Journal of Chromatography, 541 (1991) 317–331
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 981

Comparison of the isolation of adducts of 2′-deoxycytidine and 2′-deoxyguanosine with phenylglycidyl ether by high-performance liquid chromatography on a reversed-phase column and a polystyrene–divinylbenzene column

E. VAN DEN EECKHOUT* and J. COENE
College of Pharmacy, University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)
J. CLAEREBOUDT
Department of Pharmacy, University of Antwerp (U.I.A.), Universiteitsplein 1, B-2610 Wilrijk (Belgium)
F. BORREMANS
Laboratory for Organic Chemistry, University of Ghent, Krijgslaan 281, B-9000 Ghent (Belgium)
M. CLAEYS
Department of Pharmacy, University of Antwerp (U.I.A.), Universiteitsplein 1, B-2610 Wilrijk (Belgium)
E. ESMANS
Laboratory for Organic Chemistry, University of Antwerp (R.U.C.A.), Groenenborgerlaan 171, B-2020 Antwerp (Belgium)
and
J. E. SINSHEIMER
College of Pharmacy, University of Michigan, Ann Arbor, MI 48109 (U.S.A.)
(First received May 14th, 1990; revised manuscript received October 23rd, 1990)

ABSTRACT

2′-Deoxycitidine (dCyd) and 2′-deoxyguanosine (dGuo) were subjected to reaction with phenylglycidyl ether (PGE) in methanol in order to study the formation of the corresponding 2′-deoxynucleoside adducts. Separation methods were developed on analytical and semi-preparative scales using high-performance liquid chromatography with photodiode-array detection on a reversed-phase column and on a polystyrene–divinylbenzene column. The use of the latter column was prompted by decomposition of the preparatively isolated dGuo–PGE adducts on the reversed-phase column. The use of a polystyrene–divinylbenzene column solved this problem and also revealed the presence of one more peak in both the dCyd– and dGuo–PGE reaction mixtures.

The adducts of dCyd and dGuo were isolated on preparative reversed-phase and polystyrene–divinylbenzene columns and characterized by UV, fast atom bombardment mass and 360 MHz ^{1}H NMR spectrometry. The adducts of dCyd were the diastereomers of N-3-(2-hydroxy-3-phenoxypropyl)-2′-deoxycytidine and N^4-(2-hydroxy-3-phenoxypropyl)-2′-deoxycytidine whereas those of dGuo were the two diastereomers of N-7-(2-hydroxy-3-phenoxypropyl)-2′-deoxyguanosine and a third peak which appeared to be mainly N^2-(2-hydroxy-3-phenoxypropyl)-2′-deoxyguanosine.

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V.

INTRODUCTION

Aliphatic epoxides are important as commercial, industrial and laboratory chemicals; examples are styrene oxide, propylene oxide and gycidyl ethers. The U.S. National Institute of Occupational Safety and Health [1] surveyed the extent of occupational contact and potential damage from the glycidyl ether group of epoxides and established threshold limits for human exposure. Occupational and environmental vulnerability to aliphatic epoxides and *in vivo* production of epoxides and their toxicity have been reviewed by Manson [2] and Ehrenberg and Hussain [3].

It is generally believed that reactions of electrophilic reactants with sites in DNA or RNA are fundamental to the induction of mutations. Our continuing interest in the structure–mutagenicity relationship for aliphatic epoxides [4–10] and in the reactivity of these epoxides with 2′-deoxynucleosides and DNA [11–13] prompted us to extend the limited literature [14–16] on the reactivity and identification of adduct formation between phenyl glycidyl ether (PGE) and 2′-deoxynucleosides. In a recent paper, we reported on the separation and structure elucidation of the nucleoside adducts of 2′-deoxyadenosine and thymidine with PGE [17]. As an extension of that work, the adduct formation of 2′-deoxycytidine and 2′-deoxyguanosine with PGE was studied.

Chromatographic separations of purine and pyrimidine derivatives have been widely reported [18]. In general, the reversed-phase mode is used for the separation and analysis of nucleosides and bases. This mode was also used in our recent work [17] on 2-deoxyadenosine– and thymidine–PGE adducts with success. However, when a reversed-phase system was used for the preparative separation of the 2′-deoxyguanosine–PGE reaction mixtures, problems with decomposition of adducts were encountered. This phenomenon led to the use of a polystyrene–divinylbenzene column, as this type of column is well suited for the analysis of quaternary ammonium compounds [19], nucleic acids and their derivatives [20–27]. In this paper we report a comparison of the results obtained for the preparative isolation and identification of 2′-deoxycytidine–PGE and 2′-deoxyguanosine–PGE reaction mixtures on a reversed-phase column and a polystyrene–divinylbenzene column. The latter column proved to be superior for this analysis and led to the detection and identification of adducts not detected on the reversed-phase column. On-line identification during high-performance liquid chromatographic (HPLC) analysis was effected by means of a photodiode-array detector. The main adducts isolated were characterized by means of mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

EXPERIMENTAL

Materials

All solvents were of analytical-reagent grade. 2,3-Epoxypropyl phenyl ether (phenyl glycidyl ether, PGE) was obtained from Janssen Chimica (Beerse, Belgium) and was distilled *in vacuo* before use. 2′-Deoxycytidine and 2′-deoxyguanosine were purchased from Sigma (St. Louis, MO, U.S.A.). Ammonium formate was obtained from BDH (Poole, U.K.).

Reaction of 2′-deoxynucleosides with phenyl glycidyl ether

2′-Deoxycytidine (10 mg) and 2′-deoxyguanosine (10 mg) were dissolved in 3 ml

of methanol, then 1 ml of 1 *M* PGE in methanol was added. The compounds were allowed to react for 24 h at 37°C in tightly sealed test-tubes equipped with a Teflon-lined screw-cap. For preparative purposes, 100 mg of 2′-deoxynucleoside in methanol were used.

Chromatography

The HPLC system was equipped with a Waters M-45 pump. Detection was effected with a Hewlett-Packard Model 1040A photodiode-array detector equipped with a Hewlett-Packard Model 8290M flexible disk drive and a Hewlett-Packard Model 85 computer. Analytical reversed-phase chromatography for the 2′-deoxycytidine–PGE and 2′-deoxyguanosine–PGE reaction mixtures was performed on a 10 RP-18 column (25 cm × 4.6 mm I.D.) (Alltech). The eluent composition for the 2′-deoxycytidine–PGE and 2′-deoxyguanosine–PGE mixtures was 0.01 *M* ammonium formate (pH 5.1)–methanol (80:20) at a flow-rate of 2.0 ml/min. Injection was with a six-way Valco valve with a 20-μl loop. The detection wavelength was 260 nm.

Preparative chromatography was carried out on a reversed-phase 10 RP-18 (Alltech) column (25 cm × 2.2 cm I.D.) and on a polystyrene–divinylbenzene PRP-1 (Hamilton) column (30.5 cm × 7.0 mm I.D.). Injection was with a six-way Valco valve with a 100-μl loop. The detection wavelength was 260 nm.

The solvent for reversed-phase chromatography for the 2′-deoxycytidine–PGE mixture was 0.01 *M* ammonium formate (pH 5.1)–methanol (80:20) at a flow-rate of 7.0 ml/min and that for the 2′-deoxyguanosine–PGE mixture was 0.01 *M* ammonium formate (pH 5.1)–methanol (70:30) at 7.0 ml/min. With the polystyrene–divinylbenzene column, two different solvent systems were used for each reaction mixture: for the 2′-deoxycytidine–PGE mixture, 0.01 *M* ammonium formate (pH 4.25)–methanol (60:40) and (50:50) at 1.6 ml/min and for the 2′-deoxyguanosine–PGE mixture 0.01 *M* ammonium formate (pH 4.25)–methanol (60:40) at 1.6 ml/min and (30:70) at 2.0 ml/min.

NMR spectroscopy

^{1}H NMR spectra were recorded at 500.13 MHz on a Bruker AM spectrometer using 0.01 *M* solutions in [$^2H_6$]dimethyl sulphoxide (DMSO-d_6) at room temperature. No precautions were taken to exclude moisture from the samples. Therefore, the readily exchangable NH and OH protons were not observed. Chemical shifts are quoted in ppm downfield relative to TMS internal standard – DMSO-d_5 signal at 2.500 ppm. Unequivocal assignments of the NMR resonances to the specific protons were obtained from standard two-dimensional proton–proton *J* correlation spectra.

Mass spectrometry

All analyses were performed on a VG 70-SEQ hybrid mass spectrometer (VG Analytical, Manchester, U.K.), equipped with an Ion Tech saddle field atom gun. The instrument consists of a high-resolution double-focusing mass spectrometer with EB configuration (MS-I), followed by an RF-only quadrupole collision gas cell and a high-performance quadrupole mass analyser (MS-II). Xenon atoms with energies of *ca.* 8 keV and a discharge current of 1 mA were used as the ionizing beam. Positive- and negative-ion fast atom bombardment (FAB) mass spectra were recorded under control of the VG 11-250 J data system by repetitive scanning of MS-I over the range 20–600 u,

using a scan time of 2 s per decade. Daughter ion (MS–MS) spectra were obtained by collisionally activated decomposition (CAD) in the RF-only quadrupole gas cell, using argon as collision gas, and by scanning MS-II.

Ultraviolet spectroscopy

UV spectra were recorded on-line during HPLC analysis in the HPLC solvent system used with the photodiode-array detector.

UV spectra from samples isolated preparatively were taken off-line on a Perkin-Elmer Lambda 15 UV–VIS spectrophotometer equipped with a Perkin-Elmer EX-800 printer.

Dried samples were diluted in water to obtain absorbance values between 0.5 and 1.0. UV spectra were recorded at acidic pH by mixing the aqueous samples with an equal volume of 0.1 *M* hydrochloric acid or at alkaline pH by mixing with an equal volume of 0.1 *M* sodium hydroxide solution.

RESULTS AND DISCUSSION

2′-Deoxycytidine and 2′-deoxyguanosine were subjected to reaction with PGE in methanol. After 24 h at 37°C, the resulting reaction mixtures were analysed using reversed-phase HPLC with photodiode-array detection. The analytical HPLC method was used to develop separations on a preparative scale. However, for the 2′-deoxyguanosine–PGE mixture, problems of decomposition with this system were encountered. Therefore, a second HPLC system on a polystyrene–divinylbenzene column was used for the preparative isolation of adducts of both the 2′-deoxyguanosine–PGE and 2′-deoxycytidine–PGE mixtures.

Analytical reversed-phase HPLC of 2′-deoxycytidine–PGE and 2′-deoxyguanosine–PGE with photodiode-array detection

In a recent paper [17], we described analytical HPLC with photodiode-array detection for thymidine– and 2′-deoxyadenosine–PGE mixtures as a powerful tool for the structure elucidation of alkylated nucleosides. As an extension of this work, similar eluents, namely 0.01 *M* ammonium formate (pH 5.1)–methanol mixtures on a reversed-phase 10 RP-18 column were used. For both the 2′-deoxycytidine–PGE and 2′-deoxyguanosine–PGE mixtures, two main adduct peaks (as characterized by their UV spectra) were observed at $k' = 18.4$ ($t_R = 19.4$ min) and 25.30 ($t_R = 26.6$ min) for deoxycytidine–PGE and at $k' = 16.1$ ($t_R = 17.1$ min) and 21.01 ($t_R = 22.1$ min) for deoxyguanosine–PGE (Fig. 1), and were labelled dGuo 1′, dGuo 2′, dCyd 1 and dCyd 2.

UV spectra taken on-line were identical for the peaks dGuo 1′ and dGuo 2′, and also for the peaks dCyd 1 and dCyd 2, suggesting that dGuo 1′ and dGuo 2′ and also dCyd 1 and dCyd 2 are both pairs of isomers. The analytical HPLC was then adapted to a preparative scale in order to obtain off-line UV data at different pH values and mass spectral and NMR data.

Preparative reversed-phase HPLC of 2′-deoxycytidine–PGE and 2′-deoxyguanosine–PGE reaction mixtures with photodiode-array detection

Preparative HPLC of 2′-deoxycytidine–PGE and 2′-deoxyguanosine–PGE on

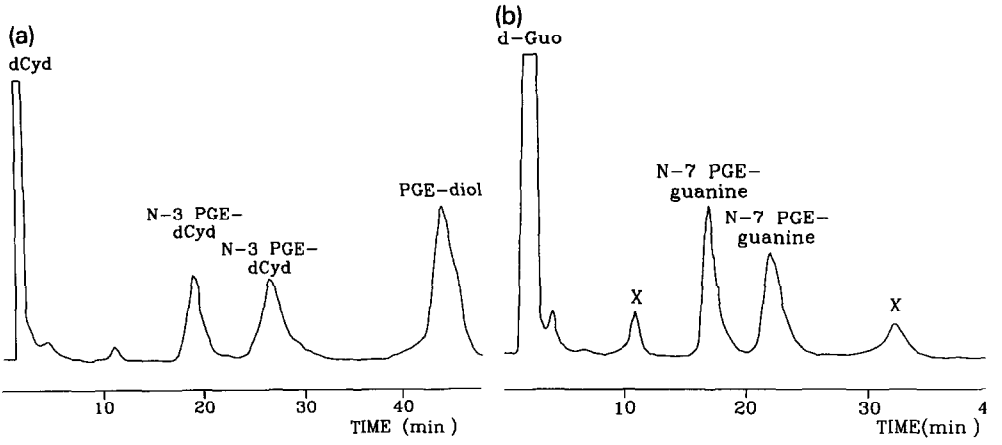

Fig. 1. Analytical reversed-phase HPLC of (a) dCyd–PGE and (b) dGuo–PGE reaction mixtures. Column, 10 RP-18 (25 cm × 4.6 mm I.D.); eluent, 0.01 *M* ammonium formate (pH 5.1)–methanol (80:20) at 2.0 ml/min. Concentration of sample: ±3 mg/ml; 0.1 a.u.f.s.

a reversed-phase RP-18 column gave similar results to the analytical HPLC. Two main adducts were isolated for both reaction mixtures at $k' = 2.7$ ($t_R = 29.0$ min) and 3.7 ($t_R = 35.5$ min) for 2′-deoxycytidine and at $k' = 2.7$ ($t_R = 29.0$ min) and 3.6 ($t_R = 36.5$ min) for 2′-deoxyguanosine.

After freeze-drying, all the adduct samples of 2′-deoxycytidine and 2′-deoxyguanosine were subjected to UV analysis off-line at different pH values in order to obtain an indication of the alkylation site. The UV data obtained off-line are given in Table I.

In previous UV studies [10–12,17] we have shown that UV spectra taken at different pH values give a strong indication of the site of alkylation of nucleosides by epoxides. The conclusions drawn from the UV studies are based on maximum wavelengths obtained for the different adduct fractions, on shifts in acidic or alkaline medium and on absorbance ratios obtained at 254 and 280 nm.

TABLE I

UV PEAK MAXIMA AND 254/280 nm ABSORBANCE RATIOS FOR PEAKS FROM DEOXYCYTIDINE–PGE AND DEOXYGUANOSINE–PGE REACTION MIXTURES

Peak	λ_{max} (nm) (ratio)		
	0.1 *M* HCl	H_2O	0.1 *M* NaOH
dCyd	279.0 (0.30)	270.4 (1.00)	270.8 (0.93)
dCyd 1	276.5 (0.40)	276.4 (0.40)	268.7 (1.05)
dCyd 2	276.5 (0.40)	276.4 (0.40)	268.7 (1.05)
dCyd 3	276.0 (0.45)	269.7 (0.93)	269.8 (0.93)
dGuo	254.5 (1.56)	252.4 (1.68)	265.6 (2.0)
dGuo 1′ (1)	257.1 (1.50)	259.0 (1.31)	266.6 (1.67)
dGuo 2′ (2)	257.1 (1.50)	259.0 (1.31)	266.6 (1.67)
dGuo 3	261.5 (1.30)	257.6 (1.55)	259.1 (1.67)

TABLE II

CAPACITY FACTORS (k'), UV PEAK MAXIMA AND 254/280 nm ABSORBANCE RATIOS AFTER PREPARATIVE HPLC OF 2'-DEOXYGUANOSINE- AND 2'-DEOXYCYTIDINE–PGE REACTION MIXTURES ON A POLYSTYRENE–DIVINYLBENZENE COLUMN

Peak	k'			λ_{max}[d]	Ratio (254/280 nm)
	A[a]	B[b]	C[c]		
dGuo	0.21			254	1.5
dGuo 1	1.04	2.37		260	1.4
dGuo 2	1.04	3.00		260	1.5
dGuo 3	8.23			258	1.6
dCyd	0.12		0.10	272	1.0
dCyd 1	1.05	2.87	0.65	278	0.4
dCyd 2	1.32	3.50	0.65	278	0.4
dCyd 3	2.87		1.35	272	0.9

[a] Solvent system: 0.01 *M* ammonium formate (pH 4.25)–methanol (50:50) at 1.6 ml/min.
[b] Solvent system: 0.01 *M* ammonium formate (pH 4.25)–methanol (60:40) at 1.8 ml/min.
[c] Solvent system: 0.01 *M* ammonium formate (pH 4.25)–methanol (40:60) at 1.6 ml/min.
[d] Wavelengths of maximum absorbances taken in the solvent system that separated the PGE adducts of 2'-deoxyguanosine and 2'-deoxycytidine.

For the two 2'-deoxycytidine adducts dCyd 1 and dCyd 2,N-3 alkylation was suggested. This hypothesis is based on Singer's research [28] in which 3-ethylcytidine displayed a λ_{max} in water of 279 and a 254/280 nm ratio of 0.40, compared with a similar λ_{max} and the same 254/280 nm ratios for both 2'-deoxycytidine adducts (Tables I and II). The lack of shift in 0.1 *M* hydrochloric acid for both dCyd 1 and dCyd 2 adducts is a further indication of N-3 alkylation. The UV spectra of dGuo 1' and dGuo 2' are very similar to that of 7-alkylated 2'-deoxyguanosine published by Singer [28]. Further, the shifts in acidic and alkaline media are very similar to those for the 7-alkyl products produced by propylene oxide, glycidol and epichlorohydrin with 2'-deoxyguanosine reported by Hemminki *et al.* [29]. An irreversible change in the spectra, whereby the maximum wavelength shifts from 259 to 266 nm, is also consistent with a 7-alkyl group. These UV assignments were then confirmed by FAB-MS and/or NMR.

Preparative HPLC of 2'-deoxycytidine–PGE and 2'-deoxyguanosine–PGE mixtures on a polystyrene–divinylbenzene column

Table II summarizes the results obtained for the preparative isolation of the deoxyguanosine- and deoxycytidine–PGE mixtures on a preparative PRP-1 column. For each reaction mixture, an additional adduct is detected, labelled dCyd 3 and dGuo 3, eluting much later than the adducts dCyd 1, dCyd 2, dGuo 1' and dGuo 2' previously detected on the reversed-phase column.

The deoxyguanosine adduct fractions on the reversed-phase column, dGuo 1' and dGuo 2', are labelled differently to those detected on the PRP-1 column, dGuo 1 and dGuo 2, as the mass spectral data which follow show that the former adducts have lost their sugar moiety, and are different from dGuo 1 and dGuo 2. For the preparative

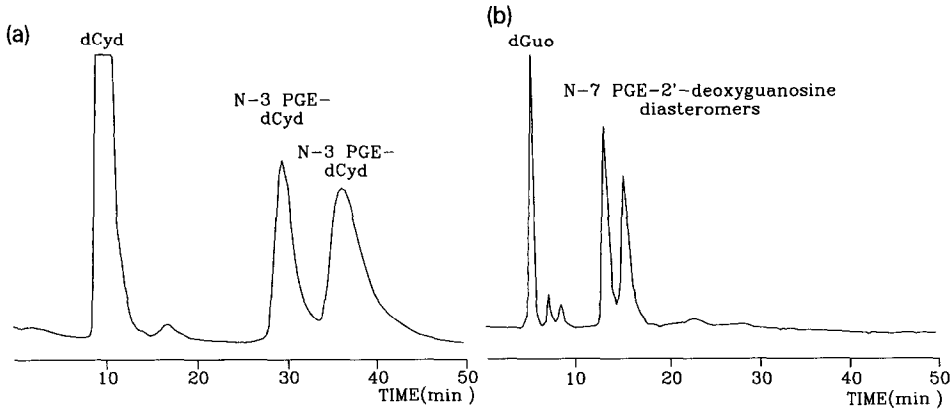

Fig. 2. Preparative HPLC on a polystyrene–divinylbenzene column of diastereomers of (a) N-3 alkylated 2'-deoxycytidine and (b) N-7 alkylated 2'-deoxyguanosine. Column, PRP-1 (30.5 cm × 7.0 mm I.D.); eluent, 0.01 *M* ammonium formate (pH 4.25)–methanol (60:40) at 1.6 ml/min. Concentration of sample: ±30 mg/ml; 0.1. a.u.f.s.

isolation of dGuo 3 and dCyd 3, a less polar solvent system in the range of 0.01 *M* ammonium formate (pH 4.25)–methanol (50:50) to (30:70) was used (Fig. 4). As expected, the diastereomers dCyd 1 and dCyd 2, dGuo 1 and dGuo 2 were not separated in this solvent system. A more polar solvent system had to be used, namely 0.01 *M* ammonium formate (pH 4.25)–methanol (60:40) (Fig. 2).

UV spectroscopy. All the adduct fractions were preparatively isolated, freeze-dried and UV spectra were taken off-line in water; 0.1 *M* hydrochloric acid and 0.1 *M*

TABLE III

MAJOR IONS OBSERVED IN THE POSITIVE- AND NEGATIVE-ION FAB MASS SPECTRA OF NUCLEOSIDE PGE ADDUCTS

Reaction mixture	HPLC column	Analysed fraction	Positive ions[a,b]		Negative ions[a,b]	
			$(M + H)^+$	Other	$(M - H)^-$	Other
PGE–2'-deoxycytidine	RP-18	dCyd 1	*m/z* 378		*m/z* 376	
		dCyd 2	*m/z* 378		*m/z* 376	
PGE–2'-deoxycytidine	PRP-1	dCyd 1	*m/z* 378		*m/z* 376	
		dCyd 2	*m/z* 378		*m/z* 376	
		dCyd 3	*m/z* 378		*m/z* 376	
PGE–2'-deoxyguanosine	RP-18	dGuo 1'	ND[c]	*m/z* 302	ND	*m/z* 300
		dGuo 2'	ND	*m/z* 302	ND	*m/z* 300
PGE–2'-deoxyguanosine	PRP-1	dGuo 1	*m/z* 418		*m/z* 416	
		dGuo 2	*m/z* 418		*m/z* 416	
		dGuo 3	*m/z* 418	*m/z* 568, *m/z* 586	*m/z* 416	*m/z* 566, *m/z* 584

[a] Fragment ions not listed because of possible interferences with matrix signals.
[b] The $(M + H)^+$ and $(M - H)^-$ ions are related to the monoalkylated nucleoside.
[c] ND = not detected, *i.e.*, negligible intensity (signal-to-noise ratio < 3).

TABLE IV

MASS NUMBERS AND RELATIVE INTENSITIES OF DAUGHTER IONS IN THE FAB-MS–CAD-MS OF THE NUCLEOSIDE–PGE ADDUCT $(M + H)^+$ IONS[a,b,c]

Compound	$(M + H)^+$	RBH_2^+	$(RBH_2 - C_6H_5OH)^+$	BH_2^+	R^+	S^+	Others
dCyd 1	378 (P)	262 (100)	168 (10)	112 (53)	151 (4)	117 (9)	244 (2); 150 (9); 133 (7); 125 (3); 107 (5); 105 (6); 99 (5); 95 (3); 73 (4); 71 (2)
dCyd 2	378 (P)	262 (100)	168 (11)	112 (51)	151 (3)	117 (9)	244 (3); 150 (8); 133 (8); 125 (4); 107 (5); 105 (7); 99 (6); 95 (2); 77 (2); 73 (4); 71 (2)
dCyd 3	378 (P)	262 (100)	168 (21)	112 (3)	151 (2)	117 (13)	244 (2); 152 (2); 150 (10); 133 (4); 125 (8); 124 (10); 107 (5); 105 (3); 99 (6); 73 (4); 71 (2)
dGuo 1	418 (P)	302 (100)	208 (19)	152 (64)	–	117 (48)	191 (3); 190 (6); 178 (2); 165 (7); 164 (4); 135 (13); 133 (15); 121 (2); 110 (3); 107 (8); 105 (13); 99 (21); 79 (2); 77 (2); 73 (11); 71 (6); 69 (4)
dGuo 2	418 (P)	302 (100)	208 (19)	152 (58)	–	117 (49)	191 (4); 190 (8); 178 (2); 165 (6); 164 (4); 135 (11); 133 (14); 110 (3); 107 (8); 105 (14); 99 (18); 95 (2); 81 (2); 79 (2); 77 (2); 73 (11); 71 (5); 69 (3)
dGuo 3	418 (P)	302 (100)	208 (28)	152 (57)	151 (1)	117 (54)	284 (2); 191 (4); 190 (11); 165 (5); 164 (5); 135 (17); 133 (17); 121 (2); 110 (3); 107 (9); 105 (16); 99 (18); 79 (2); 77 (2); 73 (13); 71 (6); 69 (2)

[a] The CAD mass spectra of the $(M + H)^+$ ions were recorded at a collision energy of 80 eV, using argon as the collision gas at a pressure of 2 mTorr (in the collision cell).

[b] The $(M + H)^+$ ions are related to the monoalkylated nucleoside.

[c] Nomenclature: M corresponds to RBS, where B = base moiety, S = sugar moiety and R = 2-hydroxy-3-phenoxypropyl substituent.

sodium hydroxide (Table I). The UV spectra for dCyd 1 and dCyd 2 were the same for the reversed-phase column and the polystyrene–divinylbenzene column, and have been discussed in the previous section. Also the UV spectra of the intact alkylated nucleosides dGuo 1 and dGuo 2 were the same as those for dGuo 1′ and dGuo 2′. It is known that the UV characteristics of deoxy bases are not influenced by the presence of their sugar moiety [30].

On the basis of the UV characteristics for dCyd 3, alkylation at O-2 could be excluded, since the peak maxima for O-2 alkylation are 262 or 263 nm for propylene oxide, trichloropropylene oxide, epichlorohydrin and glycidol-2′-deoxycytidine derivatives [11] and for ethylcytidine [28] and the 254/280 nm ratio for all the derivatives was 2.3. N^4-Ethylcytidine has λ_{max} of 272 nm (water) and 281 nm (pH 1) and a 254/280 nm ratio of 0.9. Our isolated material dCyd 3 has a λ_{max} of 272 nm (water) and a 254/280 nm ratio of 0.9. Further, the dCyd 3 adduct shows a shift towards longer wavelength in acidic medium, which is consistent with substitution on an amino group in a pyrimidine ring [30]. In contrast to dCyd 1 and dCyd 2, there is no shift in alkaline medium, which also points to alkylation on the exocyclic nitrogen. Thus, N-4 alkylation was suggested for dCyd 3.

On the basis of UV characteristics for dGuo 3, alkylation at O-6 could be ruled out, as neither the peak maxima in acidic medium nor the shape of the UV curve coincide with that of O^6-methyldeoxyguanosine [28]. Alkylation at N-2 is suggested by comparison of the UV spectra in acidic and alkaline media with N^2-methylguanosine [28]. Further, substitution of 2-aminopyridine by one or two methyl groups results in a small shift towards longer wavelengths [31], which is seen in the neutral and acidic UV spectra of dGuo 3 as compared with the unsubstituted dGuo. The HPLC behaviour of this compound is also consistent with N-2 alkylation as we also found an increase in apolarity for the N-4 alkylated material of 2′-deoxycytidine.

Mass spectra and NMR. A detailed discussion of the mass spectra will be published elsewhere [32]. The FAB mass spectra of dCyd 1 and dCyd 2 isolated on reversed-phase and polystyrene–divinylbenzene columns were identical. They yield intense peaks at *m/z* 378 in the positive-ion mode and at *m/z* 376 in the negative-ion mode (Table III). These ions can be assigned to the $(M + H)^+$ and $(M - H)^-$ ions of the monoalkylated adduct. Definite structural information as to the alkylation site is obtained by the FAB-MS–CAD-MS technique [33,34]. With this technique an ion of interest is selected by MS-I, induced to fragment by collisionally activated decomposition and a spectrum of the daughter ions is taken by MS-II. The FAB-MS–CAD-MS data for the $(M + H)^+$ and $(M - H)^-$ ions of the monoalkylated nucleosides are given in Tables IV and V. N-3 alkylation for dCyd 1 and dCyd 2 is confirmed by the daughter ion spectrum of the $(M - H)^-$ ion of the monoalkylated adducts (Fig. 3a and Table V).

Diagnostic fragment ions correspond to the RB^- ion (monoalkylated base) at *m/z* 260, $(RB - HNCO)^-$ at *m/z* 217, $(RB - phenol)^-$ at *m/z* 166 and phenolate anion at *m/z* 93. The fragment ion at *m/z* 183 originates from a retro-Diels–Alder rearrangement in the pyrimidine ring, with retention of the negative charge on the diene part of the molecule.

For dCyd 3, some differences are observed in the daughter ion spectra of the $(M - H)^-$ ions (*m/z* 376) in comparison with dCyd 1 (Fig. 3). The major fragment ion in the spectrum of dCyd 1 is detected at *m/z* 183, which is consistent with alkylation at

the N-3 position. The detection of the isocyanate anion at *m/z* 42 and the fragment ion at *m/z* 243 in the daughter ion spectrum of dCyd 3 indicates that the alkylation site for the adduct in dCyd 3 is not N-3, but N-4, as suggested by the UV data.

The positive-ion FAB mass spectra of the fractions dGuo 1′ and dGuo 2′, isolated from the 2′-deoxyguanosine–PGE reaction mixture on the reversed-phase column, were both characterized by an ion at *m/z* 302. This ion can be assigned either to the RBH_2 fragment ion or the $(M + H)^+$ ion of monoalkylated guanine. Constant neutral loss scanning of 116 u, which is a very useful method for the identification of the $(M + H)^+$ ions of modified deoxynucleosides, gives negative results for fractions dGuo 1′ and dGuo 2′. Therefore, the ions at *m/z* 302 must be attributed to the $(M + H)^+$ ions of monoalkylated guanine. This alkylated nucleobase is probably formed by depurination of the alkylated nucleoside on the RP-18 column. No differences are observed between the daughter ion spectra obtained for the ions at *m/z* 302 in the adduct samples dGuo 1′ and dGuo 2′. This indicates that the two isolated adducts are diastereomers; the alkylation site could not be determined from these MS data, but 7-alkylation was strongly suggested by the UV data.

The FAB MS data for the three adduct samples of 2′-deoxyguanosine (dGuo 1, dGuo 2 and dGuo 3), isolated on the polystyrene–divinylbenzene column (Table III), show high signal intensities for the $(M + H)^+$ ions at *m/z* 418 in the positive-ion mode and $(M - H)^-$ ions at *m/z* 416 in the negative-ion mode, indicating the presence of intact monoalkylated adducts in each of the fractions. The presence of ions at *m/z* 568 and 586 for dGuo 3 in the positive-ion FAB mass spectrum indicates the presence of two additional compounds in this fraction, namely the dialkylated adduct (MW 567) and its imidazole ring-opened derivative (MW 585). The alkylation site could not be assigned unequivocally from these MS data. The structures suggested by the UV spectra were confirmed by NMR.

TABLE V

MASS NUMBERS AND RELATIVE INTENSITIES OF DAUGHTER IONS IN THE FAB-MS–CAD-MS OF THE NUCLEOSIDE–PGE ADDUCT $(M - H)^-$ IONS[a,b,c]

Compound	$(M - H)^-$	$[(M - H) - C_3H_6O_3]^-$	$[(M - H) - C_6H_5OH]^-$	RB^-	$[(M - H) - C_6H_5OH - C_3H_6O_3]^-$
dCyd 1	376 (P)	–	–	260 (8)	192 (1)
dCyd 2	376 (P)	–	–	260 (9)	192 (1)
dCyd 3	376 (P)	286 (3)	282 (2)	260 (4)	192 (4)
dGuo 1	416 (P)	326 (12)	322 (42)	300 (9)	232 (18)
dGuo 2	416 (P)	326 (14)	322 (50)	300 (8)	232 (22)
dGuo 3	416 (P)	–	322 (62)	300 (4)	232 (14)

[a] The CAD mass spectra of the $(M - H)^-$ ions were recorded at a collision energy of 70 eV, using argon as the collision gas at a pessure of 2 mTorr (in the collision cell).

[b] The $(M - H)^-$ ions are related to the monoalkylated nucleoside.

[c] Nomenclature: M corresponds to RBS, where B = base moiety, S = sugar moiety and R = 2-hydroxy-3-phenoxypropyl substituent.

For the dCyd–PGE adducts isolated, namely dCyd 1, dCyd 2 and dCyd 3, the following NMR data were obtained [^{1}H NMR in DMSO-d_6; shifts in ppm (J in Hz)]:

dCyd 1: H-1′, 6.124 (6.8, 6.8); H-2′ and H-2″, isochronous, 2.003 (apparent couplings 4.6 and 8.0); H-3′, 4.202 (2.9, 4.5, 4.5); H-4′, 3.730 (2.8, 3.9, 3.9); H-5′, 3.532 and 3.510 (3.9, 3.9, −11.9); H-5, 5.854 (8.1); H-6, 7.340 (8.1), aromatic protons 7.263 (7.4, 8.6) *meta*, 6.910 (7.4) *para*, 6.861 (8.6) *ortho*; $CH_2(\alpha)$–$CH(\beta)$, 4.04–4.18 (ABC multiplet); $CH_2(\gamma)$ 3.88 (−10.5).

dCyd 2: H-1′, 6.116 (6.8, 6.8); H-2′ and H-2″, isochronous, 1.981 (apparent couplings 4.8, 6.7); H-3′, 4.198 (2.4, 4.7, 4.7); H-4′, 3.733 (2.4, 4.0, 4.0); H-5′, 3.532 and 3.510 (4.0, 4.0, −11.9); H-5, 5.880 (7.9); H-6, 7.376 (7.9), aromatic protons 7.26 (7.5, 8.4) *meta*, 6.906 (7.3) *para*, 6.854 *ortho*; $CH_2(\alpha)$–$CH(\beta)$, 4.031–4.180 (ABC multiplet); $CH_2(\gamma)$, 3.88 (−10.3).

dCyd 3: H-1′, 6.151 (6.0, 7.2); H-2′ and H-2″, 1.922 (6.2, 7.2, −13.1) and 2.090 (3.4, 5.9, −13.1); H-3′, 4.197 (6.0, 3.4, 3.0); H-4′, 3.750 (3.0, 4.0, 4.0); H-5′, 5.860 (7.5); H-6, 7.755 (7.5); NH-4, 7.972 (5.0, 5.0), aromatic protons, 7.278 *meta*, 6.92 *ortho* and *para*; $CH_2(\alpha)$, 3.316 and 3.480 (5, 6, −12.0); $CH(\beta)$, 3.974 (4 × 5.5); $CH_2(\gamma)$, 3.895 (6.6, −9.9) and 3.922 (4.3, −9.9).

The quasi-identical chemical shifts and couplings observed for dCyd 1 and dCyd 2 [especially the similarity in the $CH_2(\alpha)$–$CH(\beta)$–$CH_2(\gamma)$ alkyl fragment] strongly suggest their diastereomeric relationship. Monoalkylation could be deduced from these NMR data, but the alkylation site was proposed on the basis of UV and mass spectra data.

In dCyd 3, the presence of an NH proton and its J correlation to a CH_2 group is consistent with alkylation at N-4 and with the least substituted epoxide carbon atom of the PGE part of the molecule (Fig. 5).

For dGuo 3, the following NMR data were obtained: H-1′, 6.134 (7.7, 6.2); H-2′ under DMSO solvent peak 2, 5; H-2″, 2.198 (13.1, 5.8, 3.0); H-3′, 4.347 (5.6, 2.8, 2.8); H-4′, 3.814 (2.6, 4.8, 4.8); H-5′, 3.560 (11.6, 4.8); H-5″, 3.496 (11.6, 4.6); H-8, 7.94,

$(RB^- - C_6H_5OH)^-$	$C_6H_5O^-$	Others
166 (4)	93 (62)	217 (5); 183 (100); 135 (7); 123 (2)
166 (3)	93 (64)	217 (5); 183 (100); 135 (8); 123 (2)
166 (40)	93 (100)	285 (3); 243 (54); 42 (4)
206 (100)	93 (45)	265 (4); 188 (6); 176 (6); 173 (3); 164 (6); 162 (5); 150 (3); 133 (7); 89 (3
206 (100)	93 (46)	304 (3); 265 (4); 188 (5); 176 (5); 174 (3); 164 (6); 150 (4); 133 (10); 89 (
206 (100)	93 (47)	188 (4); 176 (3); 164 (4); 150 (2); 133 (8); 89 (2)

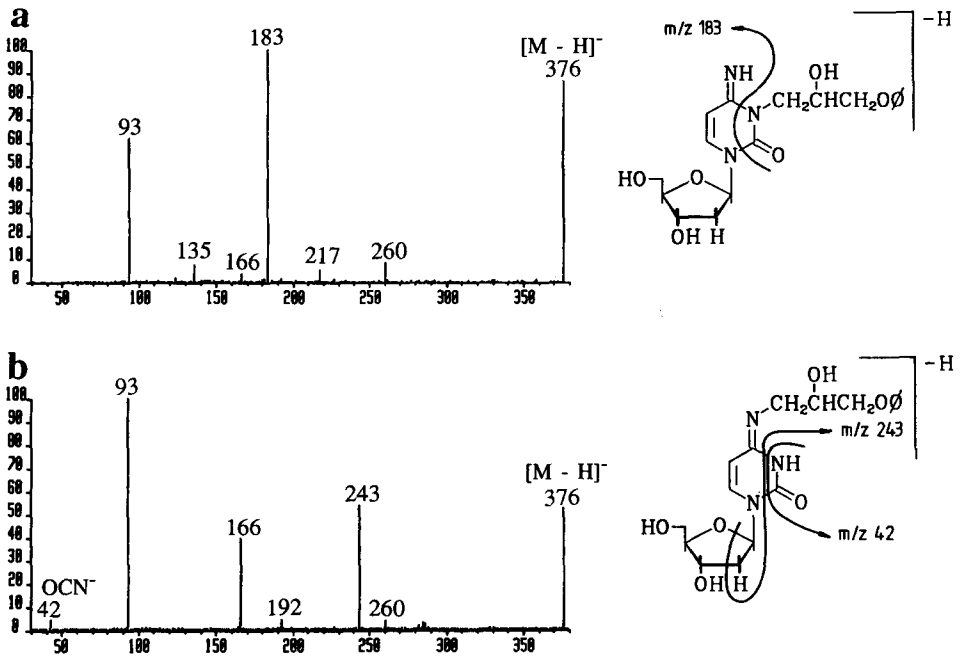

Fig. 3. Daughter ion spectra of the $(M - H)^-$ ions (m/z 376) of (a) N-3 alkylated 2'-deoxycytidine (dCyd 1 and dCyd 2) and (b) N-4 alkylated 2'-deoxycytidine (dCyd 3), obtained by CAD at E_{coll} = 70 eV and argon gas pressure = 2 mTorr.

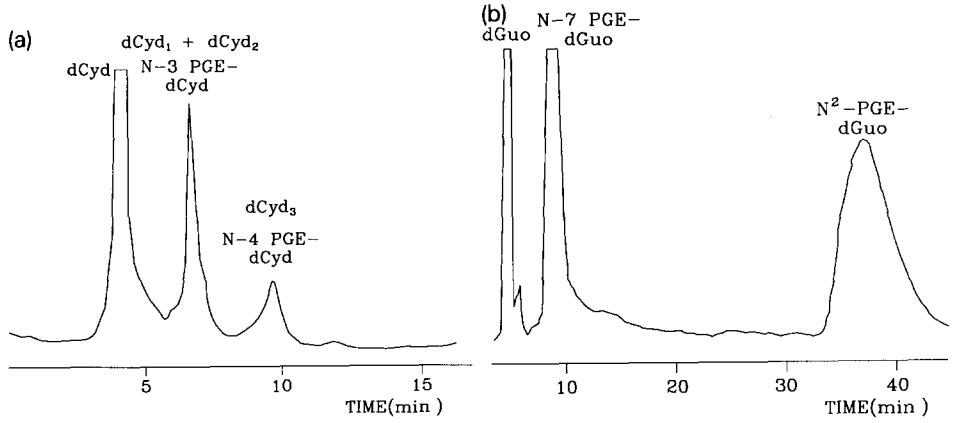

Fig. 4. Preparative HPLC on a polystyrene–divinylbenzene column of (a) dCyd–PGE and (b) dGuo–PGE reaction mixtures. Column, PRP-1 (30.5 cm × 7.0 mm I.D.); eluent for dCyd–PGE, 0.01 *M* ammonium formate (pH 4.25)–methanol (50:50) at 1.6 ml/min; eluent for dGuo–PGE, 0.01 *M* ammonium formate (pH 4.25)–methanol (30:70) at 2.0 ml/min. Concentration of sample: ±30 mg/ml; 0.1 a.u.f.s.

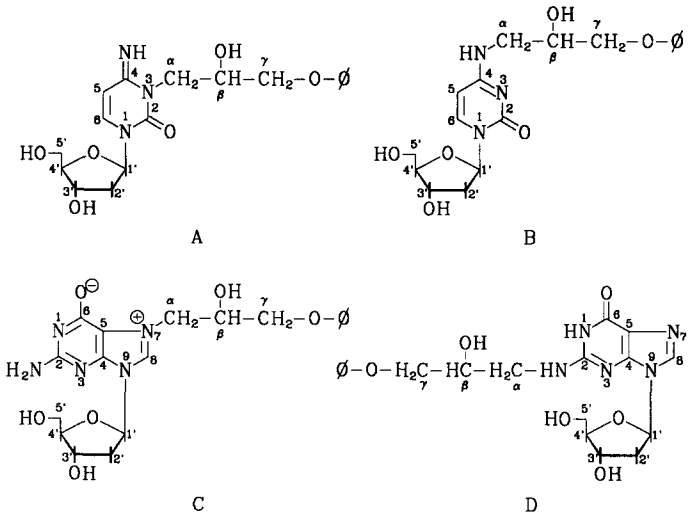

Fig. 5. Proposed structures for dCyd–PGE and dGuo–PGE adducts. (A) Diastereomers of N-3 alkylated dCyd (dCyd 1 and dCyd 2); (B) N^4 alkylated dCyd: (dCyd 3); (C) diastereomers of N-7 alkylated dGuo (dGuo 1 and dGuo 2); (D) N^2 alkylated dGuo: (dGuo 3). ϕ = Phenyl.

aromatic protons 7.282 (8.6), 6.90–6.93; CH(α), 4.243 (−14.6); CH(β), 4.163; $CH_2(\gamma)$–CH(α), 3.95 à 4.0.

The complexicity of the pattern of the proton in the region of 4.24 ppm suggest N^2 alkylation. N^2 alkylation is also consistent with the lower polarity found in the HPLC system and explains why this adduct was not detected on a reversed-phase column when a more polar solvent system than that on the PRP-1 column was used. The NMR spectrum of dGuo 3 reveals only one compound, and the minor amount of dialkylated material shown by FAB-MS is not detected. For dGuo 1 (1′) and dGuo 2 (2′), no NMR data were obtained because of the lack of sufficient material.

The instability of epoxide adducts of deoxyguanosine with trichloropropylene oxide has been observed previously [13]. Hemminki and Lax [31] noted that alkylation at N-7 of guanine in DNA, nucleosides and nucleotides greatly enhances the rate of two secondary reactions, imidazolè ring opening and depurination. These reactions are alkali- and acid-catalysed, respectively, but they are likely to proceed even under physiological conditions. The depurination reaction observed in our experiments could well be catalysed by traces of silica gel eluting from the reversed-phase column and catalysing the reaction during the preparative isolation of the 2′-deoxyguanosine adducts.

CONCLUSION

The proposed HPLC methods on reversed-phase and polystyrene–divinylbenzene columns proved to be efficient for the separation of the adducts formed between PGE and 2′-deoxycytidine and 2′-deoxyguanosine, respectively. These separations have the advantage of a shorter analysis time than existing methods for other adducts of aliphatic epoxides [11,13,31].

Further, the proposed methods separate diastereomers of N-7 alkylated 2′-deoxyguanosine and N-3 alkylated 2′-deoxycytidine. Hemminki and Lax [31] separated the diastereomers of trichloropropylene oxide–2′-deoxyguanosine products which were apolar, but not the polar glycidol–2′-deoxyguanosine adducts.

The use of a polystyrene–divinvylbenzene column showed several advantages. It solved the problem of decomposition of the two N-7 alkylated diastereomers. It also gave more symmetrical peaks than a reversed-phase column. The advantage of reduced tailing with polar samples, especially amines, was also pointed out by Smith [35] for polystyrene–divinylbenzene columns. Further, the use of a polystyrene–divinylbenzene column led to the isolation of one more adduct fraction for the 2′-deoxycytidine–PGE reaction mixture, characterized by MS and NMR as N^4 alkylated material. Also for the 2′-deoxyguanosine–PGE reaction mixture, an additional fraction was detected which appeared to be a mixture of mainly an N^2 alkylation adduct, as suggested by the NMR and UV data and a minor amount of dialkylated material and imidazole ring-opened dialkylated material as indicated by MS.

HPLC with photodiode-array detection combined with off-line UV studies at different pH values proved to be an excellent tool for preliminary identification of the adducts. The structures suggested by these UV data, *i.e.*, N-3-(2-hydroxy-3-phenoxypropyl)-2′-deoxycytidine (two diastereomers), N^4-(2-hydroxy-3-phenoxypropyl)-2′-deoxycytidine and N-7-(2-hydroxy-3-phenoxypropyl)-2′-deoxyguanosine (two diastereomers) and N^2-(2-hydroxy-3-phenoxypropyl)-2′-deoxyguanosine were confirmed by MS data and NMR for the 2′-deoxycytidine products and by MS or NMR for the 2′-deoxyguanosine products.

ACKNOWLEDGEMENT

J.E.S. acknowledges support by Grant ROI ES 03345 from the National Institute of Environmental Health Sciences DHHS. The excellent technical assistance of Hilde Cordemans is gratefully acknowledged. The typing of the manuscript was by C. Rawoens and the drawings by Jef Schrooten (R.U.C.A.), both of whom arc gratefully thanked.

REFERENCES

1 NIOSH, *Criteria for a Recommended Standard, Occupational Exposure to Glycidyl Ethers*, U.S. Department of Education and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Cincinnati, OH, 1978, p. 197.
2 M. M. Manson, *Br. J. Ind. Med.*, 37 (1980) 317–336.
3 L. Ehrenberg and S. Hussain, *Mutat. Res.*, 86 (1981) 1–113.
4 D. R. Wade, S. A. Airy and J. E. Sinsheimer, *Mutat. Res.*, 58 (1978) 217–233.
5 S. W. Frantz and J. E. Sinsheimer, *Mutat. Res.*, 90 (1981) 67–78.
6 S. H. Neau, B. H. Hooberman, S. W. Frantz and J. E. Sinsheimer, *Mutat. Res.*, 93 (1982) 297–304.
7 S. W. Frantz, E. Van den Eeckhout, J. E. Sinsheimer, M. Yashihare and M. Koreeda, *Toxicol. Lett.*, 25 (1985) 265–271.
8 L. B. Rosman, V. G. Beylin, V. Gaddamidi, B. H. Hooberman and J. E. Sinsheimer, *Mutat. Res.*, 171 (1986) 63–70.
9 L. B. Rosman, V. Gaddamidi and J. E. Sinsheimer, *Mutat. Res.*, 189 (1987) 189–204.
10 L. B. Rosman, P. K. Chakraborty, E. A. Messerly and J. E. Sinsheimer, *Mutat. Res.*, 206 (1988) 115–126.

11 Z. Djuric and J. E. Sinsheimer, *Chem. Biol. Interact.*, 50 (1984) 219–231.
12 Z. Djuric and J. E. Sinsheimer, *Chem. Biol. Interact.*, 52 (1984) 243–253.
13 Z. Djuric, B. H. Hooberman, L. Rosman and J. E. Sinsheimer, *Environ. Mutat.*, 8 (1986) 369–383.
14 K. Sugiura and M. Goto, *Chem. Biol. Interact.*, 45 (1983) 153–169.
15 K. Hemminki and H. Vaino, in B. Holmstedt, R. Lauwereys, M. Mercier and M. Roberfroid (Editors), *Mechanism of Toxicity and Hazard Evaluation*, Elsevier/North Holland Biomedical Press, Amsterdam, 1980, pp. 241–243.
16 K. Hemminki, *Arch. Toxicol.*, 52 (1983) 249–285.
17 E. Van den Eeckhout, A. De Bruyn, H. Pepermans, E. Esmans, I. Vrijens, J. Claereboudt, M. Claeys and J. E. Sinsheimer, *J. Chromatogr.*, 504 (1990) 113–128.
18 P. R. Brown (Editor), *HPLC in Nucleic Acid Research*, Marcel Dekker, New York, 1984.
19 B. M. Van Liedekerke, H. J. Nelis, W. E. Lambert and A. P. De Leenheer, *Anal. Chem.*, 61 (1989) 728–732.
20 D. P. Lee and J. H. Kindsvater, *Anal. Chem.*, 52 (1980) 2425–2428.
21 S. Ikuta, R. Chattopadhyaya and R. E. Dickerson, *Anal. Chem.*, 56 (1984) 2253–2256.
22 M. W. Germann, R. T. Pon and J. H. Van de Sande, *Anal. Biochem.*, 165 (1987) 399–405.
23 Y. Kim and P. R. Brown, *J. Liq. Chromatogr.*, 10 (1987) 2411–2422.
24 E. Quintero, R. M. Sheeley, W. J. Hurst and R. A. Martin, *J. Liq. Chromatogr.*, 10 (1987) 2145–2150.
25 C. Lacrois, P. Levert, G. Laine, J. P. Goulle and A. Gringore, *J. Chromatogr.*, 345 (1985) 436–440.
26 A. M. Rustum and N. E. Hoffman, *J. Chromatogr.*, 421 (1987) 387–391.
27 A. M. Rustum and N. E. Hoffman, *J. Chromatogr.*, 426 (1988) 121–128.
28 B. Singer, in G. D. Fasman (Editor), *CRC Handbook of Biochemistry and Molecular Biology*, CRC Press, Cleveland, OH, 1975, pp. 409–447.
29 K. Hemminki, J. Paasvirta, T. Kurkirinne and L. Virkki, *Chem. Biol. Interact.*, 30 (1980) 259–270.
30 A. Albert, in W. W. Zorbach and R. S. Tipson (Editors), *Synthetic Procedures in Nucleic Acid Chemistry*, Vol. 2, Wiley-Interscience, New York, 1971, pp. 47–123.
31 K. Hemminki and M. Lax, *Acta Pharmacol. Toxicol.*, 59 (1986) 80–85.
32 J. Claereboudt, E. Van den Eeckhout, E. Esmans and M. Claeys, *Biomed. Environ. Mass Spectrom.*, in preparation.
33 J. Claereboudt, E. L. Esmans, E. G. Van den Eeckhout and M. Claeys, *Nucleosides Nucleotides*, 9 (1990) 333–344.
34 J. Claereboudt, E. L. Esmans, E. G. Van den Eeckhout, W. Baeten and M. Claeys, *37th Annual Conference on Mass Spectrometry and Allied Topics, Miami Beach, FL, 1989*, Abstracts, p. 923.
35 R. Smith, *J. Chromatogr.*, 291 (1984) 372 376.