Identification of 3,N^4-etheno-5-methyl-2′-deoxycytidine in human DNA: a new modified nucleoside which may perturb genome methylation

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Supplementary Methods

Synthesis Scheme 1.

5′-*O*-(4,4′-dimethoxytrityl)-thymidine-3′-bis(2-cyanoethyl)phosphate (2).

A solution of the commercially available thymidine phosphoramidite (**1**) (3′-*O*-[(diisopropylamino)(2-cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-thymidine, 500 mg, 0.67 mmol) in 10 mL acetonitrile was incubated at ambient temperature with 0.5 mL 3-hydroxypropanenitrile and 2.36 mL of a 1 mM solution of tetrazole in acetonitrile. After 90 min 10 mL of a solution of 3% cumene hydroperoxide in acetonitrile was added to oxidize the phosphite moiety to a phosphate triester. After 2 h the reaction was stopped by addition of 3 mL ethanol. The resulting solution was diluted with 50 mL chloroform and washed with a concentrated aqueous solution of $NaHCO_3$ (2 × 30 mL) and water (2 × 30 mL). The organic layer was separated, dried with Na_2SO_4 and filtered; the solvent was removed *in vacuo*. The product was purified by flash chromotography (silica gel, chloroform/methanol 95/5 v/v) to yield 5′-*O*-(4,4′-dimethoxytrityl)-thymidine-3′-bis(2-cyanoethyl)phosphate (**2**) (187 mg, 38%). For $C_{37}H_{39}N_4O_{10}P$, exact mass M = 730.2404; ESI-MS: $[M+Na]^+$ *m/z* calcd. 753.2296, found 753.0 (100%).

O^4-Ethyl-5′-*O*-(4,4′-dimethoxytrityl)-thymidine-3′-bis(2-cyanoethyl)phosphate (3).

A freshly prepared solution of diazoethane (ca. 8 mmol) in diethylether (10 mL) was poured into a solution of **2** (185 mg, 0.25 mmol) in 5 mL methanol (***Caution***: *diazoethane is a hazardous alkylating compound and should be handled carefully*). After 30 min the solvents were removed *in vacuo*, and the residue was purified by flash chromatography (chloroform/ethanol: 98/2 v/v) to yield the O^4-ethyl

adduct **3** (26 mg, 13%). For $C_{39}H_{43}N_4O_{10}P$, exact mass M = 758.2716; ESI-MS (*m/z*): $[M+Na]^+$ calcd. 781.2609, found 781.2 (40%); $[2M+Na]^+$ calcd. 1539.5326, found 1539.6 (100%).

5-methyl-2′-deoxycytidine-3′-phosphate (4).

For the 5′ deprotection step, compound **3** (25 mg, 33 mmol) was dissolved in 2 mL dry nitromethane, and 2 mL of a saturated solution of $ZnBr_2$ in nitromethane was added. The reaction mixture turned bright orange. The reaction was then quenched with 1 M ammonium acetate in H_2O (40 mL) followed by extraction with chloroform (80 mL). The organic solvent was dried over Na_2SO_4, filtered and removed *in vacuo*. The residue was incubated with 10 mL ammonia for 16 h to deprotect the 3′ phosphate and to replace the O^4-ethyl group with an NH_2 group. Ammonia was roughly removed *in vacuo*, and the solution was lyophilized. Reversed-phase HPLC (Instrumentation: Hewlet Packard, column: prep-C18 reversed phase, solvent: gradient 0 to 50% acetonitrile in water over 30 min; flow rate: 1 mL/min) yielded pure 5-methyl-2′-deoxycytidine-3′-phosphate (5mdCyd-3′-*P*, **4**). For $C_{10}H_{16}N_3O_7P$, exact mass M = 321.0726; LC-ESI-MS (*m/z*): $[M–H]^-$ calcd. 320.0653, found 320.0; $[2M–H]^-$ calcd. 641.1379, found 641.0; $[M–(C_5H_7N_3O)–H]^-$ calcd. 195.0064, found 195.0 (neutral loss of 5mCyt).

3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate (5).

Compound **4** was incubated overnight in 1 M chloroacetaldehyde (CAA) in water (1 mL) and subsequently subjected to reversed-phase HPLC (conditions as above for **4**), yielding pure 3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate (ε5mdCyd-3′-*P*, **5**) (100%). This new synthetic compound was characterized by mass spectrometry and UV spectrophotometry (Fig. 1) as well as ^{1}H-NMR at 500 MHz (Table 1) as shown in Suppl. Fig. 1 below).

Suppl. Fig. 1. 500 MHz ^{1}H-NMR spectrum of 3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate (ε5mdCyd-3′-*P*, **5**), 0.5 mg in 0.4 mL D_2O, 30 °C, with Lorentz-Gauss resolution enhancement. Multiplets are labeled as in Table 1; unlabeled signals represent residual solvent impurities, e.g. acetonitrile, methanol, acetone, unknown ethyl-X with triplet at 1.28 ppm (not shown) and quartet at 3.20 ppm. Residual HDO signal was not suppressed and exhibits spinning sidebands.

500 MHz 1H–NMR: 3,N4–etheno–5–methyl–2'–deoxycytidine–3'–phosphate (5) in D2O, 30°C