Formation of Modified Cytosine Residues in the Presence of Depurinated DNA

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Instrumentation and methods ¹H and ¹³C NMR spectra were recorded in (CD₃)₂SO at 300 MHz and 75 MHz, respectively. Chemical shifts δ are given in ppm relative to the residual solvent peak; coupling constants *J* are given in Hz. Assignments were made with the aid of COSY, HMBC and HMQC 2D NMR experiments. Thin layer chromatography was performed using aluminum-backed sheets. UV spectra were collected using a single-beam spectrophotometer. Melting points were determined using a capillary melting point instrument and are uncorrected. All purchased reagents and solvents were of reagent grade or higher and were used without further purification. HPLC with UV and MS detection (HPLC-UV/MS) was performed using an HPLC system consisting of a binary pump, a degasser, a column oven and a variable wavelength UV detector. After passing through the UV detector, the column eluate was introduced directly into a single quadrupole, electrospray mass spectrometer. Oligonucleotide samples were separated using a C18 column (3 μ m, 2 $\times$ 150 mm) held at 50 °C and eluted at 0.25 mL/min with a

gradient of acetonitrile in 5 mM tributylammonium acetate (TBuAA). Mass spectra were collected in the negative ionization mode with the capillary voltage set to 4000 V, the gas temperature to 325 °C and the nebulizer pressure to 30 psig (System A). Enzyme digest samples, and the products formed by reaction of 2'-deoxycytidine with 4-oxo-2-pentenal, were analyzed at room temperature using a C18 column (3 μ m, 2 $\times$ 150 mm) eluted at 0.25 mL/min with a gradient of acetonitrile in 20 mM triethylammonium acetate. Mass spectra were collected in the positive ionization mode with the capillary voltage set to 4000 V, the gas temperature to 350 °C and the nebulizer pressure to 30 psig (System B). (MS)² analysis was performed by directly infusing samples into an ion trap, electrospray mass spectrometer. Mass spectra were collected in the positive ionization mode with the capillary voltage set to 4500 V, the gas temperature to 350 °C, the nebulizer pressure to 25 psig and the fragmentation amplitude to 0.5 V. For (MS)³ analysis, the fragmentation amplitude was set to 1.0 V and 0.5 V for the first and second fragmentation steps, respectively.

Synthesis of depurinated oligonucleotides (2a): Synthesis was performed on an OligoPilot II DNA/RNA synthesizer using 5'-O-DMT-5-methyl-2'-(2-methoxyethyl)uridine loaded Primer 200™ support (200 μ mol/g, 2.5 g). Standard 5'-O-DMT-protected-O-2-cyanoethyl-N, N-diisopropyl phosphoramidites (dA^{Bz} and T) were dissolved to a concentration of 0.2 M in anhydrous acetonitrile and activated with a 0.45 M solution of 1*H*-tetrazole in acetonitrile. Detritylation was accomplished by treatment with a 10% v/v solution of dichloroacetic acid in toluene.¹ Oxidation was achieved with a 15% v/v solution of *tert*-butyl hydroperoxide (70% v/v in water) in acetonitrile.² After completion of synthesis, the support-bound material was suspended in ammonium

hydroxide (100 mL) and the products heated at 55 °C for 16 h then allowed to cool to room temperature. The support was removed by filtration and washed with a 1:1 v/v solution of ethanol and water (100 mL). The combined filtrate and washings were concentrated under vacuum to a volume of 12 mL. To 3 mL of this solution was added concentrated hydrochloric acid (0.3 mL, 3.7 mmol). After 3 days at room temperature, aqueous sodium acetate solution (3 M, pH 8, 0.5 mL) and ethanol (40 mL) were added and the products cooled in dry ice. The formed precipitate was collected by filtration and washed with ethanol (20 mL). The material was analyzed by HPLC-mass spectrometry (System A) to confirm the completeness of the depurination reaction. (**2b**) This compound was synthesized in a similar manner using 5'-O-DMT-thymidine loaded Primer 200™ support (200 μmol/g, 2.5 g). **2a**, HPLC-MS Calcd: 2945.5 Da, found 2945.4 Da. **2b**, HPLC-MS Calcd: 2871.5 Da, found 2871.0 Da.

Synthesis of tetramers and trimers (4-10) Syntheses were performed at a 1 μmol scale on a DNA/RNA synthesizer following the manufacturer's standard 1 μmol cycle. 5'-O-DMT-protected-*O*-2-cyanoethyl-*N,N*-diisopropylphosphoramidites (dA^{Bz}, dC^{Bz}, dG^{ib} and T), nucleoside loaded controlled-pore glass supports and standard detritylation, activator, oxidation and capping solutions were used. Following synthesis, the crude DMT-off products were cleaved from the solid support and deprotected by treatment with ammonium hydroxide. These solutions were concentrated to give the title compounds that were used in subsequent experiments without purification.

Reaction of 4-10 with 2a and 2b. Tetramers 4-7 (0.25 μmol) in water (50 μL) were added separately to **2a** (10 mg, 3.4 μmol). The pH of the reaction mixtures was neutral. The mixtures were incubated at 40 °C for 5 days. Trimers **8-10** (0.5 μmol) in water (100

μL) were incubated with **2b** (10 mg, 3.4 μmol) in a similar manner. The products of each reaction were analyzed by HPLC-UV/MS (System A). For samples containing **4-7**, the extent of reaction was estimated by extracting the ions due to the -2 charge states of the parent and modified tetramers. For reaction mixtures containing **8-10**, the responses due to the -1 charge states of the parent and modified trimers were extracted. The resulting ion chromatograms were integrated, and the areas due to the parent and modified components compared. All compounds were assumed to have equivalent ionization efficiencies.

Isolation and enzymatic digestion of $\text{d}(\text{C})_4 + 80$ Da impurity The reaction mixture obtained by incubation of **5** with depurinated donor sequence **2a** was subjected to preparative HPLC purification to provide a sample enriched in the $\text{d}(\text{C})_4 + 80$ Da impurity. The crude reaction mixture was diluted to 200 μL with water and 50- μL aliquots purified using a C18 column (5 μm , 6×250 mm) held at 50 $^\circ\text{C}$ and eluted at 1 mL/min with a gradient of acetonitrile in 5 mM TBAA. The fractions eluting with 40% acetonitrile were combined and concentrated to dryness. To a solution of the residue in water (180 μL), was added an aqueous solution of snake venom phosphodiesterase (2 units in 1000 μL of water, 5 μL) and an aqueous solution of alkaline phosphatase (100 units in 1000 μL of water, 5 μL). The products were incubated at 37 $^\circ\text{C}$ for 14 h then analyzed by HPLC-UV/MS (System B). Peaks due to 2'-deoxycytidine ($t_{\text{R}} = 5$ min), thymidine ($t_{\text{R}} = 15$ min) and an unknown peak ($t_{\text{R}} = 23$ min) were observed. The $t_{\text{R}} = 23$ min component was collected and subjected to $(\text{MS})^2$ analysis. Analytical data for $t_{\text{R}} = 23$ min component: $\lambda_{\text{max}} = 278$ nm; $[\text{M}+\text{Na}]^+ = 330.1$.

References

- (1) Krotz, A. H., Cole, D. L. and Ravikumar, V. T. *Nucleosides & Nucleotides* **1999**, *18* (6 & 7), 1207-1209
- (2) (a) Jäger, A. and Engels, J. *Tet. Lett.* **1984**, *25* (14), 1437-1440; (b) Maier, M. A., Choi, Y., Gaus, H., Barchi Jr., J. J., Marquez, V. E., and Manoharan, M. *Nucl. Acids Res.* **2004** *32*, 3642-3650.

HPLC-UV analysis (System B) of a) crude reaction mixture formed by treating 2'-deoxycytidine with 4-oxo-2-pentenal; b) $t_R = 23$ min component isolated from enzyme digest of $d(C)_4 + 80$ Da impurity; c) comix

Online UV analysis of main product formed by reaction of 2'-deoxycytidine with 4-oxo-2-pentenal (**13**, solid line) and $t_R = 23$ min component isolated from enzyme digest of $d(C)_4 + 80$ Da impurity (broken line).

(MS)² analysis of a) $t_R = 23$ min component isolated from enzyme digest of d(C)₄ + 80 Da impurity b) **13**

((MS)³ analysis of a) t_R = 23 min component isolated from enzyme digest of d(C)₄ + 80 Da impurity b) **13**

¹H NMR

Current Data Parameters
NAME 1018-17
EXPNO 20
PROCNO 1

F2 - Acquisition Parameters
Date_ 20040910
Time 14.19
INSTRUM spect
PROBHD 5 mm QNP 1H/13
PULPROG zg30
TD 65536
FIDRES 0.094190 Hz
AQ 5.3084660 sec
RG 406.4
DW 81.000 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec

===== CHANNEL f1 =====
NUC1 1H
P1 8.50 usec
PL1 -2.00 dB
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 32768
SF 300.1300005 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

¹³C NMR

204.17

146.77
145.09

132.58
127.76
122.95

98.83

87.62
84.70

70.33

61.21

40.32
40.04
39.76
39.48
39.20
38.93
38.65
29.24

Current Data Parameters
NAME 1088-17
EXPNO 11
PROCNO 1

F2 - Acquisition Parameters
Date_ 20040910
Time_ 11.39
INSTRUM spect
PROBHD 5 mm QNP 1H/13
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 1024
DS 4
SWH 17985.611 Hz
FIDRES 0.274439 Hz
AQ 1.8219508 sec
RG 10321.3
DW 27.800 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
d11 0.03000000 sec
d12 0.00002000 sec

===== CHANNEL f1 =====
NUC1 13C
P1 8.25 usec
PL1 -2.00 dB
SFO1 75.4752953 MHz

===== CHANNEL f2 =====
CPDPRG2 waltz16
NUC2 1H
PCPD2 70.00 usec
PL2 -2.00 dB
PL12 15.00 dB
PL13 15.00 dB
SFO2 300.1312005 MHz

F2 - Processing Parameters
SI 32768
SF 75.4677867 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

200 180 160 140 120 100 80 60 40 20 ppm

S11

COSY NMR

Current Data Parameters
NAME Q108-13
EXPNO 13
PROCNO 1

F2 - Acquisition Parameters
Date_ 20040721
Time 0.21
INSTRUM spect
PROBHD 5 mm QNP 1H/13
PULPROG cosygpgf
TD 2048
SOLVENT DMSO
NS 1
DS 8
SWH 2332.090 Hz
FIDRES 1.138716 Hz
AQ 0.4391412 sec
RG 64
DE 214.400 usec
TE 300.0 K
D0 0.0000300 sec
D1 1.30216205 sec
d13 0.0000400 sec
D16 0.0001000 sec
IN0 0.00042880 sec
MCREST 0.0000000 sec
MCWRK 1.30216205 sec

===== CHANNEL f1 =====
NUC1 1H
P0 8.50 usec
P1 8.50 usec
PL1 -2.00 dB
SFO1 300.1313158 MHz

===== GRADIENT CHANNEL =====
GPRAM1 SINE.100
GPRAM2 SINE.100
GPT1 0.00 %
GPT2 0.00 %
GPT3 0.00 %
GPT4 0.00 %
GPT5 10.00 %
GPT6 10.00 %
P16 1000.00 usec

F1 - Acquisition Parameters
MD0 1
TD 128
SFO1 300.1313 MHz
FIDRES 18.219450 Hz
SW 7.770 PPM
FHM0DE QF

F2 - Processing parameters
SI 1024
SF 300.1300308 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
PC 1.40

F1 - Processing parameters
SI 1024
MC2 QF
SF 300.1300308 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0

HMBC NMR

Current Data Parameters
NAME Q1038-13
EXNO 21
PROCNO 1

F2 - Acquisition Parameters
Date_ 20070722
Time_ 17:54

INSTRUM spect
PROBHD 5 mm QNP 1H/13
PULPROG invgprfddg

TD 1024
SOLVENT DMSO
NS 200

DS 16
SWH 232.190 Hz
FIDRES 2.7744 Hz

AQ 0.215951 sec
RG 23170.5
DE 24.400 usec

TE 300.0 K
D0 0.00000300 sec
D1 1.40753497 sec

D2 0.06500000 sec
d13 0.00010000 sec

D16 0.00002390 sec
INVO 0.00000000 sec

WCRESST 1.40753497 sec
MCWRC

===== CHANNEL F1 =====
NUC1 1H
P1 8.50 usec
PL1 1.00 dB

===== CHANNEL F2 =====
NUC2 13C
P2 8.25 usec
PL2 -2.00 dB

SFO2 75.4752539 MHz
===== GRADIENT CHANNEL =====
GPRM1 SINE 1.00
GPRM2 SINE 1.00

GPRM3 SINE 1.00
GFX1 0.00 %
GFX2 0.00 %

GFX3 0.00 %
GFX4 0.00 %
GFX5 0.00 %

GFX6 0.00 %
GFX7 0.00 %
GFX8 0.00 %

GFX9 0.00 %
GFX10 0.00 %
GFX11 0.00 %

GFX12 0.00 %
GFX13 0.00 %
GFX14 0.00 %

GFX15 0.00 %
GFX16 0.00 %
GFX17 0.00 %

GFX18 0.00 %
GFX19 0.00 %
GFX20 0.00 %

GFX21 0.00 %
GFX22 0.00 %
GFX23 0.00 %

GFX24 0.00 %
GFX25 0.00 %
GFX26 0.00 %

GFX27 0.00 %
GFX28 0.00 %
GFX29 0.00 %

GFX30 0.00 %
GFX31 0.00 %
GFX32 0.00 %

GFX33 0.00 %
GFX34 0.00 %
GFX35 0.00 %

GFX36 0.00 %
GFX37 0.00 %
GFX38 0.00 %

GFX39 0.00 %
GFX40 0.00 %
GFX41 0.00 %

GFX42 0.00 %
GFX43 0.00 %
GFX44 0.00 %

GFX45 0.00 %
GFX46 0.00 %
GFX47 0.00 %

GFX48 0.00 %
GFX49 0.00 %
GFX50 0.00 %

GFX51 0.00 %
GFX52 0.00 %
GFX53 0.00 %

GFX54 0.00 %
GFX55 0.00 %
GFX56 0.00 %

GFX57 0.00 %
GFX58 0.00 %
GFX59 0.00 %

GFX60 0.00 %
GFX61 0.00 %
GFX62 0.00 %