Supporting Information for PNA Based Reagents for the Direct and Site Specific Synthesis of Thymine Dimer Lesions in Genomic DNA

J. Carsten Pieck, David Kuch, Friederike Grolle, Uwe Linne, Clemens Haas, and Thomas Carell*
Ludwig-Maximilians University, Department of Chemistry and Biochemistry, Butenandtstr. 5-13, 81377 Munich, Germany

General. Unless otherwise noted, starting materials were obtained from commercial sources and used without further purification; unmodified DNA oligonucleotides were purchased HPLC-purified from Metabion, Martinsried/Germany, and used as received. All enzymes, E.coli strains and antibodies were purchased from Sigma-Aldrich (Deisenhofen, Germany) and NEB (Frankfurt, Germany). Magnetic particles (Dynabeads MyOne-Streptavidin) were from Invitrogen (Karlsruhe, Germany). The positively charged nylon membrane (Immobilon-Ny+) was purchased from Millipore. [2-(9H)-Fluoren-9-ylmethoxycarbonylamino]-ethyl-amino-acetic acid-ethyl ester (5) was synthesized according to the procedure from Thomson et al. TLC analysis of reaction mixtures was carried out on Merck silica gel 60 F254 TLC plates; visualization was achieved by UV light or staining with an anisaldehyde solution. Column chromatography was performed on Merck silica gel 60 (0.040 – 0.063 mm mesh). Melting points were determined on a Büchi Smp 20 machine and are uncorrected. 1H- and 13C-NMR spectra were recorded on Bruker AMX 300 and Bruker AMX 400 spectrometers. Mass spectra were recorded on a Finnigan TSQ 7000, Finnigan MAT 95 S, PE Sciex Q-Star Pulsar i (all for ESI) and Bruker Flex III (for MALDI-TOF) machines. Matrices used for MALDI-TOF analysis were: for DNA oligonucleotides, a mixture of 2,4,6-trihydroxyacetophenone (0.5 M in ethanol), diammoniumcitrate (0.1 M in water) and ethanol (2:1:12) or 3´-hydroxypicolinic acid (0.5 M in MeCN / water 1:1); for PNA oligonucleotides a saturated sinapinic acid solution (in 0.1 % TFA in water) was used. If necessary, oligonucleotides were desalted on MF-Millipore membranes before measuring masses. IR spectra were recorded on a Bruker IFS 25 and a Bruker IFS 88 spectrometer. UV/Vis spectra were recorded on a Varian Cary 100 Bio spectrometer. HPLC purifications and analysis were performed on Merck-Hitachi machines using CC 250/4 Nucleosil 120-3 C18 and CC 250/4 Nucleosil 120-3 C8 columns from Macherey-Nagel for analytical separations and CC 250/21 Nucleosil 100-7 C18 columns for preparative separations.

Synthesis of the acetophenone-PNA monomer building block (7).

1-[4-(Bromomethyl)phenyl]ethanone (2): 4-Methylacetophenone (1) (10.0 mL, 74.5 mmol, 1.0 eq) was dissolved in acetonitrile (80 mL) under argon. NBS (14.6 g, 82.0 mmol, 1.1 eq) and AIBN (1.23 g, 7.49 mmol, 0.1eq) were added and the reaction mixture was stirred for 1.5 h at 90°C to give a yellow solution. The solvent was removed in vacuo, the residue taken up in toluene (100 mL) and the solution filtrated. After removal of the solvent, purification by silica gel column chromatography (n-pentane / EtOAc 10:1 → 5:1) yielded 2 as a white solid (15.1 g, 70.7 mmol, 95 %).
(4-Acetylphenyl)acetonitrile (3): (2) (17.1 g, 80.4 mmol, 1.0 eq) was dissolved in dioxane (140 mL) and NaCN (4.41 g, 90.0 mmol, 1.10 eq) in water (140 mL) was added. The solution was heated to reflux for 2 h (90°C) while stirring; after cooling to room temperature the deep red solution was extracted with EtOAc (3 x 180 mL), the combined organic phases were washed with brine (2 x 160 mL) and dried with MgSO₄. Solvents were evaporated and a red solid was obtained. Purification by silica gel column chromatography (n-pentane / EtOAc 2:1) gave 3 as a light red solid (8.54 g, 53.6 mmol, 67%).

Rf = 0.27 (n-pentane / EtOAc 2:1); m.p.: 83-84°C; ¹H-NMR (300 MHz, CDCl₃): δ = 2.59 (s, 3H, CH₃), 3.81 (s, 2H, CH₂), 7.42 (d, 3J = 7.8 Hz, 2H, Aryl-CH), 7.95 ppm (d, 3J = 7.6 Hz, 2H, Aryl-CH); ¹³C-NMR (75 MHz, CDCl₃): δ = 23.7, 26.7, 117.2, 128.3, 129.1, 135.1, 136.9, 197.4 ppm; MS (EI): m/z (%) = 159 [M+] (28), 144 [M+-CH₃] (100), 116 [M+-COCH₃] (28), 89 [CHC₆H₄+] (32), 43 [COCH₃+] (31).

(4-Acetylphenyl)acetic acid (4): (3) (9.40 g, 59.0 mmol) was suspended in a mixture of water (200 mL), glacial acetic acid (200 mL) and sulfuric acid (200 mL) and heated to reflux for 90 min (100°C). To the clear solution was added NaOH (50 g) and water until a total volume of 1.2 L was reached. After the NaOH was fully dissolved, the solution was allowed to cool to room temperature and extracted with EtOAc (3 x 200 mL). The combined organic phases were washed with brine (250 mL) and dried with MgSO₄. Removal of the solvent yielded (4) as a colourless solid (4.96 g, 27.8 mmol, 47 %).

Rf = 0.45 (CHCl₃ / MeOH / HCOOH 10:2:0.2); m.p.: 113-114°C; FTIR (KBr): 2929 (s), 1726 (s), 1647 (s), 1605 (m), 1422 (m), 1368 (m) 1281 (m), 1219 (m), 1171 (s), 813 (m), 594 cm⁻¹ (m); ¹H-NMR (300 MHz, CDCl₃): δ = 2.58 (s, 3H, CH₃), 3.71 (s, 2H, CH₂), 7.37 (d, 3J = 8.0 Hz, 2H, aryl-CH), 7.92 (d, 3J = 8.3 Hz, 2H, aryl-CH), 10.64 ppm (brs, 1H, COOH); ¹³C-NMR (75 MHz, CDCl₃): δ = 26.7, 41.0, 128.8, 129.8, 136.2, 138.8, 176.8, 198.2 ppm; MS (EI): m/z (%) = 178 [M+] (34), 163 [M+-CH₃] (100), 135 [M+-COCH₃] (21), 43 [COCH₃+] (14); HRMS (EI): calc. for C₁₀H₁₀O₃ [M+]: 178.0630, found: 178.0626.

tert-Butyl[[(4-acetylphenyl)acetyl]{2-[(9H-fluoren-9-ylmethoxy carbonyl)amino]ethyl}amino]acetate (6): The HCl salt of PNA backbone (5) (1.22 g, 2.81 mmol, 1.0 eq) was dissolved in CHCl₃ (200 mL), washed with saturated NaHCO₃ solution (3 x 100 mL) and dried with MgSO₄. Concentration in vacuo gave free (5) as a colourless oil which was dissolved in dry DMF (15 mL). (4) (500 mg, 2.81 mmol, 1.0 eq), TBTU (1.35 g, 4.21 mmol, 1.5 eq) and HOBt (645 mg, 4.21 mmol, 1.5 eq) were dissolved in dry DMF (20 mL), stirred for 20 min at room temperature and added, with triethylamine (782 µl, 569 mg, 5.62 mmol, 2.0 eq), to the solution of (5) in DMF. The solution was stirred for 80 min at room temperature, then diluted with CHCl₃ (300 mL) and washed with water (4 x 200 mL); the organic phases were dried with MgSO₄ and the solvents evaporated, followed by chromatographic purification (silica gel; toluene / EtOAc 10:1 → 3:1 → 1:1). (6) was isolated as a colourless solid (1.52 g, 2.73 mmol, 97 %).

Rf = 0.64 (CHCl₃ / MeOH 10:1); m.p.: 54°C; FTIR (film on NaCl): 2978 (w), 1721 (s), 1681 (s), 1650 (s), 1607 (w), 1520 (w) 1450 (m), 1367 (w), 1266 (s), 1154 (s), 759 (m), 742 cm⁻¹ (m); ¹H-NMR (400 MHz, CDCl₃): δ = 1.46 (1.47) (s, 9H, C(CH₃)₃), 2.52 (2.51) (s, 3H, CH₃ acetophenone), 3.31-3.78 (m, 2H, NH-CH₂), 3.47 (3.56) (t, 3J = 5.5 Hz, 2H, NH-CH₂-CH₂), 3.74 (3.63) (s, 2H, CH₂), 3.91 (3.97) (s, 2H, CH₂ glycine), 4.19 (t, 3J = 7.3 Hz, 1H, CH
Fmoc, 4.39 (4.33) (d, $^3$J = 7.0 Hz, 2H, CH$_2$ Fmoc), 5.99 (5.64) (t, $^3$J = 6.8 Hz, 1H, NH), 7.25-7.31 (m, 4H, aryl-CH), 7.36-7.39 (m, 2H, Aryl-CH), 7.57-7.60 (m, 2H, aryl-CH), 7.74 (d, $^3$J = 7.5 Hz, 2H, Arlyl-CH), 8.3-8.6 ppm (m, 2H, aryl-CH), (2 rotamers); 13C-NMR (100 MHz, CDCl$_3$): $\delta$ = 26.6, 28.0, 39.4 (38.7), 39.9 (40.4), 47.2 (47.3), 49.8 (49.5), 66.9 (66.7), 82.3 (83.1), 120.0 (119.9), 125.0 (125.1), 127.1 (127.0), 127.8 (127.7), 128.7 (128.9), 129.3 (129.4), 135.7 135.8, 137.8, 140.3 (139.9), 141.3 (141.2), 143.8 (143.9), 156.7, 169.3 (168.5), 171.0 (171.6), 197.8 (197.7) ppm, (2 rotamers); MS (ESI+): m/z (%) = 557 [M+H +], 579 [M+Na+]; HRMS (ESI+): calc. for C$_{33}$H$_{36}$N$_2$O$_6$ [M+Na+] : 579.2471, found: 579.2471.

((4-Acetylphenyl)acetyl)[2-(9H-fluorenyl-9-ylmethoxycarbonyl)amino]ethyl]amino acetic acid (7): The tBu-protected acetophenone-PNA monomer (6) (1.00 g, 1.80 mmol, 1.0 eq) was suspended in a mixture of TFA / water (95:5) (20 mL) and stirred for 1 h at room temperature. The solvent was removed in vacuo and the residue taken up with Et$_2$O, giving a white precipitate. Filtration and drying yielded (7) as a colourless solid (940 mg, 1.69 mmol, 94 %).

R$_f$ = 0.26 (CHCl$_3$ / MeOH / HCOOH 10:1:0.1); m.p.: 101-105°C; FTIR (KBr): 2945(m), 1681 (s), 1653 (m), 1522 (m), 1449 (m), 1414 (m), 1268 (s), 1184 (m), 741 cm$^{-1}$ (m); 1H-NMR (400 MHz, $(CD_3)_2$SO): $\delta$ = 2.54 (2.52) (s, 3H, CH$_3$ acetophenone), 3.14-3.22 (m, 2H, NH-C$_2$H$_5$), 3.43 (3.37) (t, $^3$J = 6.0 Hz, 2H, NH-CH$_2$-C$_2$H$_5$), 3.81 (3.68) (s, 2H, CH$_2$ acetophenone), 3.99 (4.12) (s, 2H, CH$_2$ glycine), 4.22 (t, $^3$J = 6.7 Hz, 1H, CH Fmoc), 4.35 (4.28) (d, $^3$J = 6.7 Hz, 2H, CH$_2$ Fmoc), 7.25-7.38 (m, 6H, aryl-CH), 7.61-7.65 (m. 2H, aryl-CH), 7.79-7.85 ppm (m, 4H, aryl-CH), (2 rotamers); 13C-NMR (75 MHz, (CD$_3$)$_2$SO): $\delta$ = 26.5, 38.1, 46.7, 47.4, 47.8, 50.2, 65.4 (64.8), 120.0, 124.9 (125.0), 127.0, 127.5, 128.0 (127.9), 129.4 (129.6), 135.1, 140.7 (140.6), 141.2, 143.7 (143.8), 156.3 (156.1), 170.1, 170.6 (171.0), 197.4 ppm (2 rotamers); MS (ESI+): m/z = 501 [M+H +], 523 [M+Na +], 539 [M+K +]; HRMS (ESI+): calc. for C$_{29}$H$_{28}$N$_2$O$_6$ [M+Na +]: 523.1845, found: 523.1825.

Methods.

PNA synthesis. PNA strands were synthesized on an Expedite 8900 Nucleic Acid Synthesis System from PerSeptive Biosystems according to well-established Fmoc-synthesis protocols. Fmoc-protected PNA monomers, HATU and Fmoc-protected XAL- and PAL-PEG solid supports were purchased from PEBiosystems. NMP and DMF were from Fluka (quality “for peptide synthesis”). Acetophenone monomers were coupled twice and with an elongated coupling time of 15 min. The PNA oligonucleotides were purified by reversed phase HPLC at 55°C; detection at 260 nm. Further characterization was achieved by MALDI-ToF mass spectrometry.

For RP-HPLC purification, the following buffers and gradient systems were used:

- analytical HPLC: Buffer A: 0.1% TFA in H$_2$O
  Buffer B: 0.1% TFA in MeCN
  Flow: 0.5 mL/min
  0 → 2 min, 100% A; 2 → 12 min, 100 → 96% A, 12 → 39 min,
  96 → 50% A; 39 → 42 min, 50 → 0% A; 42 → 47 min, 0% A; 47 →
  50 min, 0 → 100% A; 50 → 55 min, 100% A

- preparative HPLC: Buffer A: 0.1% TFA in H$_2$O
  Buffer B: 0.1% TFA in MeCN
  Flow: 20 mL/min
  0 → 10 min, 100 → 96% A, 10 → 37 min, 96 → 50% A; 37 →
  40 min, 50 → 0% A; 40 → 45 min, 0% A; 45 → 47 min, 0 →
  100% A; 47 → 50 min, 100% A
MALDI-TOF analysis of the synthesized PNA strands:

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Mass calc.</th>
<th>Mass found</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA 8</td>
<td>KK-GCG-CAT-GCA-ACG-TGC-G-KK-NH2</td>
<td>4877.1</td>
<td>4876.4</td>
</tr>
<tr>
<td>PNA 9</td>
<td>KK-GCG-TGC-AAC-GTA-CGC-G-KK-NH2</td>
<td>4877.1</td>
<td>4875.6</td>
</tr>
<tr>
<td>PNA 10</td>
<td>KK-GCG-CAT-GCA-ACG-TGC-G-KK-NH2</td>
<td>4862.1</td>
<td>4861.2</td>
</tr>
<tr>
<td>PNA 11</td>
<td>KK-GCG-CAT-GAA-AAC-ACG-G-KK-NH2</td>
<td>4901.1</td>
<td>4901.0</td>
</tr>
<tr>
<td>PNA 12</td>
<td>KK-TCA-TGC-AAC-GCG-C-KK-NH2</td>
<td>4003.3</td>
<td>4009.6</td>
</tr>
<tr>
<td>PNA 13</td>
<td>KK-GTT-GTC-AAC-GCG-T-KK-NH2</td>
<td>4049.3</td>
<td>4053.7</td>
</tr>
</tbody>
</table>

Hybridizations. All PNA:DNA and DNA:DNA hybridisations were done in an Eppendorf Mastercycler Personal. DNA:DNA hybridisations were performed in buffer A, PNA:DNA hybridisations in buffer B. Hybridisation of DNA3 and DNA4 was done in buffer C. Hybridisation of the M13 mp 18 single strand with DNA\(^B\) (5’-TCG-CCA-TTC-AGG-CTG-CGC-AAC-TGT-T\(^B\)GG-GAA-GGG-CTA-TCG-GT-3’ with \(^B\) = biotin label) was done in buffer D.

Melting curves. In order to determine the impact of mismatches in the target sequence on PNA binding, UV melting curves of PNA:DNA double strands were measured. Herein PNA13 was hybridized to a fully complementary DNA strand (DNA5) as well as to DNA strands containing one (DNA6) and two (DNA7) mismatches, respectively.

DNA5  \(^5\)’CCCAACAGTTGCGCAGC\(^3\)’
DNA6  \(^5\)’CCCAACC\(_{\text{GTTGCGCAGC}}\)\(^3\)’
DNA7  \(^5\)’CCCAACC\(_{\text{GTTG}}\)\(_{\text{GCAGC}}\)\(^3\)’

UV melting curves were recorded on a Varian Cary 100 Bio using a Cary Temperature Controller and a Sample Transport Assesory. The temperature gradient was 0.5°C/min. All melting curves were recorded at 260 and 320 nm. Concentrations of the solutions: 3 µM PNA, 3 µM DNA, 10 mM NaCl, 10 mM H\(_3\)PO\(_4\)/Na\(_2\)HPO\(_4\) (pH7).

The averaged cooling curves and the melting points are shown in Figure 1.

UV melting point studies of acetophenone containing PNA. DNA hybrid double strands without, with one and with two mismatches, showing that the reagents recognizes selectively the fully complementary target sequence in the DNA strand. The acetophenone itself destabilises the PNA:DNA hybrid like one mismatch. For typical 16mer we observe after incorporation of the acetophenone a melting point reduction of about 15°C.
Figure 1: DNA:PNA melting curves and melting points. The graphs show that one or two mismatches in
the reagent PNA cause strong melting point reduction.

Irradiation experiments. For irradiation experiments a 1000 W Hg (Xe) lamp with ca. 700 mW / cm² from L.O.T.-Oriel was used. The wavelength was controlled by a Jasco long pass filter (> 340 nm, 50 x 50 mm, 2 mm thick) that was kept at 20°C by watercooling. 200 µL of a 20 µM solution of the DNA:PNA strands was irradiated for 3 h in a fluorescence cuvette cooled at 10°C. The reaction products were separated by reversed phase HPLC at 55°C column temperature; under these conditions, DNA:PNA strands are denatured.

Restriction and total digestion. The restriction reaction of DNA3 was done in a 50 µL volume of buffer C containing 1,8 µg DNA, 50 units BstUI for 2.5h at 60°C. After RP-HPLC the different fractions were analyzed by agarose gel electrophoresis using standard conditions (Figure 2).

Figure 2: (Line 1) separated 15 mer; (Line 2) reaction mixture before RP-HPLC work up; (Line 3) separated 36 mer.
The purified DNA (20 µM) was lyophilised roughly dry and resuspended in 100 µL dd H₂O. For total enzymatic digestion, 10 µL of buffer E were added, followed by 22 U Nuclease P1 and 0.05 U Calf Spleen Phosphodiesterase II. After incubation for 3 h at 37°C, 12 µL buffer F, 10 U Alkaline Phosphatase (CIP) and 0,1 U Snake venom phosphodiesterase I were added and incubated again for 3 h at 37 °C. The reaction was stopped by adding 0.1 M HCl and incubation for 5 min. Then the reaction mixture was centrifuged at 13 g for 10 min and analyzed by HPLC-MS/MS².

**Preparation of M13 mp 18 ss.** The M13 mp 18 ss strand was prepared by standard procedures (Sambrock, Molecular Cloning) in the *E.coli* strain TB1 (Genotype: F⁻ ara Δ(lac-proAB) [Φ80dlac Δ(lacZ)M15] rpsL(Str⁵) thi hsdR).

**Analysis of CPD-Lesion in M13 mp 18 ss DNA.** PNA13 was hybridized to the M13 ss and irradiated as described above. After RP-HPLC purification, the M13 ss (~250 pmol) was hybridized to DNA flawed 50 µl buffer D. 200 µL magnetic particle dispersion were immobilized on a magnetic separator and washed twice with buffer G. The particles were then resuspended in 50 µL DNA:DNA flawed containing solution and incubated for 3 h, 600 rpm at 20 °C. The magnetic particles were separated and the supernatant was discarded. After that the magnetic particles were resuspended in 300 µL Buffer H. Subsequently 20 U BglI and 20 U PvuI were added. The reaction mix was incubated for 16 h, 400 rpm at 30 °C. The magnetic particles were separated, the supernatant concentrated and the buffer changed to buffer J. The separation was analyzed by standard agarose gel electrophoresis (Figure 3).

![Figure 3: (Line 1) marker; (Line 2) separated M13 ss; (Line 3) separated 28 mer ds.](image)

The DNA was denaturated and spotted onto a positively charged nylon membrane. The 28 mer immobilized on the magnetic particles was un-fixed by heating up the magnetic particle to 90 °C in buffer J and subsequently spotted onto a positively charged nylon membrane. The membrane was washed 3 times in buffer I, blocked with buffer K and washed 3 times for 5 min with buffer I. The membrane was incubated with the primary antibody in buffer L for 2 h. The membrane was washed again 3 times in buffer I, and then incubated with the secondary antibody in buffer M for 1 h. Finally the membrane was washed for 5 min, 10 min and 15 min with with buffer I. The Lumi-Light Blotting Substrate (Roche, Mannheim, Germany) was added and analyzed by the LAS3000 from Raytest. Only the CPD containing probe shows a black staining.

**Buffers.**

Buffer A: pH=8.0, 10 mM Tris-HCl, 100 mM NaCl
Buffer B: pH=8.0, 10 mM NaH₂PO₄, 10 mM NaCl
Buffer C: pH=7.9, 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 1 mM Dithiothreitol (DTT)
Buffer D: pH=8.0, 20 mM Tris-HCl, 7 M NaCl
Buffer E: pH=5.7, 300 mM Ammoniumacetate, 100 mM CaCl₂, 1mM ZnSO₄
Buffer F: pH=8.0, 500 mM Tris-HCl, 1 mM EDTA
Buffer G: pH=7.4, 10 mM NaH₂PO₄, 150 mM NaCl
Buffer H: pH =7.9, 50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM Dithiothreitol 100 µg /mL BSA
Buffer I: pH=8.0, 100 mM Tris, 1.5 M NaCl, 0.5% (v/v) Tween 20
Buffer J: pH=8.0, 150 mM NaCl, 15 mM Sodium citrate
Buffer K: pH=8.0, 100 mM Tris, 1,5 M NaCl, 0.5 % (v/v) Tween 20 5.0 % (w/v) Skim milk powder
Buffer L: pH=8.0, 100 mM Tris, 1.5 M NaCl, 0.5 % (v/v) Tween 20, 2.0% (w/v) Skim milk powder. 1:2000 Monoclonal Anti-Thymine Dimer-AB (Clone H3)
Buffer M: pH=8.0, 100 mM Tris, 1.5 M NaCl, 0.5 % (v/v) Tween 2, 2.0 % (w/v) Skim milk powder 1:2500 Anti-mouse-IgG1-HRP labelled