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Bioorganic Chemistry 34 (2006) 115–130
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Reaction of epichlorohydrin with adenosine, 2′-deoxyadenosine and calf thymus DNA: Identification of adducts

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Received 11 January 2006
Available online 23 March 2006

Abstract

Epichlorohydrin (a probable human carcinogen) was allowed to react with adenosine and the adducts were characterized by NMR and UV spectroscopy, and mass spectrometry. The adduct initially formed was 1-(3-chloro-2-hydroxypropyl)-adenosine, which subsequently ring closures to 1,N^6-(2-hydroxypropyl)-adenosine at neutral and basic conditions. At acid conditions, the N-1 adduct undergoes a slow deamination to yield 1-(3-chloro-2-hydroxypropyl)-inosine. Minor adducts identified were 7-(3-chloro-2-hydroxypropyl)-adenosine and 3-(3-chloro-2-hydroxypropyl)-adenosine which are easily deglycosylated, and an adduct where the epichlorohydrin residue was attached to the sugar moiety of adenosine. A diadduct, 1,N^6-(2-hydroxypropyl)-N^6-(3-chloro-2-hydroxypropyl)-adenosine was also identified. The reaction of epichlorohydrin with calf thymus DNA gave 1,N^6-(2-hydroxypropyl)-deoxyadenosine and 3-(3-chloro-2-hydroxypropyl)-adenine (major adduct).

Keywords: Epichlorohydrin; Adenosine; Deoxyadenosine; Calf thymus DNA; Structural assignment

1. Introduction

Epichlorohydrin (ECH, Scheme 1) is used as a synthetic intermediate by the industry, primarily for production of epoxy resins, but also for syntheses of glycerine and elasto-

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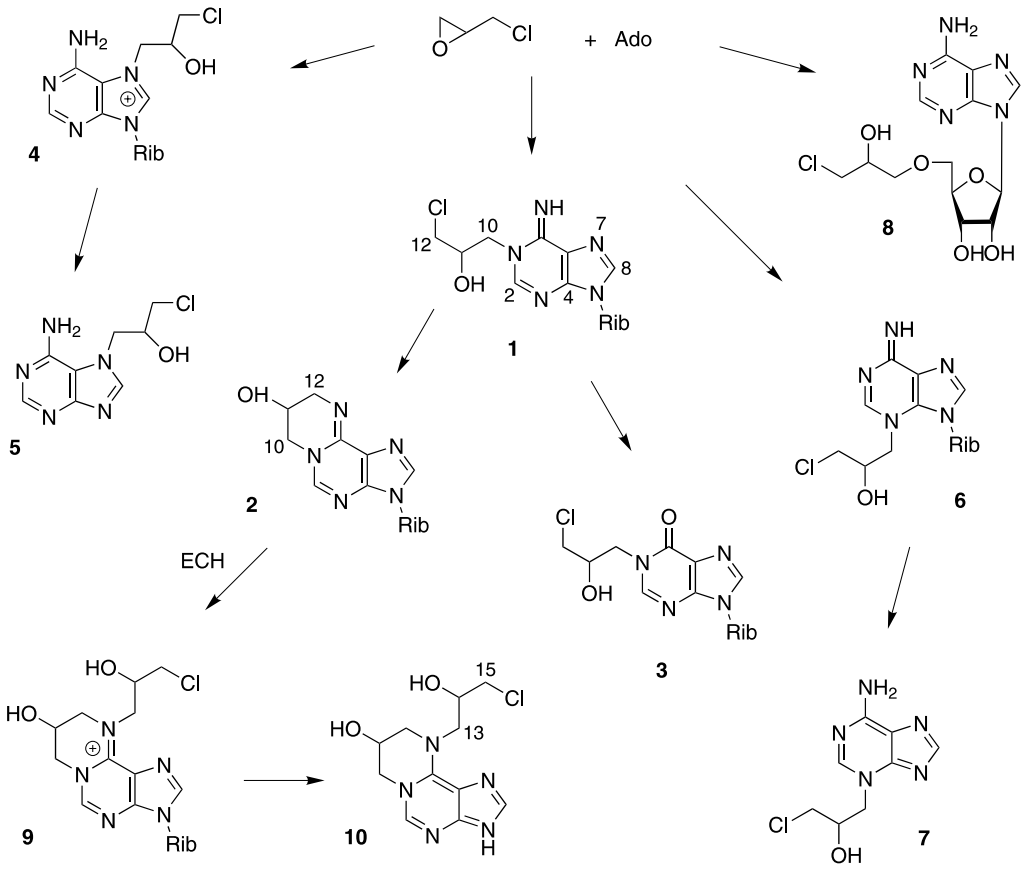
0045-2068/$ - see front matter
doi:10.1016/j.bioorg.2006.01.005



Scheme 1. Identified compounds and reaction paths. Corresponding deoxyribosyl adducts have a "**b**" appended to their number.

mers. The estimated world production [1] in 2003 was 903,000 metric tons, and in the USA 200,000–230,000 tonnes per year from 1989 to 1993 [2]. There is some proof of carcinogenity of ECH in humans [3], and ECH has been found to be carcinogenic in different test systems, causing ECH to be classified as "probably carcinogenic to humans" [4].

A review of the genetic effects of ECH has been written in 1996 by Giri [3]. ECH is a direct acting mutagen. Most tests done with bacterial test systems show mutagenic effects, both with and without metabolic activation. ECH has also been shown to be mutagenic in yeast test systems, and causing chromosomal aberrations in mice. Clastogenic effects among human populations exposed to ECH (0.5–5.0 mg/m^3) have been reported, although there are conflicting results. Cytogenetic tests on human cells and cells from various animals also show clastogenic effects.

ECH is an alkylating agent, prone to attack by nucleophiles on the less substituted ring-carbon causing ring opening. Compared to other epoxides, ECH has been shown to be several times more mutagenic [5]. Plna et al. [6] reports detection of the ECH adduct

7-CHP-guanine in DNA from white blood cells of workers handling ECH, using ^{32}P-post-labelling.

Singh et al. [2], reported on the formation of 1,N^6-(2-hydroxypropano)-2′-deoxyadenosine in the reaction of ECH with 2′-deoxyadenosine and also described the preparation of 3-(3-chloro-2-hydroxypropyl)-adenine from ECH and adenine.

The aim of our work was to do a comprehensive study of the reaction products formed when ECH is allowed to react with adenosine and deoxyadenosine. The deoxyadenosine adducts and the aglycones formed in the reactions were used as reference substances for the identification of adducts formed in the reaction of ECH with calf thymus DNA.

2. Materials and methods

Caution. "Epichlorohydrin has been found to be carcinogenic in mice and rats and has been classified as a probable human carcinogen..."

2.1. Chemicals and materials

Distilled water purified with a Millipore system (Simplicity 185, Billerica, MA, USA) was used in the reactions and for all chromatography. The epichlorohydrin was racemic. Adenosine (adenine-9-β-d-ribofuranoside) was purchased from Fluka AG, Switzerland. Deoxyadenosine (adenine-9-β-d-2′-deoxyribofuranoside) and calf thymus DNA (deoxyribonucleic acid, sodium salt) were purchased from Sigma–Aldrich Chemie, Germany.

2.2. Chromatographic methods

Analytical HPLC was performed on an Agilent 1100 Series Liquid Chromatograph equipped with a quaternary pump, a diode array detector (used wavelength range: 190–400 nm) and the Chemstation software. The column employed was a Hypersil BDS-C18, 5 μm, 4.0 × 125 mm. It was eluted isocratically for 2 min with 2% acetonitrile in 10 mM ammonium acetate (pH 7) and then with a gradient from 2 to 20% over the course of 18 min at a flow rate of 0.5 mL/min. The UV spectra were recorded while the compounds eluted from the column.

Semipreparative HPLC was performed on a Varian 5000 Liquid Chromatograph equipped with a UV detector working at 254 nm. Unless otherwise stated, the column employed was a Hypersil 8 μm Hyperprep ODS, 10 × 250 mm. It was eluted isocratically for 5 min with 2% acetonitrile in 10 mM phosphate buffer (pH 4.6) and then with a gradient from 2 to 20% over the course of 20 min at a flow rate of 3 mL/min.

2.3. Spectroscopic and spectrometric methods

The mass spectrometry analyses were performed on an Agilent 1100 Series LC/MSD SL Trap system (Agilent Technologies, Espoo/Esbo, Finland), which was equipped with an API electrospray interface and operated in the positive ion mode. Nitrogen was used as nebulizer gas (40 psi) and as drying gas (12 L/min). The drying gas was heated to 350 °C. The capillary exit offset had a value of 118.0 V and skim 1 was set at 40.0 V. In

the scan mode, the maximum ion accumulation time was 10 ms and the target (ion) value was 20,000. In the selected reaction monitoring (SRM) mode, the accumulation time was 20 ms and the target value was 50,000 ions. Scanning from m/z 100 to 500 was applied for recording of the full scan mass spectra. Collision induced dissociation (CID) experiments were coupled with multiple tandem mass spectrometry (MS^n), and employed helium as collision gas. The fragmentation amplitude was 1.0 V. An Agilent 254 nm wavelength UV detector was also coupled to the system, and the LC conditions were identical to the analytical HPLC described above, with the exception of the pump, which was of binary type.

LC-ESI-MS/MS analyses of DNA adducts were performed on a triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ES interface. The source block temperature was 120 °C and the desolvation temperature was 325 °C. Nitrogen was used as the desolvation gas (615 L/h) and the cone gas (33 L/h). Argon was used as the collision gas, at a collision cell pressure of 4.5×10^{-3} mbar. Positive ions were acquired in MRM mode with a dwell time of 0.4 s and interchannel delay of 0.05 s. The HPLC separations were performed on the Agilent 1100 system (Agilent Technologies, Espoo/Esbo, Finland) consisting of a binary pump, a vacuum degasser, an autosampler, and a thermostatted column oven. The DNA samples were chromatographed on a 5 μm, 4×125-mm reversed phase C18 analytical column (Hypersil BDS-C18, Agilent Technologies, Espoo/Esbo, Finland). The column was eluted with a gradient consisting of 10 mM ammonium acetate and acetonitrile. A gradient was applied that started from 1% acetonitrile and ended after 20 min at 30% acetonitrile. The flow rate was 0.5 mL/min. The adduct **7** was detected by monitoring the protonated molecular ion peak at m/z 228 and the fragment peak at m/z 136 corresponding to the cleavage of the 3-chloro-2-hydroxy-propyl group from the protonated molecular ion. The adduct **2b** was detected by monitoring the protonated molecular ion peak at m/z 308 and the cleavage of the deoxyribosyl moiety (116 amu) from the protonated molecular ion. The cone voltage was held at 28 V and the collision energy was 22 eV.

The electron impact high-resolution mass spectra were recorded on a Fisons ZABSpec-oaTOF instrument. The spectra were acquired using a direct insert probe and scanning from 50 to 1500 amu and using electron ionization (EI) at 70 eV energy. Accurate mass measurements were performed using a peak matching technique with PFK as a reference substance at a resolution of 8000–10000 (at 10% peak height). All measured substances but **8** and **10** were trimethylsilylated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

The high-resolution mass spectrum for compound **10** was recorded on a LC/MSD TOF mass spectrometer (Agilent G1969, Santa Clara, CA, USA). The system was operated in the positive ion mode, and it was tuned and calibrated using the automated functions, CheckTune and Calibrate, using Agilent TOF Electrospray Calibrant Solution (G1969-85001). The pressure of the nebulizer gas was 35 psig and the drying gas flow was 12 L/min. The drying gas was heated to 300 °C. Scanning from m/z 100 to 1000 was applied for recording of the full scan mass spectra. The fragmentor voltage was 150 V, skimmer voltage was 60 V and Oct RF was 250 V. The infusion rate was 10 μL/min, and masses were measured as an average of 11 measurements, with reference masses at 121.050873 and 922.009798.

To prepare NMR samples, the solutions of the pure compounds were evaporated to dryness, then residual water was removed by coevaporation twice with 5 mL acetonitrile.

DMSO-d_6, 800 μL, was added and the solutions were filtered trough a coarse-porosity sintered glass filter into NMR tubes.

The NMR spectra were recorded on a JEOL JNM A 500 spectrometer operating at 500.16 MHz for ^{1}H and 125.78 MHz for ^{13}C (JEOL, Tokyo, Japan). The spectra of compounds **9**, **9b**, and **10** were recorded on a Bruker Avance 600 spectrometer operating at 600.13 MHz for ^{1}H and 150.90 MHz for ^{13}C (Bruker Biospin Corporation). The solvent was DMSO-d_6, residual undeuterated solvent was used as internal reference for ^{1}H NMR (at 2.50 ppm). For ^{13}C NMR the solvent signal was used as reference (at 39.5 ppm). The ^{1}H NMR signal assignments were based on chemical shifts and ^{1}H–^{1}H and ^{13}C–^{1}H correlation data. Assignment of carbon signals was based on chemical shifts and ^{13}C–^{1}H correlations.

2.4. Calculation of yields

Product yields before purification were determined in the following way: quantitative ^{1}H NMR was performed, using residual undeuterated solvent as an internal standard (the concentration of undeuterated solvent was determined for the solvent lot used). HPLC standard solutions were prepared by diluting aliquots from the NMR samples. The standards and the reaction mixtures were analyzed by HPLC, and the peak areas in standards and reaction mixtures were compared at 254 nm.

The standard solutions from the NMR samples were also used for quantification by comparing the MS peak areas of the pure compounds with the areas in the DNA hydrolysate (the external standard method). To avoid MS nonlinearity effects, care was taken to use a standard with a similar adduct concentration to the concentration of the adduct in the DNA hydrolysate.

2.5. Small-scale reaction of epichlorohydrin with adenosine and 2′-deoxy-adenosine (done to observe adducts)

Adenosine, 225.7 mg (0.85 mmol) was reacted with 100 μl (1.3 mmol) ECH in 20 mL of 0.5 M phosphate buffer at room temperature (pH 4.7). The reaction with 2′-deoxy-adenosine (10.2 mg, 0.04 mmol) was carried out in 5 mL of 0.5 M phosphate buffer (pH 7) at 37 °C. Aliquots of the reactions were analyzed by LC-MS (ion trap) using the C18 analytical column.

2.5.1. Preparation of 1-(3-chloro-2-hydroxypropyl)-adenosine (*1*)

Ado, 502 mg (1.9 mmol), was dissolved in 25 mL of 0.5 M potassium phosphate buffer at pH 3.2, and 1.5 mL (19 mmol) ECH was added. After 48 h at 37 °C, the reaction mixture was purified using two consecutive semipreparative HPLC runs, first on a Hypersil 8 μm Hyperprep ODS column with the buffer at pH 4.6 and then on a RP-18, 7 μm column with the buffer at pH 2.6. The size of both columns was 10×250 mm and the buffer was 10 mM phosphate buffer. All fractions containing **1** were pooled and made into a NMR-sample. Yield before purification of the reaction mixture: 16%. UV (H_2O, pH 7): λ_{max} 211 and 259 nm; λ_{min} 235 nm. ESI$^+$-MS: *m*/*z* 362/360 (33/100%, M+H$^+$). ESI$^+$-MS^2 of *m*/*z* 360: 228 (100%, M+H$^+$ − ribosyl+H). ESI$^+$-MS^3 of *m*/*z* 360 → 228: 136 (100%, Ade+H$^+$), 120 (16). EI-HRMS (compound derivatized with BSTFA): *m*/*z* 647.2569 (M$^+$, $C_{25}H_{50}N_5O_5Si_4Cl$ calcd 647.2578). ^{1}H NMR (500.16 MHz, mix of two

diastereomers): δ 8.74 (s, 1H, H-8), 8.54 (s, 1H, H-2), 5.94 (d, H-1′), 4.49 (t, H-2′), 4.45 (d, H-10a), 4.24 (dd, 1H, H-10b), 4.15 (q, 1H, H-3′), 4.07 (m, 1H, H-11), 3.98 (q, 1H, H-4′), 3.84 (dd, 1H, H-12a), 3.73 (dd, 1H, H-12b), 3.67 (dd, 1H, H-5′a), 3.57 (dd, 1H, H-5′b). ^{13}C NMR (125.78 MHz, mix of two diastereomers): δ 150.5 (C-6), 148.6 (C-2), 147.0 (C-4), 142.4 (C-8), 119.3 (C-5), 87.8 (C-1′), 86.0(C-4′), 74.4 (C-2′), 70.1 (C-3′), 66.5 (C-11), 61.0 (C-5′), 52.9 (C-10), 47.1 (C-12).

*2.5.2. Formation of 1,N^6-(2-hydroxypropyl)-adenosine (**2**)*

An NMR-tube containing **1** (in DMSO-d_6) was held at 37 °C for 5 days, causing ring closure. UV (H_2O, pH 7): λ_{max} 213 and 264 nm; λ_{min} 236 nm. ESI$^+$-MS: *m/z* 324 (100%, M+H$^+$). ESI$^+$-MS^2 of *m/z* 324: 192 (100%, M+H$^+$ − ribosyl+H). ESI$^+$-MS^3 of *m/z* 324 → 192: 148 (100%). ^{1}H NMR (500.16 MHz, mix of two diastereomers): δ 8.77 (t, 1H, H-8), 8.65 (s, 1H, H-2), 5.94 (dd + dd, H-1′), 4.51 (dtd, 1H, H-2′), 4.46 (m, H-11), 4.42 (t + t, H-10a), 4.31, (d, 1H, H-10b), 4.16 (t, 1H, H-3′), 3.98 (q, 1H, H-4′), 3.67 (dd, 1H, H-5′a), 3.57 (H-5′b), 3.57 (H-12a), 3.42 (d, 1H, H-12b). ^{13}C NMR (125.78 MHz, mix of two diastereomers): δ 147.6 (C-2), 147.0 (C-6), 146.0 (C-4), 142.5 and 142.6 (C-8), 118.5 and 118.6 (C-5), 87.8 and 87.9 (C-1′), 86.0 (C-4′), 74.3 and 74.6 (C-2′), 70.6 (C-3′), 61.0 (C-5′), 56.7 (C-11), 52.4 (C-10), 45.0 (C-12).

*2.5.3. Preparation of 1-(3-chloro-2-hydroxypropyl)-inosine (**3**)*

Ado, 225.7 mg (0.85 mmol), was dissolved in 20 mL of 0.5 M potassium phosphate buffer at pH 4.6. ECH was added, 50 μL at a time, for 3 weeks until a total of 280 μL (3.6 mmol) had been added. The reaction mixture was held at room temperature and the proceeding of the reaction was followed by HPLC analyses. The reaction continued for a further 3 weeks after which the temperature was elevated to 37 °C and the reaction was allowed to proceed for 17 days more. The reaction mixture was purified using semipreparative HPLC and phosphate buffer as mobile phase at pH 7 (10 mM). All fractions containing **3** were pooled and made into a NMR-sample. The yield before purification of the reaction mixture was 4%. UV (H_2O, pH 7): λ_{max} 205, 250, and 274 nm (shoulder); λ_{min} 227 nm. ESI$^+$-MS: *m/z* 363 (25%, M+H$^+$), 361 (100, M+H$^+$), 231 (24, M+H$^+$ − ribosyl+H), 229 (84, M+H$^+$ − ribosyl+H). ESI$^+$-MS^2 of *m/z* 361: 229 (100%, M+H$^+$ − ribosyl+H). ESI$^+$-MS^3 of *m/z* 361 → 229: 211 (100%), 175 (8), 137 (60, hypoxanthine+H$^+$). Both diastereomers give similar mass spectra. EI-HRMS (compound derivatized with BSTFA: *m/z* 648.2409 (M$^+$, $C_{25}H_{49}N_4O_6Si_4Cl$ calcd 648.2418). ^{1}H NMR (500.16 MHz, mix of two diastereomers): δ 8.34 (s, 1H, H8), 8.24 (s, 1H, H2), 5.83 (d, 1H, H1′), 5.60 (d, 1H, 11-OH), 5.53 (d + d, 1H, 2′-OH), 5.19 (d + d, 1H, 3′-OH), 5.06 (t + t, 1H, 5′-OH), 4.49 (dd + dd, 1H, H-2′), 4.34 (t + t, 1H, H-10a), 4.13 (dd, 1H, H-3′), 4.00 (m, 1H, H-11), 3.95 (q, 1H, H-4′), 3.80 (dd + dd, 1H, H-10b), 3.70 (dd + dd, H-12a), 3.65 (m, H-5′a), 3.62 (dd + dd, H-12b), 3.56 (m, 1H, H-5′b).

*2.5.4. Preparation of 7-(3-chloro-2-hydroxypropyl)-adenosine and 3-(3-chloro-2-hydroxypropyl)-adenosine (**4** and **6**)*

Ado, 535.3 mg (2.0 mmol), was dissolved in 40 mL of 0.5 M potassium phosphate buffer at pH 4.6, and 470 μL (6.0 mmol) ECH was added. The reaction was held at room temperature for two days and then evaporated to 4 mL, allowed to stay in room

temperature overnight and then filtered. The filtrate was purified using semipreparative HPLC. The mobile phase consisted of acetonitrile and phosphate buffer (10 mM) at pH 4.1. The acetonitrile concentration was held at 2% for the first 5 min, then a gradient was applied until the concentration reached 12% at 24 min. Fractions containing **4** and **6** were collected separately. UV and MS data for compound **4** (two diastereomers): UV (H_2O, pH 7): λ_{max} 212 and 272 nm; λ_{min} 233 nm. ESI^+-MS: *m/z* 362/360 (33/100%, $M+H^+$), 230/228 (22/78, $M+H^+$ – ribosyl+H). ESI^+-MS^2 of *m/z* 360: 228 (100%, $M+H^+$ – ribosyl+H). ESI^+-MS^3 of *m/z* 360 → 228: 211 (40%), 210 (8), 193 (3), 192 (7), 174 (23), 119 (8), 136 (100, Ade+H^+). ESI^+-MS^4 of *m/z* 360 → 228 → 211: 119 (100%), 93 (37). UV and MS data for compound **6** (two diastereomers): UV (H_2O, pH 7): λ_{max} 211 and 273 nm; λ_{min} 237 nm. ESI^+-MS: *m/z* 362/360 (30/100%, $M+H^+$). ESI^+-MS^2 of *m/z* 360: 228 (100%, $M+H^+$ – ribosyl+H). ESI^+-MS^3 of *m/z* 360 → 228: 136 (100%, Ade+H^+).

*2.5.5. Preparation of 7-(3-chloro-2-hydroxypropyl)-adenine and 3-(3-chloro-2-hydroxypropyl)-adenine (**5** and **7**)*

Compounds **4** and **6** were deglycosylated in acid solution, then the volume was reduced to 5 ml by rotary evaporation. The aglycons were purified by applying identical HPLC conditions as for the purification of **4** and **6**, but for **7** the gradient was allowed to rise to 7% at 20 min. All fractions containing **5** and **7** were pooled and made into NMR-samples. The yield of **5** before purification of the reaction mixture was 0.8%. Spectroscopic and spectrometric data for compound **5**: UV (H_2O, pH 7): λ_{max} 211 and 271 nm; λ_{min} 233 nm. EI-HRMS (compound derivatized with BSTFA): *m/z* 371.1362 (M^+, $C_{14}H_{26}N_5OSi_2Cl$ calcd 371.1364). ^1H NMR (500.16 MHz): δ 8.17 (s, 1H, H-8), 8.19 (s, 1H, H-2), 6.85 (s, 2 H, NH_2), 5.90 (d, 1H, 11-OH), 4.57 (dd, 1H, H-10a), 4.25 (dd, 1H, H-10b), 3.96 (m, 1H, H-11), 3.75 (dd, 1H, H-12a), 3.65 (dd, 1H, H-12b). ^{13}C NMR (125.78 MHz): δ 152.0 (C-8), 146.6 (C-2), 69.8 (C-11), 49.5 (C-10), 47.0 (C-12). C-4, C-5, and C-6 not observed. The yield of **7** before purification of the reaction mixture was 0.2%. Spectroscopic and spectrometric data for **7**: UV (H_2O, pH 7): λ_{max} 213 and 276 nm; λ_{min} 246 nm. EI-HRMS (compound derivatized with BSTFA): *m/z* 371.1343 (M^+, $C_{14}H_{26}N_5OSi_2Cl$ calcd 371.1364) and *m/z* 336.1674 (M^+–Cl, $C_{14}H_{26}N_5OSi_2$ calcd 336.1676). ^1H NMR (500.16 MHz): δ 8.19 (s, 1H, H-8), 7.89 (br, 2 H, NH_2), 7.75 (s, 1H, H-2), 5.78 (br, 11-OH), 4.53 (dd, 1H, H-10a), 4.26 (m, 1H, H-11), 4.15 (dd, 1H, H-10b), 3.73 (dd, H-12a), 3.64 (dd, H-12b). The assignments are based on Singh et al. [2].

*2.5.6. Preparation of $O^{5'}$-(3-chloro-2-hydroxypropyl)-adenosine (**8**)*

Ado, 505.6 mg (1.9 mmol), was dissolved in 25 mL of 0.5 M potassium phosphate buffer at pH 3.2, and 1.5 mL (19 mmol) ECH was added. After 48 h at 37 °C the reaction mixture was purified using two consecutive semipreparative HPLC runs, first on a Hypersil 8 μm Hyperprep ODS column with 10 mM phosphate buffer at pH 4.6, and then on a 10 × 250 mm Thermo Hypersil Keystone BDS C18 column, using water and acetonitrile as mobile phase. In both cases, the acetonitrile concentration was held at 2% for the first 5 min, then a gradient was applied until the concentration reached 20% at 20 min. All fractions containing **8** were pooled and made into a NMR-sample. The yield of **8** before purification of the reaction mixture was 0.05%. Spectroscopic and spectrometric data (two diastereomers): UV (H_2O, pH 7): λ_{max} 208 and 260 nm; λ_{min} 228 nm. ESI^+-MS: *m/z*

362/360 (42/100%, M+H$^+$). ESI$^+$-MS^2 of *m/z* 360: 136 (100%, Ade+H$^+$). EI-HRMS: *m/z* 359.1000 (M$^+$, $C_{13}H_{18}N_5O_5Cl$ calcd 359.0996). ^{1}H NMR (500.16 MHz, mix of two diastereomers): δ 8.33 (s + s, 1H, H-2), 8.15 (s, 1H, H-8), 7.24 (br, 2 H, NH_2), 5.9 (d, 1H, H1′), 5.47 (d, 1H, 2′-OH), 5.32 (dd, 1H, 11-OH), 5.24 (d, 1H, 3′-OH), 4.58 (qd, 1H, H-2′), 4.17 (qd, 1H, H-3′), 4.02 (q, 1H, H-4′), 3.83 (qd, 1H, H-11), 3.68 (dt, 1H), 3.62 (m, 2 H), 3.53 (ddd).

2.5.7. Preparation of 1,N^6-(2-hydroxypropyl)-N^6-(3-chloro-2-hydroxypropyl)-adenosine (**9**)

Ado, 408 mg (1.5 mmol), was dissolved in 6.66 mL of 0.1 M phosphate buffer at pH 7, and 0.93 mL (12 mmol) ECH was added. After 12 h at 60 °C (stirred), the reaction was washed with 8 ml toluene, lightly evaporated, filtered, and purified using semipreparative HPLC using isocratic elution with 10 mM phosphate buffer at pH 2.6. The yield of **9** after this purification step was 15%. The fractions containing **9** were pooled, and the pH was adjusted to 7 and further purified on a 10 × 250 mm Thermo Hypersil Keystone BDS C18 column with 10 mM ammonium acetate at pH 7 as the mobile phase (isocratic elution). The fractions containing **9** were then desalted on the same column with a mobile phase of 2 mM ammonium acetate. All fractions containing **9** were pooled and made into a NMR-sample. Spectroscopic and spectrometric data of compound **9** (mix of four diastereomers): UV (H_2O, pH 7): λ_{max} 204, 218, and 274 nm; λ_{min} 242 nm. ESI$^+$-MS: *m/z* 416 (100%, M+H$^+$), 418 (50, M+H$^+$). ESI$^+$-MS^2 of *m/z* 416: 284 (100%, M+H$^+$ − ribosyl+H). ESI$^+$-MS^3 of *m/z* 416 → 284: 192 (100%, 1,N^6-HP-Ade+H$^+$). ^{1}H NMR (600.13 MHz, mix of four diastereomers, only assigned peaks listed): δ 8.80 and 8.81 (m, 1H, H-8), 8.67 and 8.68 (m, 1H, H-2), 5.96 and 5.97 (m, 1H, H-1′). ^{13}C NMR (150.90 MHz, mix of four diastereomers, only assigned peaks listed): δ 147.83 and 147.96 (C-2), 147.14 and 147.16 (C-4), 146.25, 146.26, and 146.55 (C-6), 140.78, 140.88, 141.01, and 141.14 (C-8), 118.30, 118.34, 118.36, and 118.42 (C-5), 87.30, 87.39, 87.45, and 87.46 (C-1′).

2.5.8. Preparation of 1,N^6-(2-hydroxypropyl)-N^6-(3-chloro-2-hydroxypropyl)-adenine (**10**)

The aglycon **10** was prepared from the dAdo derivative **9b** which in turn was prepared by essentially the same procedure as was used for the preparation of **9**. The yield of the **9b** before purification was 15%. The deglycosylation was achieved by storage of **9b** at pH 2 at 70 °C for 19 h. The pure compound **10** was obtained by semipreparative HPLC fractionation using 2 mM ammonium acetate as mobile phase at pH 7 (isocratic elution). All fractions containing **10** were pooled and made into a NMR-sample. The yield of **10** after purification was 5.5%. Spectroscopic and spectrometric data of compound **9b** (four diastereomers): UV (H_2O, pH 7): λ_{max} 215 and 268 nm; λ_{min} 237 nm. ESI$^+$-MS: *m/z* 400 (100%, M+H$^+$), 402 (33, M+H$^+$). Spectroscopic and spectrometric data of compound **10** (two diastereomers): UV (H_2O, pH 7): λ_{max} 215, 237 (shoulder), and 283 nm; λ_{min} 248 nm. ESI$^+$-MS: *m/z* 284 (100%, M+H$^+$), 286 (36, M+H$^+$). ESI$^+$-MS^2 of *m/z* 284: 192 (100%, 1,N^6-HP-Ade$^+$), 248 (23), 148 (20). ESI$^+$-MS^3 of *m/z* 284 → 192: 148 (100%), 137 (64). ESI-HRMS: *m/z* 284.0917 (M+H$^+$, $C_{11}H_{15}N_5O_2Cl$ calcd 284.0914). ^{1}H NMR (600.13 MHz,): δ 8.13 (s, 1H, H-2), 7.86 (s, 1H, H-8), 6.12 (br, 1H, 11-OH or 14-OH), 5.71 (br, 1H, 11-OH or 14-OH), 4.79 (dd, 1H, H-13a), 4.40 (m, 1H, H-11), 4.22 (s, 2 H, H-10a, H-10b), 4.17 (m, 1H, H-14), 3.99 (dd, 1H, H-13b), 3.82 (dd, 1H, H-12a), 3.77 (dd, 1H, H-15b), 3.68 (dd, 1H, H-15a),

3.47 (d, 1H, H-12b). ^{13}C NMR (150.90 MHz,): δ 158.3 (C-4), 153.4 (C-8), 143.7 (C-6), 141.0 (C-2), 117.8 (C-5), 68.0 (C-14), 57.0 (C-11), 54.7 (C-13), 53.5 (C-12), 50.7 (C-10), 47.1 (C-15).

2.6. Reaction of ECH with calf thymus DNA

ECH, 20 μL (0.26 mmol) was reacted with double-stranded calf thymus DNA (4.7 mg) in 5 mL of 0.1 M phosphate buffer (pH 7.4) at 37 °C. The mixture was allowed to react for 44 h and then extracted with diethyl ether (3 × 5 mL). The DNA was precipitated by addition of 5 M NaCl (1 mL), cold ethanol (15 ml), and the solution was cooled at −20 °C. The mixture was centrifuged (10 min at 900*g*) and the supernatant was collected. The supernatant was concentrated by rotary evaporation to 1.3 ml and analyzed by LC-MS (Agilent 1100 LC/MSD SL Trap). The recovered DNA was washed with cold 70% ethanol (5 mL), cold ethanol, and dissolved in H_2O (5 mL). The DNA was reprecipitated from the solution by addition of cold ethanol (15 mL), cooling to −20 °C, and recovered by centrifuging. The DNA was dried a few minutes in vacuo and dissolved in 3 mL of 100 mM bis-Tris buffer (pH 6.5) containing 2 mM $MgCl_2$. To the solution was added 0.5 mg nuclease P1 dissolved in 0.5 mL of 1 mM $ZnCl_2$ to obtain a concentration of 50 U/mL. The mixture was incubated at 37 °C for 4 h. Finally, bacterial alkaline phosphatase was added to give a final concentration of 7 U/mL, and wheat germ acid phosphatase (dissolved in bis-Tris–$MgCl_2$ buffer at a concentration of 10 mg/mL) to obtain a final concentration of 0.4 U/mL. The mixture was incubated at 37 °C for 18 h. The enzyme digest mixture was loaded into a prerinsed (H_2O) Centricon YM-3 filter (3000 NMWL cutoff) and centrifuged for 5 h at 900*g*. The ultrafiltrate was recovered and concentrated by rotary evaporation to near dryness and reconstituted in 1600 μL of a mixture of 20% methanol in water. The resulting solution was analyzed by LC-MS (Micromass, Manchester, UK).

2.6.1. Determination of rate constants at different pH for formation of 1,N^6-(2-hydroxypropyl)-adenosine (**2**) from 1-(3-chloro-2-hydroxypropyl)-adenosine (**1**)

Compound **1** was dissolved in phosphate buffer solutions at pH 3, 4, 5, 6, 7, and 8, temperature 24–26 °C, and the formation of **2** was followed by HPLC analyses with UV detection at 270 nm. A total number of nine samples from each buffer solution were analysed and one aliquot was taken from each solutions every 2 h. The resulting chromatograms were integrated, and the integral for compound **2** was divided with the integral for **2** when the reaction was complete (this value was taken from the last sample from the reaction at pH 8). Nonlinear curve fitting (Scientific Python, version 2.4.6, Levenberg–Marquardt algorithm) was then employed to fit the integral ratios for compound **2** to the model, which was the integrated rate equation for first order reactions:

$$\frac{[P]}{[P_0]} = 1 - e^{-k1*t},$$

where [P] is the product concentration, and $[P_0]$ is the product concentration when the reaction is complete.

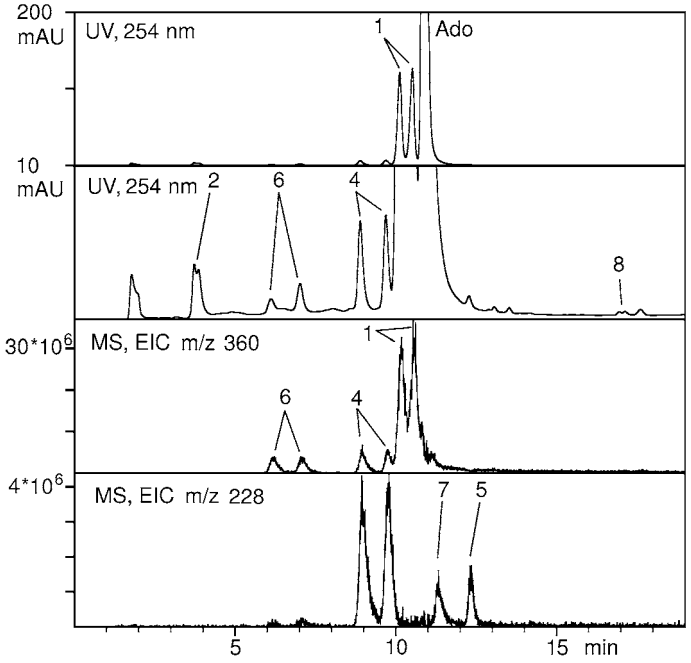


Fig. 1. LC-MS run of the reaction mixture of Ado and ECH held at pH 4.6, and room temperature. The two topmost chromatograms are 254 nm UV traces at different absorbance scale and the two following chromatograms are extracted ion current (EIC, extracted by software from mass scan data) MS chromatograms, all from the same run. The EIC chromatogram of *m*/*z* 360 represents open side-chain adenosine adducts, the lowest EIC chromatogram of *m*/*z* 228 represents open side-chain adenine adducts. The *m*/*z* 228 peaks corresponding to adduct **4** (at 9.1 and 9.9 min) are due to fragmentation in the MS source, indicating that adduct **4** is easily deglycosylated.

3. Results and discussion

HPLC analyses of the small-scale reaction of adenosine and epichlorohydrin showed the formation of seven product peaks (**1**, **2**, **4**, **5**, **6**, **7**, and **8**, Fig. 1). The corresponding product peaks were found in reaction of dAdo and epichlorohydrin (Fig. 2). The initially observed product from the reaction of epichlorohydrin and Ado/dAdo performed at neutral conditions was 1,N^6-(hydroxypropano-)-derivatives (**2** and **2b**), previously identified by Singh et al. [2]. In the reaction performed at acid condition the initially formed adduct was found to be 1-(3-chloro-2-hydroxypropyl)-adenosine (**1**). The compound is formed by attack from the nucleophilic 1-position of adenine on the β-carbon in the oxirane ring [7]. This compound was found to undergo a very fast cyclization to 2 upon storage at neutral and basic conditions. The cyclization is slower at low pH (see Table 1)—indicating that the cyclization proceeds via Dimroth rearrangement, which occurs faster at high pH conditions.

Compound **1** was identified on the basis of the HMBC correlations from C-2 and C-6 to H-10 and from C-10 to H-2, which shows that the side chain is attached to N-1 (Table 2). The presence of the chlorine atom was evident from the isotope pattern in the mass spectrum. Also supporting this conclusion is the fact that the UV-spectrum of **1** shows

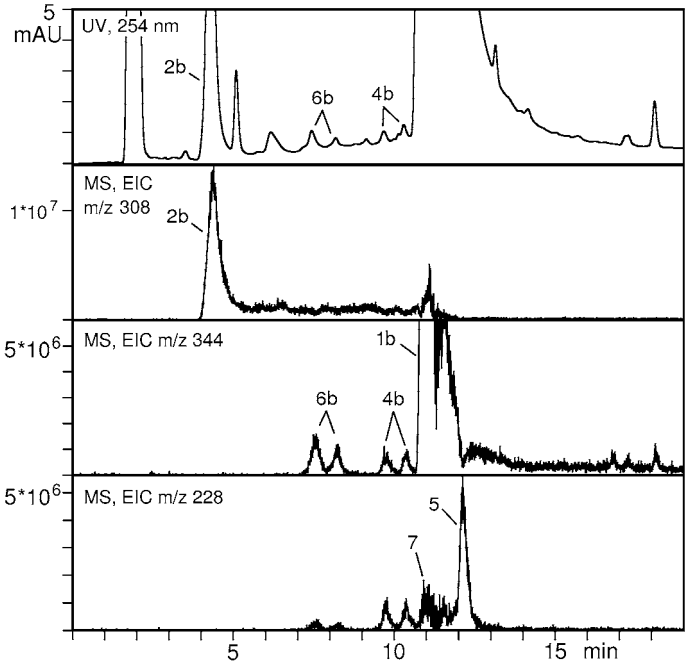


Fig. 2. LC-MS run of the reaction mixture of dAdo and ECH held at pH 7, and 37 °C. The topmost chromatogram is the 254 nm UV trace and the three following chromatograms are extracted ion current (EIC, extracted by software from mass scan data) MS chromatograms, all from the same run. The EIC chromatogram of *m*/*z* 308 represents compound **2b**, the next chromatogram (*m*/*z* 344) represents open side-chain 2′-deoxyadenosine adducts, while the last chromatogram (*m*/*z* 228) represents open side-chain adenine adducts. The *m*/*z* 228 peaks corresponding to adduct **4b** (at 9.7 and 10.3 min) are due to fragmentation in the MS source, indicating that adduct **4b** is easily deglycosylated.

Table 1
Rate constant for the formation of **2** from **1** at different pH (room temperature)

pH	Rate constant $k1$ (h^{-1})	Half life (h)
3	3.6×10^{-5}	19400
4	0.00015	4500
5	0.00094	740
6	0.01	110
7	0.07	10
8	0.28	2.5

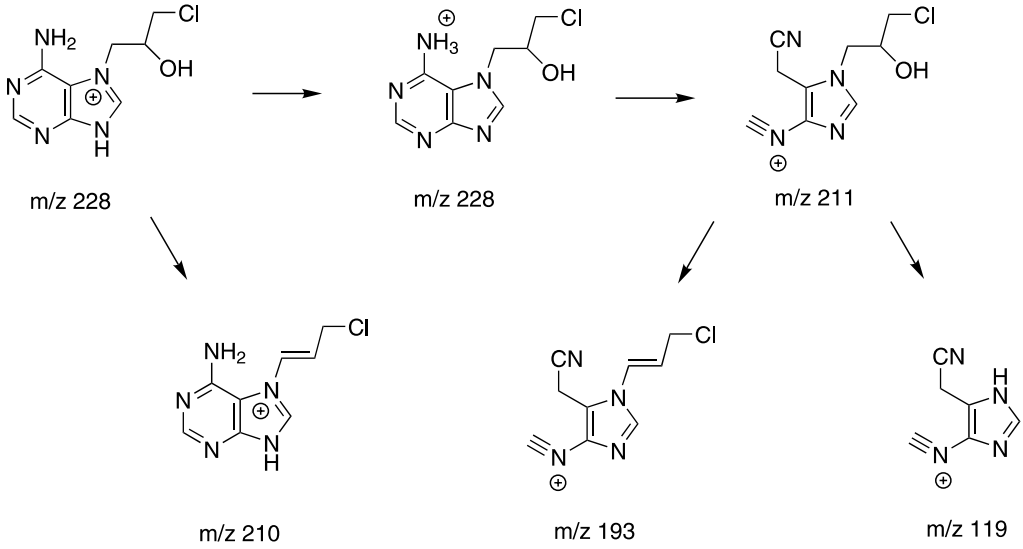
λ_{max} and λ_{min} at 259 and 235 nm, close to those of the compound [8] 1-(3-chloro-2-hydroxy-3-butene-1-yl)-2′-deoxyadenosine, having λ_{max} and λ_{min} at 262 and 235 nm. It is also consistent with N-1-adducts from adenosine and butadiene monoxide having λ_{max} at 260 nm [9].

When compound **1** is stored for prolonged times (6 weeks) at pH 4.7 it is converted to 1-(3-chloro-2-hydroxypropyl)-inosine (**3**). This slow deamination of the adenine base can be explained by steric effects of the $-CH_2-Cl$ group similar to those observed for the phenyl group in 1-(2-hydroxy-2-phenylehtyl)adenosine [10,11]. On the contrary, adenosine

Table 2
Some critical NMR shifts and correlations

Compound	Proton	δ (ppm)	Int.	Mult.	H–H correlations	Carbon	δ (ppm)	C–H correlations
1	H-8	8.74	1	s		C-8	142.4	H-8, H-1′
1	H-2	8.54	1	s		C-2	148.6	H-2, H-10a, H-10b
1						C-6	150.5	H-2, H-10b
1						C-4	147.0	H-8, H-2, H-1′
1						C-5	119.3	H-8
1	H-10a	4.45		d	H-10b	C-10	52.9	H-10a, H-10b, H-2, H-12a
2	H-8	8.77	1	t		C-8	142.6, 142.5	H-8
2	H-2	8.65	1	s		C-2	147.6	H-2
2						C-6	147.0	
2						C-4	146.0	H-2
2						C-5	118.6, 118.5	H-8
3	H-8	8.34	1	s	H-1′			
3	H-2	8.24	1	s	H-10a, H-10b			
3	H-10a	4.34	1	t + t	H-10b, H-2			
3	H-10b	3.80	1	dd + dd	H-10a, H-11, H-2			
5	H-8	8.17	1	s	H-10a, H-10b	C-8	152.0	H-8
5	H-2	8.19	1	s	NH_2	C-2	146.6	H-2
5	NH_2	6.85	2	s	H-2			
5	H-10a	4.57	1	dd	H-10b, H-11, H-8	C-10	49.5	H-10a, H-10b
5	H-10b	4.25	1	dd	H-10a, H-11, H-8, H-12a			
7	H-8	8.19	1	s				
7	NH_2	7.89	2	br				
7	H-2	7.75	1	s				
8	2′-OH	5.47	1	d	H-2′			
8	11-OH	5.32	1	d + d	H-11			
8	3′-OH	5.24	1	d	H-3′			
8	H-2′	4.58	1	qd	H-1′, H-3′, 2′-OH			
8	H-3′	4.17	1	qd	H-2′, H-4′, 3′-OH			
8	H-11	3.83	1	qd	11-OH			
10	H-2	8.13	1	s	H-10a, H-10b	C-2	141.0	H-2, H-10a, H-10b
10	H-8	7.86	1	s		C-8	153.4	H-8
10	H-10a, H-10b	4.22	2	s		C-10	50.7	H-10a, H-10b, H-2
10						C-4	158.3	H-2, H-8
10						C-6	143.7	H-2, H-8, H-13a, H-13b, H-10a, H-10b, H-12a, H-12b
10						C-5	117.8	H-2, H-8

derivatives with a primary hydroxyl group (instead of a secondary) at the 2-position in the chain at N-1 undergo deamination much faster [10,11]. In the electrospray mass spectrum of compound **3** the protonated molecular ion was observed at $m/z = 361$, i.e., one mass unit higher than that of **1**, indicating substitution of the amino group by an oxygen. Otherwise, the fragmentation pattern of the compound was similar to that of compound **1**. In the COSY NMR spectrum, long-range correlation from H-2 to H-10 was observed.



Scheme 2. Fragmentation of adduct **5** in the mass spectrometer.

One of the minor adducts observed was 7-(3-chloro-2-hydroxypropyl)-adenosine (**4**). Because **4** easily lose sugar, the NMR spectra were recorded for the aglycone (**5**). The site of alkylation was determined to be N-7 on the basis of the long-range COSY spectrum which shows a weak cross-peak from the N^6 hydrogens to a hydrogen on the purine ring system, presumably to H-2 (correlation over five bonds, "W"-configuration) rather than to H-8 (six bonds). Because the H-10 hydrogens on the side chain correlates to the other hydrogen on the purine, this hydrogen must then be H-8, and the side chain must be attached to N-7. Further, the small chemical shift difference between H-2 and H-8 ($\Delta = 0.02$ ppm) is typical for Ade alkylated at N-7 [12–15]. The UV-maximum of the compound is consistent with the value found for the corresponding styrene 7,8-oxide adduct, λ_{max} at 270 nm (pH 7) and for the corresponding diepoxybutane adduct, λ_{max} at 269 nm and λ_{min} at 235 nm [15,17]. Supporting this conclusion is also the fact that the mass spectrum of the substance shows a fragmentation pattern that is analogous to the fragmentation pattern of the corresponding PAH N-7 Ade adducts, see Scheme 2 (for a detailed mechanism, see [16]).

Another minor adduct observed was **6**, 3-(3-chloro-2-hydroxypropyl)-adenosine. Compound **6** is not as easily deglycosylated as **4**, but was nevertheless obtained as **7**, 3-(3-chloro-2-hydroxypropyl)-adenine [18,19]. The identification was based on a comparison of ^{1}H NMR and UV data to data for the same compound previously prepared by Singh et al. [2] from adenine.

The identity of adduct **8** is proposed to be $O^{5'}$-(3-chloro-2-hydroxypropyl)-adenosine on the basis of MS-fragmentation, ^{1}H NMR and COSY. The shifts of the H-2 and H-8 protons are virtually the same as those of adenosine, supporting this conclusion. Because COSY ^{1}H NMR shows that C-2′ and C-3′ have free hydroxyls, the adduct must be attached to the hydroxyl of C-5′. The third visible hydroxyl must be that of C-11, which is coupled to H-11 (the integral of H-11 is 1, eliminating the possibility that it could be H-5′a + H-5′b).

In addition, one adduct was identified that contained 2 units derived from ECH. The compound was identified as 1,N^6-(2-hydroxypropyl)-N^6-(3-chloro-2-hydroxypropyl)-adenosine, **9**. Because of severe overlap in the NMR spectrum, the structural assignment was performed using the aglycon **10**, which was obtained from **9b**, the corresponding dAdo derivative. A HMBC correlation in the NMR spectrum between C6 and the closest protons on the second, open chain ECH moiety gives the attachment point for this chain in **10** as the N^6 nitrogen.

One possible mechanism for the formation of **10** is a nucleophilic attack of **2** on ECH. This mechanism is supported by our finding of pH dependence for the formation of **10**. At low pH, **2** is protonated and the diadduct is not formed. At higher pH conditions, **2** is not fully protonated and consequently the nucleophilicity of the compound is enhanced and the oxirane ring of ECH may be attacked by N^6.

In the reaction of ECH with dAdo, the adducts **1b**, **2b**, **4b**, and **6b** was identified by their mass spectrometric characteristics ($M+H^+$ and fragmentation), UV spectra and by comparing their position in the chromatogram relative to that of the corresponding **1**, **2**, **4**, and **6** adducts. The adducts **5** and **7** (adenine adducts) were identified in the dAdo reaction by spiking with adducts in question.

The reaction of ECH with double-stranded calf thymus DNA was performed at pH 7.4 at 37 °C for 44 h, and the modified DNA was enzymatically hydrolysed for deoxynucleosides. The adducts were identified by comparison of their retention time and positive ion electrospray MS^2 fragmentation paths (using SRM) with the deoxynucleoside and aglycone standards.

The major adduct was identified as **7**, (3-(3-chloro-2-hydroxypropyl)-adenine), presumably originating from **6** in DNA [17]. This adduct was detected in the supernatant collected after precipitation of the modified DNA. The level of adduct **7** corresponded to 1200 pmol/mg DNA (one adduct in 730 adenines). Also, adduct **2b** was detected in the enzymatic hydrolysate and the level of the adduct corresponded to 27 pmol/mg DNA (one adduct in 32,000 adenines).

4. Conclusions

Epichlorohydrin can be characterized as a difunctional compound where both the epoxide ring and the C–Cl bond may be attacked by nucleophiles. Accordingly, it may be expected that the compound does not form adducts along the same paths as other epoxides and thus the reactions deserves to be explored. In this work, we have described eight novel adenosine adducts produced from epichlorohydrin. Further, we have shown that the exocyclic adduct **2** is formed from **1**, most likely through Dimroth rearrangement and cyclization by displacement of the chlorine through attack of N1. Novel structural motifs are the adduct **9** and the corresponding aglycon **10**. There are not many examples in the literature of reactions of chemical carcinogens/mutagens that yield adducts where N^6 of adenosine will be disubstituted. However, previous work in our laboratory have shown that these kinds of adduct may be formed from acrolein and from malonaldehyde–acetaldehyde conjugates [20,21].

In the reaction of calf thymus DNA, the main adduct formed was compound **7**. The corresponding N3 adduct of glycidamide has been detected in Salmon testis DNA and in liver DNA of adult male mice exposed to glycidamide [22]. Therefore, it is possible that also adduct **7** is formed in DNA in vivo. It has been shown that N3 adenine adducts are

about sixfold faster than N7-guanine adducts released from DNA [23]. The process leads to apurinic sites in DNA, which may cause mutations if not repaired [24]. The N3 adenine adduct may along with the previously known N7-guanine adduct of ECH be used as biomarkers for exposure by monitoring urine samples [25]. The exocyclic ring adduct **2b** was also found in the DNA hydrolysates. The adduct interrupts the Watson–Crick base pairing and may cause mutational effects of ECH.

Acknowledgments

We thank Mr. Markku Reunanen who made the high-resolution MS measurements, including necessary derivatizations. We also thank Dr. Rainer Sjöholm and Mr. Mattias Roslund for recording most of the NMR spectra, and Dr. Tony Munter for valuable advice concerning the DNA reactions. Financial support for the work was obtained from Magnus Ehrnrooth Foundation, Finland.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2006.01.005.

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