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Research Article

^{3}H-labelled alkyl-nucleotides, -nucleosides and -bases for the immunoanalytical quantification of DNA damage and repair

Wolfgang Drosdziok[1], Catrin Lutze[2], Kai Krüger[1], Karl-Heinz Glüsenkamp[3] and Manfred F. Rajewsky[1,*]

[1] *Institute of Cell Biology (Cancer Research), University of Essen Medical School, Virchowstrasse 173, Essen D-45147, Germany*

[2] *Ericsson GmbH, Fritz-Vomfelde-Strasse 26, Düsseldorf D-40547, Germany*

[3] *Squarix GmbH, Elbestrasse 10, Marl D-45768, Germany*

Summary

Analysis of the formation and repair of structurally modified DNA is of particular interest in the study of carcinogenesis, cancer therapy and aging. The quantification of specific DNA lesions by sensitive immunoanalytical methods requires radiotracers with high specific activity. We describe the synthesis of ^{3}H-labelled adenine-, cytosine-, guanine- and thymine-alkyl derivatives by nucleophilic *N*- and *O*-alkylation using alkyl halides and diazoalkanes: 3-alkyl-[8-^{3}H]adenine (Alkyl = Me, Et, *n*-Bu); O^{6}-alkyl-deoxy[1′,2′-^{3}H]guanosine (Alkyl = Me, Et, *i*-Pro, *n*-Bu); O^{6}-ethyl-deoxyguanosine-5′-triphosphate ([2-^{3}H-Ethyl]; [8-^{3}H]); O^{6}-alkyl-9-hydroxyhexyl-[8-^{3}H] guanine (Alkyl = Me, Et); 7-ethyl-[8,5′-^{3}H]guanosine-3′,5′-cyclic-phosphate; O^{2}-andO^{4}-alkyl-[methyl, 1′,2′-^{3}H]thymidine (Alkyl = Me, Et); the conversion of ^{3}H-labelled thymidine to the corresponding 5-methylcytidine; the synthesis of three different 8-oxo-guanine tracers; and the generation of thymidine glycol (5,6-dihydroxy-5,6-dihydro-[methyl-^{3}H]thymidine) from thymidine. All radiotracers were sucessfully employed in competitive radioimmunoassays for the quantification of defined DNA alkylation products in DNA repair analyses. Copyright © 2003 John Wiley & Sons, Ltd.

*Correspondence to: M. F. Rajewsky, Institute of Cell Biology (Cancer Research), University of Essen Medical School, Hufelandstrasse 55, D-45122 Essen, Germany. E-mail: rajewsky@uni-essen.de

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Introduction

The analysis and quantification of structurally modified DNA components is of great importance in the study of oncogenesis induced by exogenous or endogenous carcinogens, and in the DNA dosimetry and DNA repair profiling of target cells when DNA-reactive anti-cancer agents are applied in cancer therapy.[1]

Depending on the causative agent, a variety of potentially mutagenic and/or cytotoxic lesions are generated in cellular DNA. The detection and quantification of very low concentrations of specific DNA lesions and determination of the kinetics of repair of structurally altered target cell DNA require ultrasensitive analytical techniques. Antibodies directed against defined DNA modifications have proven to be particularly efficient tools in the immunoanalysis of DNA at the level of tissues, individual cells and distinct genes.[2] A variety of high affinity monoclonal antibodies have been generated for these applications. In combination with specific radiotracers—the radiolabelled analogues of modified purine and pyrimidine compounds—these antibodies can be used for radioimmunoanalysis. Here we describe the synthesis of [^{3}H]-labelled radiotracers for the quantification and repair analysis of different DNA alkylation products. The successful application of these tracers has been described.[3–17]

Experimental

Chemicals: All reagents and solvents used were of analytical grade or higher and purchased from Aldrich (Germany), Sigma (Germany), Merck (Germany), Boehringer Ingelheim (Germany), Fluka (Schweiz) and Amersham Pharmacia Biotech (Germany), if not stated otherwise. Radiochemicals were purchased from NEN (USA), and Amersham Pharmacia Biotech.

Abbreviations: Substituted alkyl groups: Me = Methyl; Et = Ethyl; *i*-Pro = iso-Propyl; *n*–Bu = *n*-Butyl.

Thin layer chromatography (TLC): Analytical and preparative TLC was performed using silica gel-coated aluminium sheets (analytical:

5×7.5 cm; preparative: 20×20 cm, Merck, Germany), if not stated otherwise. Elution conditions varied depending on the substance analysed. Labelled compounds were detected using a TLC-scanner (Berthold, Germany). For preparative isolation of labelled compounds, the product lane was scratched off and eluted with 30% methanol (v/v).

High performance liquid chromatography (HPLC): Analytical and preparative HPLC was performed using a 600E multi-solvent delivery system with a 990 photodiodearray detector and a reverse phase Nova-Pak C_{18} column (Waters, Germany). Elution conditions: 0.1 M ammonium formate (pH 5.0), linear gradient with increasing concentration of methanol. Labelled compounds were detected by liquid scintillation spectrometry (model 1410; Pharmacia-Wallac, Germany).

Affinity chromatography: Labelled tracer compounds were purified by affinity chromatography using immobilized monoclonal antibodies (mabs) as specific ligands for the respective DNA alkylation products.

Identification of radiotracers: The radiotracers synthesized were identified by co-chromatography (HPLC, TLC) using the corresponding unlabelled analytically characterized compounds for reference.

Results and discussion

Sensitive radioimmunoassays for the quantification of structurally altered DNA components not only require antibodies with high-affinity constants and substrate specificity, but also radiolabelled tracer compounds with very high specific radioactivity. Both of these criteria are important in the analysis of low levels of DNA modification and in DNA repair studies. In this communication we describe the synthesis of radiotracers successfully used in radioimmunoassays of alkylated DNA.[3–17]

Besides the synthetic route used for the production of purine- and pyrimidine- derived radiotracers, their efficient purification is of critical importance. Radiolabelled compounds may undergo radiation-induced decomposition, especially if stored in concentrated solution. Moreover, some nucleosides, e.g. thymidine derivatives, undergo isomerization in the sugar moiety, resulting in a mixture of isomeric structures while the antibody only reacts with one of the isomers. Therefore, all of the tracers synthesized were subjected to an additional immunopurification

step (affinity columns with an immobilized antibody specific for the tracer; see Experimental), and this antibody-based tracer purification was repeated at intervals.

3-Alkyl[8-^{3}H]adenine;[18,19] Alkyl = Me, Et, n-Bu

[8-^{3}H]adenine (**1**; 185 MBq; 38.5 nmol; 962 GBq/mmol; Amersham Pharmacia Biotech) was dissolved in 100 µl dry *N*,*N*-dimethylacetamide. After adding 2 µmol of alkyl iodide, the reaction mixture was stirred overnight at room temperature. It contained all possible *N*-monoalkyl adenines. The first separation of this mixture was performed by preparative TLC (Eluent: *n*-butanol/CH_3COOH/H_2O; 3:1:1, v/v/v). The main alkylation product was the target molecule (2) and finally purified by HPLC. Yields: 20–50% (Figures 1 and 2).

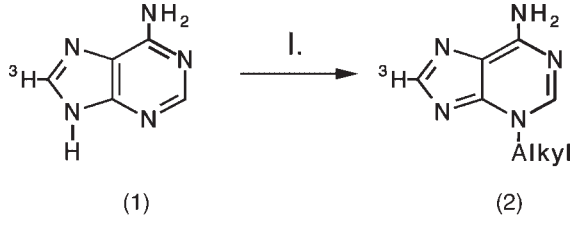

Figure 1. I. Alkyl iodide, 20°C

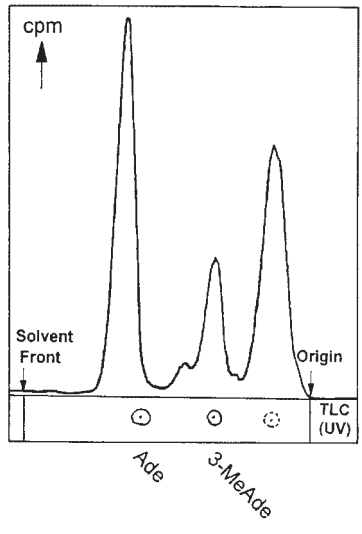

Figure 2. TLC of tritium labelled and unlabelled 3-MeAde detected by TLC-scanning (β-radiation) and UV

5-Methyldeoxy[methyl-^{3}H]cytidine[20]

This radiotracer was synthesized in two steps (Figures 3–5):

6-Thiocarbonyl-3′,5′-diacetyldeoxy[methyl-^{3}H]thymidine (**4**). Deoxy [methyl-^{3}H]thymidine (**3**; 185 MBq; 50 nmol; 740 GBq/mmol; NEN) was mixed with 50 μmol of acetic anhydride in 50 μl of absolute pyridine

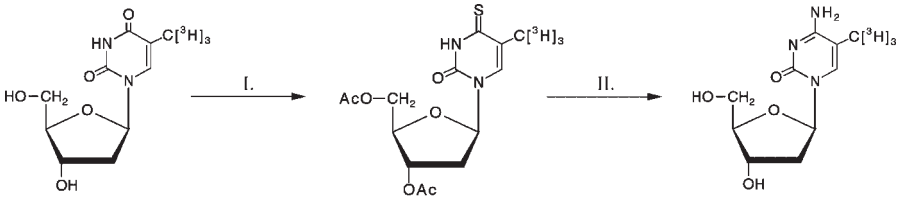

Figure 3. I. Acetic anhydride, 40°C; P_2S_5, 100°C. II. NH_3-saturated methanol, 50°C

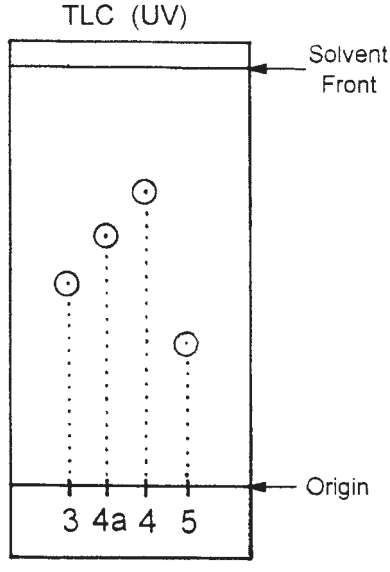

Figure 4. Reaction steps of the synthesis of 5-methyldeoxy[methyl-^{3}H]cyd monitored by TLC: 3 = deoxy[methyl-^{3}H]thymidine; 4a = 3′,5′-diacetyldeoxy [methyl-^{3}H]thymidine; 4 = 6 thiocarbonyl-3′,5′-diacetyldeoxy[methyl-^{3}H]thymidine; 5 = 5-methyldeoxy[methyl-^{3}H]cytidine

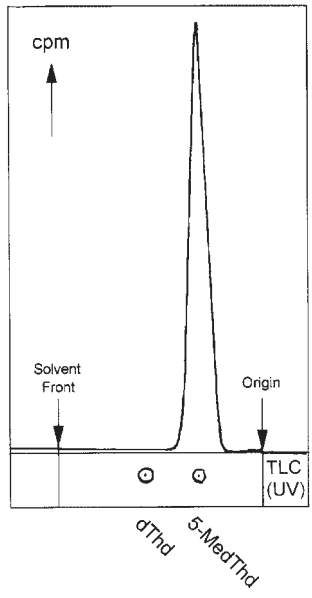

Figure 5. Comparison between labelled (^{3}H; β-scanning) and unlabelled (UV absorption; 256 nm) 5-Me-dCyd by TLC

and kept at 40°C for 40 h. The reaction was monitored by TLC (Eluent: *n*–butanol/CH_3COOH/H_2O; 3:1:1, v/v/v). The reaction mixture was evaporated to dryness and dissolved in 150 µl of pyridine. After adding 1 µmol of P_2S_5 (freshly recrystallized in chloroform), the solution was incubated at 100°C for 48 h. The reaction was controlled by TLC and terminated by evaporation to dryness.

*5-Methyldeoxy[methyl-^{3}H]cytidine (***5***).* NH_3-saturated methanol was added to the dried residue and incubated at 50°C for > 70 h. NH_3 was removed by evaporation and the target molecule was separated and isolated by HPLC as described. Yield: 60 %.

O^6-Alkyldeoxy[1′,2′-^{3}H]guanosine[21,22]; Alkyl = Me, Et, i-Pro, n-Bu

Since only the 5′–triphosphate of deoxy[1′,2′-^{3}H]guanosine possesses suffiently high specific radioactivity, the synthesis of all O^6-alkyldeoxyguanosine tracers started from deoxyguanosine-5′-triphosphate.

Deoxy[1′,2′-^{3}H]guanosine. A solution of deoxy[1′,2′-^{3}H]guanosine-5′-triphosphate (1.18 TBq/mmol; Amersham Pharmacia Biotech) was adjusted to pH 7.5 with 0.1 M NaOH and incubated with alkaline phosphatase (Roche, Germany) for 30 min at 37°C in the presence of 7 mM $MgCl_2$ and 15 mM Tris buffer (pH 7.5). Dephosphorylation was monitored by TLC (Eluent: *n*-butanol/CH_3COOH/H_2O; 3:1:1, v/v/v) and TLC-scanning (yield: 100%).

For powerful alkylation of deoxyguanosine, diazoalkanes were generated from the respective *N*-alkyl-*N*-nitroso compounds. Diazoethane was generated via decomposition of *N*-ethyl-*N*-nitrosourea in 40% KOH. To obtain diazomethane, *N*-methyl-*N*-nitroso-4-toluol-sulphonamide was used as starting material. The crude diazomethane etherate was cautiously distilled and immediately cooled on ice.

O^6-Alkyldeoxy[1′,2′-^{3}H]guanosine; Alkyl= Me, Et, n-Bu

Deoxy[1′,2′-^{3}H]guanosine (**6**; 37 MBq; 35.7–25 nmol; 1.04–1.48 TBq/mmol; Amersham Pharmacia Biotech) was dissolved in 5 ml of absolute methanol. After cooling to 0°C, 3–5 ml of dry ice-cold diazoalkane etherate were added dropwise, until the solution stopped decolourizing. The reaction was controlled by TLC as described before. Purification of the compound was performed by HPLC. Yields: 20% (Me) and 40% (Et, *n*–Bu) (Figures 6 and 7).

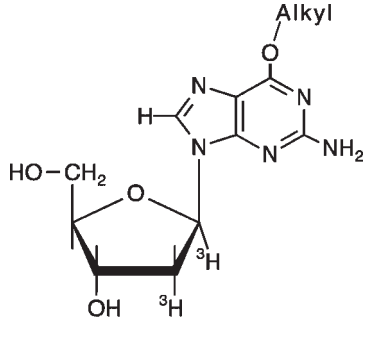

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Figure 6. O^6-alkyl-deoxy[1′,2′-^{3}H]guanosine

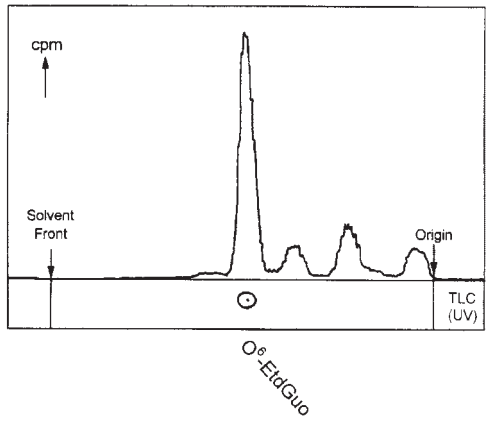


Figure 7. TLC elution profile of ^{3}H-labelled and unlabelled O^{6}-ethyldeoxyguanosine

O^{6}-Iso-propyldeoxy[8,5′-^{3}H]guanosine

A 5–10 fold molar excess of *iso*-propyl iodide was added to deoxy[8,5′-^{3}H]guanosine (37 MBq; 30 nmol; 1.22 TBq/mmol; NEN) dissolved in 100 µl of DMSO in the presence of K_2CO_3. The mixture was incubated for 3 h at room temperature in the dark. After complete alkylation, the reaction mixture was separated by TLC (Eluent: butanol/CH_3COOH/H_2O; 3:1:1, v/v/v) and the product was finally purified by HPLC. Yield: 10% (Figure 9).

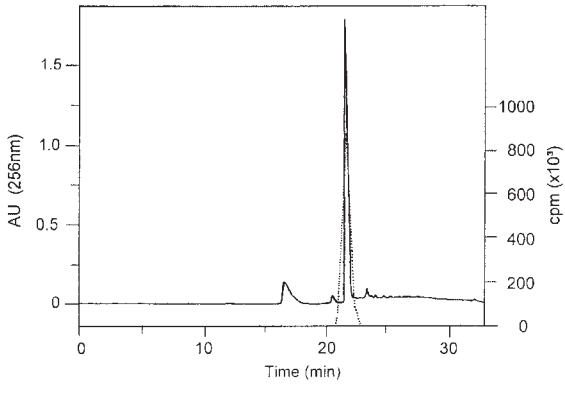


Figure 8. Elution profile (reverse phase HPLC) of ^{3}H-labelled (liquid scintillation spectrometry) and unlabelled (UV detection at 256 nm) O^{6}-methyldeoxyguanosine

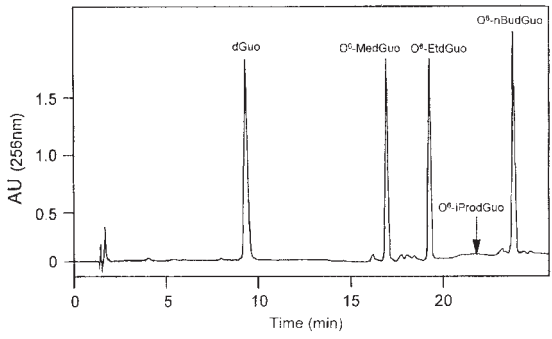


Figure 9. Elution profile (reverse phase HPLC) deoxyguanosine and four O^6-alkylation products

O^6-Ethyldeoxyguanosine-5′-triphosphate[23,24]; [2-^{3}H-ethyl] and [8-^{3}H] resp.

(*a*)*O^6-Ethyldeoxy*[8-^{3}H]*guanosine:* Deoxy[8-^{3}H]guanosine (37 MBq; 588 nmol; 62.9 GBq/mmol; Amersham Pharmacia Biotech) was ethylated to completion with diazoethane as described before (yield: 41%). After purification 'Dilution' with non-radioactive O^6-ethyldeoxyguanosine resulted in a specific radioactivity of 2.57 GBq/mmol (Figures 10 and 11).

(*b*)*O^6-*[2-^{3}H]*Ethyldeoxyguanosine:* Three to five micromoles of deoxyguanosine was incubated with 37 MBq [2-^{3}H]ethyl iodide (37 MBq; 1.3 μmol; 27.75 GBq/mmol; NEN) in dimethylformamide. Purification resulted in a yield of 12%. The specific radioactivity was adjusted to 2.7 GBq/mmol by adding of non-radioactive O^6-ethyldeoxyguanosine in a ratio of 1:10.

O^6-Ethyldeoxyguanosine-5′-monophosphate: (a) [2-^{3}H-ethyl] and (b) [8-^{3}H].

The following reaction steps are identical for both tracers and standardized to 1 mg of O^6-ethyldeoxyguanosine.

One milligram of [^{3}H-]-labelled O^6-ethyldeoxyguanosine (3.38 μmol) was dissolved in a mixture of 23 μl of triethylphosphate and 7 μl of absolute pyridine. The solution was cooled to −20°C and 8 μl of $POCl_3$ (8.6 μmol) were added. After incubation for 3 h excess $POCl_3$ was hydrolyzed and neutralized by adding 1 M triethylammonium bicarbonate buffer (pH 9). Analytical control was performed by TLC (Eluent: dioxane/iso–propanol/H_2O/NH_4OH (conc.); 4:2:4:1, v/v/v/v). The reaction mixture was separated by ion exchange chromatography using DEAE-Sephadex A-25 (Amersham Pharmacia Biotech); elution

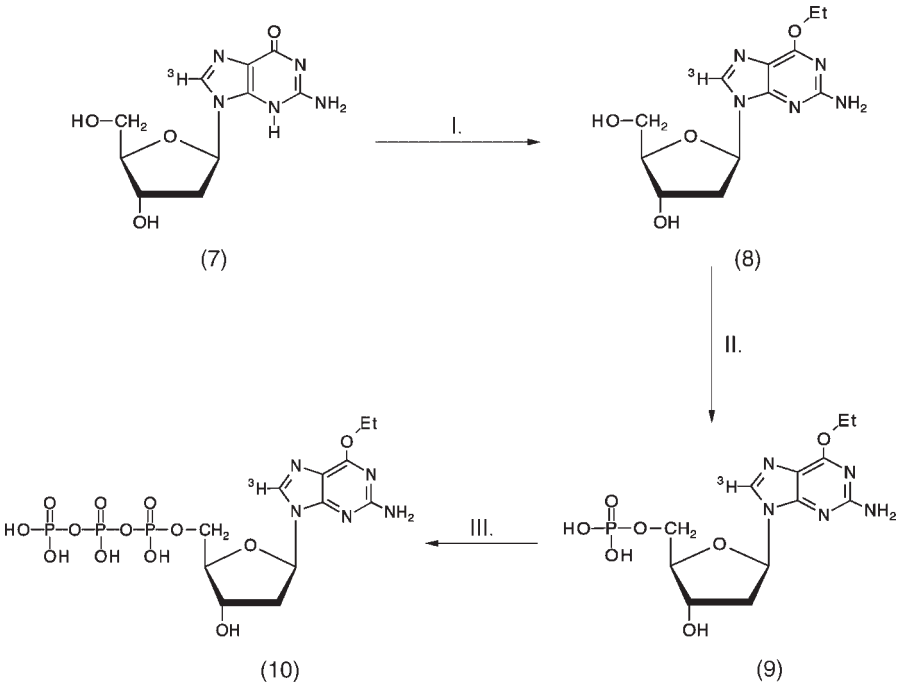


Figure 10. (a) I. Diazoethane, 20°C. (b) I. [2-^{3}H]ethyl iodide,0°C; II. $POCl_3$, −20°C; nuclease P1, 37°C; III. *N*,*N*′-carbodiimidazole, tetra-tributylammonium diphosphate, 20°C

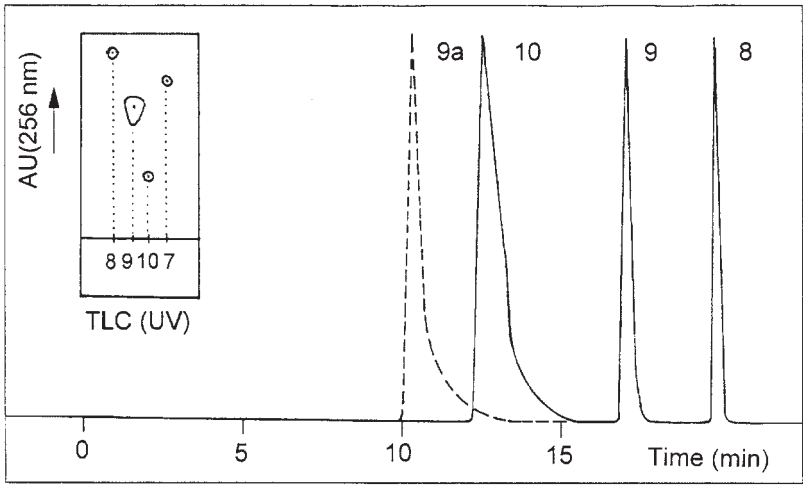


Figure 11. 5′-Triphosphorylation of ^{3}H-labelled O^6-ethyldeoxyguanosine analysed by TLC and HPLC:7 = dGuo; 8 = O^6-EtdGuo; 9a = O^6-EtdG-3′,5′-DP; 9 = O^6-EtdG-5′-MP; 10 = O^6-EtdG-5′-TP

conditions: linear gradient 0–0.8 M triethylammonium bicarbonate buffer (pH 9). In the elution profile at 280 nm the first and second peak, respectively, contained (a) O^6-ethyldeoxyguanosine-5′-monophosphate and (b) O^6-ethyldeoxyguanosine-3′,5′-dimonophosphate. The 3′-monophosphate residue was selectively removed by incubation with nuclease P1 (Roche) at pH 7.2 and 37°C in the presence of 7.5 mM CH_3COONa and 0.2 M $ZnSO_4$. This procedure gave the following yields: (a) 8% and (b) 14%.

Two milligrams of *N*,*N*′-carbodiimidazole (12.3 μmol) in 100 μl DMF were added to a solution of (a) 0.27 μmol and (b) 0.47 μmol of O^6-ethyldeoxyguanosine-5′-monophosphate (tributylammonium salt) in 200 μl of DMF. The mixture was incubated for 1 h in the dark at room temperature. Excess *N*,*N*′-carbodiimidazole was inactivated by adding 5 μl of methanol. The reaction products were monitored by TLC using PEI-cellulose F (Merck; Eluent: *iso*-propanol/NH_4OH (conc.)/H_2O, 6:3:1, v/v/v). Twenty micromoles of tetra-tributylammonium diphosphate (25.7 μmol) in 100 μl of DMF were added to the mixture and incubated overnight at room temperature. After adding 400 μl of methanol, the product was isolated using a DEAE-Sephadex A-25 matrix and a linear elution gradient of 0–1 M triethyl ammonium bicarbonate. The reaction was controlled by TLC (Eluent: dioxane/isopropanol/H_2O/NH_4OH (conc.), 4:2:4:1,v/v/v/v), TLC-Scanning showed yields of ~2.5% in either case.

O^6-Alkyl-9-hydroxyhexyl-[8-^{3}H]guanine; Alkyl = Me, Et

Starting materials were the radiotracers described above:

(a)O^6-Methyldeoxy[8,5′-^{3}H]guanosine (4.23 kBq; 9 pmol; 0.47 TBq/mmol; NEN) and
(b)O^6-Ethyldeoxy[8,5′-^{3}H]guanosine (0.56 kBq; 0.6 pmol; 0.94 TBq/mmol; NEN).

The *N*-glycosidic bond of these nucleosides was completely hydrolyzed by incubation at 70°C in 0.1 M HCl for 3 h. *N*9-alkylation was achieved by incubation of [^{3}H]-labelled O^6-alkylguanine (Me: 9 pmol, Et: 0.6 pmol) with a 10% molar excess of 1-chloro-6-hydroxyhexane in 50 μl of DMSO at 50°C overnight in the presence of K_2CO_3. Separation and isolation were carried out by HPLC. Yield: 90% (Figures 12 and 13).

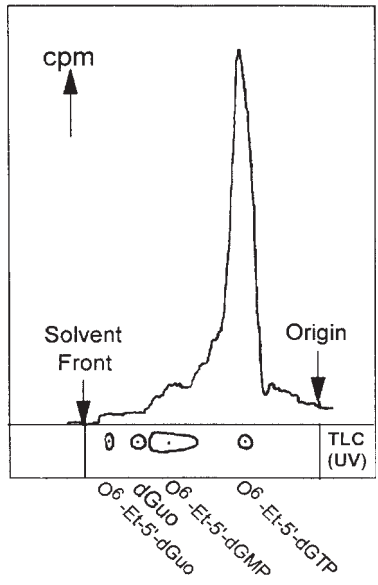
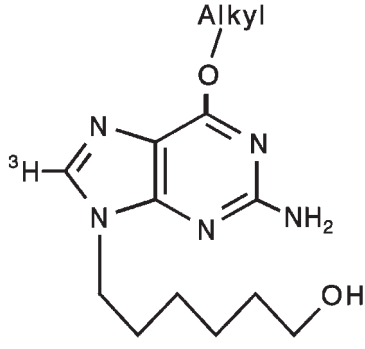


Figure 12. TLC of tritium labelled O^6-EtdG-5′-TP

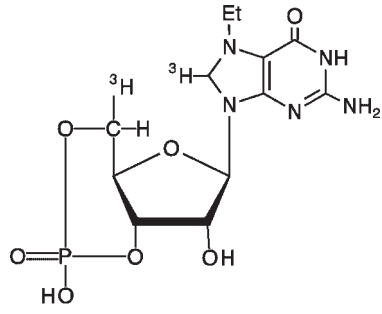


(11)

Figure 13. O^6-alkyl-9-hydroxyhexyl-[8-^{3}H]guanine

7-Ethyl-[8,5′-^{3}H]guanosine-3′:5′-cyclic monophosphate

[8,5′-^{3}H]guanosine-3′:5′-cyclic monophosphate, (37 MBq; 27.6 nmol; 1.3 TBq/mmol; NEN) was incubated with 10 µl of ethyl iodide in 500 µl of DMF at room temperature overnight. The 7-ethyl-derivative was isolated by HPLC. Yield: 17% (Figure 14).



(12)

Figure 14. 7-ethyl-[8,5′-^{3}H]guanosine-3′:5′-cyclic monophosphate

8-Oxoguanine tracer family

(a) 8-Oxo-deoxy[1′,2′-^{3}H]*guanosine:*[25] 2-deoxy[1′,2′-^{3}H]guanosine-5′-phosphate (37 GBq;29.4 nmol; 1.26 TBq/mmol; Amersham Pharmacia Biotech) was dephosphorylated as described above and purified by HPLC.

8-Carbonylation (Fenton reaction): deoxy[1′,2′-^{3}H]guanosine was dissolved in 172 µl of H_2O. Reagents were added at room temperature in the following sequence: 409 µl of freshly prepared 1.7 M ascorbic acid, 11.5 µl of 0.2 M copper sulphate solution and finally 835 µl of 31% (v/v) hydrogen peroxide. After vigorous stirring the crude product was immediately isolated via HPLC. The separation process was repeated and radiotracer (13) was stored at –20° (yield: 18%) (Figures 15 and 16).

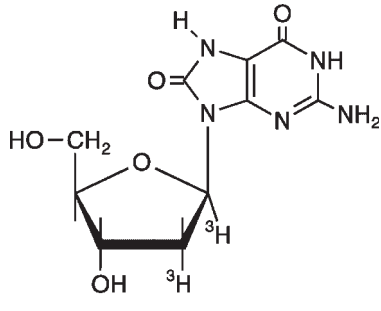


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Figure 15. 8-Oxo-deoxy[1′,2′-^{3}H]guanosine

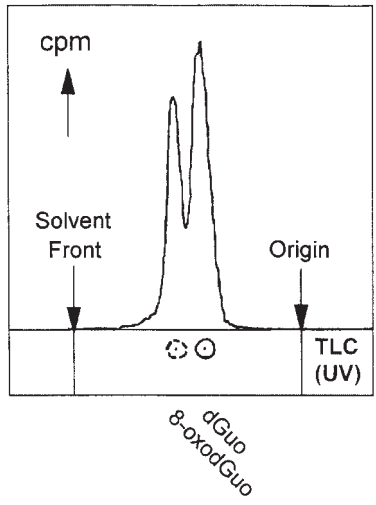

Fig. 16. TLC elution profile of tritium labelled and unlabelled 8-oxo-deoxyguanosine

(b)3-(N^2-aminohexyl-7-hydro-8-oxoguanosine)-4-([2,3,4,5,6-^{3}H]phenylalanine)-3-cyclobutene-1,2-dione:[26,27] This radiotracer was synthesized in five steps (Figure 17).

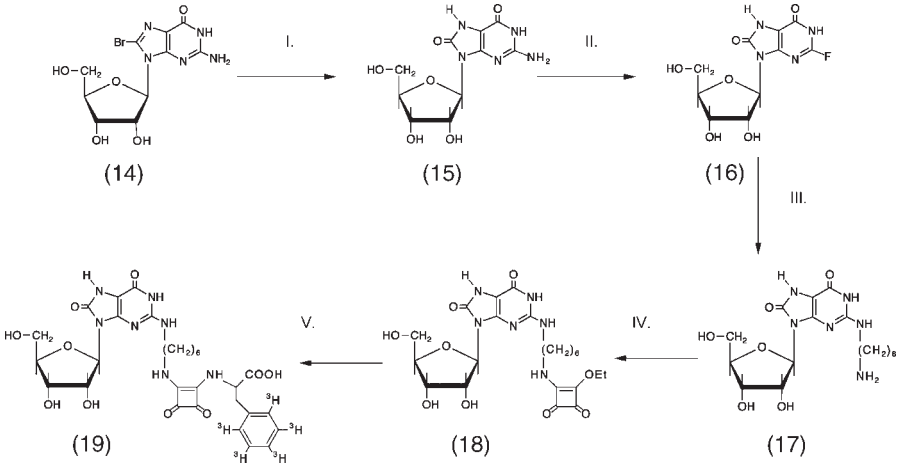

Figure 17. I. (a) CH_3COONa, CH_3COOAg, $(CH_3CO)_2O$, 1-methylimidazol, $>100^\circ C$; (b) 5% NH_4CO_3, $0^\circ C$; (c) 30% NH_4OH, $60^\circ C$; II. HBF_4, $NaNO_2$, $<0^\circ C$; III. H_2O, $NH_2(CH_2)_6NH_2$, $100^\circ C$; IV. 3,4-diethoxy-3-cyclobutene-1,2-dione, $(Et)_3N$, $20^\circ C$; V. L-[2,3,4,5,6-^{3}H]phenylalanine, $37^\circ C$

*7-Hydro-8-oxo-N^2-acetylguanosine (***15***)*. Ten millimoles of dry 8-bromoguanosine (**14**) was suspended in 120 ml of pyridine and 100 mmol of sodium acetate, and mixed with 10 mmol of silver acetate. While stirring a solution of 3.8 mmol 1-methylimidazole in 106 mmol acetic anhydride was added dropwise at 0°C and refluxed for 20 h. The reaction was controlled by TLC (Eluent: CH_2Cl_2/methanol, 9:1, v/v). Excess of acetic anhydride was removed by distillation, followed by hydrolysis with 5% (w/v) $(NH_4)HCO_3$ at 0°C. After evaporation to dryness, the solid residue was carefully extracted with CH_2Cl_2. The combined solutions, containing 7-hydro-8-oxo-N^2,2′,3′,5′-*O*-tetraacetylguanosine, were washed and evaporated to dryness. TLC analysis was perfomed as described before.

The solid residue was dissolved in 30% NH_4OH and incubated at 60°C for 16 h. After evaporation of NH_4OH, 7-hydro-8-oxo-N^2-acetylguanosine was isolated by exclusion chromatography (EC) using Sephadex G-10 (Pharmacia).

*2-Fluoro-7-hydro-8-oxoguanosine (***16***)*. A suspension of 6 mmol of 7-hydro-8-oxo-N^2-acetylguanosine (**15**) in 20 ml of 48% HBF_4 was cooled to −10°C, and a saturated solution of sodium nitrite (1.7 eq.) was added at a rate of 0.1 ml/min. Thereafter, the mixture was stirred at the same temperature for 15 min.The reaction mixture was adjusted to pH 5–7 with 50% (w/v) sodium hydroxide (<0°C). The resulting thick slurry was concentrated to a volume of one-third by distillation and the residue was washed with 70 ml of absolute ethanol. Insoluble particles were removed by filtration and washed three times with 75% (v/v) ethanol. Combined filtrates were evaporated to dryness. The crude product was purified by chromatography using a silica gel-column with $CHCl_3$/methanol/NH_4OH (conc), 2:2:1 (v/v/v) as the eluent.

*N^2-Aminohexyl-7-hydro-8-oxoguanosine(***17***)*. A suspension of 16.6 μmol (**16**) in 12.5 ml of H_2O containing 166 μmol of 1.6-diaminohexane was neutralized and refluxed for 3 h. Analysis of the reaction and preparative isolation of the product were carried out by HPLC.

*3-(N^2-Aminohexyl-7-hydro-8-oxoguanosine)-4-ethoxy-3-cyclobutene-1,2-dione (***18***)*. Compound (**17**) was dried and suspended in DMSO/ethanol, 1:1 (v/v) using ultrasound. Fifty microlitre of 3,4-diethoxy-3-cyclobutene-1,2-dione (squaric acid diethylester) and 100 μl of

triethylamine were added. After incubation for 30 min at room temperature the reaction was completed, yielding substance (**18**). The reaction products were analysed by HPLC. After filtration the squaric acid-monosubstitution product (**18**) was precipitated by diethylether and centrifuged. The sediment was carefully washed with ether.

3-(N^2-Aminohexyl-7-hydro-8-oxoguanosine)-4-([2,3,4,5,6^{-3}H]phenylalanine)-3-cyclobutene-1,2-dione (19). L-[2,3,4,5,6-^{3}H]phenylalanine (18.5 GBq; 4 μmol; 4.66 TBq/mmol; Amersham Pharmacia Biotech) was dissolved in borate buffer, pH 9 (Merck). The solution was mixed with an excess of 8-oxoderivative (**18**) dissolved in 50 μl of DMSO. After incubation for >48 h at 37°C, [^{3}H]-labelled 8-oxoguo derivative (**19**) was isolated and purified by HPLC. Yield: 49% (Figure 18).

(c)3-(N^2-Aminopentyl-7-hydro-8-oxo-9-ethyl-guanine)-4-([2,3,4,5,6-^{3}H]phenylalanine)-3-cyclobutene-1,2-dione: This 8-oxo-guanine radiotracer was synthesized in six steps, using a modified reaction sequence (Figure 19).

The reaction sequence started with 9-ethylguanine (**20**), fluorinated in position N^2 (**21**) and then substituted by 1,5-diaminopentane, as described before (**22**), followed by bromination to N^2-aminopentyl-8-bromo-9-ethylguanine (**23**).

Compound (**22**) was suspended in H_2O. At 0°C 10% (v/v) bromine in H_2O was added dropwise until no further decolourization was observed. The solid product was filtered and washed with 40% (v/v) methanol. The 8-bromo derivative (**23**) was purified on a silica gel column (methanol/30% NH_4OH; 4:1, v/v).

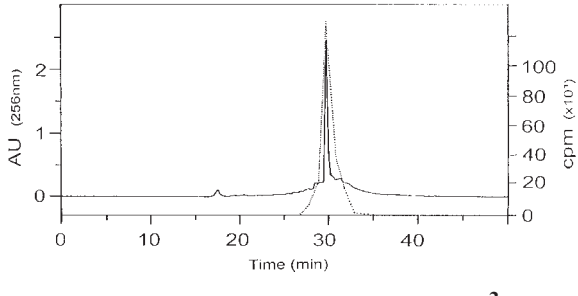

Figure 18. Elution profile (reverse phase HPLC) of 3-(N^2-aminohexyl-7-hydro-8-oxoguanosine)-4-([2,3,4,5,6-^{3}H]phenylalanine)-3-cyclobutene-1,2-dione (liquid scintillation spectrometry) with unlabelled reference (UV detection at 256 nm)

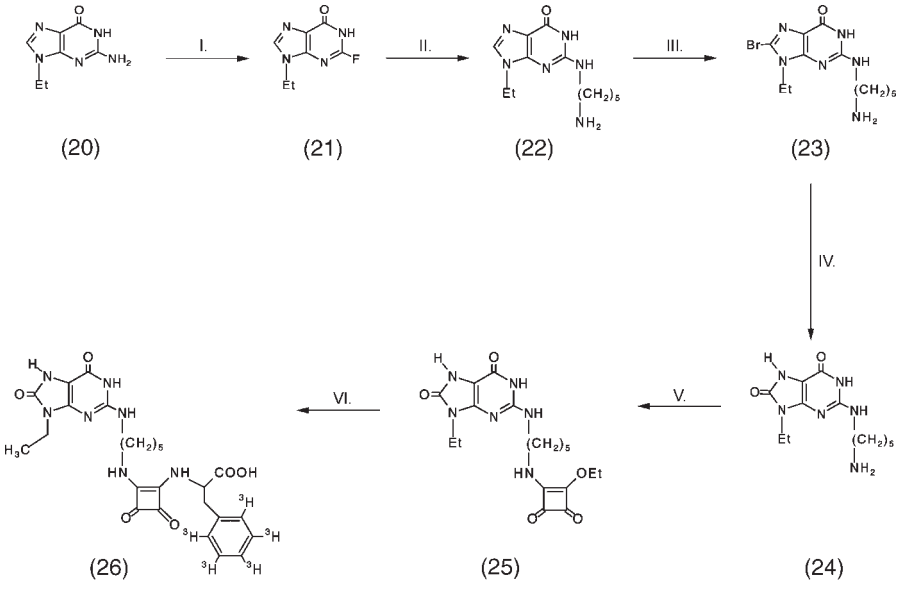

Figure 19. I. HBF_4, $NaNO_2$, $<0^{\circ}C$; II. $NH_2(CH_2)_5NH_2$, $100^{\circ}C$; III. H_2O, 10% Br_2, $0^{\circ}C$; IV. CH_3COOH, CH_3COONa, $120^{\circ}C$; V. 3,4-diethoxy-3-cyclobutene-1,2-dione, $(Et)_3N$, $20^{\circ}C$; VI. L-[2,3,4,5,6-^{3}H]phenylalanine, $37^{\circ}C$

*N^2-Aminopentyl-7-hydro-8-oxo-9-ethyl-guanine (***24***).* Twenty-nine micromoles (**23**) were dissolved in CH_3COOH (conc.) containing 287 µmol of sodium acetate and refluxed for 3–4 h at 120°C. After evaporation to dryness, the residue was dissolved in H_2O, neutralized with 0.5 M NaOH and purified chromatographically as described for compound (**23**).

*3-(N^2-Aminopentyl-7-hydro-8-oxo-9-ethyl-guanine)-4-ethoxy-3-cyclobutene-1,2-dione (***25***).* The 8-oxogua derivative (**24**) was reacted with squaric acid diethylester to the monoamide (**25**) and thereafter precipitated and isolated under the conditions described for compound (**18**).

*3-(N^2-Aminopentyl-7-hydro-8-oxo-9-ethyl-guanine)-4-([2,3,4,5,6-^{3}H] phenylalanine)-3-cyclobutene-1,2-dione(***26***).* Incubation of L-[2,3,4,5,6-^{3}H] phenylalanine with a molar excess of (**25**) under the conditions described for compound (**19**) gave the diamide (**26**).

O^2/O^4-Alkyldeoxy[methyl,1′,2′-^{3}H]thymidine; Alkyl = Me, Et

Deoxy[methyl,1′2′-^{3}H]thymidine (185 MBq; 0.417 nmol; 4.44 TBq/mmol; Amersham Pharmacia Biotech) in 5 ml of methanol was

stirred at 0°C with 4–6 ml aliquots of diazoalkane etherate, until the reaction mixture remained yellow. The reaction was controlled by TLC (Eluent: acetone). The solvent was completely removed by evaporation. The oily residue was dissolved in 0.5 ml of 30% (v/v) methanol and the products were isolated by HPLC. Yield: 2.9% (O^2-me), 8,5% (O^4-me), 13.8% (O^2-et), and 15.7% (O^4-et) (Figures 20–22).

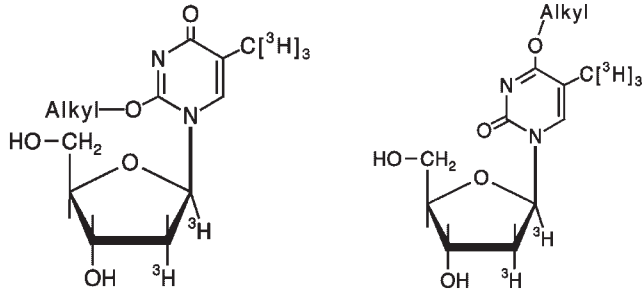

Figure 20. O^2- and O^4-alkyl-deoxy[methyl,1′2′-^{3}H]thymidine

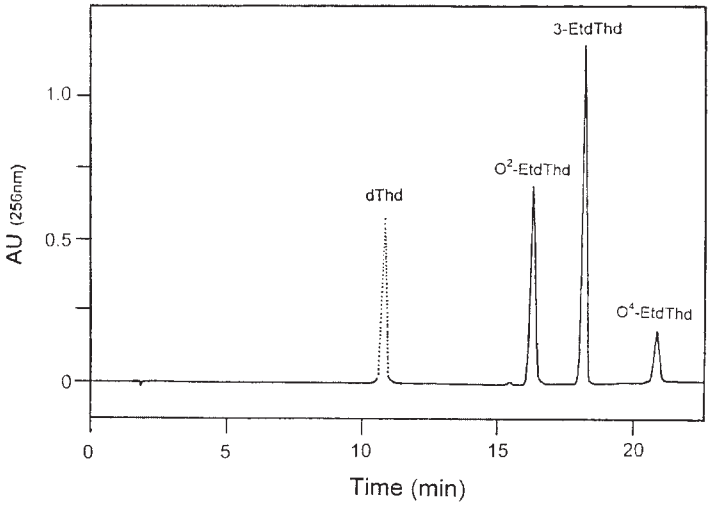

Figure 21. HPLC (reverse phase) elution profile of deoxythymidine (dThd), O^2-ethyldeoxythymidine (O^2-EtdThd), 3-ethyldeoxythymidine (3-EtdThd) and O^4-ethyldeoxythymidine (O^4-EtdThd) at 10.63, 16.10, 17.93 and 20.55 min, respectively

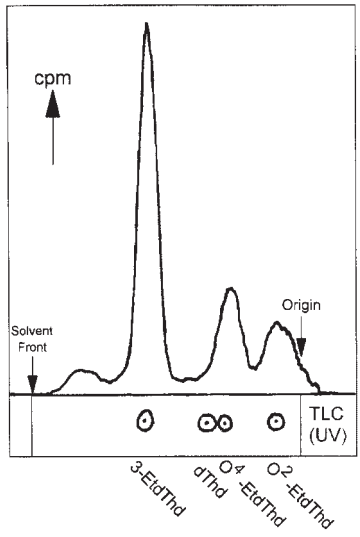

Figure 22. Ethylation of deoxy[methyl, 1′,2′-^{3}H]thymidine. The reaction mixture was separated by TLC on silicagel; β-radiation in correlation to UV-activity of the reference

5, 6-Dihydroxy-5,6-dihydrodeoxy[methyl-^{3}H]thymidine-5′-monophosphate[28]

Deoxy[methyl-^{3}H]thymidine-5′-monophosphate (9.25 GBq; 6.25 nmol; 1.48 TBq/mmol; Amersham Pharmacia Biotech) mixed with 0.5 mmol (= 183 mg) of deoxythymidine-5′-monophosphate (sodium salt) resulted in a specific radioactivity of 18.5 GBq/mmol (Figure 23).

5-Bromo-6-hydroxy-deoxythymidine[methyl-^{3}H]-5′-monophophate (**30**). Deoxy[methyl-^{3}H]thymidine-5′-monophosphate (0.5 mmol; 9.25 MBq; 18.5 GBq/mmol) (**29**) was dissolved in 4 ml of H_2O and stirred with 3.9 mmol bromine for 45 min at room temperature. The solution of bromohydrine (**30**) was extracted with an equal volume of chloroform,

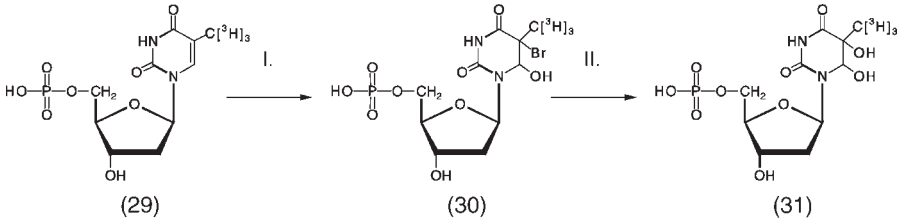

Figure 23. I. H_2O, Br_2, 20°C; AgO, 20°C

until the bromine was completely removed. Under an argon atmosphere a molar excess of freshly prepared silver oxide was added two times to the bromohydrine and stirred at room temperature in the dark overnight. After adding 4 ml of methanol the suspension was filtered. The clear filtrate was incubated with hydrogen sulphide gas for 5 min to remove silver ions by precipitation. After degassing with argon for 15 min, activated charcoal (Merck; Germany) was added and the suspension filtered. The filtrate contained [methyl-^{3}H]dTMP-glycol (**31**) at a yield of 20%.

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