Alkylation of anionic DNA bases by styrene 7,8-oxide

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Styrene 7,8-oxide (SO), a reactive epoxide that has been classified as a probable human carcinogen, was allowed to react under alkaline conditions with 2′-deoxyguanosine and thymidine monophosphates as well as with the dinucleotide dGpdT. The reaction products were separated by high-performance liquid chromatography and were characterised by UV and electrospray mass spectroscopy, the latter showing the ability to differentiate the isomerism of the hydroxyphenylethyl moiety of the adduct. The main alkylation products of the deoxyguanosine monophosphates at high pH were those reacted at the 1- (through the β-carbon of the epoxide), N^2- (α-carbon) and 7-positions (β- and α-carbons) of guanine followed by alkylation of the phosphate group. The formation of a novel diastereomeric pair of N^2-guanine adducts connected to the β-carbon of SO was identified and characterised. Furthermore, two different geometrical isomers of 1-guanine were detected. For thymidine nucleotide, base alkylation under neutral conditions was almost negligible, but at pH 10.5, alkylation at the 3-position was very prominent. The same base-adducts and the pH-effects were observed in the case of dGpdT alkylation. However, no phosphate alkylation was detected in the case of the dinucleotide.

Introduction

Styrene is an extensively used chemical in the industrial production of synthetic rubber, plastics and resins.[1] The major metabolite of styrene found in humans, is styrene 7,8-oxide (SO).[1] Studies on reactions between DNA constituents and SO are important since the compound has been shown to be mutagenic and carcinogenic in experimental animals. These properties may be due to the DNA binding properties of SO.[1] SO has been shown to react *in vitro* with all of the nucleobases at several sites.[2] In general, the covalent interaction between an electrophilic carcinogen with a nucleophilic site in DNA is considered a critical step in carcinogenesis.[3] Most of the known chemical carcinogens preferentially modify the guanine residue in DNA, the most common nucleophilic sites being the 7-, N^2- and O^6-positions.[3] The sites for alkylation vary depending on the properties of the electrophile. Agents that are not particularly ionic in nature mainly alkylate, through the bimolecular displacement (S_N2) mechanism, sites with high relative nucleophilicity, such as the 7-position in guanine. Electrophiles with more ionic character prefer to react at the less nucleophilic exocyclic sites. In addition to the sites in the DNA bases some carcinogens, such as *N*-nitrosoamines and *N*-nitrosoureas, also alkylate the phosphate groups in DNA.[4]

The relative yields of SO-alkylated products with nucleosides in aqueous buffer decrease in the sequence deoxyguanosine > deoxycytidine > deoxyadenosine > thymidine.[2] At neutral pH the dominant product is 7-alkylguanine linked either through the α- or β-carbon of the epoxide.[2,5–7] Since the α-carbon is chiral the reaction leads to a diastereomeric pair of products. The α- and β-isomers of O^6-guanine SO-adducts have been detected earlier,[2,5,6,8] the β-isomer obviously being formed through a base-catalysed rearrangement of the α-isomer.[8] The N^2-position of guanine has been found to open the epoxide only at the α-carbon.[6] Under alkaline conditions, the reactions of some alkylating agents at the 1- and N^2-positions of guanine are found to be markedly increased.[9] The same trend has also been observed for SO alkylation of 2′-deoxyguanosine 3′-monophosphate (3′dGMP),[10] but in that report only the N^2-adduct linked through the α-carbon was fully characterised, and of the other reaction products only one isomer of the 1-guanine adduct was identified, however, without complete characterisation. We have now studied in aqueous conditions the SO-alkylation of nucleotides that are deprotonated at high pH, using 3′dGMP, 2′-deoxyguanosine 5′-monophosphate (5′dGMP), thymidine 3′-monophosphate (3′TMP) and a dinucleotide containing the mentioned nucleotides (dGpdT). The reactions were monitored by high-performance liquid chromatography (HPLC), and the adducts formed were characterised by UV and electrospray ionization mass spectrometry (ESI-MS). The reaction profile under alkaline conditions was markedly different from that obtained at neutral conditions, allowing detection of some previously unidentified nucleotide adducts. In addition, we have demonstrated the possibility of using ESI-MS for determination of isomerism of the hydroxyphenylethyl moiety in SO-induced nucleotide adducts.

Results and discussion

Reaction of SO with dGMP

The treatment of 3′dGMP and 5′dGMP with SO in aqueous solution at pH 10.5 resulted in alkylation at the 1-, N^2-, 7- and O^6-positions of the guanine moiety both through the α- and β-carbon of SO (see Chart 1 for structures). 7.3% of 3′dGMP and 17.1% of 5′dGMP were transformed to SO-alkylated products. The reaction gave two diastereomeric pairs of products identified as N^2-substituted nucleotides, which comprise 25% of alkylation products for both 3′ and 5′dGMP. ESI-MS analysis of these products revealed their pseudomolecular ions $[M - H]^-$ at *m*/*z* 466. In the MS/MS experiments a product ion, derived from *m*/*z* 466, at *m*/*z* 270 was observed, corresponding to the adducted guanine base fragment after the cleavage of the glycosidic bond (Fig. 1A). For the more abundant diastereomeric pair (78% and 85% of N^2-substituted 3′ and 5′dGMP products, respectively) the assignment of positional isomerism of the hydroxyphenylethyl moiety was established by the observation that the further fragmentation of *m*/*z*

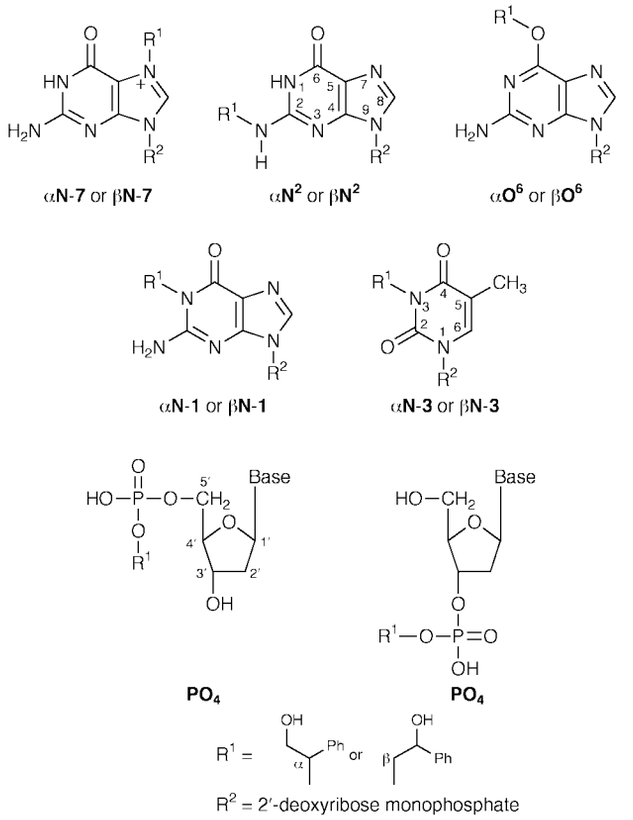

Chart 1 Structural formulae for the SO alkylation products of dGMP and TMP.

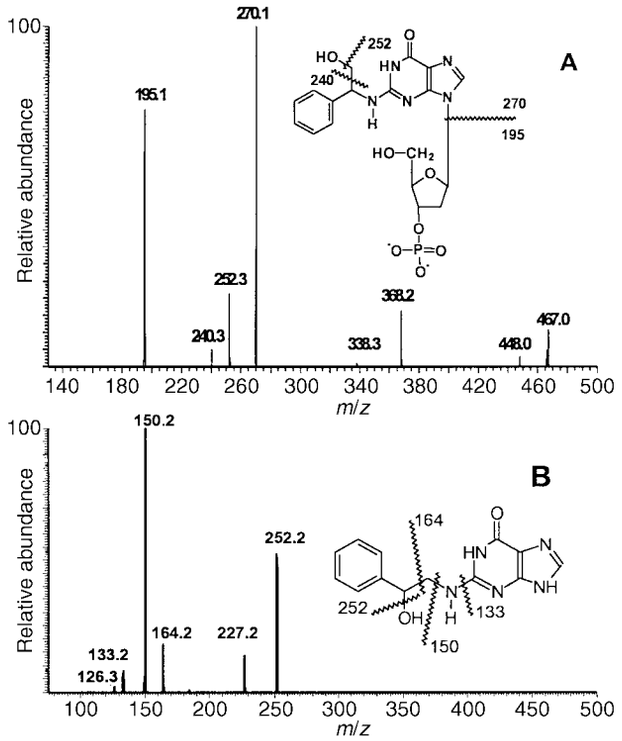

Fig. 1 Representative ESI-MS/MS spectra of SO-induced dGMP and guanine adducts. Panel A: Product ion spectrum obtained with the $[M - H]^-$ = 466 for αN^2-3′dGMP adduct as precursor ion. Panel B: Product ion mass spectrum with the $[M - H]^-$ = 270 for βN^2-guanine adduct as precursor.

270 gave a product ion at *m*/*z* 240. This ion demonstrates a neutral loss of CH_2O, giving evidence for α-substitution at SO. In the β-isomer such a fragment probably could not be formed. Depurination of either diastereomer of these N^2-(2-hydroxy-1-phenylethyl)-dGMP (α***N*²**) products gave N^2-substituted bases that eluted in HPLC-analysis at a retention time of 62 min, being chromatographically identical to an authentic standard earlier characterised by NMR and MS.[5,6] Due to the low amount obtained of the less abundant N^2-substituted products the determination of the isomerism by MS/MS analysis of the nucleotide was not successful. However, the data were obtained by MS/MS analysis on the depurinated product. The depurination products of these minor N^2-substituted dGMPs eluted by HPLC analysis at 64 min. The MS/MS experiments on $[M - H]^-$ at *m*/*z* 270 resulted in a product ion at *m*/*z* 164, which could be rationalised to the neutral loss of C_6H_5CHO (Fig. 1B). Such a fragment cannot be formed in the case of the α-isomer. Therefore the depurination product was assigned as originating from previously unidentified N^2-(2-hydroxy-2-phenylethyl)-dGMP (β***N*²**). The characteristic MS-fragments for the α- and β-isomers are in principle identical to those previously reported in MS studies involving high-energy collision-induced dissociation MS[11] and electron impact MS[5], *i.e.* CH_2OH or $C_6H_5CH_2OH$ for α- or β-isomers, respectively.

25% and 27% of alkylation products of 3′ and 5′dGMP, respectively, were those reacted at the 1-position of guanine. Again, two diastereomeric pairs of the products were identified. After acidic depurination, the more abundant 1-substituted products (78% and 85% of 1-alkylated 3′ and 5′dGMP, respectively) eluted by HPLC at 71 min. In MS/MS experiments on the alkylated base ($[M - H]^-$ at *m*/*z* 270) a fragment ion at *m*/*z* 164 was observed indicating that the fractions originated from 1-(2-hydroxy-2-phenylethyl)-dGMP (β**N-1**), as suggested for the β***N*²**-product above. Depurination products of the minor diastereomeric pair of the 1-substituted adduct eluted by HPLC at 59 min. The UV spectrum obtained was identical to that of the depurinated β**N-1** guanine adduct. These products were therefore assigned to originate from 1-(2-hydroxy-1-phenylethyl)-dGMP (α**N-1**). Due to the low amount of the α**N-1** product obtained it was not possible to detect the characteristic MS/MS-fragment of the α-isomer.

HPLC-separations of the O^6-guanine alkylation products for 3′dGMP gave three fractions, that were identified as O^6-(2-hydroxy-1-phenylethyl)-dGMP (α***O*⁶**) and a diastereomeric pair of O^6-(2-hydroxy-2-phenylethyl)-dGMP (β***O*⁶**). The different behaviour of these products under acid treatment allowed the determination of whether the epoxide had opened either at the α- or β-carbon. The β-isomer of O^6-substituted guanosine has been shown to be reasonably stable under mild acidic conditions, while the α-isomer undergoes immediate acid-catalysed hydrolysis cleaving the O^6-aralkyl ether linkage.[8] Thus, while depurination of β-isomer gave an alkylated base with a retention time of 78 min, the α-isomer was completely hydrolysed to guanine. The depurinated β***O*⁶**-guanine obtained was chromatographically identical to an authentic standard previously characterised by NMR.[8] In the case of 5′dGMP, only the β***O*⁶**-modified product was identified.

For 3′ and 5′dGMP, four phosphate alkylated products (**PO_4**) were observed constituting 11–12% of the alkylation products. The **PO_4** adducts were identified by depurinating the modified nucleotide and detecting an unmodified base by HPLC analysis, and by MS/MS, in which fragment ions at *m*/*z* 315 and 199 were observed. The former corresponds to SO-modified phosphate with a deoxyribose moiety and the latter was assigned to correspond to a fragment of SO-modified PO_3.

At neutral pH, the alkylation of guanosine by SO is well described, the major binding site being N-7 (*ca.* 80% of the alkylation products), followed by N^2- and O^6-positions, respectively.[5,6] No alkylation of the 1-position of guanine has been detected under neutral conditions. Our results demonstrate that under alkaline conditions the proportion of 1- and N^2-alkylation are clearly increased, the yields being equal to 7-alkylation (see Table 1 for details). A novel finding was the observation of the earlier unidentified β***N*²**. The reason for

Table 1 HPLC retention data of each diastereomeric product, product yields (from reactions performed at pH 10.5) and negative-ion ESI-MS data for 5′ and 3′dGMP and 3′TMP adducts

Nucleotide	Adduct	Retention times/min[a]	Yield (%)	Observed ions $[M - H]^-$ (*m*/*z*)	MS/MS fragment ions (*m*/*z*)[b]
5′dGMP	α**N-7**	32, 37	1.7	466.4	195.0, 269.9, 350.1, 251.9
	β**N-7**	35, 43	3.8	466.5	195.1, 270.1, 350.1, 252.2
	α**N²**	52, 56	3.6	466.3	368.2, 252.6
	β**N²**	55, 57	0.6	466.2	368.2, 252.2, 270.2
	α**N-1**	48, 49	0.7	466.6	449.1
	β**N-1**	61, 63	4.0	466.5	448.1, 194.8
	β**O⁶**	68[c]	0.7	466.2	—
	PO_4	37, 39, 43, 46	2.1	466.3	315.1, 150.2, 199.2, 266.1
3′dGMP	α**N-7**	33, 47	1.0	466.1/270.4	270.3, 252.2
	β**N-7**	33, 41	1.1	466.3	270.1
	α**N²**	35, 43	1.4	466.8	270.1, 195.1, 252.3, 368.2, 240.3, 448.0, 338.3, 209.2
	β**N²**	40, 46	0.4	466.4	270.1, 195.1, 368.2, 252.2
	α**N-1**	43, 45	0.4	466.2	270.1, 195.1, 449.2
	β**N-1**	55, 60	1.4	466.2	270.2, 195.1, 448.9, 252.2
	α**O⁶**	66[c]	0.2	466.5	270.1, 195.1, 252.2, 227.0
	β**O⁶**	68, 69	0.4	466.5	270.2, 195.1
	PO_4	52, 53, 54, 57	0.8	466.4	150.1, 315.1, 176.0
3′TMP	α**N-3**	60, 63	3.5	441.3	245.1, 195.1, 227.1, 185.1
	β**N-3**	66, 65	13.5	441.4	245.2, 195.1, 227.0, 162.0, 139.1, 125.0, 185.1
	PO_4	57, 58, 58[c]	2.7	441.2	315.1, 199.1, 176.9, 125.1

[a] Retention times are given for the two different diastereomers of each isomeric form of the adduct, except for the phosphate adducts, for which the isomerism was not studied. [b] MS/MS fractions are obtained either by fragmentation of $[M - H]^-$ or from an MS/MS experiment on the depurinated base product. [c] Diastereomers not resolved by HPLC.

β-alkylation, as well as for the increased level of αN^2-product at high pH, may be explained by the delocalization of the negative charge in the anionic form of guanine. Thus, a considerable proportion of the negative charge is delocalized between O^6, N-1 and maybe N-3 leading to increased electron density also in the N^2-group. The increased nucleophilicity of the exocyclic amino-group makes the epoxide opening possible at the β-carbon, the extent of reaction, however, being much lower compared to that of the α-carbon. The formation of the two isomers of 1-substituted dGMP was also a new finding. The results obtained indicate that the N-1 binding of SO is predominantly through the β-carbon, the reaction obviously following the S_N2 mechanism, as suggested for the ring nitrogen.[6,12] N-3 would also be a site for alkylation, but no 3-substitution of either 3′ or 5′dGMP was observed. It remains to be studied whether this is due to steric hindrance, or an intramolecular or intermolecular hydrogen bonding between the 2′-deoxyribose-monophosphate moiety and 3-guanine.

Reaction of SO with TMP

At neutral pH the SO treatment of 3′TMP was found to result almost exclusively in the alkylation of the phosphate group. These adducts were assigned in MS/MS experiments by detecting the product ion at *m*/*z* 315, as described above for **PO_4** of dGMP. Only small fractions of the products were found to be base modified adducts (recovery < 0.1%). However, at pH 10.5, the base alkylation at the 3-position of 3′TMP was drastically increased. The most prominent 3-alkylation products were verified by ESI-MS to be 3-(2-hydroxy-2-phenylethyl)-TMP (β**N-3**). Thus, the $[M - H]^-$ at *m*/*z* 441 was first fragmented to a product ion *m*/*z* 245, which corresponds to SO-alkylated thymine. This ion was further fragmented to give a product ion at *m*/*z* 139 indicating loss of C_6H_5CHO from the β-substituted SO-alkylated thymine. For the other 3-substituted products the typical fragment of the α-adduct was not observed. However, it is assumed that these are 3-(2-hydroxy-1-phenylethyl)-TMP (α**N-3**).

ESI-MS also revealed a dialkylation product of 3′TMP. A $[M - H]^-$ of *m*/*z* 561 was observed, corresponding to a TMP with two hydroxyphenylethyl moieties. The pseudomolecular ion was cleaved into two fragments corresponding to alkylated thymine and alkylated deoxyribose 3′-phosphate. The alkylated thymine was the major fragment, and further fragmentation of it verified that it was a β-isomer. The base alkylation was assigned to be the β3-thymine since the UV-spectral properties for this product were identical to those of the mono-substituted 3-TMP adducts. Also other rather minor alkylation products having $[M - H]^-$ at *m*/*z* 441 were detected (at retention times 52 and 54 minutes as well as 77 and 78 minutes). These products, however, remained unidentified. The UV spectra of earlier eluting pairs gave maxima of 255 nm, 255 nm and 264 nm and for later eluting pairs 258 nm, 257 nm and 258 nm, under neutral, alkaline and acidic conditions, respectively. A possible nucleophilic site for alkylation might involve the oxygen atoms at the 2- and 4-positions. However, UV spectroscopy did not support at least the O^4-alkylation.

Since thymine is known to be a poor nucleophile towards SO[2,13] it was quite expected that at neutral pH the alkylation took place mainly at the phosphate group. However, when thymine is deprotonated the alkylation occurs preferentially at the 3-position (69% and 18% of the mono-alkylation products for β**N-3** and α**N-3**, respectively). 3-Alkylation at 7.4 has earlier been identified,[2] however, without characterisation of the isomerism. The alkylation at the oxygen atoms might have been expected to be more prominent since after deprotonation the negative charge can be considered to be delocalized between the O^2-, O^4- and N-3 atoms, thus increasing the nucleophilicities of the oxygen atoms. However, no adducts at the oxygens were verified.

Reaction of SO with the dinucleotide dGpdT

In the reaction of SO with the dinucleotide dGpdT the same base-substituted products were observed as in the case of the corresponding mononucleotides (see Fig. 2). However, no alkylation of the intervening phosphodiester was observed. At pH 8.0 the alkylation at the 7-position of guanine prevailed while at pH 10.5 these products constituted only a rather minor fraction of the products. Under alkaline conditions the alkylation at N^2- and 1-positions of guanine was much more prominent. These products were identified by acidic depurination followed by HPLC analysis. The dinucleotide products migrating at *ca.* 70 min (fractions 8 and 9 in Fig. 2B) did not show any clear depurination products in addition to unmodified guanine. This indicates thymine alkylation since pyrimidine adducts are

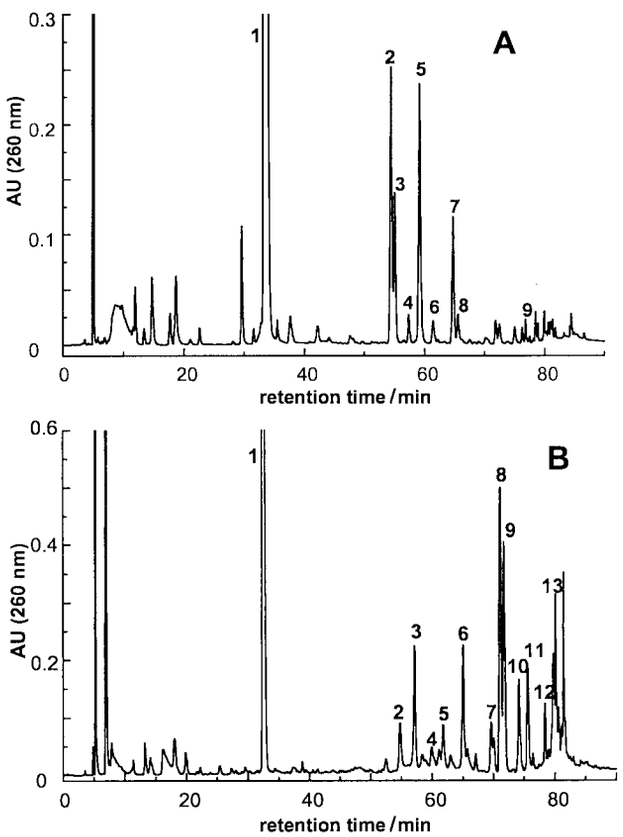

Fig. 2 HPLC separations of SO-dGpdT adducts. Panel A: Reaction between SO and dGpdT performed at pH 8.0. Peaks: 1, dGpdT; 2 and 5, β**N-7**; 3 and 7, α**N-7**; 4 and 8, α***N*²**; 6, N-7 guanine; 9, ***O*⁶**. Panel B: Reaction performed at pH 10.5. Peaks: 1, dGpdT; 2 and 4, β**N-7**; 3 and 6, α***N*²**; 5 and 7, β***N*²**; 8 and 9, β**N-3** (thymine); 10 and 11, β**N-1**; 12, ***O*⁶**; 13, α***N*²/N-3**.

known to be depyrimidated only under strongly acidic conditions. We interpreted these products as resulting from the alkylation of the 3-position of thymine because at pH 8.0 the alkylation level was very low, but at pH above the pK_a-value of thymine the alkylation at the 3-position is very facile. In the HPLC separation of the reaction mixture of pH 10.5, two additional prominent peaks were detected at retention times of *ca.* 80 min. The late migration by HPLC suggested dialkylation of the dinucleotide. Depurination of these fractions in mild acidic conditions gave only N^2-guanine products. However, because the 3-thymine fractions were found to be the major ones, it can be assumed that the dialkylation products obviously correspond to alkylation at both N^2-guanine and 3-thymine.

Conclusions

In the anionic state, guanine in dGMP is alkylated at oxygen and every nitrogen atom, except N-3 or N-9, through both α- and β-carbons of the epoxide. At the ring nitrogens the reactions take place preferentially through the less hindered β-carbon, as expected for S_N2-alkylating agents. For TMP only 3-alkylation was observed even though O^2 and O^4 could be considered as nucleophilic sites. Phosphate alkylation was observed for mononucleotides, but surprisingly the dinucleotide was not modified in the intervening phosphate. The lack of alkylation of the phosphodiester suggests that when considering the *in vivo* monitoring of DNA-alkylation by SO the phosphate adducts should not be a target. Further, we have shown that ESI-MS can be used for the investigations of isomerism of the hydroxyphenylethyl moiety in the SO adduct.

Experimental

Chemicals were used as purchased from the manufacturer. The mononucleotides were from Sigma Chemical Co. (St. Louis, MO), dGpdT from CyberGene AB (Huddinge, Sweden), racemic styrene 7,8-oxide (>97% pure) from Aldrich Chemie (Steinheim, Germany). Gradient grade methanol was from Merck (Darmstadt, Germany). All other chemicals were either from Sigma or Merck. The HPLC analyses were performed on a Beckman HPLC system Gold with a 168 diode array UV-detector module. UV spectra were recorded by a Beckman DU-640 spectrophotometer. ESI-MS spectra were obtained using the Finnigan LCQ LC/MS^n system (equipped with an ion trap mass analyser), connected to a Waters 2690 separation module.

Reactions of dGMP with SO

5′dGMP and 3′dGMP (2 mg ml^{-1}, each) were treated with 100 mM SO in 20 mM ammonium bicarbonate–ammonia (pH 10.5) and 30% methanol by incubating at 37 °C for 20 h. The excess SO was extracted twice with ethyl acetate (1 vol.), and the mixtures were evaporated to dryness under vacuum. The dried mixtures were dissolved in water and the reaction products were separated by HPLC equipped with a C-18 column (Kromasil, 250 × 4.5 mm, 5 μm) using a binary gradient consisting of 50 mM ammonium formate pH 4.6 and methanol (see Table 1 for retention times). The separation conditions have been described earlier.[7] The collected reaction products were evaporated to dryness under vacuum and were identified by ESI-MS and UV spectroscopy, and by depurination of the modified nucleotides. The depurination was performed by adding to an aliquot of the products in water an equal volume of 0.25 M hydrochloric acid and by keeping the mixture in a boiling water bath for 30 min. Because of their acid lability,[8] the O^6-products were kept in the water bath for only 5 min. After neutralisation with 0.25 M sodium hydroxide the depurination mixture was analysed by HPLC, as above.

Reaction of 3′TMP with SO

3′TMP (2 mg ml^{-1}) was treated with 100 mM SO either in 50 mM Tris-HCl (pH 7.4) and 30% methanol or in 20 mM ammonium bicarbonate–ammonia (pH 10.5) and 30% methanol by incubating at 37 °C for 23 h. The separation of the reaction products was performed as above.

Reaction of dGpdT with SO

dGpdT (1 mg ml^{-1}) was treated with 100 mM SO either in 50 mM Tris-HCl (pH 8.0) and 30% methanol or in 20 mM ammonium bicarbonate–ammonia (pH 10.5) and 30% methanol by incubating at 37 °C for 20 h and 16 h, respectively. The separation of the reaction products was performed in the same way as for dGMP (Fig. 2). The identification of the products was mainly based on depurination and subsequent detection of the alkylated bases by HPLC, in the same way as for dGMP adducts.

UV spectrometry

The UV spectra were measured first under neutral conditions in water, followed by measurements at alkaline (0.1 M sodium hydroxide) and then at acidic (0.1 M hydrochloric acid) conditions (Table 2). The site for base alkylation was determined by comparing the spectra to those of similar alkylation products described in the literature.[9,14] The concentration of the products was determined by using published molar extinction coefficients.[9,14]

Mass spectrometry

ESI-MS spectra of samples were obtained in the negative mode (Table 1). Samples (approximately 10–100 ng) were dissolved in 10 μl of the eluent (water–acetonitrile–ammonia = 49 : 50 : 1%)

Table 2 UV absorption data of the SO-alkylated nucleotides

Adduct	λ_{max}/nm			
	neutral	0.1 M NaOH	0.1 M HCl	from HPLC separations[a]
α**N-7**/β**N-7**	257	266	267	260
α***N*²**/β***N*²**	254	259	258	257
α**N-1**/β**N-1**	254	256	258	256
α***O*⁶**/β***O*⁶**	247/280	246/278	283	250/285
PO$_4$ dGMP	252	264	255	254
α**N-3**/β**N-3**	268	268	268	269
PO$_4$ TMP	266	265	266	266
N-7 dGpdT	261	265	271	264
N-1 dGpdT	258	258	262	261
*N*² dGpdT	258	264	263	260
*O*⁶ dGpdT	256/272	248/269	272	254/275
N-3 dGpdT	256	265	258	258

[a] λ_{max} from the UV spectrum obtained by diode-array detector during the HPLC-separation.

and injected *via* a loop into the running solvent, at a flow-rate of 0.2 ml min^{-1}. For negative-ion MS/MS experiments, precursor ions were selected in the iontrap analyser and fragmented. The full scan data were acquired for 70–1000 *m*/*z* and for MS/MS data 70–500 *m*/*z*. Electrospray voltage was 5.0 kV and capillary temperature 250 °C.

In order to confirm elemental composition, high resolution mass spectra (HRMS) were obtained for the novel adducts β***N*²** and α**N-1** as well as for β**N-3** by electrospray in the negative ion mode on a Micromass Autospec mass spectrometer with a mass resolution of 8000. HRMS Calc. for β***N*²** $C_{18}H_{21}N_5O_8P$ [M − H]: 466.1128, found 466.1139; Calc. for α**N-1** $C_{18}H_{21}N_5O_8P$ [M − H]: 466.1128, found 466.1141; Calc. for β**N-3** $C_{18}H_{22}N_2O_9P$ [M − H]: 441.1063, found 441.1054.

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