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Reactions of 9-substituted guanines with bromomalondialdehyde in aqueous solution predominantly yield glyoxal-derived adducts

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Reactions of 9-ethylguanine, 2′-deoxyguanosine and guanosine with bromomalondialdehyde in aqueous buffers over a wide pH-range were studied. The main products were isolated and characterized by ^{1}H and ^{13}C NMR and mass spectroscopy. The final products formed under acidic and basic conditions were different, but they shared the common feature of being derived from glyoxal. Among the 1 : 1 adducts, 1,N^2-(*trans*-1,2-dihydroxyethano)guanine adduct (**6**) predominated at pH < 6 and N^2-carboxymethylguanine adduct (**10a**,**b**) at pH > 7. In addition to these, an N^2-(4,5-dihydroxy-1,3-dioxolan-2-yl)methylene adduct (**11a**,**b**) and an N^2-carboxymethyl-1,N^2-(*trans*-1,2-dihydroxyethano)guanine adduct (**12**) were obtained at pH 10. The results of kinetic experiments suggest that bromomalondialdehyde is significantly decomposed to formic acid and glycolaldehyde under the conditions required to obtain guanine adducts. Glycolaldehyde is oxidized to glyoxal, which then modifies the guanine base more readily than bromomalondialdehyde. Besides the glyoxal-derived adducts, 1,N^2-ethenoguanine (**5a**–**c**) and N^2,3-ethenoguanine adducts (**4a**–**c**) were formed as minor products, and a transient accumulation of two unstable intermediates, tentatively identified as 1,N^2-(1,2,2,3-tetrahydroxypropano) (**8**) and 1,N^2-(2-formyl-1,2,3-trihydroxypropano) (**9**) adducts, was observed.

Introduction

Human beings are exposed every day to numerous alkylating agents of environmental origin. Such compounds can also be formed endogenously as a result of metabolism. Alkylation of DNA bases is deleterious for living organisms.[1] Reactions of nucleophilic amine functions with electrophiles block the normal hydrogen bonding within double-helical DNA, resulting in miscoding during the DNA synthesis and, hence, formation of a mutation. Cyclic DNA adducts obtained by reactions with bifunctional electrophiles, such as malondialdehyde,[2–4] acrolein,[5–8] crotonaldehyde,[7–10] halogenated acetaldehydes,[11–15] halogenated malondialdehydes,[16–18] vinyl chloride and chlorooxirane,[19–21] and mucohalo acids,[22–25] have been extensively studied over several decades. Such studies serve a dual purpose. On one hand, development of sensitive methods for detection of mutations within DNA depends on an understanding of the structure and stability of the adducts.[26–31] On the other hand adducted nucleosides are useful diagnostic tools. Some cyclic DNA adducts, such as 1,N^6-ethenoadenine,[12,32] 3,N^4-ethenocytocine,[32] N^2,3-ethenoguanine,[33] and 1,N^2-propenoguanine,[2–4] are fluoresescent and can be used as biomarkers.[3,12,16,32,33]

Reactions between nucleic acid bases and malondialdehydes,[28] halogenated malondialdehydes[18] and their congener, triformylmethane,[34–36] have previously been studied in our laboratory. The reaction of bromomalondialdehyde (BMA) with adenosine has been shown to yield, under slightly acidic conditions, two products, *viz.* 1,N^6-ethenoadenosine and its formyl derivative, suggested to be formed *via* a common acyclic intermediate.[18] As a continuation of the mechanistic work on the reactions of halogenated malondialdehydes with nucleic acid bases, the present work studies the reactions of BMA with 9-substituted guanines. It has been reported that 1,N^2-etheno and 1,N^2-formyletheno[25] products are formed in reactions with 3-chloro-4-dichloromethyl-5-hydroxy-2(5*H*)-furanone, similar to the situation with adenine nucleosides. Formation of N^2,3-etheno products is of particular interest, since such adducts are potentially mutagenic[37] and exceptionally susceptible to depurination.[38]

The adduct formation was studied over a wide pH-range, and the predominant stable products were isolated and characterized. The results showed that under any conditions employed, neither the etheno nor formyletheno products were the predominant products, but those derived from glyoxal actually prevailed. More detailed kinetic investigations were carried out under neutral and slightly acidic conditions to study the mechanism of the formation of these unexpected adducts and to compare the results to those previously obtained with 9-substituted adenines.

Results

Kinetic measurements

To avoid the complications that depurination of guanine nucleosides or their adducts could possibly result in, 9-ethylguanine (**1a**) was used as a model compound in most of the kinetic studies. At pH > 5, the reactions of 2′-deoxyguanosine (**1b**) were also studied. The reactions were usually carried out at 60 °C in 10 mmol dm^{-3} solutions of BMA, the pH of which was adjusted with 0.1 mol dm^{-3} buffer solution. The progress of the reactions was followed by analyzing aliquots withdrawn at appropriate intervals by RP-HPLC. The initial concentration of the starting material was lower than 0.5 mmol dm^{-3} and, hence, its disappearance obeyed pseudo first-order kinetics. The rate constants obtained as a function of pH are shown in Fig. 1. The data previously obtained with 9-methyladenine is included for comparative purposes.

As seen from Fig. 1, the reaction of 9-ethylguanine with BMA exhibits an inverse dependence of the rate on acidity at pH < 5, a broad maximum around pH 6, and a rate-retardation at pH > 7. The overall effect of pH on the reaction rate is, however, rather modest; at pH < 5 the rate constant is decreased by approximately one order of magnitude when the hydronium ion concentration becomes thousand-fold. As discussed below in more detail, several parallel reactions in all likelihood contribute to the disappearance of the starting material and most of them are preceded by complicated transformation reactions

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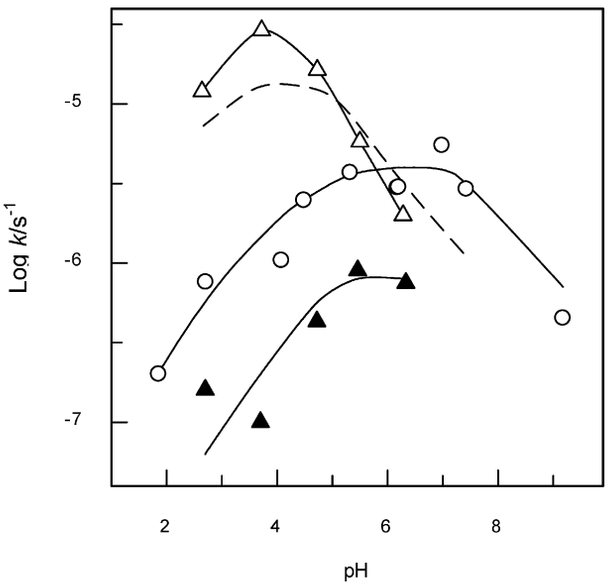


Fig. 1 pH-Rate profiles for the reactions of various 9-substituted guanines (< 0.5 mmol dm^{-3}) with BMA (10 mmol dm^{-3}) at 60 °C. Notation: 9-ethylguanine (**1a**;○), 9-ethyl-O^6-methylguanine (**2**; Δ), 9-ethyl-1-methylguanine (**3**; ▲). The dotted line refers to the reaction of 9-methyladenine with BMA.[18]

of BMA. Accordingly, it is not possible to give a simple mechanistic explanation for the shape of the rate profile.

For comparative purposes, the reactions of 9-ethyl-O^6-methylguanine (**2**) and 9-ethyl-1-methylguanine (**3**), which may be regarded as models of the two tautomeric forms of **1a**, were studied under the same conditions. As seen from Fig. 1, the pH – rate profile of the reaction of 9-ethyl-1-methylguanine with BMA closely resembles that of the corresponding reaction of 9-ethylguanine, but the rate constants are only one fifth of those obtained with **1a**. The reaction of 9-ethyl-O^6-methylguanine, mimicking the rare tautomer of **1a**, is more than one order of magnitude faster than that of **1a**, and the shape of the pH–rate profile resembles that of the reaction of 9-methyladenine, exhibiting a maximum at pH 4. Accordingly, 9-ethylguanine appears to react through its major tautomeric form. 2′-Deoxyguanosine (**1b**) reacts with BMA as fast as 9-ethylguanine at pH > 5 (data not shown) and, hence, seems to be a relevant model for the studies of the reactions of guanine nucleosides under neutral and alkaline conditions. In more acidic solutions, 2′-deoxyguanosine undergoes depurination.

Product analyses

Fig. 2 shows the RP-HPLC chromatograms obtained for the reaction of 9-ethylguanine with BMA at pH 4.1 and 7.4. As seen, the previously reported etheno products[22,25] are present in the product mixture (*cf.* Table 1), but only as minor components. The fluorescent N^2,3-etheno product (**4a**) was observed at a retention time clearly shorter (t_R 8.5 min) than that of its non-fluorescent 1,N^2-etheno isomer (**5a**) (t_R 17 min). Low pH favours the formation of the 2,N^3-adduct compared to the 1,N^2-adduct and the other products. No clear evidence for the formation of the corresponding formyletheno products could be obtained, in contrast to the situation with 9-methyladenine.[18]

In addition to the signals of the etheno derivatives, three UV-absorbing peaks appeared. The one with t_R 4.2 min predominated during the early stages of the reaction (Fig. 3). PR-HPLC-ESI/MS analysis revealed that this signal actually resulted from co-elution of two compounds having MH^+ values 284 and 296, respectively. On prolonged treatment, these products disappeared. Neither of them was sufficiently stable to be preparatively isolated. The other two signals that appeared at t_R 5.0 and 5.9 min, both exhibited a MH^+ value of 238. The

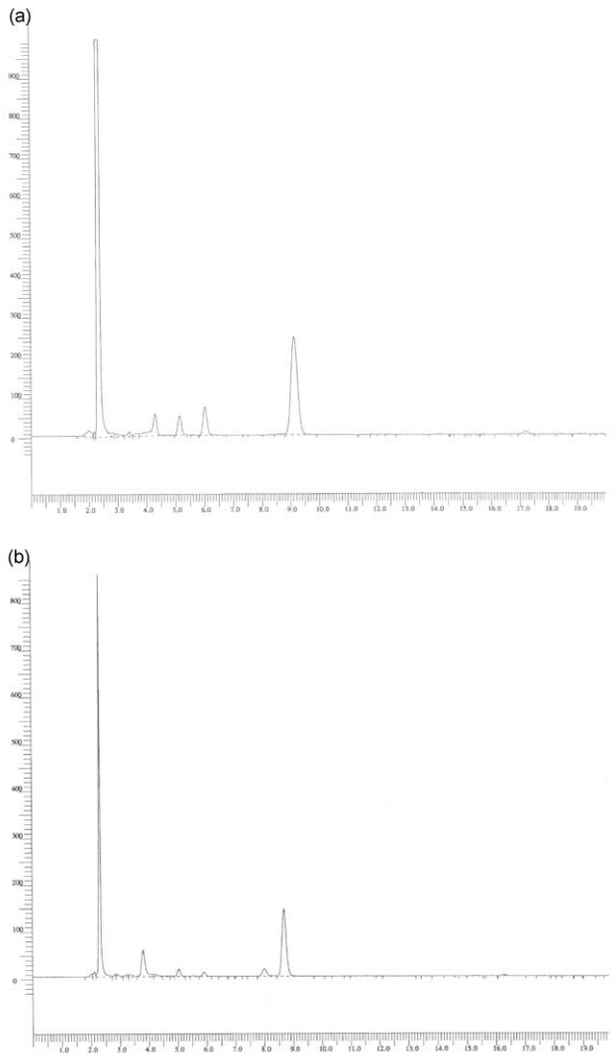
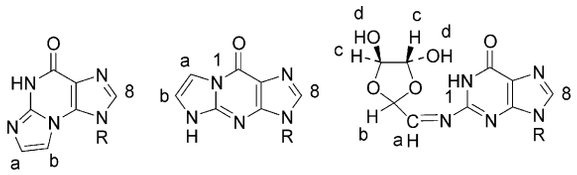


Fig. 2 RP-HPLC chromatograms of the reaction mixtures of 9-ethylguanine (**1a**; < 0.5 mmol dm^{-3}) with BMA (10 mmol dm^{-3}) at pH 4.1 and 60 °C. Annotation: t_R 4.3 min: 1,N^2-(1,2,2,4-tetrahydroxypropano)-9-ethylguanine and 1,N^2-(2-formyl-1,2,3-trihydroxypropano)-9-ethylguanine (**8** + **9**), t_R 5–6 min 1,N^2-(1,2-dihydroxyethano)-9-ethylguanines (**6** and **7**), t_R 8 min N^2,3-etheno-9-ethylguanine (**4a**) and t_R 9 min 9-ethylguanine (**1a**). b: RP-HPLC chromatograms of the reaction mixtures of 9-ethylguanine (**1a**; < 0.5 mmol dm^{-3}) with BMA (10 mmol dm^{-3}) at pH 7.4 and 60 °C. Annotation: t_R 4.3 min: 1,N^2-(1,2,2,4-tetrahydroxypropano)-9-ethylguanine and 1,N^2-(2-formyl-1,2,3-trihydroxypropano)-9-ethylguanine (**8** + **9**), t_R 5–6 min 1,N^2-(1,2-dihydroxyethano)-9-ethylguanines (**6** and **7**), t_R 9 min 9-ethylguanine (**1a**) and t_R 16 min 1,N^2-etheno-9-ethylguanine (**5a**).

concentration of the respective products continuously increased with time, the dependence of peak area on time showing a slight upward curvature during the early stages of the reaction (Fig. 3). Hence these products clearly predominated during the late stages of the reaction. They were isolated from the reaction mixture obtained at pH 5.5 (60 °C) and were characterized by ^{1}H and ^{13}C NMR spectroscopy (Tables 2 and 3). The isolated compound turned out to be predominantly 1,N^2-(*trans*-1,2-dihydroxyethano)-9-ethylguanine (**6**). Shapiro *et al.*[39] have shown previously that this adduct may be obtained in a solid form from glyoxal and guanine. The identity of **6** formed in the reaction of 9-ethylguanine with BMA was verified by spiking with a sample prepared in such a manner from glyoxal and 9-ethylguanine.

Since the product having MH^+ 238 appeared as two signals in the original HPLC chromatogram, the occurrence of **6** as various stereoisomers was studied by ^{1}H NMR spectroscopy. The cyclic *trans* adduct proved to be the thermodynamically

Table 1 ^{1}H NMR chemical shifts for the adducts of 9-substituted guanines[a]



	4a	5a	11b
1-H			
N^2-H			
8-H	7.93(s)	7.89(s)	8.13(s)
a-H	7.82(d, *J* 1.8)	7.56(d, *J* 2.6)	7.60(d, *J* 2.6)
b-H	7.14(d, *J* 1.8)	7.37(d, *J* 2.6)	7.41(d, *J* 2.6)
c-H			7.43
d-H			7.90
Others	[b]	[c]	[d]

[a] As ppm from TMS in DMSO-d_6. Coupling constants in Hz. [b] Ethyl group resonances at 1.41(t, *J* 7.3) and 4.42(q, *J* 7.3). [c] Ethyl group resonances at 1.37(t, *J* 7.3) and 4.08(q, *J* 7.3). [d] For the *–HOH–CHOH–* moiety an AA′XX′ proton spectrum was observed: $^3J_{\text{HC–CH}}$ 6.7, $^3J_{\text{HO–CH}}$ 8.4, $^4J_{\text{HO–C–CH}}$ 1.1.[41] Sugar proton resonances at 5.82(d, *J* 5.8), 4.48(q, *J* 5.5), 4.12(q, *J* 4.4), 3.91(q, *J* 4.1), 3.64(m) and 3.55(m). Sugar hydroxy resonances 5.39(d, *J* 6.1), 5.12(d, *J* 4.7) and 5.06(t, *J* 5.3).

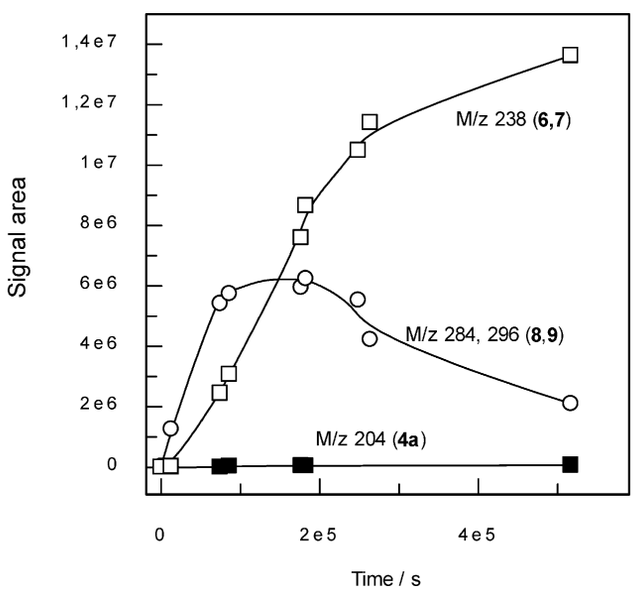


Fig. 3 Time-dependent product distribution for the reaction of 9-ethylguanine (**1a**; <0.5 mmol dm^{-3}) with BMA at pH 5.3 and 60 °C. Notation: 1,N^2-(*trans*-1,2-dihydroxyethano)-9-ethylguanine (**6**, □), 1,N^2-(1,2,2,4-tetrahydroxypropano)-9-ethylguanine and 1,N^2-(2-formyl-1,2,3-trihydroxypropano)-9-ethylguanine (**8** + **9**; ○) and N^2,3-etheno-9-ethylguanine (**4a**; ■).

stable form, but in addition the *cis*-form (**7**) could be observed immediately after dissolution of a solid product prepared from **1a** and glyoxal in DMSO-d_6. Accordingly, the product having MH$^+$ 238 may well appear as two separate signals in HPLC chromatograms. Consistent with the structure of **6**, the coupling constants between the carbon bonded hydrogens are small compared to the couplings to adjacent hydroxy protons, as reported earlier for a cyclic glyoxal-2′-deoxyguanosine adduct.[40]

As mentioned above, the products having MH$^+$ 284 and 296 could not be isolated. Accordingly, it is not clear whether they lie on the pathway to the final product having MH$^+$ 238. In other words, their degradation to non-chromophoric products cannot be strictly excluded. We tentatively suggest that these compounds are 1,N^2-(1,2,2,3-tetrahydroxypropano) (**8**, MH$^+$ 284) and 1,N^2-(2-formyl-1,2,3-trihydroxypropano) (**9**, MH$^+$ 296) adducts. Consistent with the suggested structures, **8** exhibited ESI-MS signals at *m*/*z* 266 (MH$^+$ − H_2O), 248 (MH$^+$ − 2H_2O) and 220 (MH$^+$ − H_2O–HCOOH) and **9** at 180 (MH$^+$ of **1a**). Their UV-absorption maxima were rather similar to those of 9-ethylguanine. Accordingly, **8** exhibited, at pH 4.5, an absorption maximum at 252 nm with a shoulder at 278 nm (*cf.* **1a** 253 nm with shoulder 275 nm), and **9** at pH 7.4 a maximum at 256 nm with a shoulder at 283 nm (*cf.* **1a** 254 with shoulder 274 nm). While **6** was obtained by reacting 9-ethylguanine with either BMA or glyoxal, products **8** and **9** could only be obtained from BMA; the reaction of 9-ethylguanine with glyoxal gave no such products. The plausible mechanisms for the formation of **6**, **8** and **9** are discussed below.

The products obtained under alkaline conditions (pH > 8) were different. Again products having MH$^+$ 238 and 296 were formed, but they were not identical with **6** or **9**, respectively. The product having MH$^+$ 238 was isolated and assigned by ^{1}H and ^{13}C NMR spectroscopy as N^2-carboxymethyl-9-ethylguanine (**10a**) (Tables 2 and 3). The corresponding adduct, N^2-carboxymethylguanosine (**10b**), was obtained on treating guanosine with BMA in a phosphate buffer at pH 10 and 70 °C for 72 h (**10b**). Consistent with structures **10a** and **10b**, the coupling between the N^2H and the two methylene protons is clearly detected (*J* 3.9 Hz and *J* 4.1 Hz, respectively). The presence of a carboxylic acid group may well explain the fact that the retention time of **10a** is very sensitive to the acidity of the eluent.

The product having MH$^+$ 296 is in all likelihood N^2-(4,5-dihydroxydioxolan-2-yl)methylene-9-ethylguanine (**11a**). The respective guanosine adduct (**11b**) was isolated from the same reaction mixture as **10a** and characterized by ^{1}H and ^{13}C NMR spectroscopy (Tables 1 and 3). The structure proposed for **11b** is consistent with the appearance of an AA′XX′ type proton spectrum for the *–HOHCHOH–* moiety[41] and the fact that the UV spectrum is rather similar to that of 9-ethylguanine. In addition to **10a** and **11a**, a 1 : 2 adduct, N^2-carboxymethyl-1,N^2-(*trans*-1,2-dihydroxyethano)-9-ethylguanine (**12**) was formed (Tables 1 and 3). The mechanisms for the formation of **10a**, **11a** and **12** are discussed below.

The products which the reaction of 2′-deoxyguanosine with BMA yielded at pH > 5 were similar to those described above for 9-ethylguanine (Fig. 4). As mentioned above, at pH < 5 the predominant reaction was depurination, and significant amounts of 1,N^2-ethenoguanine (**5d**) and N^2,3-ethenoguanine

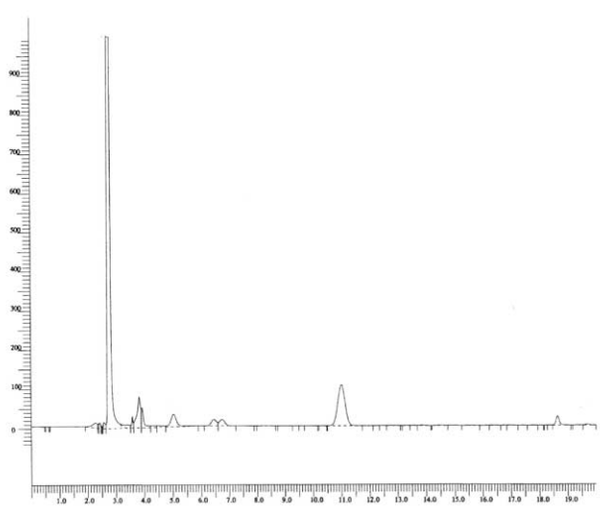
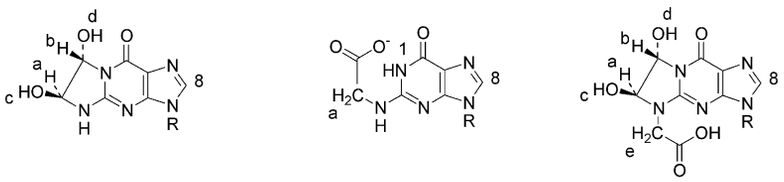


Fig. 4 RP-HPLC chromatogram of the reaction mixture of 2′-deoxyguanosine (**1b**; <0.5 mM) with BMA (10 mM) at pH 7.4 and 60°. Annotation: t_R 5 min: 1,N^2-(1,2,2,4-tetrahydroxypropano)-2′-deoxyguanosine and 1,N^2-(2-formyl-1,2,3-trihydroxypropano)-2′-deoxyguanosine (**8** + **9**), t_R 6–7 min 1,N^2-(1,2-dihydroxyethano)-2′-deoxyguanosines (**6** and **7**), t_R 11 min 2′-deoxyguanosine (**1b**) and t_R 19 min 1,N^2-etheno-2′-deoxyguanosine (**5b**).

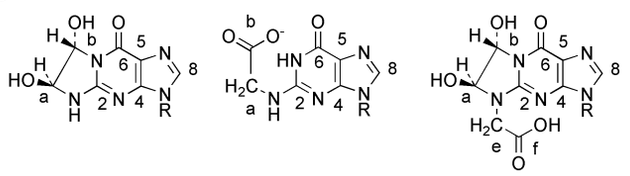
Table 2 ^{1}H NMR chemical shifts for the adducts of 9-substituted guanines[a]



	6	10a	10b	12
1-H		8.41(br s)	8.20(br s)	
N^2-H	8.75(d, *J* 0.7)	6.61(t, *J* 3.9)	6.69(t, *J* 4.1)	
8-H	7.74(s)	7.65(s)	7.88(s)	7.74(s)
a-H	4.85(dd, *J* 7.1, 0.7)	3.49(t, *J* 3.9)	3.67(d, *J* 4.1)	4.94(s)
b-H	5.46(d, *J* 6.4)			5.38(s)
c-H	6.48(d, *J* 7.1)			6.67(br s)
d-H	7.18(d, *J* 6.4)			7.29(br s)
e-H				3.80; 4,06 [e]
Others	[b]	[c]	[d]	[f]

[a] As ppm from TMS in DMSO-d_6. Coupling constants in Hz. [b] For the minor *cis*-isomer **7**: N^2-H 8.42, a-H 5.07, b-H 5.68, c-H 6.77 and d-H 7.01. Ethyl group resonances at 1.32(t, *J* 7.3) and 3.98(q, *J* 7.3). [c] Ethyl group resonances at 1.33(t, *J* 7.3) and 3.96(q, *J* 7.3). [d] Sugar proton resonances as 5.69(d, *J* 5.8), 4.52(t, *J* 5.3), 4.12(t, *J* 4.4), 3.88(q, *J* 4.1), 3.62(dd, *J* 11.7, 4.3) and 3.52(dd, *J* 11.7, 4.6). Sugar hydroxy resonances at 5.35(d, *J* 6.4), 5.12(d, *J* 4.4) and 4.95(t, *J* 5.3). [e] An AB proton spectrum was observed: $^{2}J_{\mathrm{H-C-H}}$ 17.0. [f] Ethyl group resonances at 1.33(t, *J* 7.3) and 3.99(q, *J* 7.3).

Table 3 ^{13}C NMR chemical shifts for the adducts of 9-substituted guanines[a]



	6	10b	12
C-2	154.6	152.2	153.2
C-4	150.8	151.1	150.8
C-5	117.4	116.8	117.4
C-6	155.0	157.1	155.1
C-8	137.2	136.2	137.3
C-a	83.6	45.2	88.2
C-b	84.0	171.6	82.4
C-e			47.9
C-f			171.9
Others	[b]	[c]	[d]

[a] As ppm from TMS in DMSO-d_6. [b] Ethyl group resonances at 15.4 and 37.9. [c] Sugar carbon resonances at 87.1, 85.3, 73.4, 70.5, 61.7. [d] Ethyl group resonances at 15.3 and 37.9.

(**4d**) were formed. The sigmoid product distribution curves (data not shown) clearly showed that it is the guanine base released upon depurination that reacted with BMA, yielding the etheno products. Accordingly, unsubstituted guanine appears to form etheno products considerably faster than 2′-deoxyguanosine.

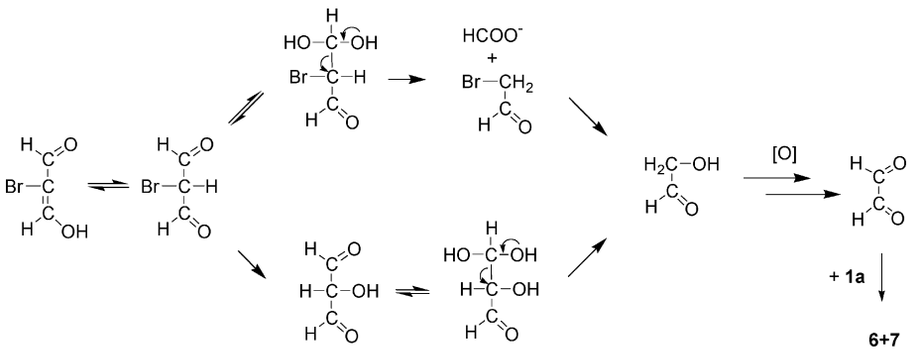
Routes to the glyoxal-derived adducts: evidence for intermediary formation of glyoxal from BMA

A priori, two alternative pathways may be suggested for the formation of the glyoxal-derived adducts (**6**,**7**,**10**–**12**). Either, the starting material reacts with BMA and the adducts are formed upon decomposition of an intermediate initially formed, or alternatively, BMA is decomposed to glyoxal under the experimental conditions. As indicated below, the results of the kinetic experiments on the formation of the dihydroxyethano adducts (**6**,**7**) are rather consistent with the latter pathway: BMA is decomposed to glyoxal, and 9-substituted guanines react with glyoxal considerably more readily than with BMA.

Firstly, BMA was decomposed to a significant extent during the time period that the reactions of 9-ethylguanine were followed. The disappearance of BMA can actually be followed by RP-HPLC by using an acidic sodium formate buffer as an eluent. An experiment in the absence of any nucleosidic substrate shows that the half-life of the disappearance of BMA under slightly acidic conditions at 60 °C is five days, the rate of disappearance being rather independent of pH. Unfortunately the products formed could not be observed by UV-detection. When the decomposition of BMA was followed by ^{1}H NMR spectroscopy, formate ion was the only product that could be definitely assigned.

Secondly, indirect evidence for the decomposition of BMA was obtained by a series of experiments where a reaction solution (10 mmol dm^{-3} BMA in 0.1 mol dm^{-3} acetic acid buffer pH 5.5) was incubated at 60 °C from one to seven days before the reaction was started by adding the 9-ethylguanine substrate. When 9-ethylguanine was added at the same time as BMA, the high molecular weight products **8** and **9** were clearly observed. In contrast, when the reaction was started after seven days incubation of BMA in the buffer solution, only traces of these products were formed. Furthermore, the latter reaction was nearly 20 times as fast as that started immediately after the preparation of the reaction solution. This increased reactivity may well be accounted for by a significant amount of glyoxal being formed in the reaction solution. In fact, the reaction rate was comparable to that observed for the reaction of 9-ethylguanine with 2 mmol dm^{-3} glyoxal at pH 5.5; a value of $(5.9 \pm 0.2) \times 10^{-5}$ s^{-1} was obtained for the pseudo first-order rate constant. Under these conditions, the reaction with glyoxal yielded only the dihydroxyethano adducts (**6**, **7**).

There is another piece of indirect evidence that suggests glyoxal to be formed in the reaction solutions. The dihydroxyethano adducts (**6**,**7**) are unstable in aqueous solutions, being decomposed to glyoxal and 9-ethylguanine. This happened when **6** isolated from the reaction solution was dissolved in a freshly prepared solution of 10 mmol dm^{-3} BMA in an acetic acid buffer, pH 5.2. However, if the BMA solution was kept at 60 °C for nine days before **6** was added, only 20% of the adduct decomposed, suggesting that an equilibrium between the adduct and free 9-ethylguanine and glyoxal had been settled. Addition of a small amount of glyoxal resulted in total recovery of **6** and complete disappearance of 9-ethylguanine.



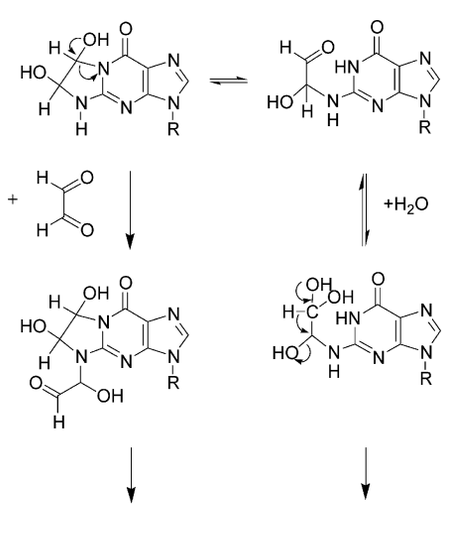
Scheme 1

Discussion

Mechanisms for the formation of the reaction products

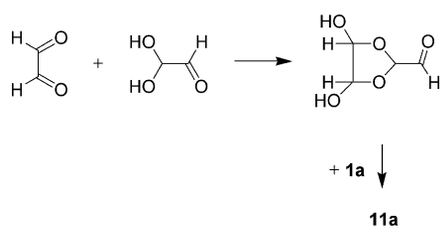
As mentioned above, disappearance of BMA was observed to be accompanied by formation of formate ion. As shown in Scheme 1, the other expected product, hence, is bromoacetaldehyde or its hydrolysis product, glycolaldehyde, while oxidation is required to obtain glyoxal. It has been shown previously that glycolaldehyde, when reacted with 2′-deoxyguanosine, gives diastereomeric glyoxal adducts similar to **6** under aerobic but not under unaerobic conditions.[42] Evidently glyoxal formed by oxidation of glycoladehyde eventually reacts with 9-ethylguanine, giving the dihydroxyethano adduct **6**. The latter reaction is well known.[39,43]

The carboxymethyl derivative **10a** obtained under alkaline conditions may be formed from the dihydroxyethano adduct (**6**), as indicated in Scheme 2. This kind of a ring opening reaction followed by a hydride ion migration has previously been reported for a glyoxal-arginine adduct,[44] and a similar carboxymethyl derivative has also been obtained by treating guanosine with glucose under oxidative conditions.[45] Compound **12** may be formed by the reaction of glyoxal with **10a**. Product **11a** may simply be obtained by a nucleophilic attack of N^2 of the guanine base on a glyoxal dimer (Scheme 3), the formation of which is a well documented reaction.[46,47]



Scheme 2

As discussed above, adducts **8** and **9** were obtained on using fresh solutions of BMA, while increasing of the glyoxal content by ageing favoured the formation of **6** or **10a**, depending on pH. It should also be noted that neither **8** nor **9** were formed on treating 9-ethylguanine with glyoxal. Accordingly, these



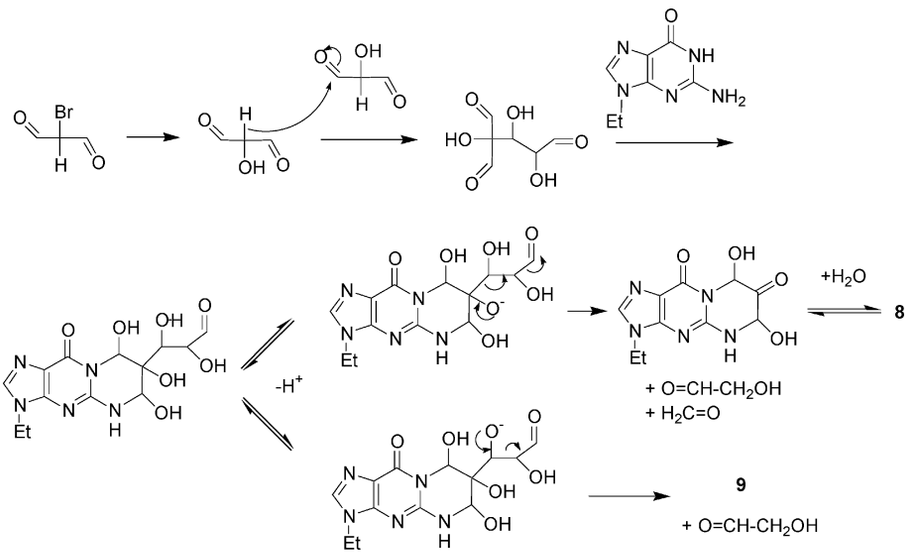
Scheme 3

products appear to be obtained by dimerization of BMA. The effect of the BMA concentration on the product distribution is consistent with such a mechanism. Although the overall disappearance of **1a** was approximately first-order in the concentration of BMA, high BMA concentrations clearly favoured the formation of **8** and **9** over **6**. At a low BMA concentration (2 mmol dm^{-3}), these products were not observed at all, whereas **6** was formed to a significant extent. Malondialdehyde is known to undergo dimerization by an aldol condensation mechanism.[48] If 2-hydroxymalonaldehyde, obtained by hydrolysis of BMA, behaves similarly, formation of **8** and **9** could be expected to take place as shown in Scheme 4.

Implications to reactions of BMA with adenosine

The results of the present work apparently argue against those reported previously for the reaction of BMA with adenosine, according to which the adducts are formed by a reaction between the adenine base and intact BMA. This apparent difference, however, results from the different inherent reactivity of these two bases towards BMA. The amidine group of the adenine base appears to favour halogen substituted carbonyl compounds over 1,2- or 1,3-dicarbonyl compounds. 9-Methyladenine, for example, reacts readily with halogenenated malondialdehydes giving etheno products ($t_{½}$ < 1d in 10 mmol dm^{-3} aq. BMA at pH 5 and 60 °C[18]), whereas no reaction with glyoxal can be observed at pH 5.4 and 60 °C in one week (the present work). Since the half-life for the decomposition of BMA under such conditions is five days, it is clear that 9-substituted adenines react with intact BMA, and not with glyoxal that possibly is present at low levels. Consistent with this, a marked rate retardation was observed when the BMA solution was incubated at 60 °C for 5 days before the adenosine substrate was added, but even in this case the etheno adducts were the only products observed.

In striking contrast to 9-substituted adenines, 9-substituted guanines react preferably with 1,2-dicarbonyl compounds: the half-lives of the reaction of 9-ethylguanine with 2 mmol dm^{-3} glyoxal and 10 mmol dm^{-3} chloroacetaldehyde at pH 5.4 and 60 °C are 2 h and 2 d, respectively. Accordingly, even minor conversion of BMA to glyoxal is sufficient to make glyoxal-derived adducts as the main products. In this respect, 1,3-dicarbonyl and α,β-unsaturated carbonyl compounds also



Scheme 4

seem to favour 9-substituted guanines over adenines: malondialdehyde,[3] triformylmethane,[35,36] acrolein,[5] and cronaldehyde[9] all react faster with guanosine than with adenosine. α-Haloacetaldehydes, in turn, react less readily with guanosine than with adenosine.[11,13,20] Accordingly, it is expected that besides glyoxal-derived adducts, those obtained by the reactions through the 1,3-carbonyl groups (**8**, **9**) predominate during the early stages of the reaction of 9-ethylguanine with BMA.

Consistent with the preceding discussion, the tautomeric form of the guanine base is crucial in dictating the reactivity. 9-Ethyl-O^6-methylguanine (**2**), which exists in a tautomeric form similar to that of 9-substituted adenine, reacts like adenosine: the rate of reaction with BMA is the same, and the reaction reaches its maximal rate under the same conditions. Etheno adducts are formed as predominant products. 9-Ethyl-1-methylguanine (**3**), which mimics the stable tautomer of 9-ethylguanine (**1a**), exhibits a pH-rate profile similar to that obtained with **1a**. The 1-methyl group, however, prevents the reactions at this site, and hence the reaction of **3** with BMA is nearly one order of magnitude slower than that of 9-ethylguanine. Consistent with previous observations on the reactions of 9-substituted guanines with malondialdehyde[2–4,49] or triformylmethane,[35] the formation of cyclic N^2,3-adducts is a less favourable process than that leading to 1,N^2-adducts.

Experimental

General

The HPLC analyses were carried out on a Perkin-Elmer Integral 4000 HPLC equipped with a diode array detector and the HPLC-ESI/MS analyses on a Perkin Elmer Sciex API 365 LC/MS/MS triple quadrupole mass spectrometer or on a Fissions ZabSpectraTOF instrument.

The NMR spectra were acquired on a JEOL Alpha 500 NMR spectrometer equipped with a 5 mm normal configuration tunable probe or a 5 mm inverse z-axis field-gradient probe operating at 500.16 for ^{1}H, and 125.78 MHz for ^{13}C. The deuterium of the solvent was used as lock signal. The spectra were recorded at 30 °C in DMSO-d_6. The ^{1}H and ^{13}C spectra were referenced internally to tetramethylsilane. The 1D proton spectra were acquired with single-pulse excitation, 45° flip angle, pulse recycle time of 9 sec and with spectral widths of 8 kHz consisting of 65 k data points (digital resolution 0.11 Hz per pt), zero-filled to 128 k prior to Fourier transformation. 1 Hz of exponential weighting was usually applied prior to Fourier transformation. 2D Homonuclear H,H-correlation experiments were acquired using double quantum filtered COSY in phase-sensitive mode with spectral widths appropriately optimized from the 1D spectra, and processed with zero-filling (×2, ×4) and exponential weighting (1 Hz) applied in both dimensions prior to Fourier transformation.

1D Carbon spectra were acquired with single-pulse excitation, 45° flip angle, pulse recycle time of 3.5 sec, and with spectral widths of 34 kHz consisting of 64 k data points (digital resolution 0.52 Hz per pt), zero-filled to 128 k and with 1 Hz exponential weighting applied prior to Fourier transformation. 2D Heteronuclear one-bond correlation experiments were acquired using carbon detected CH-shift correlation (with partial homonuclear decoupling in the f1 dimension) with spectral widths appropriately optimised from the 1D spectra and processed with zero-filling (×2, ×4), a 2Π/3-shifted sinebell function, and exponential weighting applied in both dimensions prior to Fourier transformation. A $^1J_{HC}$ coupling of 145 Hz was used.

Materials

2′-Deoxyguanosine, guanosine, O^6-methylguanine, and 9-ethylguanine were products of Sigma and 1-methylguanine a product of Fluka. They all were used as received. HPLC grade solvents were used for HPLC analyses and purifications. All the electrolytes and buffer constituents were of reagent grade. BMA was prepared by the method of Trofimenko.[50]

9-Ethyl-O^6-methylguanine (2) and 9-ethyl-1-methylguanine (3)

Compounds **2** and **3** were synthesized by a procedure modified from that of Kohda *et al.*[51] The reaction of O^6-methylguanine (0.30 mmol) with ethyl iodide (0.75 mmol) in the presence of excess of K_2CO_3 (10 cm^3 DMF; 50 °C; 1 d) gave 9-ethyl-O^6-methylguanine (**2**). Similarly, 1-methylguanine (1.5 mmol) when reacted with ethyl iodide (2.3 mmol) in the presence of excess of K_2CO_3 (46 cm^3 DMF; 50 °C; 20 h) yielded 9-ethyl-1-methylguanine (**3**). The products were purified by semipreparative RP-HPLC on a LiChrospher®100 RP-18 (250 × 10 mm, 5μm) column and characterized by ^{1}H NMR and mass spectroscopy. The UV spectrum of both compounds was identical to that of the corresponding 9-methylated compound.[51] 9-Ethyl-O^6-methylguanine: ^{1}H NMR (DMSO-d_6) δ 7.86 (s, 1H, 8-H), 6.36 (s, 2H, NH_2), 4.02 (q, 2H, J 7.3 Hz, $-CH_2-$), 3.94 (3H, $-OCH_3$), 1.33 (t, 3H, J 7.3 Hz, $-CH_3$). ESI-MS: m/z 194 (MH^+). 9-Ethyl-1-methylguanine: ^{1}H NMR (DMSO-d_6) δ 7.70 (s, 1H, 8-H), 6.98 (s, 2H, NH_2), 3.96 (q, 2H, J 7.3 Hz, $-CH_2-$), 2.50 (3H, $-OCH_3$), 1.31 (t, 3H, J 7.3 Hz, $-CH_3$). ESI-MS: m/z = 194 (MH^+).

1,N^2-(1,2-Dihydroxyethano)-9-ethylguanine (6)

Compound **6** was prepared by a procedure described for the synthesis of 1,N^2-(1,2-dihydroxyethano)guanine.[39] To 5 cm^3 of water, 9-ethylguanine (0.05 mmol) and glyoxal (0.5 mmol as 30 wt% solution in water) were added. The suspension was stirred for 42 h at 60 °C. 20 mm^3 of acetic acid was added after 18 h. The suspension was concentrated and kept at 7 °C for one day and filtered. The product was washed with water and dried in a vacuum. The ^1H and ^{13}C NMR spectroscopic data is given in Tables 1 and 2. ESI-MS: *m*/*z* = 260 (MNa^+), 238 (MH^+), 220 ($MH^+ - H_2O$), 192 ($MH^+ - HCOOH$). UV_{max} 251 nm, sh. 280 nm.

The same compound was isolated from a reaction mixture of 9-ethylguanine and BMA obtained as follows. BMA (20 mmol) in 0.2 mol dm^{-3} acetic acid buffer (5 cm^3; pH 5.5) was heated in a sealed tube for one week at 60 °C. 9-Ethylguanine (6 mmol) was added, and the suspension was kept at 60 °C for one week. The product was purified by semipreparative HPLC on a LiChrospher®100 RP-18 (5μm) column. In order to prevent decomposition of the product, the elute was kept on an ice bath or in the freezer and evaporations were made in a lyophilizator.

Isolation of 9-ethylguanine adducts 10a and 12

BMA (1.51 mmol; 230 mg) in 0.5 mol dm^{-3} phosphate buffer (pH 7.3, 50 cm^3) was heated at 60 °C for one week. A mixture of BMA (230 mg) and 9-ethylguanine (1.50 mmol; 300 mg) was then added and the incubation was continued for an additional 5 d. The solution was concentrated to 10 cm^3, filtered and eluted through a LiChroprep RP-18 column (37 × 440 mm, 40–63 μm) with 0.1 mol dm^{-3} aqueous ammonium acetate containing 3% MeCN (*v*/*v*). Two main fractions were collected, concentrated to 5 cm^3 and subjected to desalting on a Hyperprep HS-18 column (10 × 250 mm, 8 μm) using aqueous acetonitrile as eluent.

Isolation of guanosine adducts

Guanosine (0.57 g, 2 mmol) and BMA (0.61 g, 4 mmol) were stirred in a phosphate buffer (20 mL; pH 10.0; 70 °C, 72 h). The solution was cooled to room temperature and evaporated to dryness under reduced pressure. The crude mixture was dissolved in 10 cm^3 of water, the insoluble materials were filtered off, and the filtrate was applied onto an RP-18 column (40 μm). The products were eluted with a mixture of MeOH and a phosphate buffer increasing the MeOH content in a stepwise manner: 0% (20 min), 5% (1 h), 11% (1 h), 22% (30 min), 25% (3 h), and 30% (1 h). The main products were collected as follows: **10b** and **11b** as a single peak with 11% MeOH, and **12** with 30% MeOH. The fractions were concentrated under a reduced pressure and desalted by semipreparative RP-chromatography using 7% aqueous acetonitrile as an eluent. Compounds **10b** and **11b** were separated during the desalting. The desalted fractions were evaporated to dryness and analyzed by NMR spectroscopy.

The ^1H and ^{13}C NMR spectroscopic data is given in Tables 1 and 2. Compounds **10b**: UV_{max} (H_2O) 260 nm, UV_{min} (H_2O) 226 nm. FAB-MS *m*/*z* (relative intensity): 683.2 [$(2M + H)^+$, 45%], 364.2 (MNa^+, 40%), 342.3 (MH^+, 100%), 210.2 ($MH^+ - C_5H_8O_4$, 40%). Compound **11b**: UV_{max} (H_2O) 260 nm, UV_{min} (H_2O) 222 nm. FAB-MS *m*/*z* (relative intensity): 400.0 (MH^+, 100%), 268.2 ($MH^+ - C_5H_8O_4$, 25%).

Reaction solutions

The pH of the reaction solutions was adjusted with formic acid (pH 3–4), acetic acid (pH 4–5.5), MES (pH 5.5–6.5), HEPES (pH 6.5–8) and CHES (pH 8.5–10) buffers. Below pH 3, aqueous hydrogen chloride was employed for the same purpose. The pH of the reaction solutions was checked with a pH-meter at 60 °C. Ionic strength was adjusted with $NaNO_3$ or NaCl.

Kinetic measurements

Reactions were carried out in stoppered tubes, which were immersed in a water bath, the temperature of which was kept at 60.0 ± 0.1 °C. Aliquots were withdrawn at appropriate intervals, immediately cooled down on an ice bath and stored in the freezer until analyzed. The aliquots were analyzed by RP-HPLC on a Hypersil ODS column (250 × 4 or 4,6 mm, 5 μm) using UV-detection at wavelengths 260 nm for 2′-deoxyguanosine and 9-ethylguanine, 279 nm for 9-ethyl-O^6-methylguanine and 255 nm for 9-ethyl-1-methylguanine. Eluents were mixtures of MeCN and 0.05 mol dm^{-3} acetic acid buffer (pH 4.3, I = 0.1 M with NH_4Cl).

Calculation of the rate constants

Pseudo first order rate constants for the disappearance of the starting material were calculated by using the integrated first order rate law. The calculation was based on the decrease of the signal area as a function of time.

References

1 P. M. Dansette, R. Snyder, M. Delaforge, G. G. Gibson, H. Greim, D. J. Jollow, T. J. Monks and I. G. Sipes, *Advances in Experimental Medicine and Biology*, vol. 500, Kluwer Academic/Plenum Publishers, 2001.
2 R. C. Moschel and N. J. Leonard, *J. Org. Chem.*, 1976, **41**, 294–300.
3 H. Seto, T. Okuda, T. Takesue and T. Ikemura, *Bull. Chem. Soc. Jpn*, 1983, **56**, 1799–1802.
4 A. K. Basu, S. M. O′Hara, P. Valladier, K. Stone, O. Mols and L. J. Marnett, *Chem. Res. Toxicol.*, 1988, **1**, 53–59.
5 E. Eder and C. Hoffman, *Chem. Res. Toxicol.*, 1993, **6**, 486–496.
6 G. R. Reddy and L. J. Marnett, *Chem. Res. Toxicol.*, 1996, **9**, 12–15.
7 F.-L. Chung, R. Young and S. S. Hecht, *Cancer Res.*, 1984, **44**, 272–282.
8 D. W. Boerth, E. Eder, S. Hussain and C. Hoffman, *Chem. Res. Toxicol.*, 1998, **11**, 284–294.
9 E. Eder and C. Hoffman, *Chem. Res. Toxicol.*, 1992, **5**, 802–808.
10 F.-L. Chung and S. S. Hecht, *Cancer Res.*, 1983, **43**, 1230–1235.
11 N. K. Kotchetkov, V. N. Shibaev and A. A. Kost, *Tetrahedron Lett.*, 1971, 1993–1997.
12 J. A. Secrist, III, J. R. Barrio, N. J. Leonard and G. Weber, *Biochemistry*, 1972, **11**, 3499–3506.
13 K. Kayasukada-Mikado, T. Hashimoto, T. Negishi, K. Negishi and H. Hayatsu, *Chem. Pharm. Bull.*, 1980, **28**, 932–939.
14 J. T. Kusmierek and B. Singer, *Chem. Res. Toxicol.*, 1992, **5**, 634–638.
15 F. P. Guengerich and M. Persmark, *Chem. Res. Toxicol.*, 1994, **7**, 205–208.
16 V. Nair, R. J. Offerman and G. A. Turner, *J. Org. Chem.*, 1984, **49**, 4021–4025.
17 L. Kronberg, S. Karlsson and R. Sjöholm, *Chem. Res. Toxicol.*, 1993, **6**, 495–499.
18 S. Mikkola, N. Koissi, K. Ketomäki, S. Rauvala, K. Neuvonen and H. Lönnberg, *Eur. J. Org. Chem.*, 2000, 2315–2323.
19 F. P. Guengerich, *Chem. Res. Toxicol.*, 1992, **5**, 2–5.
20 M. K. Dosanjh, A. Chenna, E. Kim, H. Fraenkel-Konrat, L. Samson and B. Singer, *Proc. Natl. Acad, Sci. USA*, 1994, **91**, 1024–1028.
21 F. P. Guengerich, M. Persmark and W. G. Humpreys, *Chem. Res. Toxicol.*, 1993, **6**, 635–648.
22 L. Kronberg, R. Sjöholm and S. Karlsson, *Chem. Res. Toxicol.*, 1992, **5**, 852–855.
23 D. Asplund, L. Kronberg, R. Sjöholm and T. Munter, *Chem. Res. Toxicol.*, 1995, **8**, 841–846.
24 L. Kronberg, D. Asplund, J. Mäki and R. Sjöholm, *Chem. Res. Toxicol.*, 1996, **9**, 1257–1263.
25 T. Munter, F. Le Curieux, L. Kronberg and R. Sjöholm, *Chem. Res. Toxicol.*, 1999, **12**, 42–52.
26 P. Yi, X. Sun, D. R. Doerge and P. P. Fu, *Chem. Res. Toxicol.*, 1998, **11**, 1032–1041.
27 M. Müller, F. J. Belas, I. A. Blair and F. P. Guengerich, *Chem. Res. Toxicol.*, 1997, **10**, 242–247.
28 K. Hakala, S. Auriola, A. Koivisto and H. Lönnberg, *J. Pharm. Biomed. Anal.*, 1999, **21**, 1053–1061.
29 M. Otteneder, J. P. Plastaras and L. J. Marnett, *Chem. Res. Toxicol.*, 2002, **15**, 312–318.

30 I. Terashima, N. Suzuki and S. Shibutani, *Chem. Res. Toxicol.*, 2002, **15**, 305–311.
31 O. J. Schmitz, C. C. T. Wörth, D. Stach and M. Wiessler, *Angew. Chem., Int. Ed.*, 2002, **41**, 445–448.
32 J. R. Barrio, J. A. Secrist, III and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, 1972, **46**, 597–604.
33 P. D. Sattsangi, N. J. Leonard and C. R. Frihart, *J. Org. Chem.*, 1977, **42**, 3292–3296.
34 K. Neuvonen, C. Zewi and H. Lönnberg, *Acta Chem. Scand.*, 1996, **50**, 1137–1142.
35 N. Koissi, K. Neuvonen, T. Munter, L. Kronberg and H. Lönnberg, *Nucleosides, Nucleotides, Nucleic Acids*, 2001, **20**, 1761–1774.
36 K. Neuvonen, N. Koissi and H. Lönnberg, *J. Chem. Soc., Perkin Trans. 2*, 2002, 173–177.
37 B. Singer, S. J. Spengler, F. Chavez and J. T. Kusmierek, *Carcinogenesis*, 1987, **8**, 745–747.
38 C. Glemarec, Y. Besidsky, J. Chattopadhyaya, J. Kusmierek, M. Lahti, M. Oivanen and H. Lönnberg, *Tetrahedron*, 1991, **47**, 6689–6704.
39 R. Shapiro, B. I. Cohen, S.-J. Shiuey and H. Maurer, *Biochemistry*, 1969, **8**, 238–245.
40 R. N. Loeppky, W. Cui, P. Goelzer, M. Park and Q. Ye, in *Exocyclic DNA adducts in mutagenesis and carcinogenesis*, eds. B. Singer, and H. Bartsch, International Agency for Research on Cancer, Lyon, 1999, pp. 155–168.
41 R. Laatikainen, M. Niemitz, S.-P. Korhonen and T. Hassinen, PERCH NMR Software, version 2000, Kuopio University NMR Research Group, Department of Chemistry, University of Kuopio, P.O. BOX 1627, FIN-70211, Kuopio, Finland.
42 M. Awada and P. C. Dedon, *Chem. Res. Toxicol.*, 2001, **14**, 1247–1253.
43 R. Shapiro and J. Hachmann, *Biochemistry*, 1966, **5**, 2799–2807.
44 M. A. Glomb and G. Lang, *J. Agric. Food. Chem.*, 2001, **49**, 1493–1501.
45 W. Seidel and M. Pischetsrieder, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 2017–2022.
46 E. P. Whipple, *J. Am. Chem. Soc.*, 1970, **92**, 7183–7186.
47 C. Rabiller, *New J. Chem.*, 1987, **11**, 419–424.
48 B. T. Golding, N. Patel and W. P. Watson, *J. Chem. Soc., Perkin Trans. 2*, 1989, 668–669.
49 L. J. Marnett, A. K. Basu, S. M. O′Hara, P. E. Weller, A. F. M. M. Rahman and J. P. Oliver, *J. Am. Chem. Soc.*, 1986, **108**, 1348–1350.
50 S. Trofimenko, *J. Org. Chem.*, 1963, **28**, 3243–3245.
51 K. Kohda, K. Baba and Y. Kawazoe, *Tetrahedron Lett.*, 1987, **28**, 6285–6288.