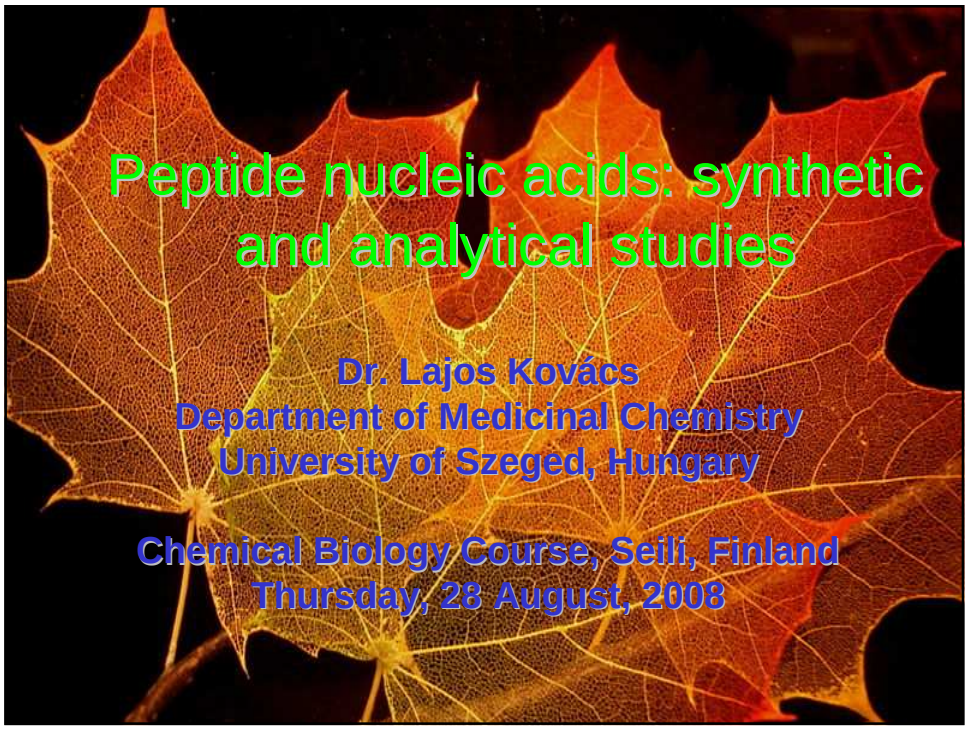
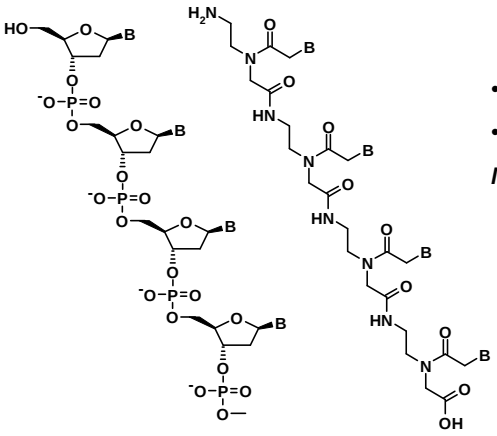


Peptide nucleic acids: synthetic and analytical studies

Dr. Lajos Kovács
Department of Medicinal Chemistry
University of Szeged, Hungary

Chemical Biology Course, Seili, Finland
Thursday, 28 August, 2008

What are peptide nucleic acids (PNA)?



DNA PNA

- **Radically different DNA mimics**
- **Possessing a neutral and achiral *N*-(2-aminoethyl)glycine backbone**

The overview of PNAs

- **Discovered in 1991 by Egholm, Nielsen, Berg, and Buchardt**
- **Synthesis chemistry commercialized in 1993**
- **More than 1,000 PNA publications in the fields of chemistry, molecular biology, diagnostics, and therapeutics**
- **A variety of products are on the market today**
- **A lot of sequence limitations in PNA oligomer synthesis**
- **Expensive**
- **PNA comes of age**

Characteristics of PNA

- **Higher affinity to complementary nucleic acid (DNA, RNA)**
- **Strong hybridization independent of salt concentration**
- **Greater specificity and sensitivity of interaction**
- **Thermal and chemical stability**
- **Resistance to nucleases and proteases**
- **Poor cellular uptake**

Higher affinity to complementary nucleic acid (DNA, RNA)

a,t,g,c : PNA A, T, G, C : DNA

Sequence	T_m [°C]		Buffer
	DNA	RNA	
H-tgt acg tca caa cta	69.5	72.3	A
5'-TGTACGTCACAACTA	53.3	50.6	A
H-aca tca tgg tcg	58.7	62.3	B
5'-ACATCATGGTCG	47.9	44.5	B
5'-ACATCA tgg tcg	52.6	52.1	B

Buffer A: 100 mM NaCl, 10 mM NaH_2PO_4, 0.1 M EDTA, pH 7.0
Buffer B: 140 mM KCl, 10 mM NaH_2PO_4, 0.1 M EDTA, pH 7.4

Strong hybridization independent of salt concentration

Thermal stability 15-mer PNA vs. DNA

NaCl [mM]	PNA/DNA T_m (°C)	DNA/DNA T_m (°C)
0	72	38
100	69	54
140	69	56
1000	65	65

10 mM phosphate buffer, 0.1 M EDTA, pH = 7.0

Greater specificity and sensitivity of interaction

ΔT_m for single mismatch

15-mer PNA / DNA 8 ~ 20 °C
15-mer DNA / DNA 4 ~ 16 °C

Summary: PNA versus DNA

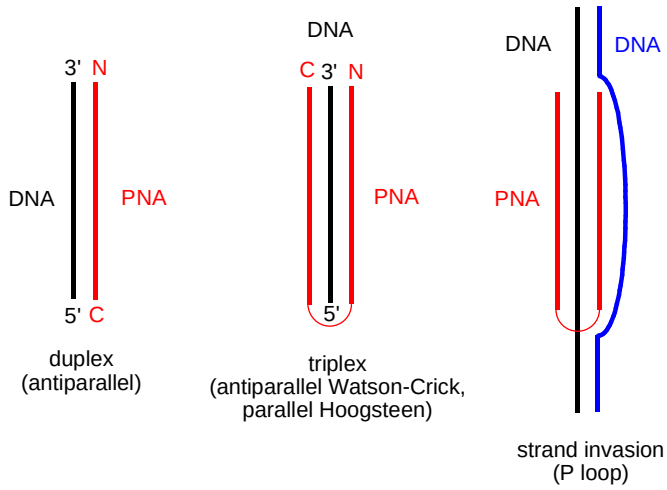
	DNA	PNA
Hybridization affinity with DNA		At least 1 °C higher per base
Hybridization rate with DNA		100 - 5000 times
Salt concentration for hybridization	Dependent	Independent
T_m single mismatch	Lowering 10 °C	Lowering 15 °C
Chemical stability	Unstable in acid and base	Stable
Water solubility	Soluble	Restricted solubility
Maximum base length	No limit	18 bases: aggregation
Required base length for diagnosis	20 - 30	13 - 17
Biological stability	Degradation in nuclease	Stable
Thermal stability	Moderate	Good

PNA applications

1. **Molecular tool** in molecular biology and biotechnology
2. **Lead compound** for gene-targeted drugs (antisense & antigene)
3. **Diagnostic** purpose and development of **biosensor**
   - Self reporting PNA
     - LightSpeed probes
     - LightUp probes
     - Molecular Beacon
   - PCR Tools
     - PCR clamping
     - Q-PNA

   More than 25 probes for clinical and industrial microbiology
4. **The study of basic chemistry –** improvement of basic architecture

PNA-oligonucleotide structures



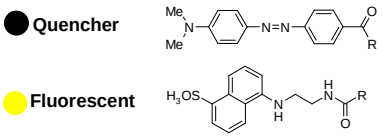
A molecular tool in molecular biology and biotechnology

- Enhanced PCR amplification
- Pre-gel hybridization – A rapid alternative to Southern blotting
- PNA-assisted rare cleavage
- Artificial restriction enzyme system
- Determination of telomere size
- Nucleic acid purification

LightSpeed probe

Diagnostics

PNA probe with minimized hydrophobic surface area

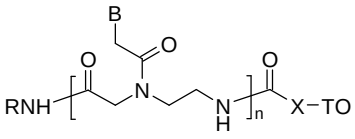


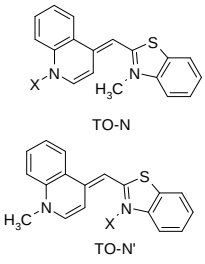
Developed by Boston Probes
http://www.appliedbiosystems.com/press_releases/license/

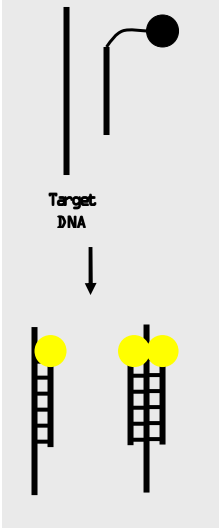
Light-up Probe

Diagnostics

- A hybridisation probe based on a DNA analogue tethered to a dye
- Non-fluorescent when free in solution but becomes brightly fluorescent upon binding to the target nucleic acid.
- To date, an asymmetric cyanine dye as the reporter group
- The most frequently employed DNA analogue is PNA (No charge, sequence specific, thermal stability of PNA/DNA)
- Most useful for real time fluorescent detection of PCR products

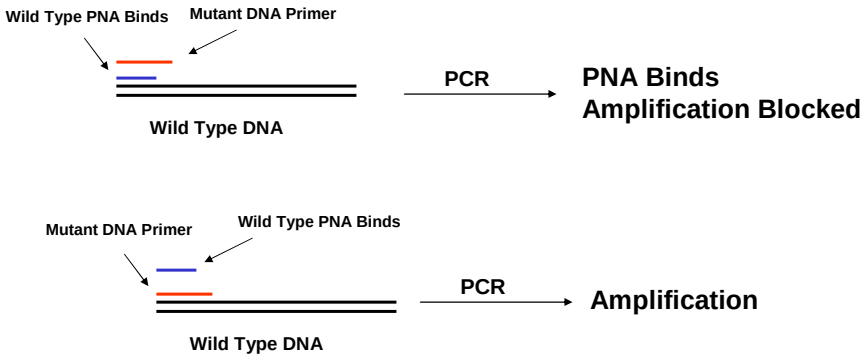






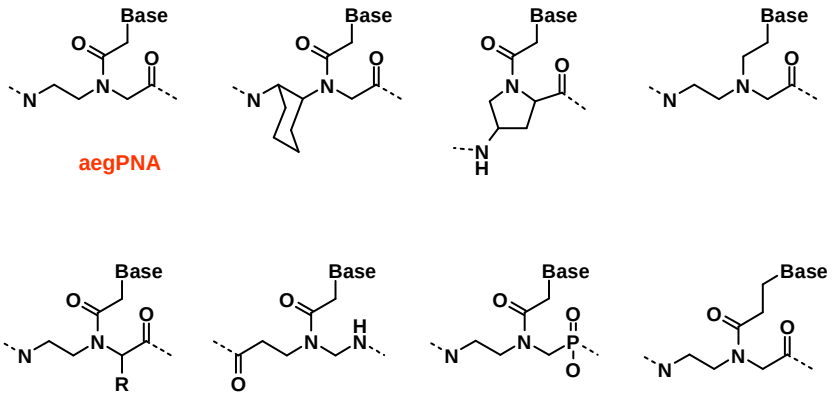
Developed by LightUp Technologies
http://www.lightup.se

PCR Clamping

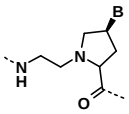


Analysis of point mutation

Modifications of PNA



K. N. Ganesh, P. E. Nielsen (2000): Peptide nucleic acids: Analogs and derivatives.
Curr. Org. Chem. **4**, 931-943.



aepPNA (2S/R, 4S)

PNA Sequence	DNA antiparallel		DNA parallel	
	(2*S*,4*S*)	(2*R*,4*S*)	(2*S*,4*S*)	(2*R*,4*S*)
H-GTA GAT CAC T-NH$(CH_2)_2$COOH	43.8		40.3	
H-GT**a** GAT CAC T-NH$(CH_2)_2$COOH (1)	53.8	53.1	50.2	50.2
H-GTA GA**t** CAC T-NH$(CH_2)_2$COOH (2)	56.6	33.0	34.0	26.2
H-GTA **g**AT CAC T-NH$(CH_2)_2$COOH (3)	43.2	53.1	58.3	28.3
H-GTA GAT **c**AC T-NH$(CH_2)_2$COOH (4)	55.2	62.3	31.2	34.0

a, **t**, **g**, and **c** represent (2*S*/*R*, 4*S*)-aepPNA unit

T_m (°C) of Mismatched PNA/DNA Duplex

DNA (5'-3')	aegPNA	(2S,4S)-aepPNA
AGT GAT CCA C	35.4 (-8.4) (1)	44.4 (-9.4)
AGT G*T*T CTA C	36.8 (-7.0) (2)	26.9 (-29.7)
AGT GAT *A*TA C	39.6 (-4.2) (3)	24.7 (-18.5)
AGT *T*AT CTA C	36.8 (-7.0) (4)	43.6 (-11.6)

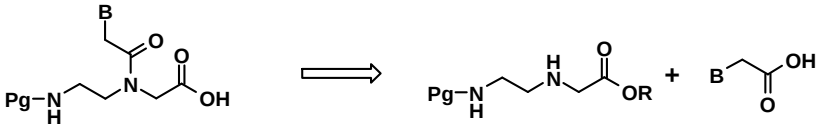
Synthesis of PNA

- Monomers
- Oligomers

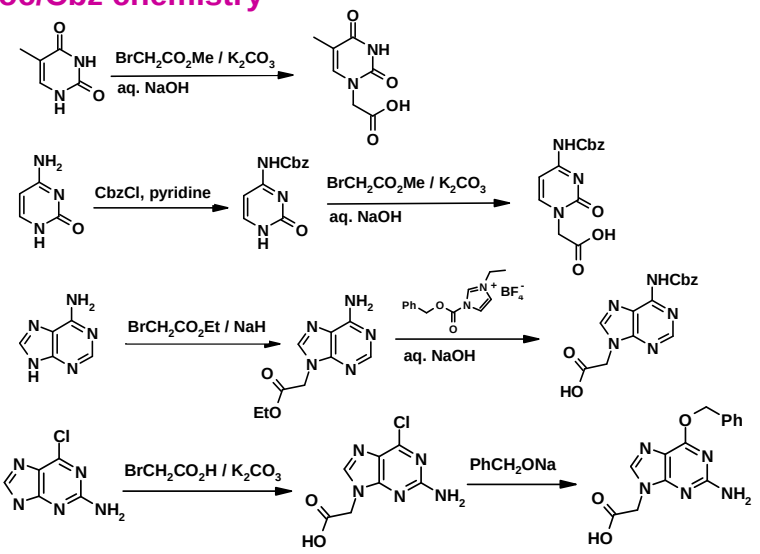
Synthesis of PNA monomers

Protecting groups[a]	Compatibility
Boc/Cbz[b](Benzyl)	peptide
Fmoc/Cbz[b]	peptide
Fmoc/Bhoc[c]	peptide
Fmoc/Mmt[d]	peptide
Fmoc/acyl	ON[e]
Mmt[d]/acyl	ON[e]

a. The first abbreviation denotes the protecting group of the backbone while the second one that of the nucleobase; *b.* Cbz: benzyloxycarbonyl; *c.* Bhoc: benzhydr yloxycarbonyl; *d.* Mmt: monomethoxytrityl.

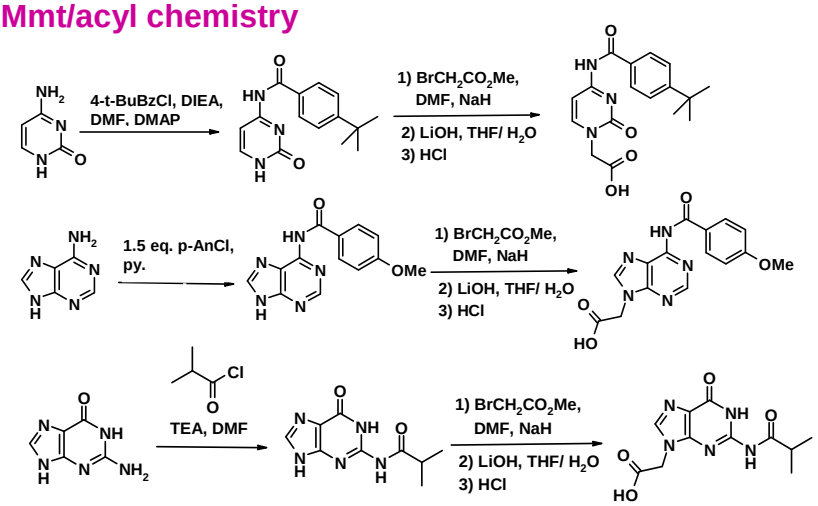


Boc/Cbz chemistry



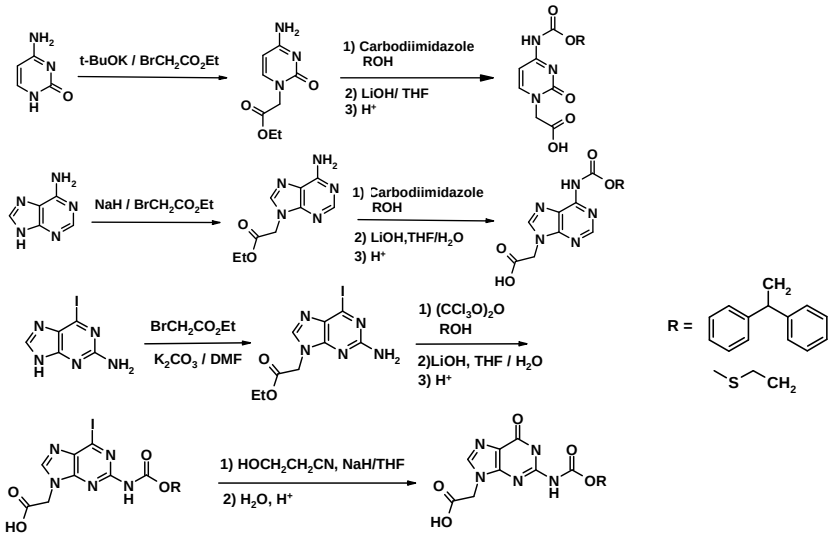
K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt. *J. Org. Chem.,* 1994, **59**, 5767-5773.

Mmt/acyl chemistry



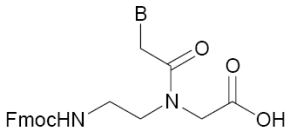
G. Breipohl, D. W. Will, A. Peyman, E. Uhlmann. *Tetrahedron,* 1997, **53**, 14671–14686.

Fmoc/Bhoc chemistry



T. Stafforst, U. Diederichsen. *Eur. J. Org. Chem.* **2007**, 681-688.

Fmoc/acyl chemistry

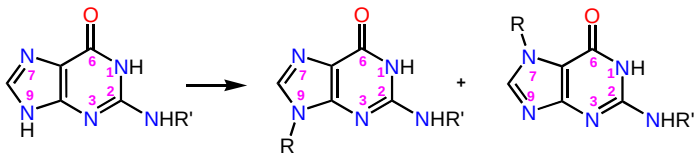


B[a]	Thy	4-tBuBz Cyt	An Ade	Ibu Gua
Code[b]	t	c	a	g

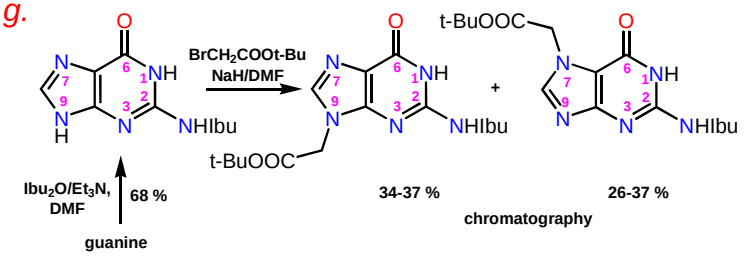
a. Thy: thymin-1-yl,
4-tBuBzCyt: *N*4-(4-*tert*-butylbenzoyl)-cytosin-1-yl,
AnAde: *N*6-anisoyladenin-9-yl,
IbuGua: *N*2-isobutyrylguanin-9-yl.
b. Abbreviated notation of unprotected or protected monomer units, depending on the context.

- PNA monomers: Z. Timár, L. Kovács, G. Kovács, Z. Schmél, *J. Chem. Soc. Perkin Trans.* 1, **2000**, 19-26.

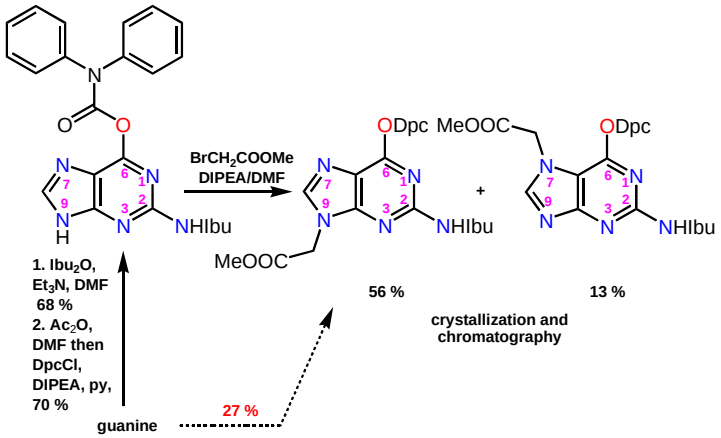
Guanine alkylation: the usual case



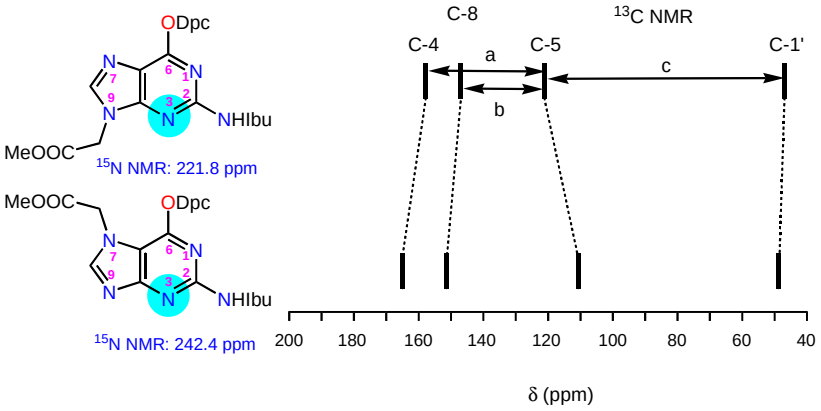
e.g.



Guanine alkylation: a better solution



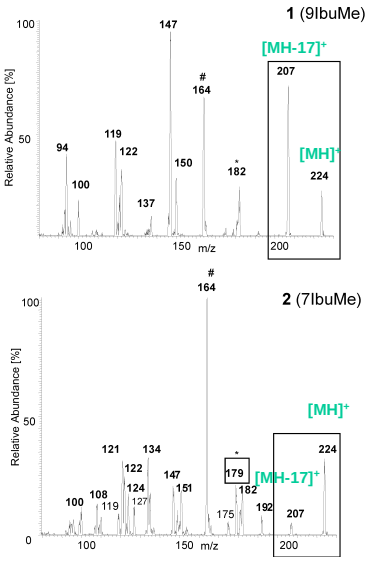
NMR methods for quantitation of N^9/N^7 ratio of alkylated guanine isomers

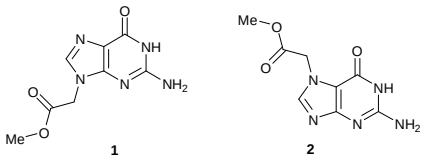


R. Marek, J. Brus, J. Tousek, L. Kovács, D. Hocková, *Magn. Reson. Chem.* 2002, **40**, 353–360.

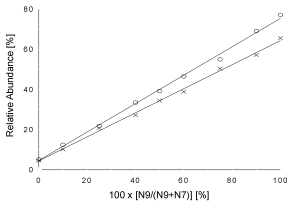
Z. Timár, L. Kovács, G. Kovács, Z. Schmél, *J. Chem. Soc. Perkin Trans.* 1, **2000**, 19-26.

An ESI-MS/MS method for quantitation of N^9/N^7 ratio of alkylated guanine isomers





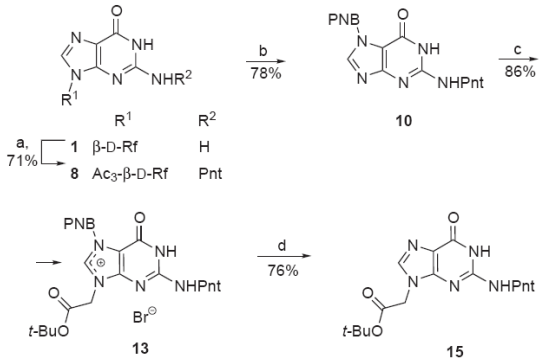
MW: 223



the abundance of $[MH-17]^+$ ion (ammonia loss) is in linear correlation with N^9 isomer content

G. Ferenc, Z. Kele, L. Kovács,
Rapid Commun. Mass Spectrom. 2005, **19**, 236-240

Synthesis of a guanine monomer

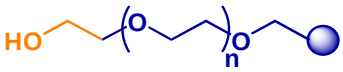


	R^1	R^2
1	β-D-Rf	H
8	Ac_3-β-D-Rf	Pnt

a. 1. TMSCl, pyridine, r.t., 1 h; 2. 1.25 equiv. pent-4-enoic anhydride, pyridine, r.t., 16 h; 3. water, 0–5 °C, 5 min; 4. aq. NH_3, r.t., 30 min; 5. Ac_2O, pyridine, DMF, r.t., 16 h.
b. 4 equiv. 4-nitrobenzyl bromide, DMF, r.t., 60 h.
c. 3 equiv. *tert*-butyl bromoacetate, DMF, 70 °C, 16 h.
d. 1. 4 equiv. $Na_2S_2O_4$, aq. acetone, pH 7.0, r.t., 30 min; 2. 70 °C, 16 h. β-D-Rf = ββ-D-ribofuranosyl, Ac_3-β-D-R_f = 2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl

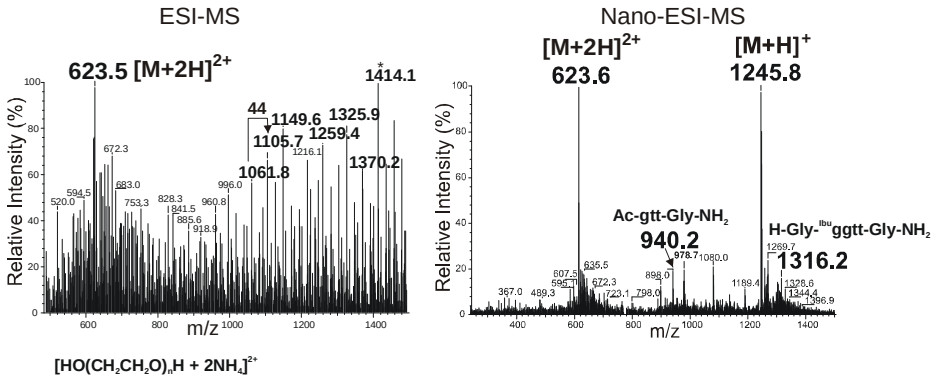
G. Ferenc, P. Forgó, Z. Kele, L. Kovács, *Collect. Czech. Chem. Commun.* 2005, **70**, 85-102.

PNA oligomer synthesis on a TentaGel™ support



ethylene glycol oligomers (“leakage” of the support);
nano-ESI-MS: capped sequences and $[M_x+70]^+$
(incomplete isobutyryl removal)

H-Gly-ggtt-Gly-NH_2



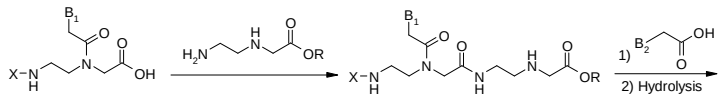
$[HO(CH_2CH_2O)_nH + 2NH_4]^{2+}$

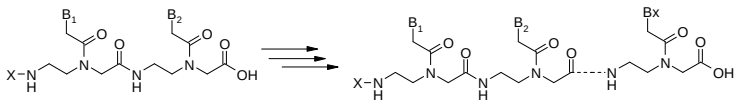
G. Kovács, Z.Timár, Z. Kupihár, Z. Kele, L. Kovács, *J. Chem. Soc. Perkin Trans.* 1, **2002**, 1266-1270.

Synthesis of PNA Oligomers

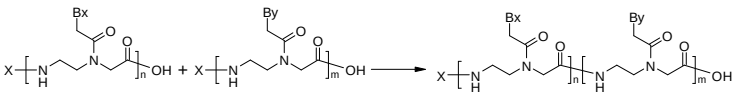
Liquid-Phase Synthesis of PNA

Convergent Approach



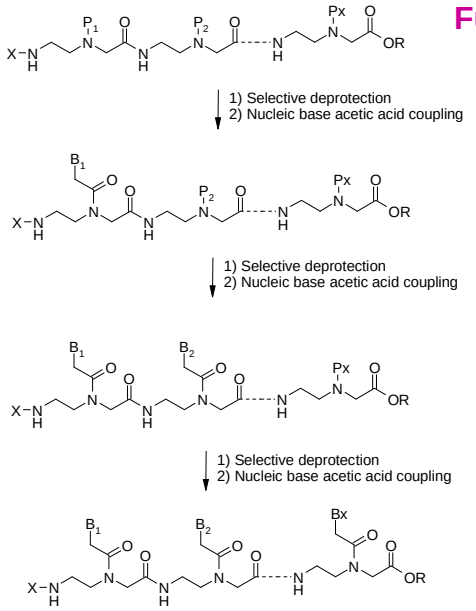


Divergent Approach



C. Di Giorgio, S. Pairot, C. Schwergold, N. Patino, R. Condom, A. Farese-Di Giorgio, R. Guedj. *Tetrahedron,* 1999, **55**, 1937-1958.

Fully protected polyamide backbone approach

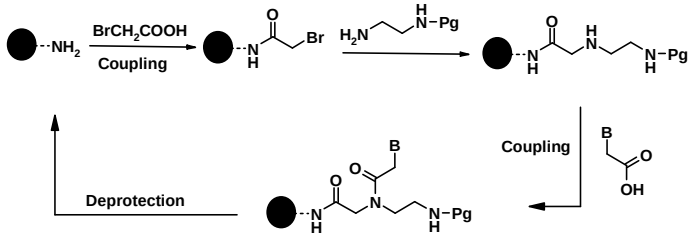


P_1, P_2, P_x : protecting groups

C. Schwergold, G. Depecker, C. Di Giorgio, N. Patino, F. Jossinet, B. Ehresmann, R. T erreux, D. Cabrol-Bass, R. Condom. *Tetrahedron,* 2002, **58**, 5675-5687.

Solid phase synthesis of PNA oligomers

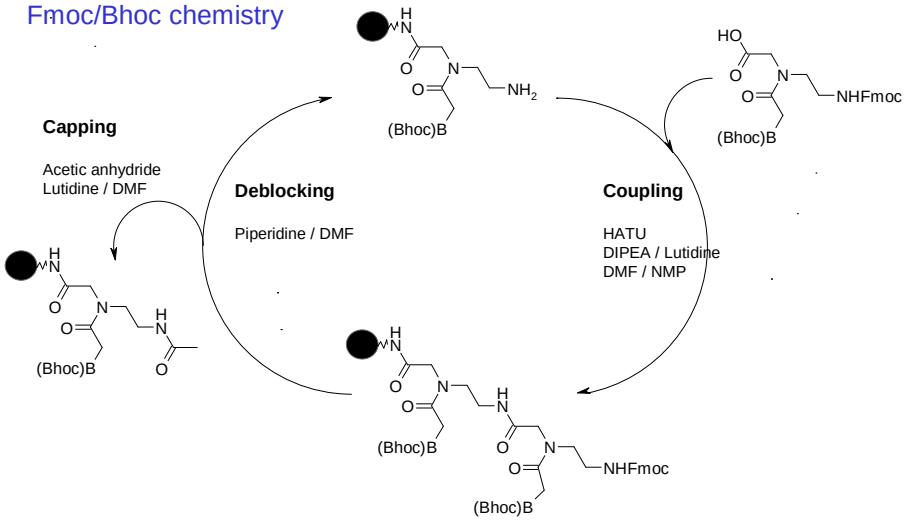
Submonomer solid phase synthesis



L. S. Richter, R. N. Zuckermann. *Bioorg. Med. Chem. Lett.,* 1995, **5**, 1159-1162.

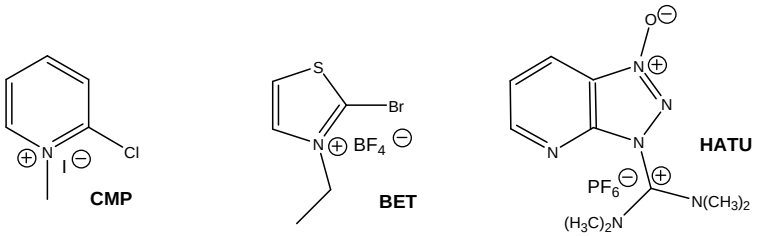
Solid phase synthesis of PNA oligomers

Fmoc/Bhoc chemistry



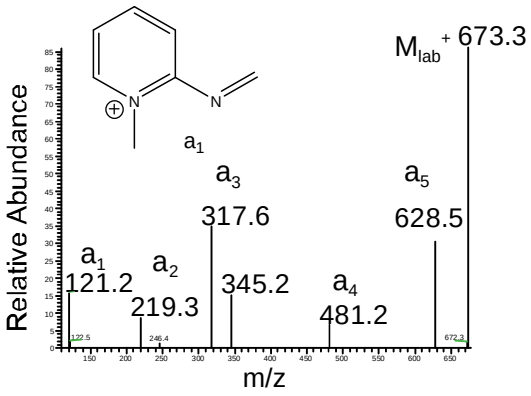
Variables affecting the effectiveness of a PNA oligomer synthesis

- nature of solid supports and monomers including the protecting groups of the latter
- coupling reagents and conditions
  - *e.g.* bases, preactivation, reaction time, mixing sequence, monomer excess, repetition of couplings *etc.*
- order of reactivity of coupling agents *in solution*: CMP > BET >> HATU
- *on solid phase*: side reactions with CMP and BET
- CPG/HATU/3 equiv./20min/(98%/cycle)



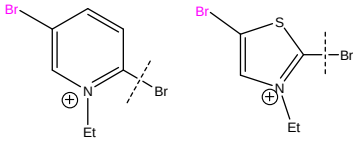
G. Kovács, Z.Timár, Z. Kupihár, Z. Kele, L. Kovács, *J. Chem. Soc. Perkin Trans.* 1, **2002**, 1266-1270.

Labeling apolar peptides



MP-GPVYF-OH peptide
(MP = 1-methyl-2-pyridyl)

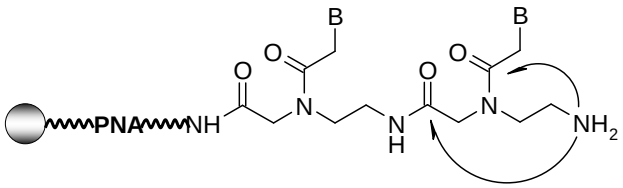
$\mathbf{M}_{lab}$=M-H+93 (M=581);
a_2-a_1=98 (Pro);
a_3-a_2=99 (Val);
a_4-a_3=163 (Tyr);
a_5-a_4=147 (Phe)



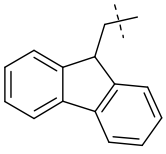
Extension to other peptides and $^{79}Br/^{81}Br$-containing labels:
G. Ferenc, P. Pádár, T. Janáky, Z. Szabó, G. Tóth, L. Kovács, Z. Kele *J. Chrom. A*, 2007, **1159**, 119-124.

Sequence limitations

1) Piperidine catalyzed reactions of the amine terminus (transacylation: A > G > T, Lutidine < DIEA < DBU < Piperidine < n-PrNH$_2$)



2) π-π Stacking of Fmoc aromatic rings with nucleobases



Synthesis limitations

- Total purine content<85%
- Not more than 10 consecutive purines
- Less than 5-consecutive G's
- If high purine contents, add a linker
- Avoid probes with high self-complementarity

Technical challenges in synthesis of PNA oligomers

Minimization of transacylation

No Further Reaction with Transacylated Product

Minimization of Aggregation

No Capping

Cost Effectiveness

Convenience for the Synthesis of Oligomer

Ease in Purification

Selected readings

Book

Peter E. Nielsen (ed.): Peptide nucleic acids. Protocols and applications, 2nd. ed. Horizon Bioscience, Wymondham, 2004.

Reviews and papers

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- S. Pensato, M. Saviano and A. Romanelli (2007): New peptide nucleic acid analogues: synthesis and applications. *Expert Opin. Biol. Ther.* **7**, 1219-1232.
- F. Wojciechowski and R. H. E. Hudson (2008): A convenient route to *N*-[2-(Fmoc)aminoethyl]glycine esters and PNA oligomerization using a bis-*N*-Boc nucleobase protecting group strategy. *J. Org. Chem.*, **73**, 3807–3816.

Availability of this lecture

- http://www.mdche.u-szeged.hu/~kovacs/PNA_Seili.pdf
- kovacs@ovrisc.mdche.u-szeged.hu