



Synthesis and improved cross-linking properties of C5-modified furan bearing PNAs

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Solid Phase Diels-Alder optimization	
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In order to optimize the Diels-Alder reaction conditions on the solid phase, a small PNA sequence was N-capped with 2-furanpropionic acid (Fur-GATCT-Gly-NH₂, loading 7,45 mg/µmol).

Solvent and maleimide concentration optimization

General protocol

1- Load a 200 μL Eppendorf with 0.2 μmol resin (approx. 1.86 mg);

- 2- Prepare desired solutions of N-(N-Boc-2-amminoethyl)maleimide, and transfer them to the Eppendorf tube;
- 3- Allow Diels-Alder reaction for 15 hours at 90°C
- 4- Transfer the resin beads in a 1 mL SPE tube equipped with PE frit and wash the resin with DMF/DCM;
- 5- Add 100 μ L of a 10% m-cresol solution in TFA and allow to react for 1h30';
- 6- Percolate the solution in a 2 mL Eppendorf tube, and wash the resin with 80 µL TFA;
- 7- Add 1.8 mL ethyl ether and allow to precipitate for 2h at -20 °C;
- 8- Recover the precipitate by centrifugation, and wash the pellet with ethyl ether;
- 9- Redissolve the dry pellet in 500 μL mQ, transfer in a 500 μL Eppendorf tube and heat for 15 hours at 90 °C;
- 10- Submit the samples to UPLC-MS analysis.

Solution used

	Eq. maleimide / Concentration	Solvent: Toluene	Solvent: DMF
	100 eq / 2 M	Test A1	Test B1
50 eq / 1 M 20 eq / 0.5 M		Test A2	Test B2
		Test A3	Test B3
	10 eq / 0.25 M	Test A4	Test B4
	5 eq / 0.125 M	\	Test B5

Target identification

PNA \ Charge	No charge	2+	3+	4+
Target	1546.5	774	516	387
Alkylated	1712.7	857	571	429
Cycloaddition by-prod	1686.6	844	563	422

<u>Results</u>: when toluene is used as a solvent, it is not possible to prevent furan alkylation or the formation of a by-product with an additional unit of maleimide connected to the PNA strand. When using DMF it is possible to prevent the alkylation (20 eq / 0.5 M seems enough) but not the formation of the extra by-product. In all cases the alkylation of the furan is inversely proportional to the amount of maleimide used, while the formation of the by-product is directly proportional.

Molecules **2017**, 22, x FOR PEER REVIEW

MHP1-17-B5	Scan ES+	MHP1-17-A4 Scan ES+
100 2.702.97 100 3.30 100 5.02 5.53 5.84 7.04 7.48	TIC 5.00e9	$\begin{bmatrix} 2.77 \\ 5.55 \\ 5.87 \end{bmatrix}$
	· · · · 1	
2.00 4.00 8.00 8.00 10.00 MHP1-17-B4	Scan ES+	2.00 4.00 8.00 8.00 10.00 MHP1-17-A3 Scan ES+
2.78 2.95 4.27,5.02,5.55,84 7.02,7.48	TIC 5.53e9	$\begin{bmatrix} 100\\ 8 \end{bmatrix} = 0.92 \pm 1131.78 \sqrt{2.97} 4.27 5.04 \sqrt{5.53} 5.87 7.04 7.77 8.04 4.66e9 4.66e9 $
0^{-1} 0		
MHP1-17-B3	Scan ES+	MHP1-17-A2 Scan ES+
2.78 2.95 4.37 5.87 7.04 7.778 27 8.91	5.95e9	$\begin{bmatrix} 100 \\ 8 \\ 9 \\ 100 \\ $
0 ¹ ,, 1.12 2.00 4.00 6.00 8.00 10.00)	2.00 4.00 6.00 8.00 10.00
MHP1-17-B2	Scan ES+	MHP1-17-A1 Scan ES+
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	5.41e9	$\begin{bmatrix} 100 \\ 0.44 \\ 0.96 \\ 1.11 \\ 1.71 \\ 3.28 \\ 5.05^{5.57} \\ 7.047 \\ 51 \\ 8.25 \\ 8.91 \\ 6.04e9 $
2.00 4.00 6.00 8.00 10.00)	2.00 4.00 6.00 8.00 10.00
MHP1-17-B1	Scan ES+ TIC	MHP1-17-0 Scan ES+
3.24 3.24 4.37 5.87 8.26 8.91	5.39e9	0.48 1.02 3.30.3.45 4.23 5.07 5.91 6.76 8.188.20 9.92e8
2.00 4.00 6.00 8.00 10.00) Iime	2.00 4.00 6.00 8.00 10.00
MHP1-17-A4 162 (2 .766) C m (145:215)	Scan ES+	MHP1-17-R5 158 (2.698) Cm (145:215) Scan ES+
100 516.33	6.75e6	100 1 456 16 1 516.33 607.68 636.09 742.00 773.82 5.68e6
456.16 563.14 007.00 698.21 843.94 911.35		844.13 911.35 925.15
MHP1-17-A3 163 (2.783) Cm (145:215)	Scan ES+	MHP1-17-B4 163 (2,784) Cm (145:215) Scan ES+
100 516.33 450.40 562.01.607.68 774.07 843.94	8.83e6	100 516.33 774.07 7.93e6
		0
MHP1-17-A2 163 (2.784) Cm (145:215)	Scan ES+	MHP1-17-B3 163 (2,783) Cm (145:215) Scan ES+
100 516.33 563.01 607 68 cm c 200 00 774.07 844.38	9.96e6	100 516.33 774.07 8.53e6
0 ⁻¹ 456.09 914.37	984.37	
MHP1-17-A1 163 (2,783) Cm (145:215)	Scan ES+	MHP1-17-B2 163 (2,784) Cm (145:215) Scan ES+
100 516.33 774.07 843.94 563.01 607 74 555 51 608 52 774.07	9.53e6	100 516.33 773.88 6.54e6 6.54e6
	984.37	0 406.18 914.37 984.24 0 407 10 10 10 10 10 10 10 10 10 10 10 10 10
MHP1-17-0 160 (2.732) Cm (145:215)	Scan ES+	MHP1-17-B1 163 (2783) Cm (145:215) Scan ES+
100 405.19 \$42.17 517.08 662.74 698.34 773.88 763.73 000.36	6.9065	100 563.14 563.14 609.70 656.28 608.28 774.07 843.87 6.4666
0-1 - 0-1	m/z 1000	400 500 600 700 800 900 1000 1000
MHP1-17-A4 325 (5.550) Cm (280:360) 100- 571.70	Scan ES+ 1 7.28e6	MHP1-17-B5 324 (5.533) Clm (280:360) Scan E5+ 100- 571.70 7.13e6
443.12 464.16 528.99 577.75618.39660.47 773.75 857.36 876.38		415.14 464.16 528.93 577.82 618.83660.35 774.07 857.36 908.83
	0	MUD4 17 D4 225 (5 550) Cm (290-260)
100-2 571.70	Scan ES+ 1.05e7	100 ₃ 415.14 571.70 2.88e6 2.88e6
415.14 528.99 563.01 577.69 618.45 660.35 773.75 857.36 927.35		⁶ 464.16 529.05 618.39660.41702.50 773.88 856.92 908.83 927.48
MUD4 47 A0 000 (5 507) On (000/200)	Com EQ.	MHD1-17-B3 344 (5 874) Cm (280-360) Scop ES+
100 - 571.70	4.74e6	415.21 571.96 7.23e5
415.21 529.18 52 95 577.69618.39 665.07 773.88 857.29 927.42		8 441.23 516.33 559.99 11 606.42618.33 001.90
MHP1-17-01 326 (5 567) Cm (280-360)	Scan ES :	MHP1-17-B2 342 (5 840) Cm (280/360) Scan ES4
100-1	3.02e6	$\begin{array}{c} 415.14 \\ 516 \\ 359.73 \\ 516 \\$
\$ 415.14 529.24 505.14 577.69618.39 665.20 774.07 857.29 927.42		
MHP1-17-0 326 (5 567) Cm (280:360)	Scan ES	MHP1-17-B1 344 (5.874) Cm (280:360)
100 ₃ 571.64	1.83e6	100 415.08 559.80 577.82 606.23 665.52 2.85e5
8 443.12 528.99 660.35 774.26 856.98 875.88	m/z	of the second se
	1000	400 500 600 700 800 900 1000

Figure S1. UPLC analysis of products obtained for Fur-GATCT-Gly-NH₂ PNA synthesis obtained under various conditions (A1–A4 and B1–B5 tests reported above) using *N*-(*N*-Boc-2-amminoethyl)maleimide as protecting group. Left column: tests A, reference; Right column: tests B. Top panels: UPLC-MS traces; middle panels: MS spectra of 2.40–3.30 minutes region; bottom panels: MS spectra of 5.00–6.00 minutes region. MW target (red boxes) = 1546.5; $[MH_2]^{2+}$ = 774.3; $[MH_3]^{3+}$ = 516.5; MW alkylated product = 1712,7 (blue boxes); $[MH_2]^{2+}$ = 857.4; $[MH_3]^{3+}$ = 571.9 MW cycloadduct = 1686,6 (green boxes); $[MH_2]^{2+}$ = 844.3; $[MH_3]^{3+}$ = 563.2.

Maleimide concentration and equivalents optimization

General protocol

- 1- Load a 200 µL Eppendorf with 0.2 µmol resin (approx. 1.86 mg);
- 2- Prepare desired solutions of N-(N-Boc-2-amminoethyl)maleimide, and transfer them to the Eppendorf tube;
- 3- Run each condition in triplicate;

- 4- Allow Diels-Alder reaction to proceed for 7.5 hours at 90°C
- 5- Transfer the resin beads in a 1 mL SPE tube equipped with PE frit and wash the resin with DMF/DCM;
- 6- Add 100 μL of a 10% m-cresol solution in TFA and allow to react for 1h 30 min;
- 7- Percolate the solution in a 2 mL Eppendorf tube, and wash the resin with 80 µL TFA;
- 8- Add 1.8 mL ethyl ether and allow to precipitate for 2h at -20 °C;
- 9- Recover the precipitate by centrifugation, and wash the pellet with ethyl ether;
- 10- Redissolve the dry pellet in 500 μL mQ, transfer in a 500 μL Eppendorf tube and heat for 5 hours at 90 °C;
- 11- Submit the samples to UPLC-MS analysis.
- 12- Data analysis

Solution used

Maleimide concentration	30 eq	20 eq	10 eq
0.5 M	\	Test B1	Test C1
0.25 M	Test A2	Test B2	Test C2
0.1 M	Test A3	Test B3	\

Target identification

PNA \ Charge	No charge	2+	3+	4+
Target	1546.5	774	516	387
Alkylated	1712.7	857	571	429
Cycloaddition by-prod	1686.6	844	563	422

<u>Data analysis</u>: For each single UPLC-MS trace three different XIC where obtained extracting the signal relative to the 2+ and 3+ m/z signals. Peaks were then integrated using MassLynx 4.0 algorithm. <u>Results</u>: 20 equivalent of maleimide at a concentration of 0.25 M gives the best results in term of by-product formation.



Figure S2. Relative by-product formation for Fur-GATCT-Gly-NH₂ PNA synthesis obtained under various conditions (A1–A4 and B1–B5 tests reported above) using *N*-(*N*-Boc-2-amminoethyl)maleimide as protecting group. Quantities are normalized to target product intensity in the UPLC-MS analysis (carried out as in figure S1). Data presented are an average of 3 different experiments.

Maleimide equivalents and reaction time optimization

General protocol

- 1- Load a 200 µL Eppendorf with 0.2 µmol resin (approx. 1.86 mg);
- 2- Prepare the desired 0.25 M solution of N-(N-Boc-2-amminoethyl)maleimide, and transfer the correct amount to the Eppendorf tube;
- 3- Run each condition in triplicate;
- 4- Allow Diels-Alder reaction to proceed for the specified time at 90°C
- 5- Transfer the resin beads in a 1 mL SPE tube equipped with PE frit and wash the resin with DMF/DCM;
- 6- Add 100 μ L of a 10% m-cresol solution in TFA and allow to react for 1h30';
- 7- Percolate the solution in a 2 mL Eppendorf tube, and wash the resin with 80 µL TFA;
- 8- Add 1.8 mL ethyl ether and allow to precipitate for 2h at -20 °C;
- 9- Recover the precipitate by centrifugation, and wash the pellet with ethyl ether;
- 10- Redissolve the dry pellet in 500 μL mQ, transfer in a 500 μL Eppendorf tube and heat for 5 hours at 90 °C;
- 11- Submit the samples to UPLC-MS analysis.
- 12- Data analysis

Conditions used

Maleimide amount	le amount 15 h at 90°C		
10 eq	Test A1	Test B1	
20 eq	Test A2	Test B2	
30 eq	Test A3	\	

Target identification

PNA \ Charge	No charge	2+	3+	4+
Target	1546.5	774	516	387
Alkylated	1712.7	857	571	429
Cycloaddition by-prod	1686.6	844	563	422

<u>Data analysis</u>: For each single UPLC-MS trace three different XIC were obtained extracting the signal relative to the 2+ and 3+ m/z signals. Peaks were then integrated using MassLynx 4.0 algorithm. <u>Results</u>: The use of 10 equivalents of maleimide at a concentration of 0.25 M gives the best results in terms of by-product formation, if the Diels-Alder reaction is conducted for a reduced time (5h).



Figure S3. Relative by-product formation for Fur-GATCT-Gly-NH₂ PNA synthesis obtained under various conditions (A1–A4 and B1–B5 tests reported above) using *N*-(*N*-Boc-2-amminoethyl)maleimide as protecting group. Quantities are normalized to target product intensity in the UPLC-MS analysis (carried out as in figure S1). Data presented are an average of 3 different experiments.

Visualization of retro-DA reaction time course



Figure S4. Time-course of the retro-DA reaction. UPLC-MS chromatograms (XIC with m/z selected for the Diels alder product (2.77 min) and for the Retro-Diels-Alder product (3.28 min) for reactions carried out for Fur-GATCT-Gly-NH₂ PNA synthesis obtained using 20 equivalents of N-(N-Boc-2-amminoethyl)maleimide in the protection step (7.5 h at 90°C), and different times of the retro-DA reaction at 90°C after cleavage. a) before retro-DA; b-e: after 1, 3, 4, and 5 h respectively.





Figure S5. Overview of PNA sequences and furan-PNA monomers.



Figure S6. HPLC-DAD-HRMS chromatogram of purified **PNA 2**. HPLC-UV at 254 nm trace (top), MS spectrum of the corresponding peak at 15.58 min (center) and mathematical deconvolution of the multicharged signals (insert). (MW: 3158.2976; Molecular formula: C132H169N69O36).



Figure S7. HPLC-DAD-HRMS chromatogram of purified **PNA 3**. HPLC-UV at 254 nm trace (top), MS spectrum of the corresponding peak at 15.93 min (center) and mathematical deconvolution of the multicharged signals (insert). (MW: 3186.3051; Molecular formula: C132H169N71O36).



Figure S8. HPLC-DAD-HRMS chromatogram of purified **PNA 5**. HPLC-UV at 254 nm trace (top), MS spectrum of the corresponding peak at 15.70 min (center) and mathematical deconvolution of the multicharged signals (insert). (MW: 3296.3502; Molecular formula: C127H163N65O35).



Figure S9. HPLC-DAD-HRMS chromatogram of purified **PNA 6**. HPLC-UV at 254 nm trace (top), MS spectrum of the corresponding peak at 16.03 min (center) and mathematical deconvolution of the multicharged signals (insert). (MW: 3324.3570; Molecular formula: C127H163N67O35).



Figure S10. HPLC-DAD-HRMS chromatogram of purified **PNA 8**. HPLC-UV at 254 nm trace (top), MS spectrum of the corresponding peak at 15.60 min (center) and mathematical deconvolution of the multicharged signals (insert). (MW: 3420.3795; Molecular formula: C137H173N71O38).



Figure S11. HPLC-DAD-HRMS chromatogram of purified **PNA 9**. HPLC-UV at 254 nm trace (top), MS spectrum of the corresponding peak at 15.77 min (center) and mathematical deconvolution of the multicharged signals (insert). (MW: 3448.3870; Molecular formula: C137H173N73O38).

PAGE analysis

Crosslink samples were analyzed on a 20% polyacrylamide gel (acrylamide:bisacrylamide 19:1) prepared in 1x Tris-Borat-EDTA (TBE) buffer containing 7 M urea. The temperature of the gel was stabilized with a Julabo F12 at 25°C. The power supply used for gel electrophoresis was a consort EV202 and a constant voltage of 230 V was used to run the gels. Gels were stained with SYBR gold (Thermo Fisher Scientific, Life Technologies) and pictures were taken with an Autochemi imaging system (UVP). 4 μ L of the crosslink solution (10 μ M) were mixed with 16 μ L formamide and from this mixture 8 μ L were loaded on the gel. It should be noted that staining of PNA is considerably more difficult and much less sensitive that DNA staining. As a result individual ssPNA strands cannot be visualized on PAGE and can thus not be seen in the figures below. Consequently also PNA-DNA crosslinked complexes (boxes in purple) are only visualized with low intensity.

A DNA impurity occurring in all samples appears in all gels as a slower running spot between the cross-linked complex and the ssDNA spots, due to the considerably higher sensitivity of DNA for SybrGold staining



Figure S12. PAGE analysis: Cross-link experiment between **ON 1-4** and **PNA 1-3**. Analysis was done on a 20% polyacrylamide gel (acrylamide:bisacrylamide 19:1) prepared in a TBE buffer containing 7M urea. A constant voltage of 230V was applied at a temperature of 25 °C. Gels were stained with a SYBR gold solution and gels were analysed using an Autochemi Imaging system.



Figure S13. PAGE analysis: cross-link experiment between **ON 1-4** and **PNA 4-6**. Analysis was done on a 20% polyacrylamide gel (acrylamide:bisacrylamide 19:1) prepared in a TBE buffer containing 7M urea. A constant voltage of 230V was applied at a temperature of 25 °C. Gels were stained with a SYBR gold solution and gels were analysed using an Autochemi Imaging system.



Figure S14. PAGE analysis: cross-link experiment between **ON 1-4** and **PNA 7-9**. Analysis was done on a 20% polyacrylamide gel (acrylamide:bisacrylamide 19:1) prepared in a TBE buffer containing 7M urea. A constant voltage of 230V was applied at a temperature of 25 °C. Gels were stained with a SYBR gold solution and gels were analysed using an Autochemi Imaging system.



Figure S15. PAGE analysis. **A)** Strand displacement experiment of **ON1** and **PNA 1-9**. **B)** Strand displacement experiment between **ON 2** and **PNA 1-9**. Analysis was done on a 20% polyacrylamide gel (acrylamide:bisacrylamide 19:1) prepared in a TBE buffer containing 7M urea. A constant voltage of 230V was applied at a temperature of 25 °C. Gels were stained with a SYBR gold solution and gels were analysed using an Autochemi Imaging system.

MALDI analysis

Prior to MALDI analysis, the crosslinked product was purified using reverse phase HPLC (RP-HPLC). Purification was performed on an Agilent 1200b System equipped with a Waters X-bridge 130 Å Oligonucleotide C18 column (2.5 μ M, 4.6 mm x 50 mm) at either a column temperature of 50 °C. Acetonitrile and 0.1 M TEAA buffer with 5% acetonitrile were used as mobile phase and were applied through a gradient of 0-18% MeCN in 20 minutes. The isolated crosslinked samples was dissolved in 5 μ L milliQ. Sample and matrix (2,5-dihydroxybenzoic acid; 15 mg in 100 μ L milliQ and 50 μ L MeCN with 1% TFA) were spotted in a 1:1 ratio onto the MALDI plate. The PNA-DNA crosslinked samples were analyzed by MALDI-TOF on an ABI Voyager DE-STR system equipped with a high performance nitrogen laser (337 nm). PNA-DNA crosslinked samples were analyzed in linear, positive mode.



Figure S16. MALDI analysis of the cross-link product of ON 2 and PNA 4. The labeled peak is attributed to the DNA-PNA cross-linked duplex (Expected mass: 6591 Da).



Figure S17. HPLC trace of the ICL experiment (top panel) and MALDI analysis (bottom panel) of the cross-link product of **ON 1** and **PNA 2** (peak at 11.00–11.50 min). The labeled peak is attributed to the DNA-PNA cross-linked duplex (Expected mass: 6477 Da). Peak at 8.5 min correspond to unreacted **ON 1**.



Mechanism of Interstrand Crosslink formation



Figure S18. Schematic representation of interstrand crosslink formation between PNAs bearing **F** building block (**PNA 1-3**) and **ON 2** (upper panel) and PNAs bearing **f** building block (**PNA 4-6**) and **ON 1** (lower panel). a) Activation of the furan unit; b) reaction of the keto-enal with the exocyclic amine; c) ring closure reaction; d) re-aromatization reaction, through dehydration.