy solution was added to the aqueous solution at 40 °C. After stirring for 2 h at 40 °C the mixture was concentrated *in vacuo*, extracted with Et_2O (5 × 1 ml) and evaporated to dryness. The crude product was redissolved in H_2O and purified by preparative HPLC with method C [t_R (dG5′P-2 CA) = 16.5 min; t_R (dG5′P-4CA) = 16.0 min]. The remaining residue was lyophilized.

dG5′P-2CA: yield: 4.9 mg (10.3 µmol, 4.0%); UV (H_2O): λ_{max} = 276 nm, λ_{min} = 236 nm; UV (MeOH : H_2O = 1 : 4): λ_{max} = 277 nm, λ_{min} = 234 nm.

dG5′P-4CA: yield: 8.0 mg (16.85 µmol, 6.6%); UV (H_2O): λ_{max} = 282, 224 nm, λ_{min} = 254 nm; UV (MeOH : H_2O = 1 : 4): λ_{max} = 283 nm, λ_{min} = 242 nm.

Reaction of nucleotide adducts with enzymes

The enzyme reactions were monitored by HPLC with method A. All nucleotide adducts were converted to the corresponding nucleoside adducts. The identity of the products was confirmed by the UV-spectra and by co-elution with the corresponding synthesized nucleoside adducts.

Reaction of 2′-deoxyguanosine-5′-monophosphate adducts with alkaline phosphatase

Alkaline phosphatase from calf intestine (0.5 mg, 8.8 units) was suspended in 1 ml of buffer (0.1 mM zinc sulphate, 1.0 mM magnesium dichloride, 3.2 M ammonium sulphate). The enzyme solution (10 µl) was mixed with the nucleotide solutions and incubated for 90 min at 40 °C.

Reaction of 2′-deoxyguanosine-3′-monophosphate adducts with nuclease P1

The enzyme nuclease P1 (10 units) from *Penicillium citrinum* was dissolved in 0.03 M sodium acetate buffer pH 5.3. This enzyme solution (5 µl) was added to the nucleotide solutions (50 µl) with 30 mM sodium acetate buffer pH 7.3 (200 µl) and 10 mM zinc sulphate (20 µl) and incubated for 2 h at 40 °C.

Hydrolysis of DNA adducts

Calf thymus DNA in H_2O (1 ml) was placed in a glass tube (15 × 100 mm, Schott, Germany) with a screw cap. The screw cap was fitted with a Teflon liner. The solution was degassed by bubbling a gentle stream of nitrogen through it for 1 min. A methanolic solution of the synthesized dG adduct, the internal standard (the corresponding deuterated arylamine) and 10 M NaOH (100 µl) were added. The sample tubes were heated in an oil bath at 110 °C overnight. The extraction, derivatization and GC/MS analysis was performed by a modification of a procedure developed in our laboratory (Sabbioni and Beyerbach 1995). The hydrolysis solution was cooled to room temperature and the amines were extracted with hexane (1 × 3 ml). The organic extract was dried by passing through a Pasteur pipette filled with anhydrous sodium sulphate. The sodium sulphate was rinsed with hexane (1 ml). The combined hexane phases were derivatized with pentafluropropionic anhydride (5 µl). After 10 min at 25 °C, 2,4-difluoroaniline (100 ng in 10 µl hexane) was added and the hexane solution was evaporated in a gentle stream of nitrogen to dryness. When the last drop of solvent had disappeared the residue was taken up in EtOAc (15 µl). The GC/MS analysis was carried out as previously described (Sabbioni and Beyerbach 1995).

Results and discussion

Synthesis of DNA adducts

The DNA adducts of arylamines were synthesized from the corresponding *N*-acetoxyarylamine and the nucleoside or nucleotide (Figure 1). We obtained the *N*-acetoxy compounds after reaction of the corresponding *N*-hydroxyarylamines with acetyl cyanide at –40 °C. The freshly prepared solution of the *N*-acetoxy amine was added to the aqueous solution of dG, dG5′P or dG3′P respectively. The resulting mixture was prepurified by solvent extraction to remove most by-products. An enrichment of the adduct was carried out either by dissolving the residue in hot methanol with elimination of the precipitate by centrifugation or filtration or by repeated HPLC purification. In all reactions only the C-8 adduct could be isolated. Methanolic solutions of the dG adducts and aqueous solutions of the nucleotide adducts were kept frozen at –20 °C. They showed no decomposition after several months.

This synthetic route was based on observations by Lobo *et al.* (1985) and Prabhakar *et al.* (1982) on the reaction of *N*-acetoxyarylamines with nucleophiles under basic conditions. *N*-Acetoxy compounds have been isolated and characterized by NMR and IR spectroscopy (Prabhakar *et al.* 1982, Lobo *et al.* 1985, Famulok and Boche 1989, Famulok *et al.* 1989, Meier and Boche 1990). The reaction mechanism of the dG adduct formation has been studied by Humphreys *et al.* (1992). Their investigations on the adduct formation of *N*-acetoxy-2-aminofluorene with guanine derivatives have provided chemical evidence for an attack of activated aromatic amines at the N-7 position of guanine residues. After deprotonation of C-8 a Stevens-type rearrangement can occur, resulting in the C-8 adduct.

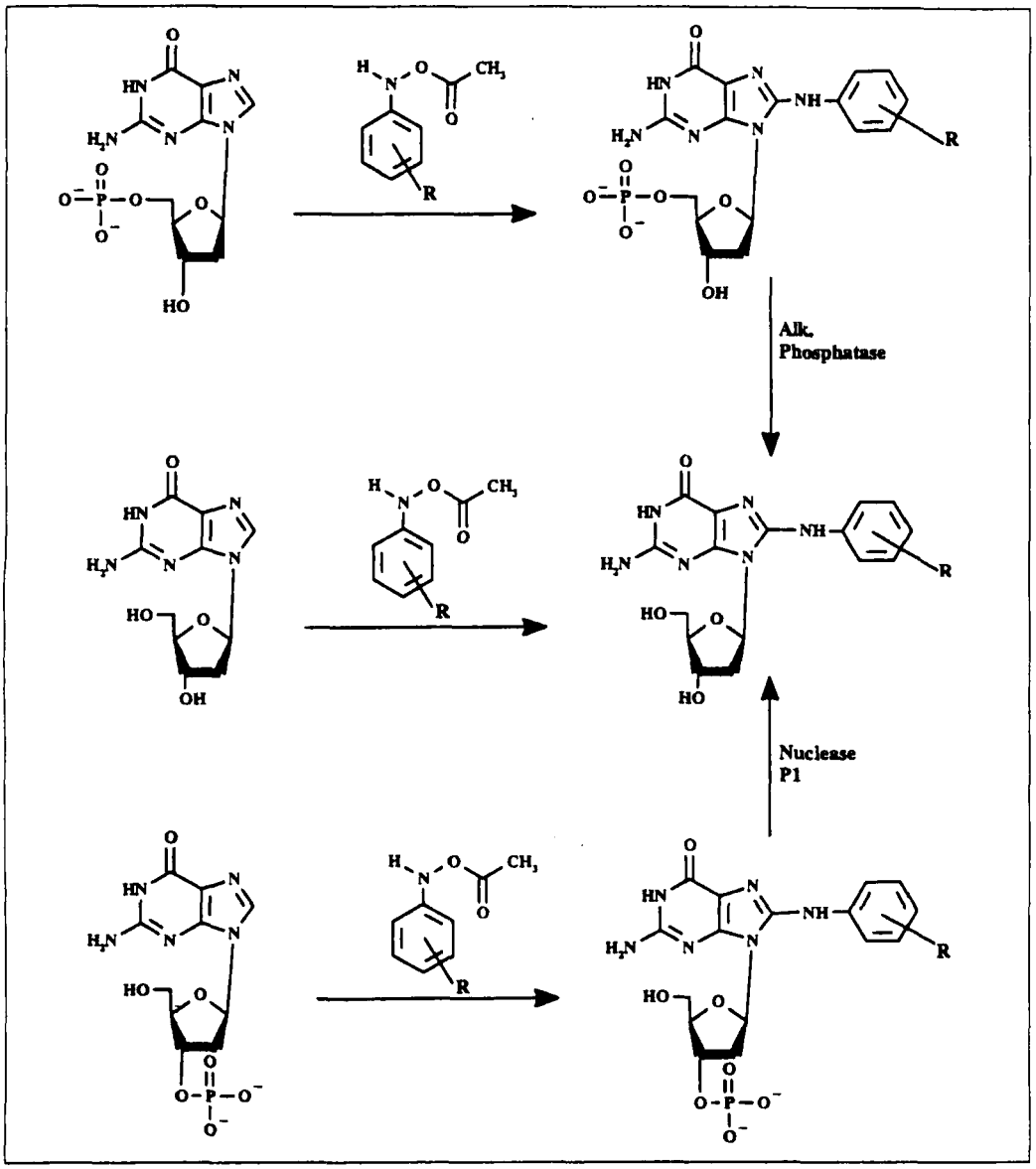

Figure 1. Reaction of *N*-acetoxy-arylamines with dG, dG3′P, and dG5′P and structural identification of the nucleotide adducts by enzyme-catalysed hydrolysis to the corresponding dG adducts.

The yields of the dG adducts were determined by ^{1}H-NMR spectroscopy with ethyl acetate added to the sample as a standard. All dG adducts were characterized by NMR spectroscopy (Figures 2 and 3) and positive FAB-MS. The dG3′P adducts were identified by ESI-MS. The dG3′P and dG5′P adducts were characterized after cleaving the phosphate group with alkaline phosphatase and nuclease P1, respectively. The HPLC retention times and the UV-spectra were identical to the synthesized dG adducts. The peak area of dG-24DMA from nuclease P1 reaction was 95% of the peak area of the injected dG3′P-24DMA before nuclease P1 reaction, and 93% for the corresponding 26DMA derivatives. The dephosphorylated reaction products were quantified by an external calibration curve obtained with the corresponding dG-adduct solutions. The concentrations of the parent nucleotide were determined, assuming that the yields of the enzymatic reactions were 100%.

Spectroscopic characterization of the DNA adducts

NMR spectroscopy

All NMR experiments were performed with [D_6]DMSO as solvent. The signals were assigned according to the spectra of dG-adducts of other arylamines (Kriek 1972, Famulok and Boche 1989, Famulok *et al.* 1989, Beland and Kadlubar 1990, Meier and Boche 1990). Typical for all C-8 dG adducts is the shift of the C-8 signal from 135.7 ppm for the unmodified dG to 143–146 ppm for the adducts. The binding site at C-8 could be determined unambiguously by a DEPT experiment. The C-8 resonance changed from a signal for a methine carbon in the dG to a signal for a quarternary carbon in the adducts. In the ^{1}H-NMR spectra of the adducts the 8-H signal at 7.9 ppm disappears and the NH signal of the arylamine shifts downfield by 4 ppm. The ^{1}H- and ^{13}C-NMR spectroscopic data of the sugar or the guanine moieties, respectively, are not very

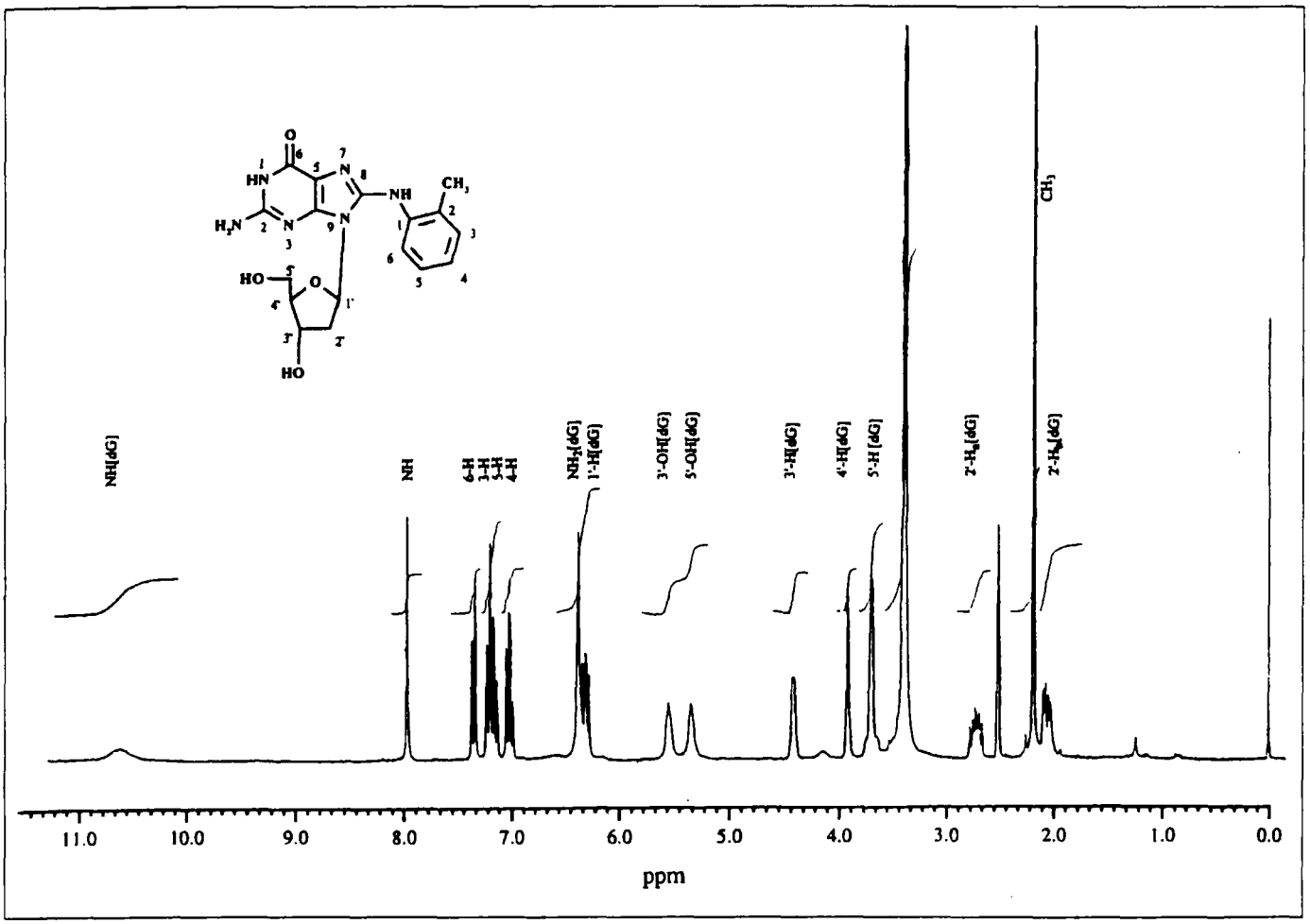

Figure 2. ^{1}H-NMR (250 MHz, [D$_6$]DMSO) spectrum of dG-2MA.

different for dG and C-8 dG-adducts (Tables 1 and 3). Substituents in the *ortho* position of the aniline nitrogen shift the resonances of the 2′-H$_a$ protons slightly downfield (about 0.1 ppm) compared with the same resonance in adducts with no *ortho* substituent. We observed this for the methyl group by comparing the chemical shifts of dG-2MA and dG-4MA, and for the chlorine atom by comparing the chemical shifts of dG-2CA and dG-4CA. The same effect was noticed for dG-24DMA and dG-26DMA. The second methyl group in the *ortho* position as in the dG-26DMA adduct does not further increase the downfield shift. This shift might be an indication of a conformational change of the glycosidic bond to a preferred *syn* conformation as postulated by Evans *et al.* (1980). Detailed and conformational studies with dG and dG5′P-adducts of 4-methylaniline (4MA) and 4-methoxyaniline (Meier and Boche 1991) showed that in nucleotides the glycosidic bond was *anti* and in nucleosides *syn*. Therefore, NMR studies on nucleosides are not appropriate for the prediction of the conformation found *in vivo*. Biologically significant conformational studies using NMR have been performed with DNA adducts of oligonucleotides with 4ABP Lasko *et al.* 1987) and *N*-acetyl-2-aminofluorene (O'Handley *et al.* 1993).

In the ^{13}C-NMR spectra the C-2′ signal of the sugar in the adducts is shifted slightly upfield by 1 ppm and the resonance of C-5 is shifted upfield by 4 ppm. Noteworthy is the influence of the adduct formation on the ^{13}C-NMR values of the arylamines (Tables 2 and 4). In the ^{13}C-NMR spectra the C-1 of the arylamines are shifted upfield by about 7 ppm, and the *para* positions are shifted downfield for aniline (A), 4MA, 2MA, 24DMA and 26DMA by 4, 5.5, 7.2, 8.4 and 9.6 ppm, respectively. These shifts are comparable to the shifts for *N*-acetylated arylamines, where 6.79, 7.06, 9.39, 10.17 and 11.14 ppm were registered for A, 4MA, 2MA, 24DMA and 26DMA, respectively. This might be explained by the steric hindrance of the *ortho* substituents which increase the dihedral angle between the plane of the guanine and the aromatic amine ring and therefore decrease the conjugation with the nitrogen. As expected the signals for the aromatic carbons in *meta* positions do not change substantially. The largest downfield shift is seen for the carbons in *ortho* positions. The signals for the carbon atom in the *ortho* position for A, 4MA, 2MA, 24DMA, and 26DMA shift downfield by 2.6, 2.96, 9.34, 10.09 and 14.48 ppm, respectively. The chemical shifts of the *ortho* carbon atoms in the corresponding *N*-acetylated arylamines are at 4.68, 5.15, 11.03, 10.96 and 14.62 ppm to lower field

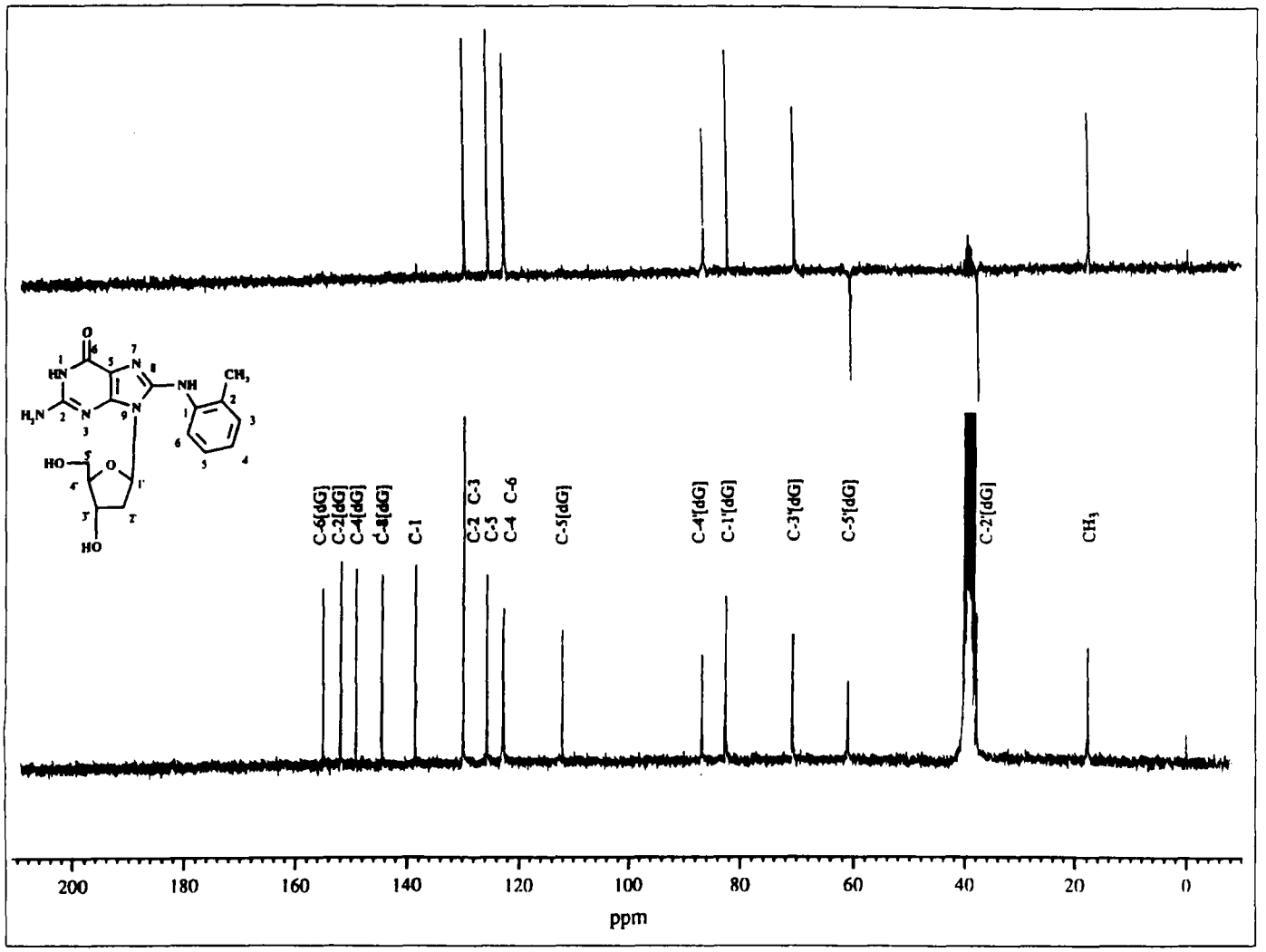

Figure 3. ^{13}C-NMR (63 MHz, [D_6]DMSO) spectrum of dG-2MA.

Compound	^{1}N-H	NH_2	1'-H	2'-H_a	2'-H_b	3'-H	4'-H	5'-H_a	5'-H_b
dG	10.73	6.51	6.13	2.50	2.20	4.35	3.71	3.53	3.53
dG-4MA[a]	10.70	6.40	6.25	2.45	1.92	4.34	3.84	3.73	3.69
dG-2CA	10.60	6.40	6.23	2.72	2.08	4.36	3.89	3.65	3.65
dG-2MA	10.59	6.34	6.27	2.71	2.05	4.38	3.88	3.67	3.67
dG-4OMeA[a]	10.69	6.38	6.29	2.48	1.98	4.39	3.91	3.70	3.74
dG-24DMA	10.59	6.33	6.27	2.69	2.04	4.41	3.88	3.67	3.67
dG-4CA[a]	10.45	6.46	6.32	2.49	2.01	4.41	3.92	3.74	3.70
dG-26DMA	10.52	6.31	6.34	2.73	2.05	4.42	3.92	3.67	3.67
dG-A[b]	10.83	6.45	6.33	–	2.02	4.43	3.93	3.76	3.76
dG-4ABP[c]	10.9	6.52	6.56	2.74	2.14	4.49	3.99	–	–

Table 3. ^{1}H-NMR data of the guanine and sugar (2'-deoxyribose) moiety in dG and dG adducts. Chemical shifts are given in ppm.

Key: [a] Meier and Boche (1990); [b] Famulok and Boche (1989); [c] Kadlubar *et al.* (1982).

Compound	NH	2-H	3-H	4-H	5-H	6-H
dG-2MA	7.93	–	7.17	6.99	7.12	7.32
2MA	4.77	–	6.92	6.45	6.90	6.48
dG-24DMA	7.87	–	7.00	–	6.94	7.19
24DMA	4.54	–	6.72	–	6.69	6.50
dG-26DMA	8.00	–	7.04	7.04	7.04	–
26DMA	4.47	–	6.79	6.40	6.79	–
dG-2CA	8.11	–	7.75	7.03	7.30	7.44
2CA	5.30	–	7.18	6.54	7.02	6.83
dG-4CA[a]	8.77	7.79	7.30	–	7.30	7.79
4CA	5.22	6.55	7.01	–	7.01	6.55
dG-4MA[b]	8.54	7.48	7.16	–	7.16	7.48
4MA	4.77	6.47	6.82	–	6.82	6.47
dG-A[c]	8.63	7.73	7.25	6.90	7.25	7.73
A	5.00	6.51	7.02	6.60	7.02	6.51

Table 4. ^{1}H-NMR data of the aryl-protons of the arylamines and of the corresponding dG adducts. Chemical shifts are given in ppm.

Key: [a] The same values were obtained by Meier and Boche (1990); [b] Meier and Boche (1990); [c] Famulok *et al.* (1989).

compared with the arylamines. Therefore, dG substitution decreases the electron donating effect of the amino group as with an acetyl group substitution.

Ultraviolet spectroscopy

The maximum of the UV spectra of dG adducts are shifted to longer wavelengths by 16–36 nm compared with dG, depending on the adduct. The maxima have molar extinction coefficients in the range of 10^4, which is typical for monocyclic arylamines. The UV spectra of the dG adducts support the hypothesis of a partial double bond between dG C-8 and the *N*-aryl atom which leads to a larger conjugation system (Meier and Boche 1990). However, the two chromophores are not coplanar. Otherwise the observed bathochromic shift would be larger. This result is in accord with the NMR data discussed above. In MeOH/H_2O the spectra of the nucleoside adducts are similar to those of the nucleotide adducts, with identical maxima. The phosphate group of the nucleotide adducts has no influence on the UV spectra.

Mass spectrometry

Mass spectrometric experiments confirmed the structure of the synthesized adducts. Typical for all adducts are fragments due to the loss of sugar by the cleavage of the glycosidic bond. The nucleotide adducts release a fragment with *m/z* 97 from phosphoric acid, confirming the presence of phosphate. The dG adducts dG-2MA, dG-24DMA and dG-4CA were examined using positive FAB-MS in a *p*-toluene sulphonic acid/glycerol matrix. They gave strong $[MH]^+$ ions and fragments because of

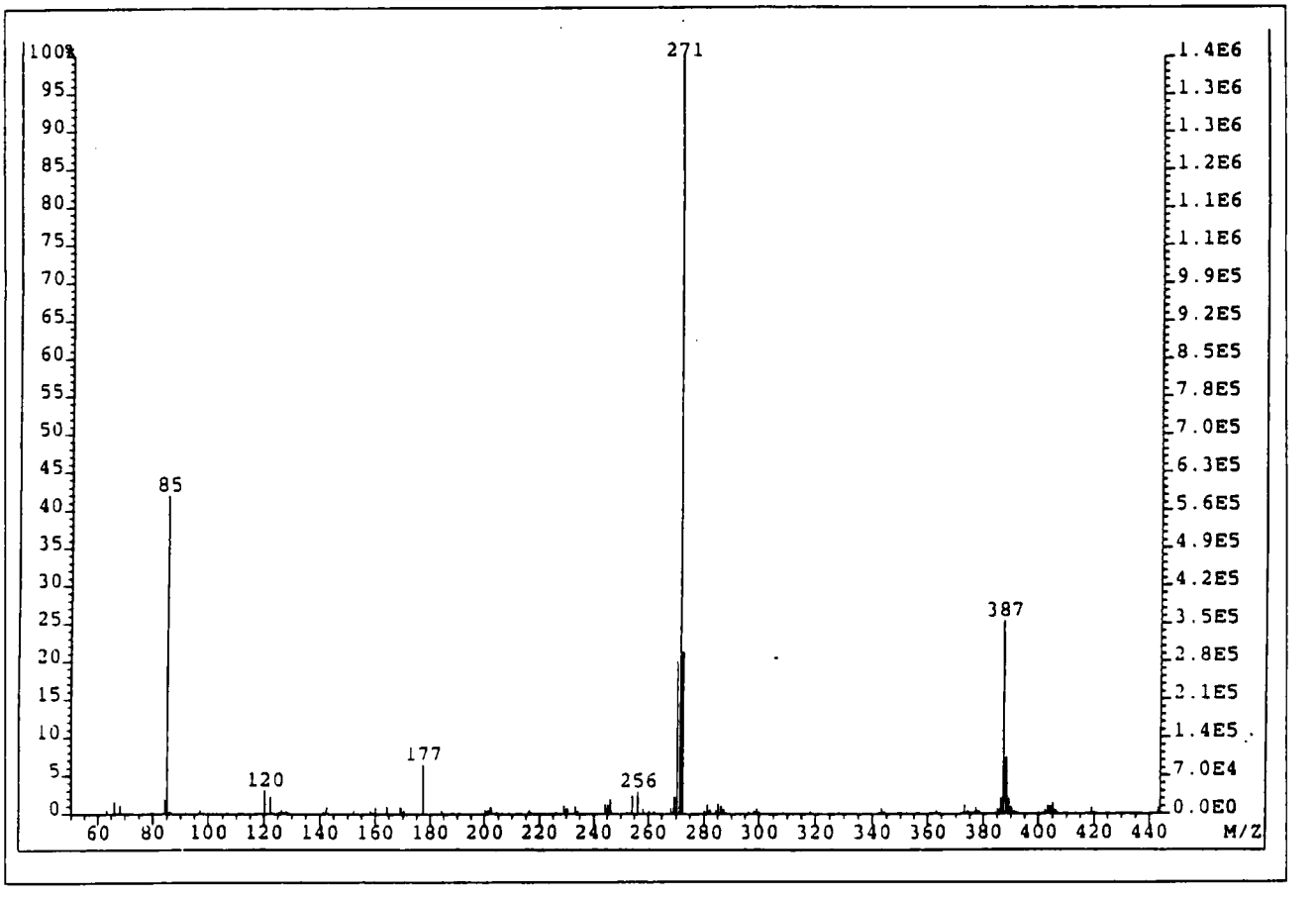

Figure 4. Positive ion FAB spectrum of dG-24DMA (6.14 nmole, 2.38 µg). *m/z* = 85: $[MH]^+$ of $[D_6]$–DMSO.

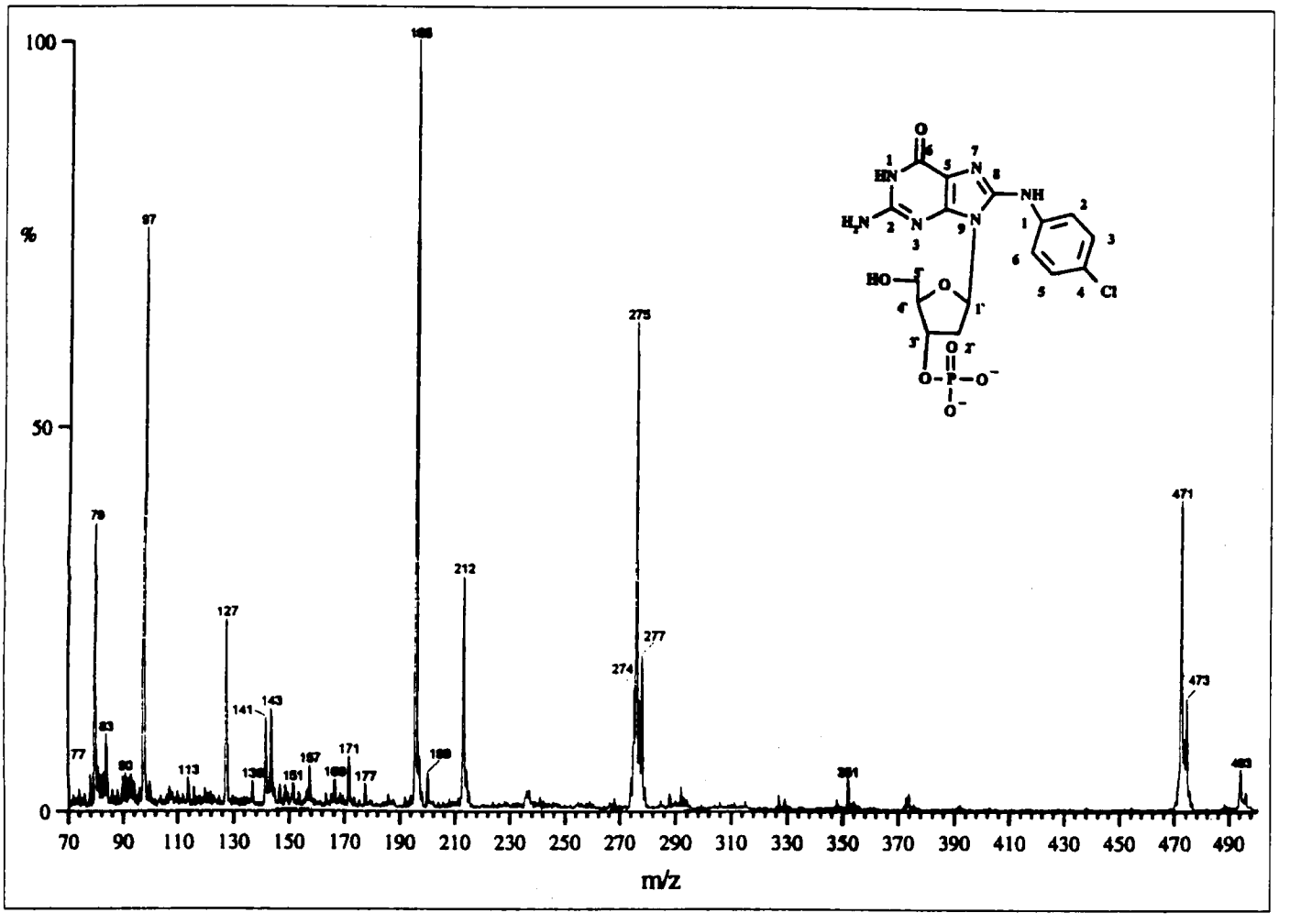

Figure 5. Negative ESI spectrum of dG3′P-4CA (loop injection of 3.84 nmole, 1.81 µg).

loss of the sugar $[MH-116]^+$ (Figure 4). Negative FAB-MS analyses of dG3′P-2MA, dG3′P-24DMA, dG3′P-26DMA and dG3′P-4CA in aminopropane diol showed $[M-H]^-$ ions, which were not very strong. But with negative electrospray ionization MS (ESI-MS) the $[M-H]^-$ ion was the main fragment (Figure 5). For dG-26DMA we observed a strong $[M-H]^-$ ion with negative ESI-MS and with positive ESI-MS $[MH]^+$ ion a fragment $[MH-116]^+$ which corresponds to the loss of the sugar moiety. Full scan spectra of dG-26DMA could be registered down to 25 pmol with positive and negative ESI-MS. In the single ion monitoring mode a signal for $[M-H]^-$ was detectable down to 1 pmol. It is conceivable that with sample introduction by capillary electrophoresis the detection limit could be lowered 100-fold.

Hydrolysis of DNA adducts

We investigated the release of arylamines from DNA adducts by alkaline hydrolysis. To verify the efficiency of the hydrolysis, we determined the amount of arylamine released from the dG adducts (Figure 6). For the investigation of the reaction kinetics the DNA solution was spiked with dG adducts. At intervals of 2 h aliquots of the reaction mixture were analysed for the arylamine released from the dG adduct. The corresponding ring deuterated arylamines and $^{13}C_6$-4CA were used as recovery standards. These studies showed that optimal conditions were 0.9 M NaOH overnight at 110 °C. Friesen *et al.* (1995) and Lin *et al.* (1994) used lower concentrations of sodium hydroxide (0.05 M) but at 130 °C. For the present study, DNA was spiked with 20 fmol to 38 pmol of dG-2MA, dG-24DMA, dG-26DMA and dG-4CA. The recoveries of the released arylamines were 85±7% 2MA (from 149 fmol to 29.8 pmol dG-2MA), 87±9% 24DMA (from 154 fmol to 30.7 pmol dG-24DMA), 63±4% 26DMA (from 127 fmol to 25.4 pmol dG-26DMA), and 53±5% 4CA (from 190 fmol to 38 pmol dG-4CA) (Figure 7). Using alkaline hydrolysis-GC/MS, the limit of detection was determined to be 100±12 fmol of arylamines per mg of DNA (3.13±0.38 adducts per 10^8 nucleotides). All samples were performed in duplicate. These detection limits are comparable to the GC–MS methods presented for the quantification of dG-adducts of PhIP and 4ABP. The detection limit for 4ABP released with sodium hydroxide from DNA was 10 fmoles 4ABP per mg DNA, corresponding to 0.32 adducts per 10^8 nucleotides (Lasko *et al.* 1987). For release of PhIP from modified DNA, Friesen *et al.* (1995) reported 3 fmol of PhIP per 100 µg DNA, corresponding to 1 PhIP adduct per 10^8

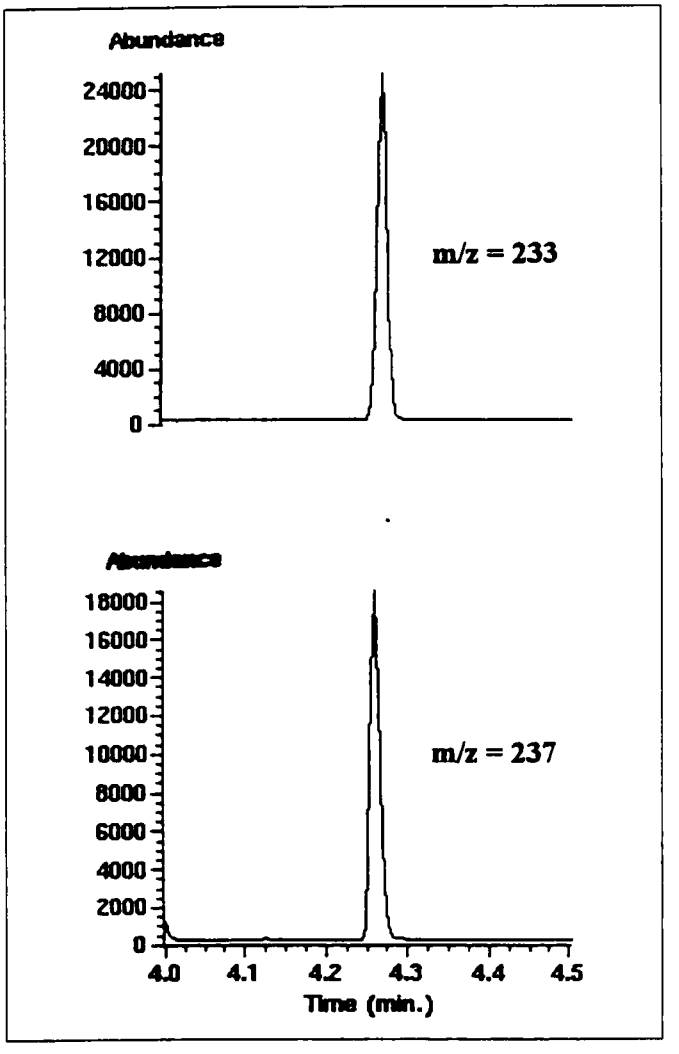

Figure 6. GC chromatogram of 2MA (*m/z* = 233) released from dG-2MA by NaOH and [D_4]2MA (*m/z* = 237)

nucleotides. These detection limits are lower than the detection limits obtained by FAB-, ESI- and tandem MS of the intact nucleoside or nucleotide adducts, although the dG C-8 adduct of 2-acetylaminofluorene in calf thymus DNA was detected in the lower femtomole range using capillary liquid chromatography coupled to a tandem mass spectrometer (Wolf and Vouros 1994) or FAB-MS (Wolf and Vouros 1995).

Conclusion

In the present study several dG3′P adducts and dG adducts of monocyclic arylamines were synthesized, which can be used to develop ^{32}P-postlabelling and mass spectrometric methods for low level detection in biological material. We showed that the arylamines can be released in good yields and quantified to levels down to 0.4 adducts per 10^8 nucleotides. Preliminary experiments with ESI-MS demonstrated that the detection limits may be reduced to similar levels in the near future. Although ^{32}P-postlabelling is still the most sensitive technique these new mass spectral methods could become an alternative for the ^{32}P-postlabelling technique.

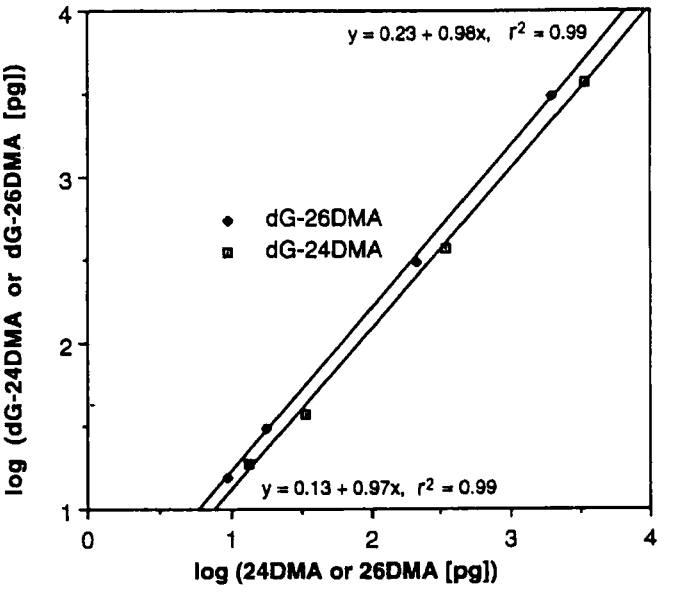

Figure 7. Recoveries of 24DMA and 26DMA from their dG adducts.

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