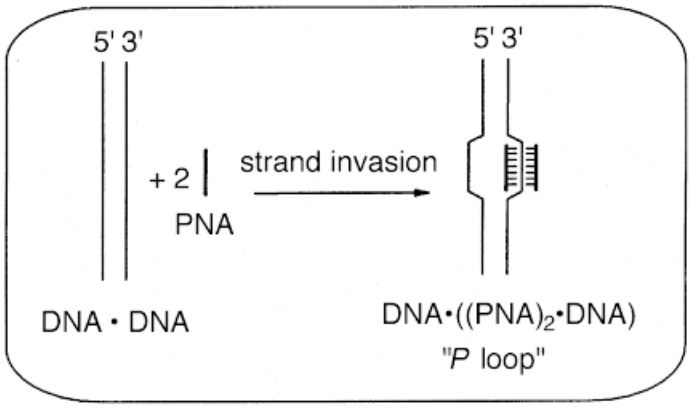
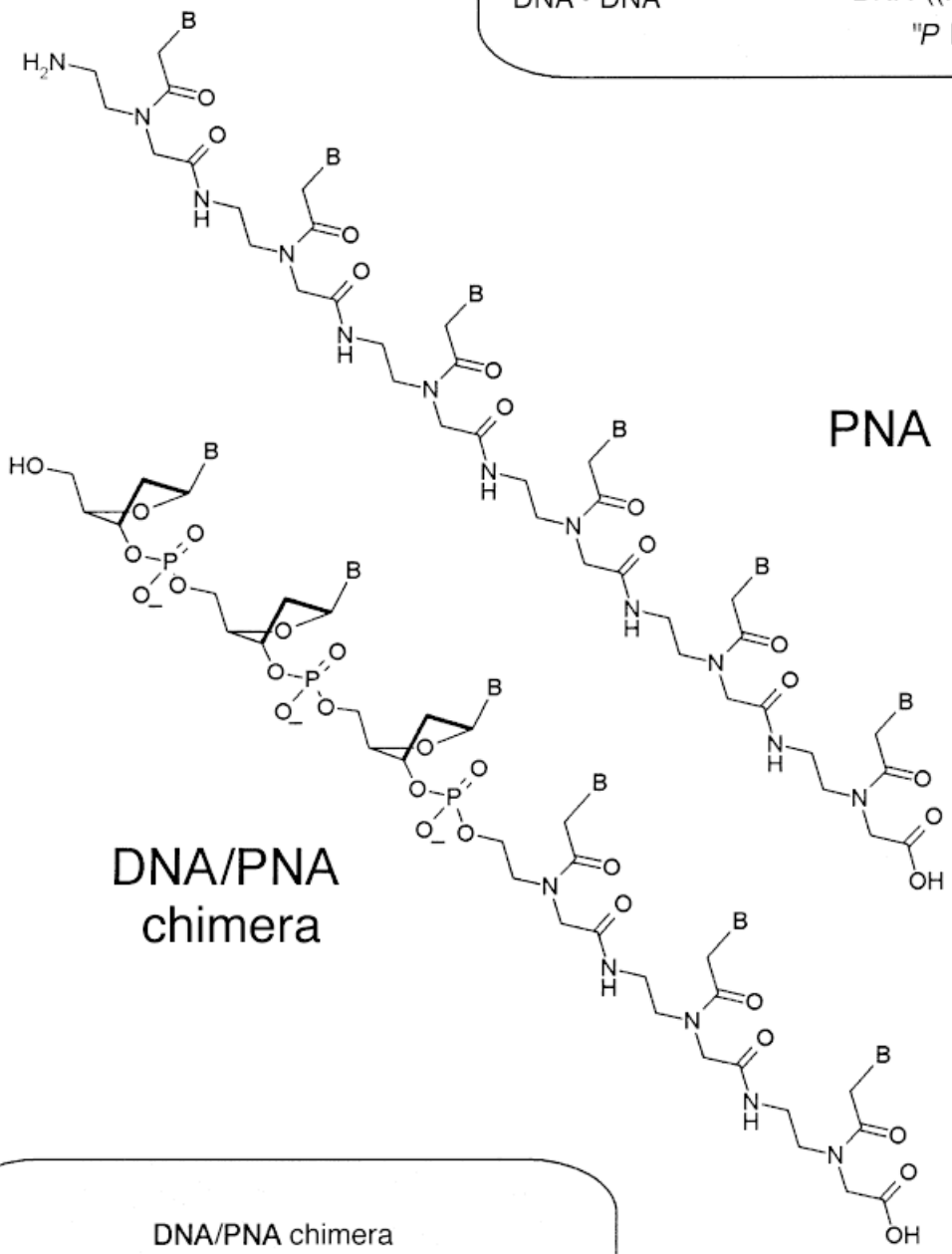
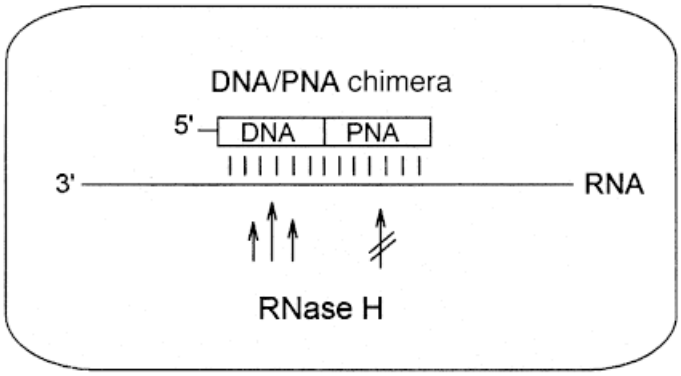
Polyamide or peptide nucleic acids (PNAs) bind with higher affinity to complementary nucleic acids than their natural congeners.







DNA/PNA chimeras can assume biological functions, such as the activation of ribonuclease H for the cleavage of RNA.

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PNA: Synthetic Polyamide Nucleic Acids with Unusual Binding Properties**

Eugen Uhlmann,* Anusch Peyman, Gerhard Breipohl, and David W. Will

Dedicated to Professor Wolfgang Pfleiderer on the occasion of his 70th Birthday

Since the investigation of oligonucleotides as potential therapeutics that target nucleic acids was initiated, the search for nucleic acid mimetics with improved properties, such as strengthened binding-affinity to complementary nucleic acids, increased biological stability, and improved cellular uptake, has accelerated rapidly. In 1991 Nielsen et al. first described what is undoubtedly one of the most interesting of the new derivatives, the polyamide or peptide nucleic acids (PNAs), in which the entire sugar–phoshate backbone is replaced by an *N*-(2-aminoethyl)glycine polyamide structure. Since even minor structural changes in oligonucleotides, such as the replacement of an oxygen atom by sulfur (phosphorothioates), or by a neutral methyl group (methyl phosphonates), result in a decrease in binding affinity, it was even more astonishing to find that the drastic structural changes in PNAs result in nucleic acid mimetics with higher binding-affinity to complementary DNA and RNA than unmodified oligonucleotides. The remarkable binding properties of PNAs have spawned a rapidly expanding new field of research, where the targets are the synthesis of PNAs and PNA analogues, and their application as therapeutics, DNA diagnostics, and tools in biotechnology. In addition, investigation of PNAs and PNA/DNA chimeras can be used to generate information on the structural and biological properties of DNA and RNA themselves. Furthermore, they may trigger the generation of new ideas on models for alternative living systems and potential transitions between different genetic systems.

Keywords: antisense agents · DNA recognition · gene technology · molecular recognition · peptide nucleic acids

1. Introduction

The most important molecular recognition event in nature is the base-pairing of nucleic acids, which guarantees the storage, transfer, and expression of genetic information in living systems. The highly specific recognition through the natural pairing of the nucleobases has become increasingly important for the development of DNA diagnostics and for oligonucleotide therapeutics in the form of antisense and antigene oligonucleotides.[1–4] In the last few years, attempts to optimize the properties of oligonucleotides have resulted in the synthesis and analysis of a huge variety of new oligonucleotide derivatives[5] with modifications to the phosphate group, the ribose, or the nucleobase. The most radical change to the natural structure, however, was made by the Danish group of Buchardt, Nielsen, Egholm, and Berg[6–9], who replaced the entire sugar–phosphate backbone by an *N*-(2-aminoethyl)glycine-based polyamide structure (Figure 1). The astonishing discovery that these polyamide or peptide nucleic acids (PNAs) bind with higher affinity to complementary nucleic acids than their natural counterparts,[10] and obey the Watson–Crick base-pairing rules resulted in the rapid establishment of a new branch of research focussed on diagnostic and therapeutic applications of this highly interesting compound class.[11–14] Soon after, the ability of PNAs to displace one strand of a DNA double-helix, an inefficient process with natural oligonucleotides, was reported.[15] The combination of PNA and DNA to form

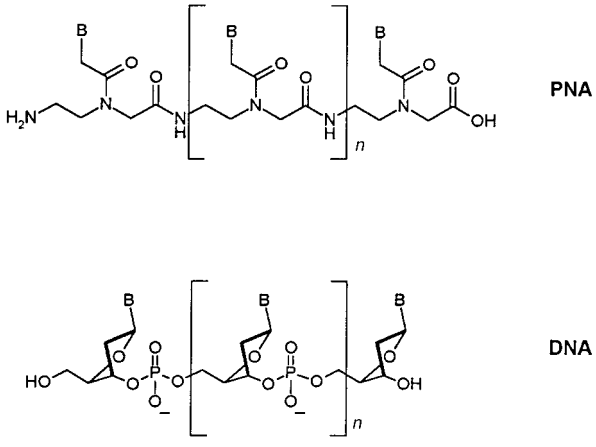

Figure 1. Structures of PNA and DNA. B = nucleobase.

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[**] A list of abbreviations used is provided in the Appendix.

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PNA/DNA chimeras results in new structures, which, in addition to excellent binding, can also assume biological functions, such as a primer function for DNA polymerases.[16] Furthermore, as self-organising structures PNAs are of fundamental interest in material sciences, as well as in the study of evolution of potential alternative life-forms.[16–18] In the following sections the synthesis, properties, and possible applications of PNAs and PNA/DNA chimeras are described.

2. Monomeric Building Blocks for the Synthesis of PNAs

A variety of different monomeric building blocks have been used for the synthesis of PNAs and their structural analogues. These differ from each other in the type of protecting group (PG) for the amino function of the backbone and/or for the nucleobase, and also in the structure of the backbone. Other achiral and chiral backbone building blocks have been employed in addition to *N*-(2-aminoethyl)glycine. The structures of the most important of the monomeric building blocks and their synthesis are described below. The selection is limited to monomers where a detailed description of the synthetic route has been reported, and to those monomers that have been employed for the synthesis of oligomers.

2.1. PNA Monomeric Building Blocks with an Aminoethylglycine Backbone

The "classical" PNA backbone consists of the amino acid *N*-(2-aminoethyl)glycine, where the secondary amino func-

Eugen Uhlmann was born in Bad Schussenried (Germany) in 1953 and studied chemistry at the University of Konstanz where he worked under the supervision of Professor W. Pfleiderer on the development of new protecting groups for oligonucleotide synthesis. After receiving his Ph.D in organic chemistry in 1981, he joined Hoechst AG in Frankfurt and established a laboratory for nucleotide synthesis. In 1984/1985 he spent one year in the Department of Molecular Biology and Genetics at the Massachusetts General Hospital and Harvard Medical School in Boston. Since 1986 he has been employed in the pharmaceutical division of Hoechst AG (now Hoechst Marion Roussel) where he works on the synthesis of new nucleic acid derivatives and their therapeutic use as antisense agents.

E. Uhlmann

A. Peyman

Anusch Peyman was born in Freiburg (Germany) in 1958, he studied chemistry at the University of Freiburg and completed his Ph.D. on the stereoselective dimerization of free radicals under the supervision of C. Rüchardt. From 1987–1988 he carried out postdoctoral research with J. R. Knowles at Harvard University on the reaction mechanism of triosephosphate-isomerase. In 1988 he joined the Peptide/Nucleotide group of Hoechst AG pharmaceutical division, and in 1991 spent one year working on ribozymes with J. Szostak at Harvard Medical School. In 1992 he returned to the medicinal chemistry division of Hoechst (now Hoechst Marion Roussel) and carries out research on antisense oligonucleotides and peptidomimetics.

G. Breipohl

D. W. Will

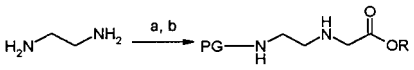
Gerhard Breipohl was born in Bielefeld in 1951 and studied chemistry at the Westfälische-Wilhelms University in Münster where he worked under the supervision of Professor B. Franck on the biosynthesis of mycotoxins. After receiving his Ph.D. in 1982 he worked for one year as a postdoctoral fellow at the Massachusetts Institute of Technology with G. Buechi. In 1983 he joined the peptide/nucleotide group of pharmaceutical research at Hoechst (now Hoechst Marion Roussel). There his research areas were the synthesis of enzyme inhibitors, hormone analogues, receptor antagonists, non-natural amino acids, peptide mimetics, PNAs and also the development of linkers and coupling methods for solid phase synthesis. In 1996 he moved to Chemical Development at HMR and works on developing and optimizing synthetic procedures for drug candidates.

David William Will was born in Bathgate (Scotland) in 1967, studied chemistry at the University of Edinburgh and completed his Ph. D. on the synthesis of modified oligonucleotides under the supervision of Professor Tom Brown in 1992. From 1992–1993 he carried out postdoctoral research on oligonucleotide mimetics and antisense therapeutics with Dr. Eugen Uhlmann in the Peptide/Nucleotide group of Hoechst AG pharmaceuticals division in Frankfurt. In 1994 he moved to the European Molecular Biology Laboratory in Heidelberg and worked on the synthesis of modified ribozymes with Dr. Brian Sproat. He continued this work at Ribonetics GmbH in Göttingen. In late 1994 he returned to Hoechst AG and continued his research on antisense therapeutics. In 1997 he joined the Automated Synthesis and New Technologies team of Hoechst Marion Roussel in Frankfurt.

tion is acylated with a nucleobase acetic acid derivative. Acid- or base-labile protecting groups are normally used for temporary protection of the primary amino function. The preparation of the monomers can be divided into the synthesis of a suitably protected *N*-aminoethylglycine, and the nucleobase acetic acid derivatives. Their syntheses are described in the following sections.

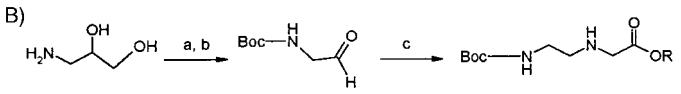
Figure 2 shows the three most important synthetic routes to aminoethylglycine: alkylation of 1,2-diaminoethane with halogenoacetic acid derivatives[19–22] (Figure 2A), reductive

A)



PG	R	a	b	Ref.
Boc	Me	1. $ClCH_2COOH$ 2. MeOH/HCl	$(Boc)_2O$, dioxane/H_2O	[19]
Boc	*t*Bu	$(Boc)_2O$, NEt_3, CH_2Cl_2	$ClCH_2COOtBu$, NEt_3, CH_2Cl_2	[22]
Fmoc	*t*Bu	$BrCH_2COOtBu$, CH_2Cl_2	Fmoc-ONSu, DIEA	[20]
Mmt	Et	1. $ClCH_2COOH$ 2. EtOH/HCl	Mmt-Cl, NEt_3, CH_2Cl_2	[21]

B)



R	a	b	c	Ref.
Me	$(Boc)_2O$, NaOH, H_2O	KIO_4, H_2O	$H_2NCH_2COOMe{\cdot}HCl$, NaOAc, H_2, Pd/C	[23]
Et	$(Boc)_2O$, NaOH, H_2O	KIO_4, H_2O	$H_2NCH_2COOEt{\cdot}HCl$, NaOAc, H_2, Pd/C	[23]
Et	$(Boc)_2O$, NaOH, H_2O	$NaIO_4$, H_2O	$H_2NCH_2COOEt{\cdot}HCl$, $NaBH_3CN$, MeOH	[24]

C)

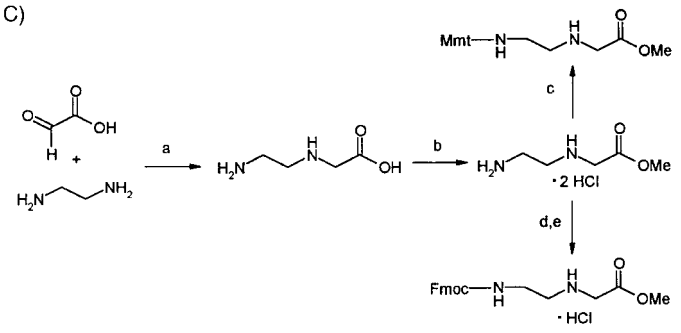


Figure 2. A) Synthesis of Aeg derivates by alkylation of 1,2-diaminoethane. B) Synthesis of Boc–Aeg derivates by reductive amination from Boc-aminoacetaldehyde and glycine esters. C) Synthesis of Aeg derivates by reductive amination from 1,2-diaminoethane and glyoxylic acid:[25,26] a) H_2/Pd/C, H_2O; b) MeOH/HCl; c) Mmt-Cl, DMF, NEt_3; d) Fmoc-(ONSu), dioxane/H_2O; e) MeOH/HCl.

amination with glycine esters and protected aminoacetaldehyde[23, 24] (Figure 2B), and reductive amination using 1,2-diaminoethane and glyoxylic acid[25, 26] (Figure 2C). Further reaction steps lead to protected aminoethylglycine derivatives with unprotected secondary amino groups to which the nucleobase acetic acid derivatives can be coupled.

The synthesis of nucleobase acetic acid derivatives is shown in Figures 3–6. Thyminyl acetic acid, which does not require an additional base protecting group, can be prepared either by alkylation of thymine with halogenoacetic acid esters[6, 27] followed by saponification, or by direct alkylation with bromoacetic acid[28] (Figure 3). We prefer the latter method because of its simplicity.

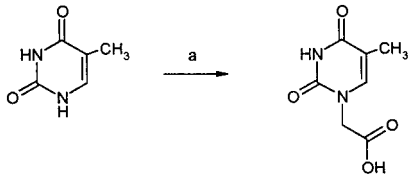
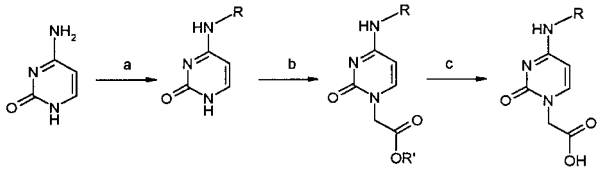


Figure 3. Synthesis of thyminyl acetic acid. Method 1:[27] a) 1. $BrCH_2COOMe$, K_2CO_3, DMF; 2. NaOH, H_2O. Method 2:[28] a) $BrCH_2COOH$, KOH, H_2O.

A common feature in the synthesis of cytosyl acetic acid derivatives (Figure 4) is that the protecting group on the exocyclic 4-amino function of cytosine is introduced in

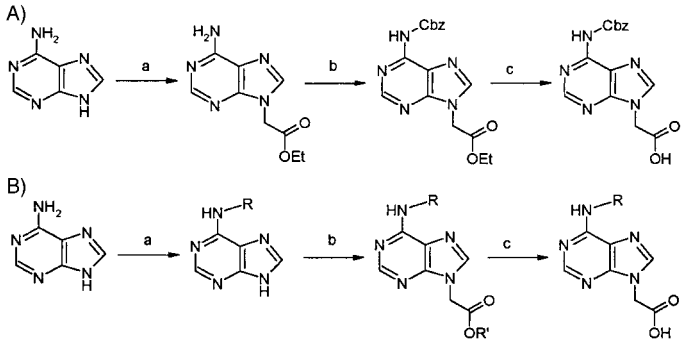


R	R'	a	b	c	Ref.
Cbz	Me	Cbz-Cl/ pyridine	$BrCH_2COOMe$, K_2CO_3, DMF	NaOH (aq.)	[27]
Cbz	*t*Bu	Cbz-Cl, DMAP, pyridine	$BrCH_2COOtBu$, K_2CO_3, Cs_2CO_3, DMF	HCl/dioxane, CH_2Cl_2	[20]
Mmt	Me	Mmt-Cl, pyridine, NEM	$BrCH_2COOMe$, NaH/ DMF	NaOH, H_2O/dioxane,	[25]
*t*BuBz	Me	*t*BuBz-Cl/ pyridine	$BrCH_2COOMe$, NaH/ DMF	NaOH, H_2O/ dioxane	[26]
An	Me	anisoyl chloride/ pyridine	$BrCH_2COOMe$, NaH/ DMF	NaOH, H_2O/ dioxane	[36]

Figure 4. Synthesis of cytosyl acetic acid derivatives.

the first step by using the appropriate acid chloride or Mmt chloride in pyridine. The resulting benzyloxycarbonyl,[9, 20, 27] 4-*tert*-butylbenzoyl, anisoyl,[22, 26] and Mmt derivatives[25] are then N^1-alkylated with methyl bromoacetate[25–27] or *tert*-butyl bromoacetate[20]. Finally, cleavage of the ester group under basic or acidic conditions gives the corresponding cytosyl acetic acid derivative.

The synthesis of the adenyl acetic acid derivatives is shown in Figure 5. The first synthetic route (Figure 5a)[27] involves N^9 alkylation of adenine by ethyl bromoacetate. Attempts to protect the exocyclic 6-amino functionality with Cbz-Cl resulted in a complex mixture of products. However, the use of *N*-(benzyloxycarbonyl)-*N'*-ethylimidazolium tetrafluoroborate (Rapoport's reagent)[29] proceeds smoothly to give the desired product. The other synthetic routes (Figure 5b) involve the initial protection of the 6-amino group of adenine. In this case, the reaction with Cbz-Cl is unproblematic.[20] Introduction of the Mmt[25] or anisoyl groups[26] in pyridine



R	R'	a	b	c	Ref.
Cbz	*t*Bu	NaH, Cbz-Cl, DMF	$BrCH_2COOtBu$, K_2CO_3, Cs_2CO_3, DMF	TFA, CH_2Cl_2, Et_3SiH	[20]
Mmt	Me	Mmt-Cl, pyridine, NEM	$BrCH_2COOMe$, NaH/ DMF	NaOH, H_2O/dioxane	[25]
An	Me	anisoyl chloride/ pyridine	$BrCH_2COOMe$, NaH/ DMF	NaOH, H_2O/ dioxane	[26]

Figure 5. A) Synthesis of Cbz-protected adenyl acetic acid according to Dueholm et al.:[27] a) $BrCH_2COOEt$, NaH, DMF; b) *N*-(benzyloxycarbonyl)-*N*′-ethylimidazolium tetrafluoroborate, DMF, CH_2Cl_2; c) NaOH (aq.). B) Synthesis of adenyl acetic acid via the N^6-protected adenine.[20, 25, 26]

also proceeds smoothly. The resulting protected adenine derivatives are then N^9-alkylated with methyl or *tert*-butyl bromoacetate. The desired protected adenyl acetic acid derivatives are obtained by alkaline saponification, or by ester hydrolysis under acidic conditions, with the addition of triethylsilane to prevent partial loss of the Cbz group.

The preparation of the guanyl acetic acid derivatives (Figure 6) presents the greatest synthetic challenge. The difficulty of obtaining selective N^9 alkylation while avoiding N^7 alkylation is well known in nucleoside chemistry. This selectivity problem can often be diminished by the use of 2-amino-6-chloropurine in alkylation reactions. Figure 6a shows the synthesis of the first protected guanyl acetic acid derivative to be used in PNA monomer synthesis.[27] 2-Amino-6-chloropurine is alkylated in moderate yield with bromoacetic acid. The desired O^6-benzylguanyl acetic acid is then obtained by exchange of the chlorine atom with sodium benzyloxide. However, this derivative is no longer in use because of solubility and stability problems under the conditions necessary for Boc cleavage.[27]

N^2-Cbz guanyl acetic acid is obtained by the procedure described in Figure 6b.[20] Reaction of 2-amino-6-chloro purine with allyl bromide results in a 3:1 ratio of N^9/N^7-alkylated products, which gives 9-allyl guanine after chromatographic separation and acid hydrolysis. The N^2 amino functionality of this compound is then protected by reaction with Cbz-imidazolide and KH in the presence of [18]crown-6 as a phase transfer catalyst. After ozonolysis of the allyl function and oxidative work-up the desired N^2-Cbz-guanyl acetic acid is obtained.

The synthesis of guanyl acetic acid protected on N^2 by the Mmt group, which can be cleaved under weakly acidic conditions, is depicted in Figure 6c.[25] Again, 2-amino-6-chloropurine is used for the synthesis. The Mmt group is introduced on N^2 after alkylation with methyl bromoacetate. Under the conditions of the subsequent alkaline saponification of the methyl ester the chlorine in position 6 is also exchanged, and the desired guanyl acetic acid is obtained.

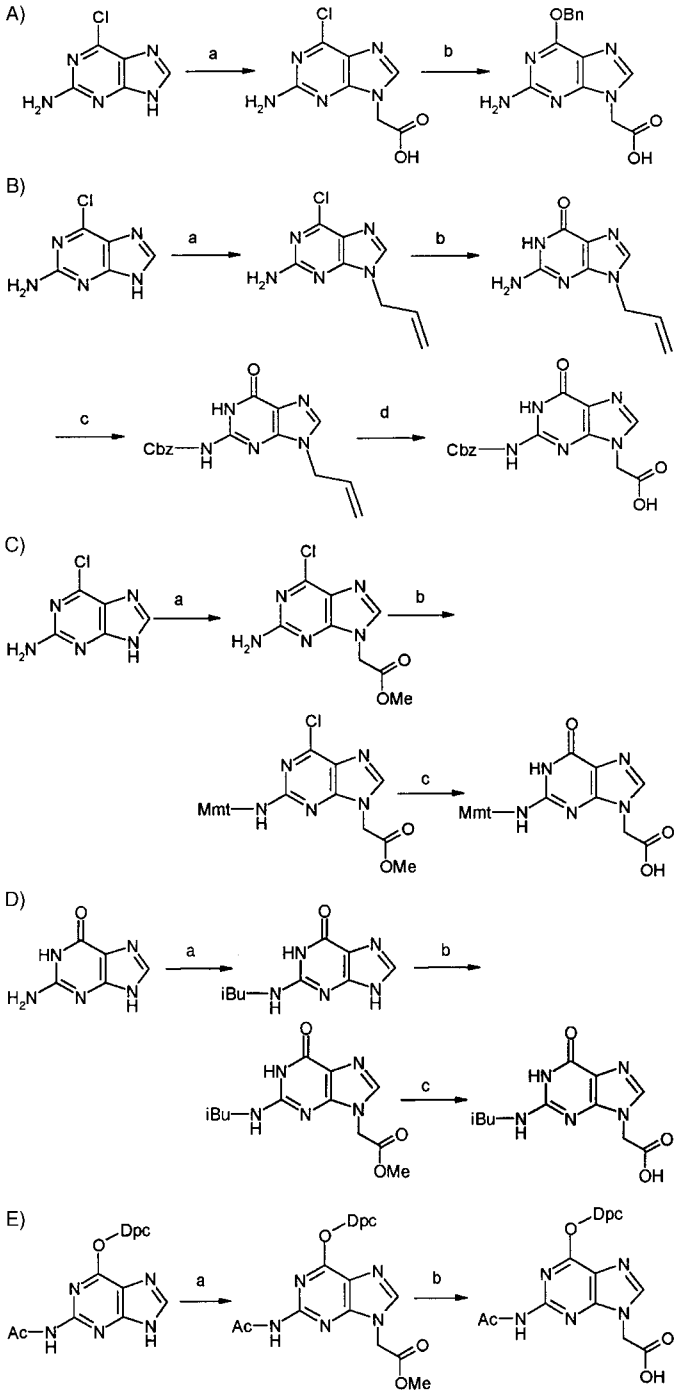


Figure 6. A) Synthesis of O^6-benzylguanyl acetic acid:[27] a) $BrCH_2COOH$; b) $PhCH_2ONa$, DMF. B) Synthesis of N^2-Cbz-guanyl acetic acid:[20] a) $BrCH_2CH{=}CH_2$, K_2CO_3, DMF; b) 1N HCl, reflux; c) Cbz-imidazole, [18]crown-6, KH, THF; d) 1. O_3, CH_2Cl_2, MeOH; 2. Me_2S; 3. $NaClO_2$, NaH_2PO_4, H_2O, THF, *t*BuOH, 2-methyl-2-butene. C) Synthesis of N^2-Mmt-guanyl acetic acid:[25] a) $BrCH_2COOMe$, NaH/DMF; b) Mmt-Cl, pyridine, NEt_3; c) 10% NaOH (aq), reflux. D) Synthesis of N^2-iBu-guanyl acetic acid:[26] a) Isobutyryl chloride, NEt_3/ DMF; b) $BrCH_2COOMe$, NaH/DMF; c) NaOH, H_2O/dioxane. E) Synthesis of N^2-Ac-O^6-Dpc-guanyl acetic acid:[22] a) $BrCH_2COOMe$, DMF, DIEA; (b) NaOH/ MeOH/ dioxane/ H_2O.

Figure 6d shows the synthesis of guanyl acetic acid carrying a base-labile isobutyryl protecting group on the 2-amino

function.[26]. N^2-Isobutyrylguanine is readily obtained by acylation of guanine with isobutyryl chloride in triethylamine/DMF or pyridine. This compound can then be alkylated with methyl bromoacetate under standard conditions to give a mixture of N^7/N^9-alkylated products. After chromatographic separation and alkaline saponification of the methyl ester the desired N^2-Ibu-guanyl acetic acid is obtained.

A useful alternative method to prevent N^7 alkylation is the use of N^2-acetyl-O^6-(diphenylcarbamoyl)purine[30] (Figure 6e). Alkylation of this compound with methyl bromoacetate in DIEA/DMF, and subsequent precipitation gives exclusively the N^9-alkylated product.[22] Subsequent alkaline saponification gives N^2-Ac-O^6-Dpc-guanyl acetic acid in good yield.

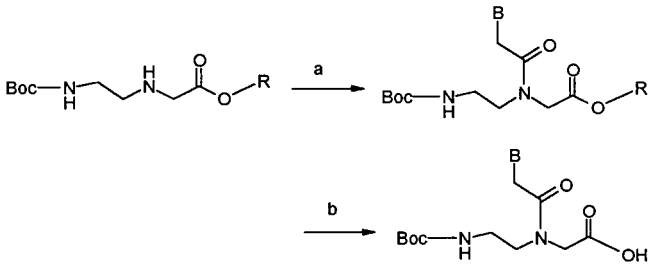
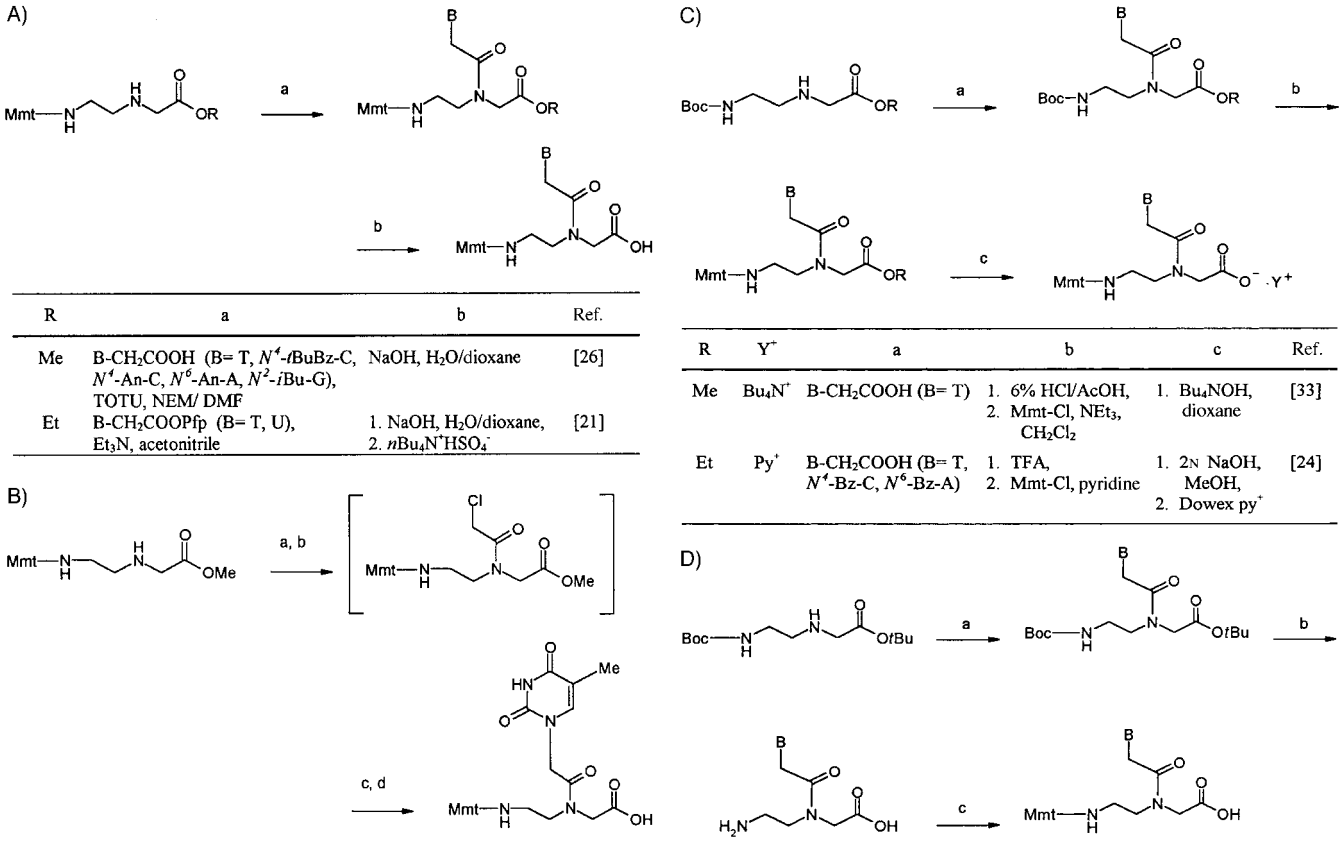


Figure 7. Synthesis of Boc–PNA monomers:[27] a) B-CH_2COOH (B = T, N^4-Cbz-C, N^6-Cbz-A, O^6-Bn-G, N^2-Cbz-G), DCC/HOObt[160] or PyBrop/DIEA; b) LiOH/THF/H_2O or NaOH/MeOH/H_2O

2.1.1. Monomers with Acid-labile N-Protecting Groups

The first reported syntheses of PNA[6] used monomers with the trifluoroacetic acid-labile Boc group for N-terminal temporary protection[8, 9, 27]. The exocyclic functional groups of the nucleobases are protected with Cbz and/or benzyl groups, which can be cleaved with liquid hydrofluoric acid or trifluoromethanesulfonic acid. The synthesis of PNA monomers with this protecting group combination and the building blocks described above is shown in Figure 7. Standard peptide coupling reagents, such as DCC/HOBt[31] or PyBrop,[32] are used to attach the nucleobase acetic acid derivatives to the secondary amino function of the Boc-protected aminoethylglycine ester.

These Boc-monomers are unsuitable for the synthesis of PNA/DNA chimeras, as the strongly acidic conditions (TFA/HF) necessary for deprotection would lead to depurination of the DNA-part of the oligomers. The Mmt-protected monomers described by us,[26] and others[21, 24, 33, 34] (Figure 8) can be deprotected under much milder conditions (trichloroacetic acid), and thus do not have this disadvantage. In particular, the combination with base-labile protecting groups for the exocyclic amino functionalities of the nucleobases cytosine, adenine, and guanine allows deprotection conditions that are compatible with standard oligonucleotide synthesis. Protect-



R	a	b	Ref.
Me	B-CH_2COOH (B= T, N^4-*t*BuBz-C, N^4-An-C, N^6-An-A, N^2-*i*Bu-G), TOTU, NEM/ DMF	NaOH, H_2O/dioxane	[26]
Et	B-$CH_2COOPfp$ (B= T, U), Et_3N, acetonitrile	1. NaOH, H_2O/dioxane, 2. $nBu_4N^+HSO_4^-$	[21]

R	Y^+	a	b	c	Ref.
Me	Bu_4N^+	B-CH_2COOH (B= T)	1. 6% HCl/AcOH, 2. Mmt-Cl, NEt_3, CH_2Cl_2	1. Bu_4NOH, dioxane	[33]
Et	Py^+	B-CH_2COOH (B= T, N^4-Bz-C, N^6-Bz-A)	1. TFA, 2. Mmt-Cl, pyridine	1. 2N NaOH, MeOH, 2. Dowex py^+	[24]

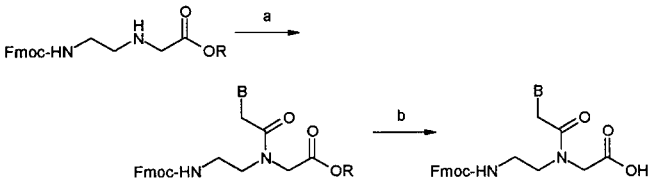
Figure 8. A) Two routes for the synthesis of Mmt monomers.[21, 26] B) Alternative synthesis of Mmt monomers:[36] a) $ClCH_2COCl$, NEt_3, THF; b) DMF, THF ↑ ; c) thymine, K_2CO_3, DMF; d) NaOH, H_2O/dioxane. C) Synthesis of Mmt monomers from the corresponding Boc monomers.[24, 33, 34] D) Synthesis of Mmt monomers via intermediates which can be precipitated:[22] a) B-CH_2COOH (B = T, N^4-An-C, N^6-An-A, N^2-*i*Bu-G, N^2-Ac-O^6-Dpc-G), TOTU, NEM/DMF or propylphosphonic anhydride, EtOAc, NEt_3; b) 95% TFA, c) Mmt-Cl, NEt_3, DMF.

ing groups established in oligonucleotide synthesis, such as the benzoyl-, anisoyl-, and *tert*-butyl benzoyl-groups for cytosine and adenine, or the isobutyryl- and acetyl-groups for guanine, are especially suitable in this context. The synthesis of these monomers, which are universally applicable for the synthesis of PNAs and PNA/DNA chimeras, is shown in Figures 8 A – C. The synthetic route[26] we follow utilizes TOTU[35] to couple the nucleobase acetic acids to Mmt-Aeg-OMe. The monomer synthesis is concluded by saponification of the methyl ester with sodium hydroxide in water/dioxane and subsequent purification by chromatography on silica gel with an eluent that contains triethylamine. Stetsenko et al.[21] coupled thyminyl and uracil acetic acids, as their pentafluorophenyl active esters, to Mmt-Aeg-OEt. After saponification of the ethyl ester with sodium hydroxide the monomers are converted into their tetrabutylammonium salts. Figure 8 B shows an attractive alternative method[36] employed by us for the synthesis of the thymine derivative. Acylation of Mmt-Aeg-OMe with chloroacetyl chloride in THF, gives a chloroacetyl derivative that can be used, after solvent exchange from THF to DMF, to alkylate thymine directly. A further synthetic route (Figure 8 C), reported by van der Laan et al.[33, 34] and Finn et al.[24], gives the Mmt-Aeg-esters by a Boc to Mmt "protecting group exchange", starting from the Boc-Aeg-esters. After saponification the monomers are converted into their tetrabutylammonium or pyridinium salts.

The route shown in Figure 8 D is used by us[22] for the large-scale synthesis of monomers. It avoids the need for chromatographic purification steps, as the fully protected intermediates that result from the coupling reaction are readily purified by precipitation.

2.1.2. Monomers with Base-labile N-Protecting Groups

So far, only Aeg monomers incorporating the base-labile Fmoc protecting group have been described. The synthesis of these monomeric building blocks is shown in Figure 9.



	a	b	Ref.
*t*Bu	B-CH_2COOH (B= T, N^4-Cbz-C, N^6-Cbz-A, N^2-Cbz-G), EDC, DMF or BOP, HOBt, DMF	HCl/dioxane or TFA, CH_2Cl_2, Et_3SiH	[20]
Me	B-CH_2COOH (B= T, N^4-Mmt-C, N^6-Mmt-A, N^2-Mmt-G), TOTU or PyBOP/ DMF /NEt_3	NaOH/dioxane/H_2O	[25]

Figure 9. Synthesis of Fmoc monomers.

Thomson et al.[20] combined the Fmoc group with the Cbz-nucleobase protection strategy of Nielsen et al.[6, 27] The monomers are obtained by the coupling of the nucleobase acetic acid derivatives to Fmoc-Aeg-OtBu, using for example BOP[37] as the activator. Treatment with HCl/dioxane or TFA results in cleavage of the *tert*-butyl ester. The addition of triethylsilane in this step is necessary to prevent partial Cbz cleavage in the adenine building block. The problem remains, however, that strongly acidic conditions (HF, TFMSA) are required to cleave the Cbz-protecting groups employed in these monomers.

A combination of the Fmoc group with the Mmt group, which can be cleaved under mildly acid conditions, gives monomers that allow deprotection under substantially milder conditions (Figure 9).[25] Although synthesis of these monomers, by the coupling of the nucleobase acetic acid derivatives with TOTU[35] or PyBOP[38], is unproblematic, the subsequent cleavage of the methyl ester in the presence of the Fmoc group is rather more challenging. The partial Fmoc-cleavage observed during this procedure can be readily reversed by the addition of a small quantity of Fmoc(ONSu) before work up.

2.2. Monomeric PNA Building-blocks with Modified Backbones

Structures that are either homologues or other modifications of the *N*-aminoethylglycine backbone have also been synthesized. In addition, monomers that incorporate chiral building blocks have been described and used for oligomer synthesis either in combination with aminoethylglycine monomers, or for the synthesis of homo-oligomers.

2.2.1. Monomers with Achiral Backbones

Phosphonic ester nucleic acids (PHONAs)[39] are PNA analogues with an *N*-(2-hydroxyethyl)aminomethylphosphonic acid backbone. PHONA monomer synthesis (Figure 10) involves reaction of O-Mmt protected aminoethanol with

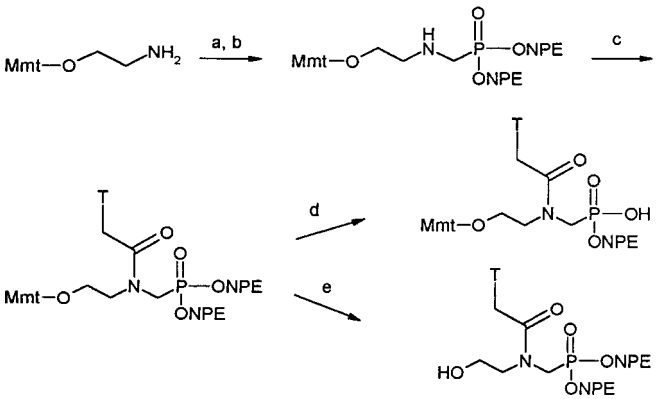


Figure 10. Synthesis of PHONA monomers:[39a] a) CH_2O, MeOH; b) $H(O)P(ONPE)_2$, 100 °C; c) thyminyl acetic acid, HOBt, NEM, DIC, DMF; d) 0.1 M DBU, pyridine; e) 80 % AcOH.

formaldehyde and phosphonic acid-bis[ethyl (4-nitrophenyl) ester]. The protected aminomethylphosphonic acid backbone unit is then coupled with thyminyl acetic acid. Subsequent selective cleavage of one of the NPE protecting groups with DBU[40] gives the desired thymine monomer that is employed

for the synthesis of PHONA homo-oligomers, and for incorporation into PNA and DNA oligomers.

Hyrup et al.[41–43] described monomers (Figure 11) based on homologues of aminoethylglycine: aminoethyl-β-alanine (**A**), aminopropylglycine (**B**) and aminopropyl-β-alanine (**C**).

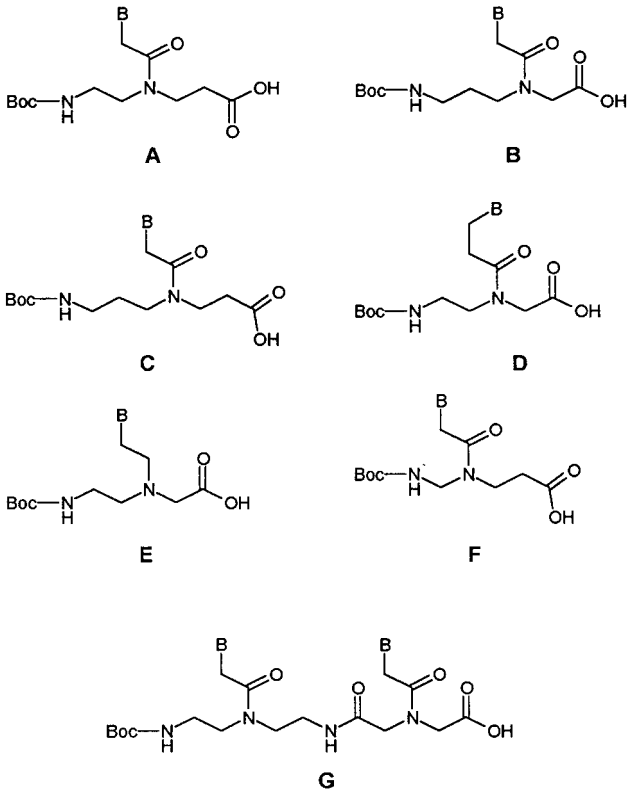


Figure 11. Monomers with modified backbone structures (**A**–**E**)[41–43] and special PNA building blocks for "retro-inverse"-PNAs (**F**[44] and **G**[45]).

Variation in the spacing (**D**) and type of attachment (**E**) of the nucleobase to the Aeg backbone were also described. These compounds are synthesized in an analogous manner to the procedures described for the Aeg derivatives.

Special monomers that are used to prepare "retro-inverse" PNAs[44] are shown in Figure 11 (**F**). Heterodimers of *N*-(2-aminoethyl)ethylenediamine and iminodiacetic acid (**G**)[45] are also used for this purpose.

2.2.2. Monomers with Chiral or Olefinic Backbones

Monomers with a chiral center in the backbone (Figure 12) have been described by several research groups. The amino acid derivatives that constitute the backbone are assembled through reductive amination of Boc-alaninal with glycine methyl ester[28] (**H**) or of Boc-glycinal with amino acid esters[46, 47] (**I**), followed by coupling of the nucleobase acetic acid derivatives to give the desired monomeric building blocks.

Further backbone variations based on ornithine (**J**)[48, 49], proline (**K**, **L**),[50–53] the conformationally constrained diaminocyclohexane (**M**),[54, 55] and the phophoramidite of 2-aminopropanediol (**N**)[56] have recently been described, as well as monomers for olefinic PNAs (**O**),[57] in which the central amide bond is replaced by a configurationally defined C–C double bond.

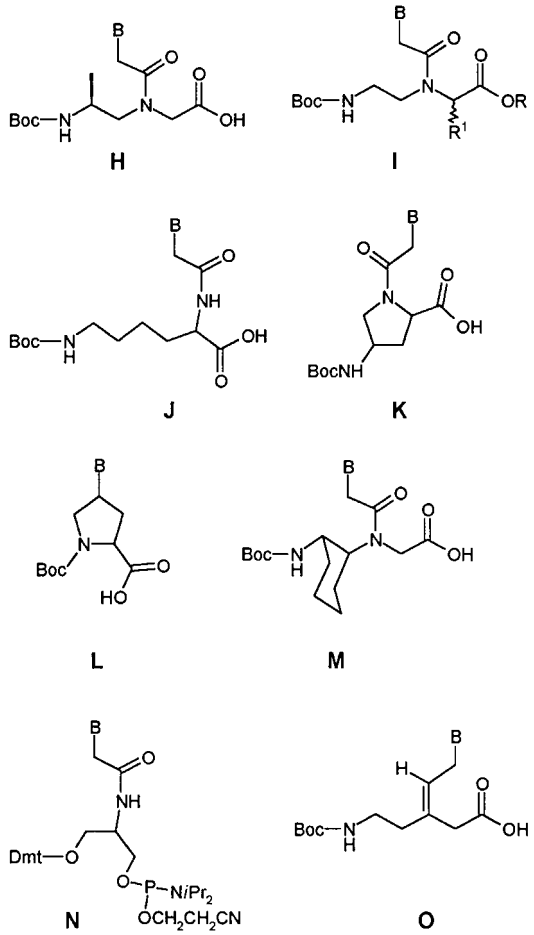


Figure 12. Monomers **H**,[28] **I** (example of R_1: CH_3, $CH_3CH_2CH(CH_3)$, CH_2OH, $CH_2CH_2CO_2H$, $(CH_2)_4NH_2$)[46, 47], **J** (based on ornithine),[48, 49] **K, L** (based on proline),[50–53] **M** (based on diaminocyclohexane)[54, 55] and **N** (based on 2-aminopropan-1,3-diol)[56] with chiral backbones and monomers based on a Z-olefinic structure (**O**).[57]

3. PNA Oligomer Synthesis

The following possibilities for preparing PNAs are summarized in Table 1.

3.1. PNAs with the Aminoethylglycine Backbone

3.1.1. The tert-Butyloxycarbonyl/Benzyloxycarbonyl (Boc/Cbz) Protecting Group Strategy

The earliest PNAs to be synthesized were homopolymers of thymine.[6, 8] They were prepared by Merrifield solid-phase synthesis on a Boc-L-lysine-derivatized MBHA polystyrene resin (Figure 13a) and were thus obtained as C-terminal lysine amides. The positive charge on the lysine side-chain helps to suppress the undesirable tendency of PNAs to self-aggregate, and simplifies their purification and characterization. The monomers described in Section 2.1.1 were used for PNA synthesis as preformed pentafluorophenyl active esters. The synthesis cycle consisted of cleavage of the N-terminal Boc temporary protecting group with TFA, followed by monomer coupling to the N terminus, which

Table 1. Summary of selected PNA synthesis methods.

Ref.	N-terminal protecting group	Base and protecting group	Solid support type	Coupling method (yield)	Capping	Cleavage and deprotection
[58]	Boc	T: none, C: N^4-Cbz, A: N^6-Cbz, G: N^2-Cbz	MBHA-polystyrene (Fig. 13a)	HBTU, diethylcyclohexylamine in DMF/pyridine (97%)	N^1-benzyloxycarbonyl-N^3-methylimidazole triflate	TFA/TFMSA
[59]	Boc	T: none, C: N^4-Cbz, A: N^6-Cbz, G: N^2-Cbz	MBHA-polystyrene	HATU, DIPEA in NMP/pyridine (99%)	acetic anhydride/NMP/pyridine, piperidine	TFA/TFMSA
[20]	Fmoc	T: none, C: N^4-Cbz, A: N^6-Cbz, G: N^2-Cbz	MBHA- or Rink-resin	pentafluorophenyl active ester (95–99%)	acetic anhydride/DIEA in NMP	HF/anisole
[25]	Fmoc	T: none, C: N^4-Mmt, A: N^6-Mmt, G: N^2-Mmt	Breipohl linker-polystyrene (Fig. 13d) or aminohexyl-succinyl-CPG	PyBOP, NEM in DMF (94%)	–	95% TFA or NH_3/H_2O
[68]	Fmoc	T: none, C: N^4-Bz, A: N^6-Bz, G: N^2-*i*Bu	hydroxydodecanoic acid-CPG (Fig. 13b)	HATU, DIPEA in DMF (not reported)	acetic anhydride in DMF	NH_3/EtOH
[26]	Mmt	T: none, C: N^4-*t*BuBz, A: N^6-An, G: N^2-*i*Bu	aminohexylsuccinyl-CPG or -Tentagel (Fig. 13c)	HATU or PyBOP, NEM or DIEA in DMF (95–99%)	acetic anhydride/lutidine/*N*-methylimidazole in THF	NH_3/H_2O

[a] The abbreviations are shown in the appendix.

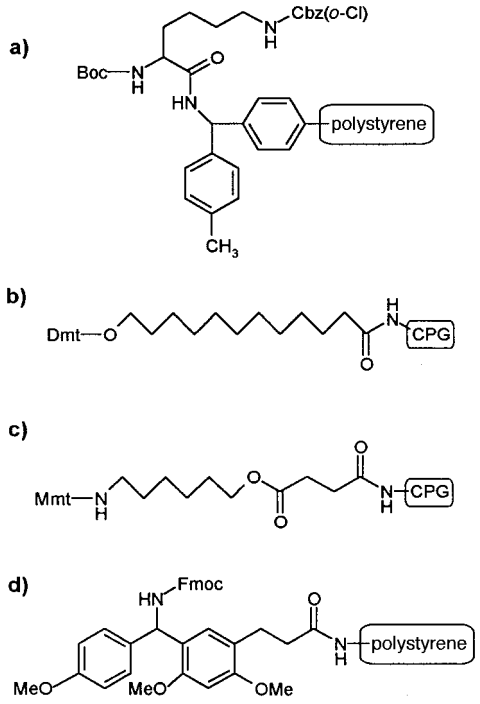


Figure 13. Supports for the solid-phase synthesis of PNA and PNA/DNA chimeras: a) Boc-Lys(Cl-Cbz)-MBHA-polystyrene;[6, 8, 27] b) dimethoxytrityl-12-hydroxydodecanoic acid-CPG;[68] c) monomethoxytrityl aminohexylsuccinate-CPG;[25, 26] d) Breipohl linker.[25]

proceeded in 94–99% yield. On completion of chain elongation the PNA oligomer was cleaved from the support with HF. Remarkably, even in these early reports PNA-nitrobenzylamido-acridinium conjugates[6, 8] were synthesized as photonuclease/intercalator ligands to provide evidence for binding.

The use of monomeric building blocks based on Boc-protected thymine, (N^4-benzyloxycarbonyl)cytosine,[7] (N^6-benzyloxycarbonyl)adenine, and (O^6-benzyl)guanine[9] in combination with in situ activation by DCC in 50% DMF/dichloromethane was reported to give coupling efficiencies of 98–100% per cycle. In 1994 the first detailed description of the preparation of these monomers and their use in PNA synthesis was published.[27] Highly efficient coupling, measured by a quantitative ninhydrin test, was achieved for all four monomers by in situ activation with DIC. Cleavage of the N-terminal Boc-group was carried out with 50% TFA in dichloromethane and, after coupling, any unreacted amino functionalities were capped with acetic anhydride in pyridine/dichloromethane. Cleavage of these mixed sequence PNA oligomers from the solid support was carried out with HF/2.5% thioanisole, and the products were purified by reversed-phase HPLC.

A systematic investigation to find the optimal synthesis conditions for a 17mer model PNA sequence, using in this case an N^2-Cbz-protected guanine monomer, was carried out by Christensen et al.[58] who found that the highest coupling efficiencies were obtained with HBTU. They also found that the use of N^1-benzyloxycarbonyl-N^3-methylimidazolium triflate instead of acetic anhydride in the capping reaction generated fewer undesirable side products. The most important side-reaction, however, was found to be the migration of the nucleobase acetic acid from the secondary amino function to the free N-terminal amino function of the aminoethylglycine backbone under basic or neutral conditions (Figure 14a). In a subsequent study the synthesis of the same PNA

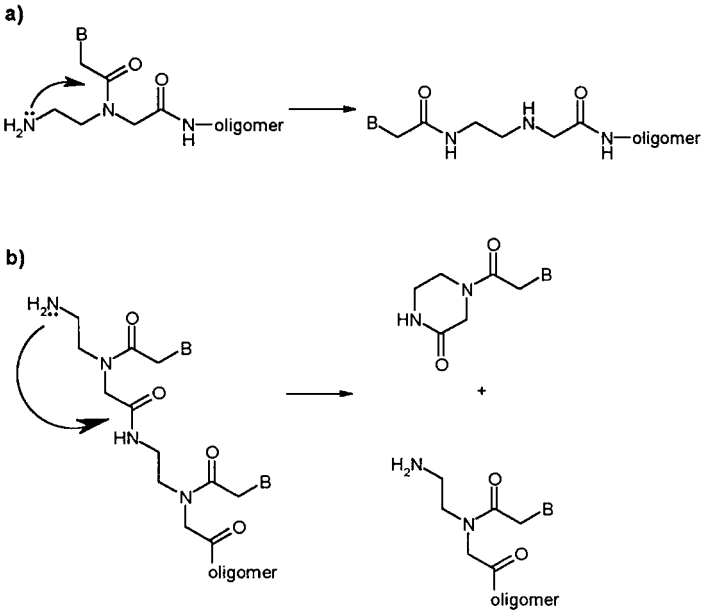


Figure 14. a) *N*-Acyl transfer reaction;[58] b) N-terminal deletion reaction.[20]

heptadecamer[59] was found to be even more efficient by using Boc/Cbz-protected monomers and HATU as the coupling reagent. Average coupling efficiencies of 99.4% per cycle were achieved on a commercial peptide synthesizer (5 μmol scale). The coupling step used a sevenfold monomer excess and a short preactivation with HATU/DIEA. Capping was carried out with acetic anhydride/NMP/pyridine, followed by a piperidine wash step to destroy any highly reactive acetylated nucleobase derivatives that can be formed in the capping step.[59]

The Boc/Cbz protected PNA monomers can also be used in conjunction with HATU activation for the synthesis of PNAs on a polyethylene glycol-polystyrene graft copolymer[60] and on polystyrene-grafted polyethylene films. [61]

The incorporation of various nonstandard nucleobases in PNAs has also been reported (Figure 15). Pseudoisocytosine[62] has been incorporated into PNAs to investigate the

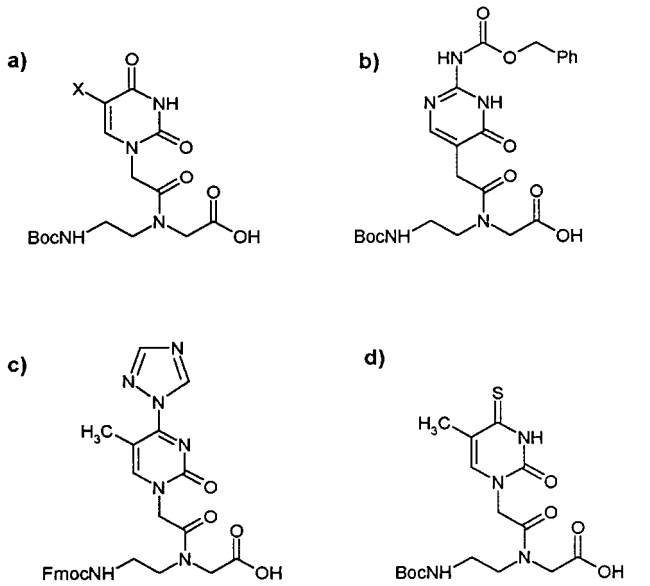


Figure 15. PNA monomeric building blocks with modified nucleobases. a) 5-Bromo- (X = Br) or 5-Iodo-uracil (X = I); b) pseudoisocytosine; c) triazolothymine; d) 4-thiothymine.

binding properties of bis(PNAs) (see Section 4.3.2), in which two different PNA sequences are linked "head-to-tail" by a flexible 8-amino-3,6-dioxaoctanoic acid linker.[63] A second nonstandard nucleobase, 5-iodouracil, has been incorporated into a bis(PNA)/peptide chimera.[64] The iodine atom was used to provide phase information for the X-ray crystal structure determination of a bis(PNA)/oligonucleotide complex. Similarly, the incorporation of 5-bromouracil[65] facilitated the structure determination of PNA · PNA duplexes. Structural investigations using photochemical reactions can be carried out on PNAs containing 4-thiothymine.[66] By using the Fmoc strategy we incorporated 5-methyl-4-(*3H*-(1,2,4)triazol-1-yl)-3,4-dihydro-*1H*-pyrimidin-2-one into PNAs.[25] This nucleobase should have served as an intermediate for the synthesis of 5-methylcytosine or thymine derivatives, however, in contrast to the analogous reaction in oligonucleotides,[67] attempts to exchange the triazolyl function in N-terminal deprotected PNAs by ammonia, ethylenediamine, or sodium hydroxide were unsuccessful.

3.1.2. The 9-Fluorenylmethyloxycarbonyl (Fmoc) Protecting Group Strategy

PNA monomers that utilize the Fmoc group for the temporary protection of the N-terminal in combination with the protection of the benzyloxycarbonyl nucleobase have been synthesized and used for PNA oligomer synthesis.[20] PNAs were obtained as their C-terminal amides or C-terminal acids when MBHA/MBHA-Rink or chlorotrityl polystyrene supports were used, respectively, for solid phase PNA synthesis. Cleavage of the N-terminal Fmoc group was carried out with 30% piperidine in 20% DMSO/NMP. The monomers, as their pentafluorophenyl active esters, were used in twofold excess in the coupling step. The synthesis cycle was completed with a capping step that used acetic anhydride/DIEA in NMP. Yields of 95 to 99% per cycle were obtained. A comparison of the pentafluorophenyl coupling method with HBTU/HOBt activation of the free acids of the monomers demonstrated the superiority of the former method.[20]

On completion of the oligomer synthesis the final N-terminal Fmoc group was retained, and the PNA was cleaved from the support with HF/anisole. The intact terminal Fmoc group could be used as a lipophilic handle to facilitate separation of the full-length PNA from failure sequences by reversed-phase HPLC, and could be removed after purification by treatment with piperidine. However, cleavage of the N-terminal PNA unit (Figure 14b) was observed as a new side-reaction on longer treatment with piperidine. On the other hand the N-acyl transfer reaction (Figure 14a) that had been reported previously[58] was not detected under these conditions.

Although the Fmoc strategy is undoubtedly milder than the Boc/Cbz strategy, its combination with the MBHA-solid phase linker and the benzyloxycarbonyl group for nucleobase protection still necessitates the use of harsh acidic conditions for the cleavage and deprotection of the PNA oligomer. Use of the Fmoc group as the temporary protecting group in combination with the Mmt group for protection of the exocyclic amino functions of the nucleobases[25] is more advantageous. The Mmt group gives the monomeric building blocks excellent solubility in organic solvents, and can be removed by treatment with 80% acetic acid on completion of the PNA oligomer synthesis. Oligomer synthesis can be carried out with our amide linker (Figure 13d), which allows simultaneous cleavage of the PNA amide from the solid support and deprotection of the Mmt groups with trifluoroacetic acid. Alternatively, the use of strongly acidic conditions can be avoided completely by the use of our base-labile aminohexylsuccinyl linker,[26] which allows cleavage of the deprotected PNA-hydroxyhexylamide from the support with concentrated aqueous ammonia.

A further variation of the Fmoc PNA synthesis strategy has been described by Bergmann et al.[68] for the synthesis of PNA/DNA chimeras. An Fmoc/acyl protecting group strategy was proposed for the N^4-benzoylcytosine-, N^6-benzoyladenine- and N^2-isobutyrylguanine-derivatized monomeric building blocks. Coupling was carried out with HATU/DIEA in DMF, and capping with acetic anhydride in DMF. The use of a 12-hydroxydodecanoic acid-derivatized aminoalkyl-CPG support (Figure 13b) allowed the attachment of the first PNA

unit through an ester group. The synthesized oligomer was cleaved, together with the acyl protecting groups of the nucleobase, by treatment with a solution of anhydrous ethanolic ammonia.

3.1.3. The 4-Methoxyphenyldiphenylmethyl/Acyl (Mmt/Acyl) Protecting Group Strategy

In an effort to find a milder method for PNA synthesis that would be compatible with conditions for DNA synthesis, we developed a new synthesis strategy that used the Mmt group for the temporary protection of the N-terminus and acyl protecting groups for the nucleobases.[26] The Mmt/acyl monomeric building blocks were described in section 2.1.1 (Figure 8). The lipophilic Mmt group improves the solubility of the monomers in polar organic solvents, such as DMF or acetonitrile. The solubility of the monomers could be further improved by the use of the *tert*-butylbenzoyl or anisoyl groups instead of the more commonly used unsubstituted benzoyl protecting group.

The synthesis cycle for the Mmt/acyl strategy is shown in Figure 16. The temporary protecting group Mmt can be cleaved under very mild conditions (3% trichloroacetic acid in dichloromethane), and the coupling efficiency can be determined very easily by measurement of the coloured Mmt cations released. The coupling step itself was carried out in DMF by using a short preactivation with HATU or PyBOP in the presence of DIEA. Capping, in an analogous manner to DNA synthesis, was carried out with acetic anhydride/lutidine/N-methylimidazole in THF. The use of a base-labile aminohexylsuccinyl linker between the CPG- or Tentagel solid-support and the PNA (Figure 13c) allowed the simultaneous cleavage of the desired PNA oligomer from the support and deprotection of its nucleobases with concentrated aqueous ammonia. To avoid potential side reactions (Figure 14) the final N-terminal Mmt protecting group was left intact during the ammonia treatment and subsequently removed by treatment with 80% acetic acid. Alternatively, the PNA strand could be converted into its base-stable N-terminal acetyl form by cleavage of the final Mmt group and subsequent capping.

The PNAs were thus obtained in the form of their C-terminal hydroxyhexylamides. The N-terminal Mmt group can be used as a lipophilic handle, in an analogous manner to the Dmt group in oligonucleotide purification, for so called "Trityl-on" reversed-phase HPLC purification. We also found that analysis and purification of N-terminal acetylated PNAs that contained more than three thymine or guanine residues can be carried out by ion-exchange HPLC or FPLC at pH 12, since at this high pH both guanine and thymine nucleobases are deprotonated. HPLC under alkaline conditions, which is especially useful for self-aggregating guanine-rich PNA sequences, has also been described by Bohler et al.[18] and Schmidt et al.[69] Similarly to oligonucleotides, PNA/DNA

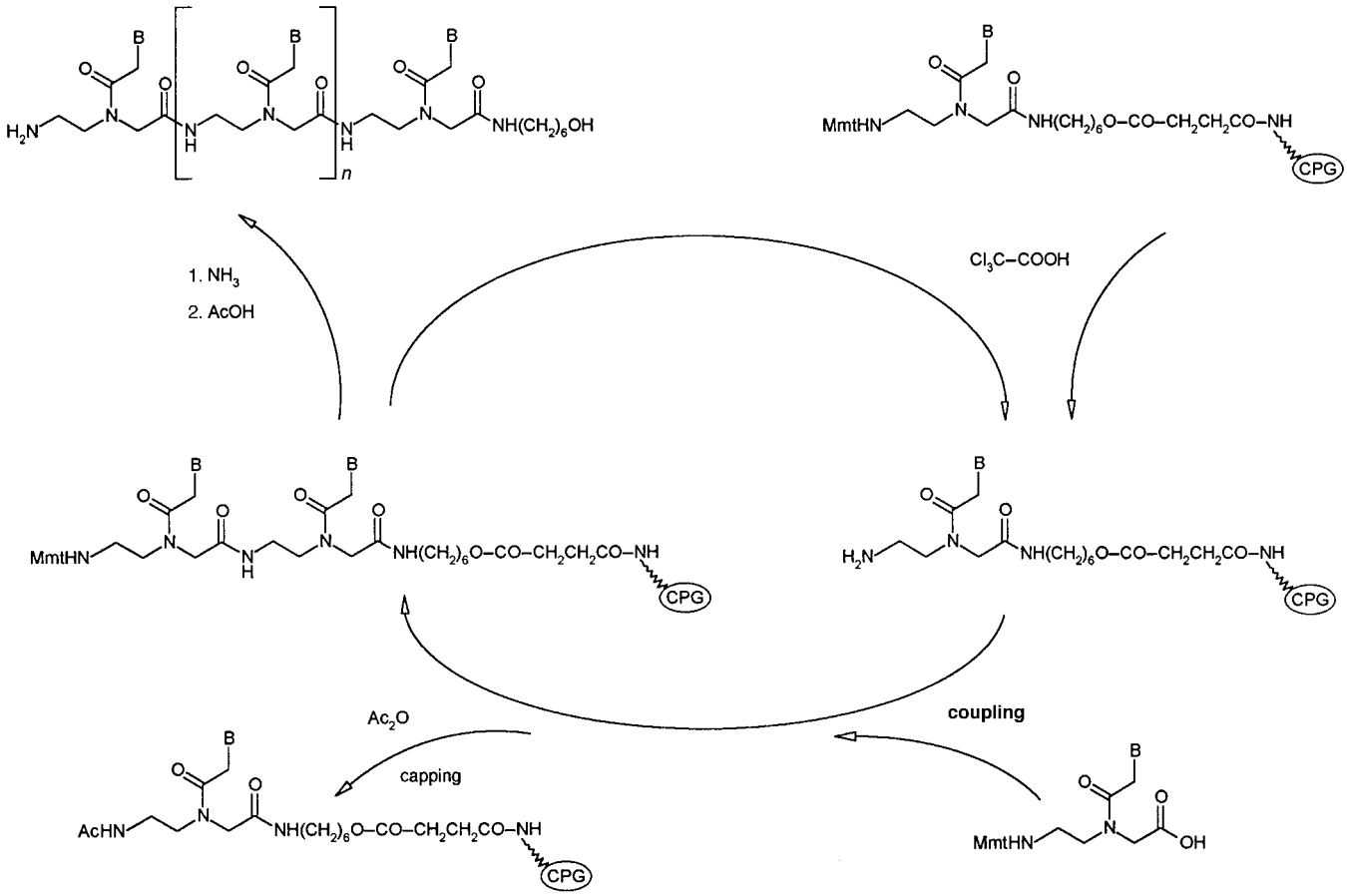


Figure 16. PNA synthesis cycle for the Mmt/Acyl-protecting group strategy.[26] Favored PNA coupling reagents are HATU, HBTU, and PyBOP.

chimeras have a multiple negative charge, (see Section 3.3) and can be analysed and purified by polyacrylamide gel electrophoresis.[70]

We use the Mmt/Acyl strategy routinely for the synthesis of PNAs and PNA/DNA chimeras on a 3 to 5 μmol scale on a modified Eppendorf Biotronik Ecosyn D300 DNA synthesizer[71] or in one to two μmol scale on an ABI (Applied Biosystems) DNA synthesizer similarly to van der Laan et al.[34]

3.1.4 Other Strategies

There is one preliminary report of the synthesis of H-ttttt-Gly-NH_2 that uses a PNA monomer with the thiol-labile dithiasuccinate (Dts) group for N-terminal temporary protection.[72]

A more exotic method for the synthesis of PNAs, which uses submonomers that do not contain the nucleobase,[73a] is outlined in Figure 17. The strategy involves the stepwise assembly of the aminoethylglycine PNA backbone units by solid-phase synthesis and acylation of the secondary amino functionality with nucleobase acetic acids. This strategy, which is reminiscent of peptoid synthesis,[73b] was applied to the synthesis of Ac-$(t)_8$-NH_2. It remains to be seen if this method in which, apart from the nucleobase acetic acid derivatives, only one monomeric building block must be synthesized, can be more generally applied to the synthesis of mixed-sequence PNAs.

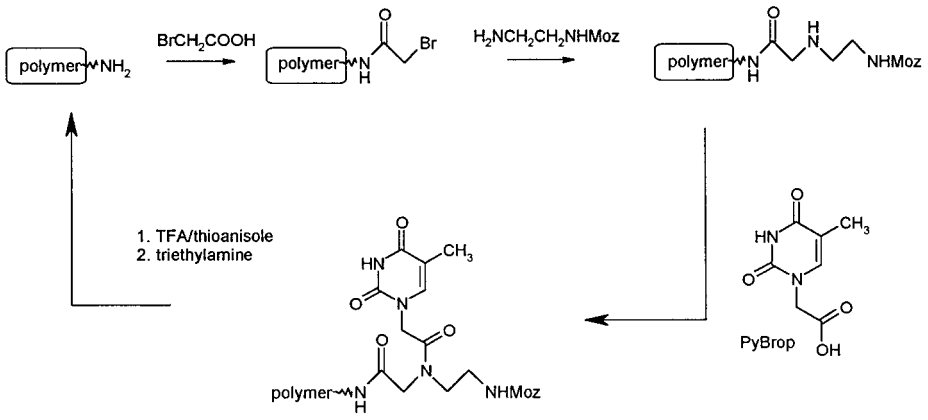


Figure 17. PNA synthesis cycle using submonomers.[73a] (Moz = 4-methoxybenzyloxycarbonyl).

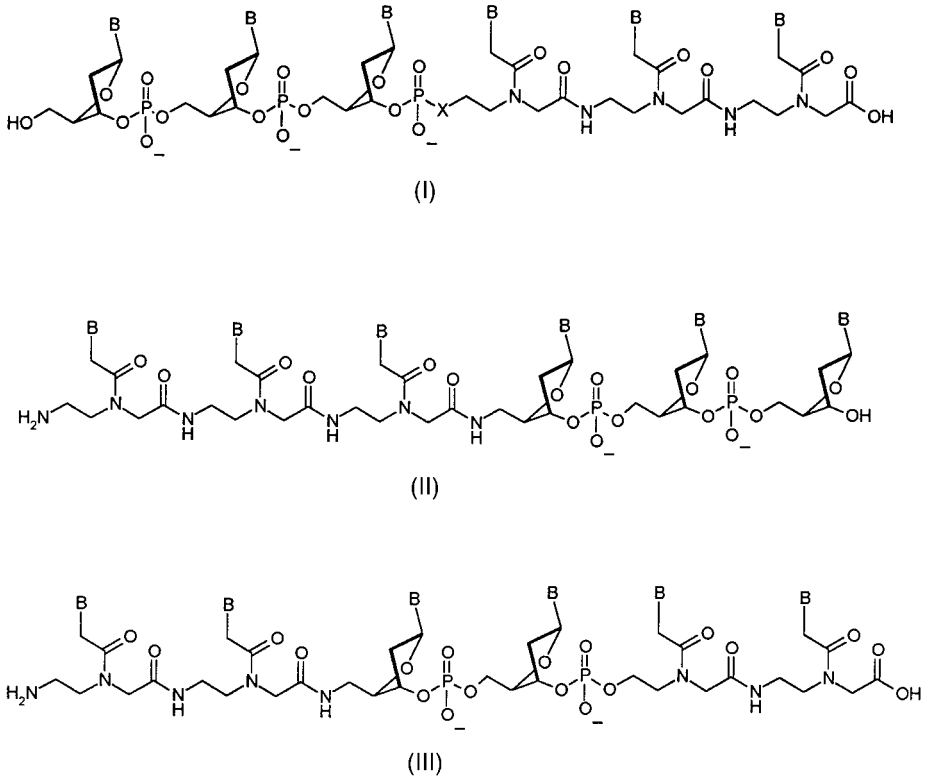


Figure 18. PNA/DNA chimeras: (I) 5′-DNA-(linker)-PNA-(*pseudo*-3′), X = NH or O; (II) (*pseudo*-5′)-PNA-(linker)-DNA-3′; (III) (*pseudo*-5′)-PNA-(linker)-DNA-(linker)-PNA-(*pseudo*-3′).

3.2. PNAs with Modified Backbones

The synthesis of backbone-modified PNAs is, in most cases, carried out in analogous ways to the methods described above. For instance, hetero-oligomeric PNAs composed of normal aminoethylglycine and aminoproline building blocks were assembled using the Boc strategy.[51, 74] This is not the case, however, for PNA derivatives that deviate strongly from the basic aminoethylglycine structure, such as the PHONAs where special synthetic methods had to be developed.[39, 75, 76]

3.3. Synthesis of PNA/DNA Chimeras

Although pure PNAs have truly remarkable binding properties, their handling during work-up, analysis, and purification is often difficult because of their tendency to self-aggregate. The synthesis of chimeric molecules composed of PNA and DNA (Figure 18) is one possible approach to addressing this problem, and would simultaneously improve the cellular uptake and the RNase H activation properties of PNAs (see Section 5).

The two principle strategies for the synthesis of PNA/DNA chimeras are "block condensation" of presynthesized PNA and DNA oligomers in solution, and stepwise "on-line" solid-phase synthesis with suitably protected

monomeric building blocks. The successful synthesis of PNA/DNA chimeras by the block condensation approach is fraught with difficulties, which include insufficient solubility and low yields,[77] and has not been realized so far. The on-line solid-phase synthesis strategy has proven to be the most successful and flexible method for the synthesis of PNA/DNA chimeras. However, to ensure full compatibility of the conditions for PNA and DNA synthesis, this method entails the development of special protecting group strategies. It also entails the synthesis of linker molecules (Figure 19) to connect PNA to DNA and vice versa.

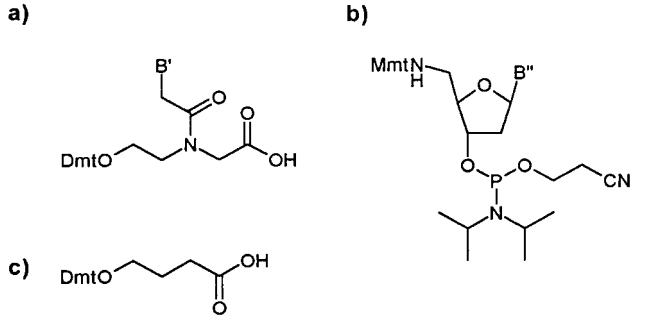


Figure 19. Types of linker molecules:[81] a) *N*-(2-hydroxyethyl)glycine PNA – DNA linker (B′ = T, N^4-An-C, N^6-An-A, N^2-iBu-G); b) 5′-amino-2′,5′-dideoxynucleoside-DNA-PNA linker (B″ = T, N^4-Bz-C, N^6-Bz-A, N^2-iBu-G); c) simple, nonhybridizing PNA – DNA linker.

So far the synthesis of three different structural classes of PNA/DNA chimeras have been described (Figure 18): (I) 5′-DNA-(linker)-PNA-(*pseudo*-3′); (II) (*pseudo*-5′)-PNA-(linker)-DNA-3′; and (III) PNA-DNA-PNA chimeras, which are a combination of (I) and (II). To avoid confusion we use lower-case letters for PNA units, and upper-case letters for DNA units when writing the sequences of PNA/DNA chimeras.

The synthesis of the homothymine oligomer (*pseudo*-5′)-(acetyl)-tttt-TTTT-3′ (T: 5′-amino-5′-deoxythymidine) of structure type (II) was carried out by van der Laan et al.[33] through on-line solid-phase synthesis on CPG. The 3′-DNA part was synthesized by standard DNA synthesis methods[78] on a 10-μmol-scale. The 3′-phosphoramidite of 5′-*N*-(4-monomethoxytrityl)amino-5′-deoxythymidine (Figure 19b), described by Bannwarth[79] and Mag et al.,[80] was used as the linker between the DNA and PNA, and was coupled to the 5′-end of the thymidylate trimer through the standard DNA synthesis cycle. Cleavage of the terminal Mmt protecting group with 2% trichloroacetic acid in dichloromethane was followed by the stepwise assembly of the PNA tetramer part in over 85% yield per cycle by using an Mmt-protected thymine PNA monomer and HBTU/DIEA activation. The terminal Mmt group was removed after the final synthetic cycle and the amino terminus was capped with acetic anhydride. Finally, treatment with ammonia removed the β-cyanoethyl protecting groups from the internucleotide bridges and cleaved the oligomer from the solid support. Unfortunately, no binding data were reported for the purified and characterized chimera.

Three PNA/DNA chimeras of structure type (I) have been synthesized by Petersen et al.[77] After initial unsuccessful attempts to couple nucleoside 3′-phosphoramidites to a hydroxyl-modified PNA oligomer in solution, they too changed to the on-line solid-phase synthesis strategy. The PNA – DNA linker utilized was *N*-(2-hydroxyethyl)-*N*-(thymin-1-ylacetyl)glycine (Figure 19a, B′ = T). Although the use of the Boc-protected thymine PNA monomer is possible for homopyrimidine chimeric sequences, it is unsuitable for chimeras containing purine bases in the DNA part; the use of HF to cleave the benzyloxycarbonyl protecting groups of cytosine, guanine, and adenine would lead to depurination in the DNA part. For this reason only homopyrimidine chimeras could be synthesized with this protecting group strategy. Synthesis was performed on a CPG solid support derivatized with a glycine linked to 4-(hydroxymethyl)benzoic acid. The PNA part was assembled from Boc/Cbz protected monomers.[58] The Dmt-protected hydroxyethylglycine – thymine linker building block was then attached, which after detritylation facilitated the assembly of the DNA part of the chimera according to standard DNA synthesis methods.[78] The resulting chimeras were linked in a "head-to-tail" manner, that is with the 3′-end of the DNA part attached to the N-terminus of the PNA part. Conversely, the use of nucleoside-5′-phosphoramidites allowed the synthesis of "head-to-head"-linked chimeras, in which the 5′-end of the DNA part is attached to the N-terminus of the PNA part. Since the amino terminus of PNAs corresponds to the 5′-end of DNA, a change of orientation takes place at the PNA – DNA junction in this type of chimera. After solid-phase synthesis the chimeras that bore a C-terminal glycine unit were cleaved with 0.1M aqueous sodium hydroxide. The benzoyl protecting groups of deoxycytidine-containing chimeras were removed by treatment with concentrated ammonia at 55°C overnight.

Bergmann et al.[68] synthesized PNA/DNA chimeras of types (I) and (III). A sarcosine-linked CPG solid support was used for the synthesis of homopyrimidine (*pseudo*-5′)-PNA-(linker)-DNA-3′ chimeras. Interestingly, the DNA part was assembled from special nucleoside 3′-phosphoramidites containing palladium-, or base-labile allyl protection, instead of the more usual cyanoethyl phosphate protection. Synthesis of the DNA strand was terminated with an Mmt-protected 5′-amino-5′-deoxynucleoside-3′-phosphoramidite[79] (Figure 19b), which serves as the DNA – PNA linker. The PNA part of the homopyrimidine chimeras was then synthesized from Boc/Cbz-protected PNA monomers. The oligopyrimidine DNA part is stable enough to survive the strongly acidic conditions required for deprotection of the PNA part. The chimeras were completely deprotected by treatment with concentrated ammonia. The use of Fmoc/acyl-protected PNA building blocks for the synthesis of mixed sequences with acid-sensitive purine nucleosides in the DNA part was suggested.[68] However, neither the preparation of these monomeric building blocks, nor their use in the synthesis of purine-containing mixed sequences have been described in detail.

The synthesis of chimeras of the type (I) starts by preparation of an alkylamino-CPG support derivatized with Dmt-protected 12-hydroxydodecanoic acid (Figure 13b).[68]

The first PNA unit was coupled to the unmasked hydroxyl function to give an ester linkage, which could be cleaved on completion of the chimera synthesis by treatment with anhydrous ethanolic ammonia. The PNA chain was extended with Fmoc/acyl-protected monomers. The DNA part of the chimera was then assembled by the coupling of standard cyanoethyl-protected DNA monomers directly onto the N-terminus of the PNA part, to give a phosphoramidate PNA – DNA linkage. The negatively charged chimeras were purified by preparative polyacrylamide gel electrophoresis, and characterized by mass spectrometry. Their nuclease stability (Section 5.1) and binding properties (Section 4.3) were then investigated.

Finn et al.[24] described the synthesis of two PNA/DNA chimeras of type (II) by using an approach based on the chimera synthesis strategy of van der Laan et al.[33] and the Mmt PNA synthesis strategy of Will et al.[26] The DNA – PNA linker employed was a 5′-amino-5′-deoxynucleotide, as described by Bergmann et al.[68] Unfortunately only the synthesis of homopyrimidine sequences was reported. The strong tendency of such sequences to form triple helices, however, complicates the study of their duplex formation with complementary nucleic acids (see Section 4.3).

The first synthesis of uracil-containing PNA/DNA chimeras was reported by Stetsenko et al.[21] An Mmt protected uracil PNA monomer was used to assemble oligomers of type (II), again by using 5′-amino-5′-deoxythymidine as the DNA – PNA linker. T_m measurements of these chimeras indicated that the DNA part was involved in cooperative binding to the complementary nucleic acid.

We recently reported the synthesis of PNA/DNA chimeras of all three structural types, which contained all four nucleobases in both the PNA and the DNA parts.[81] The previous year we introduced the Mmt/acyl protected PNA monomers, whose protecting group combination was chosen with the aim of synthesizing PNA/DNA chimeras.[26] It was of critical importance to us that the assembly of the DNA part could be carried out with commercially available standard DNA monomers. Chimeras of type (I) were synthesized on a CPG solid support derivatized with a 6-aminohexylsuccinate linker (Figure 13c), and were thus obtained as C-terminal hydroxyhexylamides. The PNA – DNA linkers employed were Dmt-protected, nucleobase-derivatized hydroxyethylglycine derivatives (Figure 19a). Alternatively, linkers that do not interact with complementary nucleic acids, such as Dmt-protected 4-hydroxybutyric acid (Figure 19c), could be used. After cleavage of the Dmt protecting group by 3 % trichloroacetic acid in dichloromethane, the DNA part was assembled by standard DNA synthesis methods.[78] Chimeras of type (II) were synthesized on a CPG solid support by utilizing 5′-amino-2′,5′-dideoxynucleoside-3′-phosphoramidites as DNA – PNA linkers (Figure 19b). The synthesis of chimeras of structure type (III), in which two structural transitions occur in a single sequence, were also reported for the first time. The enormously strong binding of these PNA-DNA-PNA chimeras to complementary DNA is especially interesting, and occurs when one PNA part binds through Watson – Crick and the other PNA part through Hoogsteen base-pairing (see Section 4.3.2).

3.4. Synthesis of PNA/Peptide Chimeras

The synthetic methods reported so far for PNA synthesis are mostly modifications and extensions of "standard" peptide synthesis strategies. It is thus no surprise that chimeras of PNA and peptides can be synthesized "on-line" by, for example, the Boc and Fmoc methods. Koch et al.[82] synthesized a PNA pentadecamer with a heptapeptide attached to the N-terminus through a tris(8-amino-3,6-dioxaoctanoic acid) by using the Boc/Cbz PNA synthesis strategy combined with Boc peptide synthesis. The chimera was found to have a slightly higher T_m on hybridization with an overhanging DNA template, which was attributed to the presence of two positively charged arginine units in the peptide. Circular dichroism (CD) experiments showed that the presence of the peptide did not significantly affect the structure of the PNA – DNA duplex. As the peptide was a substrate for protein kinase A the chimera could be enzymatically labelled with a radioactive phosphate group by kinazing the serine side-chain residue.

A PNA/peptide/PNA chimera that consisted of two oligopyrimidine PNA nonamers linked by a -His-Gly-Ser-Ser-Gly-His- peptide was synthesized by Betts et al.[64] The structure of the stable triple-helical complex formed by this chimera with an oligopurine-DNA strand was solved by X-ray crystallography. The synthesis of similar bis(PNA) structures that utilize a positively charged lysine hairpin has been described by Griffith et al.[83] PNAs with N-terminal oligohistidine peptides have been prepared by Boc/Cbz PNA synthesis followed by Fmoc peptide synthesis.[84] The resulting $(His)_6$-linker-PNA-chimeras could be bound to a nickel-derivatized column material and the capture of specific DNA and RNA sequences on the immobilized PNA was demonstrated.

4. Chemical and Physical Properties of PNAs and Chimeras

4.1. Chemical Stability

With the exception of the nucleobases, PNAs and DNA have no functional groups in common. As a result of this the chemical stability of the two compound classes is completely different. In contrast to DNA, which depurinates on treatment with strong acids, PNAs are completely acid stable. It is thus possible to synthesize PNAs by using standard protecting groups from peptide chemistry that require cleavage with trifluoromethanesulfonic acid or anhydrous HF.[27] PNAs are also sufficiently stable to weak bases that the classical, ammonia-cleavable nucleobase protecting groups, or the piperidine-labile Fmoc amino protecting group can be applied in PNA synthesis.[20, 25] The only noteworthy chemical instability of PNAs is caused by the free amino functionality at the N-terminus. A slow N-acyl transfer of the nucleobase acetic acid, or a cleavage of the N-terminal PNA unit by ring closure can occur[58] (see Figure 14), predominantly under basic conditions. This side-reaction can be suppressed by capping the final N-terminal amino function, for example with an

acetyl group. Acid-labile amino protecting groups are thus more suitable for PNA synthesis.[26]

The chemical stability of PNA/DNA chimeras is determined by the stability of the individual parts of the molecule, for example by the acid-lability of the DNA part. On the other hand, there are cooperative stabilization effects; the DNA part of 5′-DNA-PNA chimeras protects the PNA part from the deletion reaction shown in Figure 14b.

4.2. Solubility

Pure PNAs are neutral compounds with a tendency for self-aggregation and limited water solubility. However, the introduction of charged groups, for instance a C-terminal lysine amide, greatly improves their properties. For example H-$(t)_{10}$-Lys-NH_2 is water soluble at concentrations in excess of 1.5 mM.[8] PNA solubility drops with increasing length and purine:pyrimidine ratio.[13] Surprisingly, however, we found a homoadenine PNA polymer to be highly water soluble. Positive charges can also be introduced by modification of the PNA backbone, for instance by replacement of the glycine by a lysine unit.[47] The incorporation of only two such groups greatly increases the solubility of the oligomers. Alternatively, negative charges can be introduced, such as in the PNA–DNA chimeras, which show enhanced water solubility.[81]

4.3 Binding Affinity

PNAs bind superbly well to complementary DNA and even better to complementary RNA. The strongest binding affinity is to PNA itself. As PNAs are uncharged they appear to be predestined to form triple helical structures. The original design of PNAs[6] was based on a computer model of a DNA triple helix, in which the sugar–phosphate backbone of the third strand was replaced by the PNA scaffold. In spite of this, these PNA · $(DNA)_2$ triple helices are only observed for certain sequences (see Section 4.3.2). The formation of $(PNA)_2$ · DNA hybrids is favored, sometimes under strand displacement in double-stranded DNA. If the sequence is inappropriate for the formation of triple helices, then PNA · DNA, PNA · RNA or, if applicable, PNA · PNA duplexes are formed.

4.3.1. Duplex Formation with Complementary DNA and RNA

PNAs obey the Watson–Crick rules on hybridization with complementary DNA and RNA. In contrast to DNA they can, however, bind in both the parallel and antiparallel orientation,[10, 81] whereby the PNA C-terminus corresponds to the 3′ end, and the N-terminus to the 5′ end of normal oligonucleotides. As shown in Table 2, the antiparallel orientation is favored. Antiparallel PNA · DNA hybrids are considerably more stable than the corresponding DNA · DNA complexes. The increased stability results in an increase in T_m of approximately 1 K/base. Antiparallel PNA · RNA duplexes are even more stable, with a T_m increase of about 1.5 K/base relative to DNA · RNA hybrids. Consequently, PNA · RNA duplexes are on average 0.2–0.5 K/base more stable than the corresponding PNA · DNA duplexes.[85] The stability of parallel PNA · DNA and PNA · RNA duplexes is almost exactly the same as that of (antiparallel) DNA · DNA and DNA · RNA duplexes, respectively. There are dramatic differences in the kinetics of formation of duplexes of differing orientation. A kinetic study of PNA complex formation, carried out by Rose[86] using capillary electrophoresis, showed that the antiparallel complex was formed immediately (< 30 s), whereas the formation of the parallel complex required several hours.

Table 2. Melting temperatures of PNA and PNA/DNA-chimeras with complementary DNA or RNA in parallel (p) and antiparallel orientation (ap). The amino (H)-terminus of PNA corresponds to the 5′-terminus of DNA.

Sequence	T_m [°C]				Buffer[d]	Ref.
	DNA^{ap}	DNA^{p}	RNA^{ap}	RNA^{p}		
H-tgt acg tca caa cta[a]	69.5	56.1	72.3	51.2	A	[10]
5′-TGT ACG TCA CAA CTA	53.3	–	50.6	–	A	[10]
H-aca tca tgg tcg[b]	58.7	47.6	62.3	43.5	B	[81]
5′-ACA TCA TGG TCG	47.9	–	44.5	–	B	[81]
5′-ACA TCA t̲gg tcg[b, c]	52.6	< 25	52.1	< 25	B	[81]

[a] Terminal carboxamide. [b] Terminal 6-(hydroxyhexyl)carboxamide. [c] t̲: T-hydroxyethylglycine as PNA–DNA linker. [d] 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.

An especially interesting aspect of PNA · DNA duplex formation is its dependence on ionic strength. In a careful study by Tomac et al.,[87] the T_m of DNA · DNA hybrids was shown to increase considerably (more than 20 K for a 10-mer) with increasing salt concentration (0.01 to 0.5 M NaCl), whereas the T_m of PNA · DNA duplexes decreases (ca. 8 K), so that duplex stability is almost identical at 0.5 M NaCl. Increasing in the salt concentration even further has only a weak destabilizing effect on both PNA · DNA and DNA · DNA duplexes. The contrasting effect of ionic strength on duplex formation can be explained by the association of counterions in the case of DNA · DNA duplex formation, and by displacement of counterions in the case of PNA · DNA duplex formation.

Base-pair mismatches result in a reduction of the T_m value of 8–20 K.[10] This discrimination is, in some cases, approximately double that observed for DNA · DNA duplexes.

PNA/DNA chimeras also obey the Watson–Crick rules on hybridization with complementary DNA and RNA[34, 81] (Table 2). The T_m value of chimera · DNA duplexes usually lies between that of the corresponding PNA · DNA and DNA · DNA duplexes. In detail, the T_m value is strongly dependent upon the PNA:DNA ratio in the chimera, the sequence, and also the nature and position of the linker molecule between DNA and PNA. Exclusively antiparallel-oriented complexes are formed between 5′-DNA–PNA chimeras with an hydroxyethylglycine-type linker and DNA and RNA under physiological conditions. Base-pairing mismatches in either the PNA or the DNA part result in a similar reduction in the melting temperature, whereas a base-pairing mismatch at the PNA–DNA linker results in a somewhat smaller drop in the T_m value,[34, 81] which suggests that the

duplex is slightly destabilized at the DNA – PNA transition. A stronger structural perturbation occurs at the PNA – DNA junction of duplexes with chimeras of type (II). In this case, the transition consists of an amide bond between the PNA carboxy group and the 5′-amino function of the terminal nucleotide. Interestingly, the reduction in the T_m value of the duplex with RNA is much smaller than that of the duplex with DNA. The fact that PNA · RNA duplexes are structurally more similar to DNA · RNA duplexes than PNA · DNA duplexes are to DNA · DNA duplexes may explain the improved tolerance of the perturbation caused by the transition in duplexes with RNA. The introduction of a PNA unit at the ends of a chimera perturbs the duplex structure much less than its introduction in the middle of the sequence.[68, 88] In this context, self-complementary DNA/PNA chimeras, such as the Dickerson-type oligomer 5′-CGCGAAttcgcg, are noteworthy. In the very stable duplexes formed by these chimeras each PNA unit is paired with a DNA unit, which apparently results in a relatively uniform duplex structure.[81]

Great caution is required in the interpretation of binding studies of PNA/DNA chimeras that contain a homothymine – PNA part, as these compounds have a tendency to form at least partial triple-helical structures. For example, Finn et al.[24] reported that the introduction of a mismatch in the PNA part of the PNA – DNA chimera Ac-tttcttTGCCAT-3′ resulted in a dramatic reduction of 37.3 K in the T_m value, whereas a mismatch in the DNA part resulted in a reduction of only 1.9 K. In our opinion, the only explanation for this large difference in destabilization is the formation of a partial triple-helical structure in the PNA part (Figure 20), since it is

```
      1      2
      ↓      ↓
         tttcttTGCCAT-3' Hs (p)
5'-ATGGCAAAGAAA-3'
3'-TACCGTttcttt-5'      WC (ap)

1: G --> C ⇒ ΔTm 1.9 K
2: G --> A ⇒ ΔTm 37.3 K
```

Figure 20. Partial triple-helix structure (Upper case: DNA, lower case: PNA; T: 5′-amino-5′-deoxythymidine). Hs = Hoogsteen base-pairing, WC = Watson – Crick base-pairing.

well known that a $(t)_n \cdot (dA)_n$ duplex is an exceptionally good acceptor for an additional PNA strand and forms a $(t)_n \cdot (dA)_n \cdot (t)_n$ triple helix. The binding data for a (homopyrimidine PNA)/DNA chimera reported by Petersen et al.[77] cannot be unambiguously interpreted, presumably for the same reasons. The T_m data of Stetsenko et al.,[21] on the other hand, show unambiguously that the DNA part of uracil-containing PNA/DNA chimeras participates in binding to complementary DNA.

The replacement of the glycine unit in PNA building blocks by other amino acids has various effects on duplex stability that depend upon the amino acid side-chain.[46, 47] In all cases the D-configuration is most favored. Small, uncharged side-chains, such as those of D-alanine and D-serine, do not influence duplex stability. Positively charged side-chains, such as in D-lysine, have a stabilizing effect of approximately 1 K/unit, whereas negatively charged side-chains, such as in D-glutamic acid, have a destabilizing effect.

4.3.2. Triple Helix Formation

(Homopyrimidine PNA) oligomers and PNA oligomers with a high pyrimidine:purine ratio bind to complementary DNA normally by formation of unusually stable $(PNA)_2 \cdot$ DNA triple helices.[6] However, in the case of C-rich PNAs and GC-rich DNA duplexes, PNA · $(DNA)_2$ triple helices are observed. The T_m value of H-$(t)_{10} \cdot (dA)_{10}$ is 73 °C, whereas that of the corresponding $(dT)_{10} \cdot (dA)_{10}$ hybrid lies below 23 °C.[6] However, it should be borne in mind that the PNA decamer forms a triple-helical structure, while the DNA forms a duplex. As a rule of thumb the T_m value is increased by approximately 10 K per base pair in $((t)_n)_2 \cdot (dA)_n$ complexes.[8] A very strong hysteresis, of up to 30 K,[62, 83] in the UV – melting curves of $(PNA)_2 \cdot$ DNA triple helices is an indication of the rather slow kinetics of triple-helix formation under these conditions.[89] Base-pairing mismatches result in a drop in melting temperature of 14 – 25 K.[7] Demidov et al.[90] concluded from a detailed kinetic investigation that both the binding efficiency and the sequence specificity are kinetically controlled. The sequence specificity of triple-helix formation is based on the selectivity of formation of the intermediate PNA · DNA duplex, whereas binding of the third strand contributes only slightly to selectivity.

The melting temperature of triplexes containing cytosine in their sequences is, as expected, pH-dependent (Table 3).[7]

Table 3. pH-dependence of the melting temperature of $(PNA)_2 \cdot$ DNA triple helices.

PNA[a]	DNA	T_m [°C]		
		pH 5.0	pH 7.2	pH 9.0
H-ttt tct ttt t	5′-d(AAA AGA AAA A)	80	74	71
H-ttt ttt ttt t	5′-d(AAA AAA AAA A)	71	73	71

[a] Terminal carboxamide.

The triple helix is most stable at pH 5 (C^+GC). Surprisingly the CGC base arrangement still contributes to the stability of the complex even at pH 9, where protonation of cytosine is no longer expected. A possible explanation for this behavior is a local increase in the pKa of cytosine in the triple-helical structure, or Hoogsteen base pairing through only one hydrogen bond. The pH-dependence can be eliminated in "bis(PNAs)" (see below) by the introduction of pseudoisocytosine in the Hoogsteen strand (Figure 21),[62] as has been demonstrated previously for DNA triple helices.[91, 92]

If only one PNA sequence is used to form a $(PNA)_2 \cdot$ DNA triple helix then both strands are necessarily either parallel or antiparallel to the DNA strand. Although the literature describes both orientations as being equally stable,[62, 93] we found for the sequence (*pseudo*-5′)-tctctt-(*pseudo*-3′) that the antiparallel orientation (T_m: 30 °C) was more stable than the parallel orientation (T_m: 22 °C) at pH 5. The antiparallel duplex formed initially is both kinetically and thermodynamically favored and hence determines the orientation of the strands in the triple helix. When two different homopyrimidine PNA sequences are used, the most stable complex is formed when the Watson – Crick PNA strand is oriented antiparallel and the Hoogsteen strand is parallel to the purine strand of the DNA.[62]

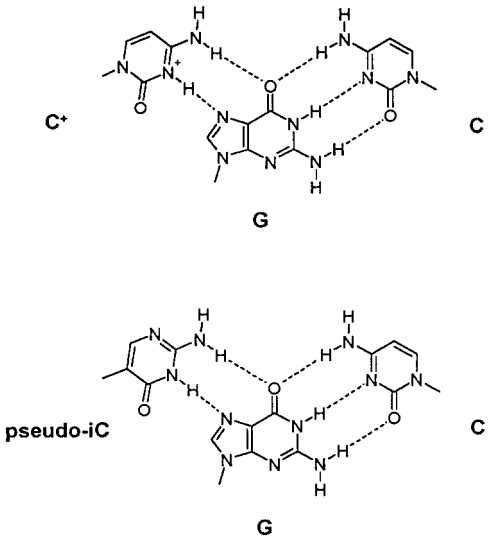


Figure 21. Hoogsteen binding with protonated cytosine (top) and with pseudoisocytosine (bottom).[62]

DNA triple helices can also be formed with the base motifs A*AT and G*GT (* represents Hoogsteen base-pairing), whereby the homopurine Hoogsteen strand is antiparallel to the Watson–Crick homopurine strand.[94] In contrast, the analogous $(PNA)_2 \cdot DNA$ triple helices are not formed by homopurine PNA.[95]

PNAs also form stable $(PNA)_2 \cdot RNA$ triple helices with RNA.[96–98] Although this type of hybrid is less-well investigated than that with DNA, the T_m values appear to be similar to those of $(PNA)_2 \cdot DNA$ triple helices. The PNA decamer H-ttcttctttt-Lys-NH_2 was found to bind to complementary RNA with a T_m of 69 °C.[98] So far, proof of triple-helix formation has mostly been provided by indirect methods, such as biological experiments. For example, the translation of CAT–mRNA was successfully inhibited by the triple helix forming PNA described above, whereas a different duplex-forming PNA had no inhibitory activity, in spite of its higher T_m value.[98]

In "bis(PNAs)" the Watson–Crick PNA strand, which binds antiparallel to DNA, and the parallel Hoogsteen strand are joined by a linker to form a single molecule (Figure 22a).[62, 64, 83]

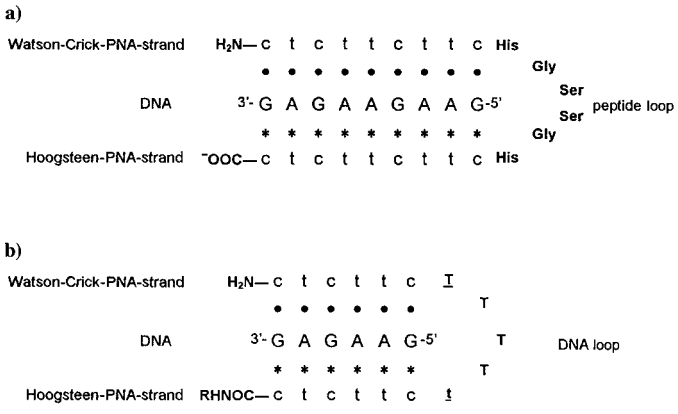
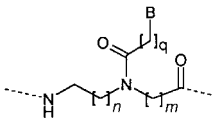


Figure 22. a) Peptide-[64] and b) DNA-bridged[81] bis(PNAs). Watson–Crick base-pairing: ●; Hoogsteen base-pairing: *; t: hydroxyethylglycine intermediate building block; T: 5′-amino-5′-deoxythymidine intermediate building block.

This leads to a small increase in the melting temperature, which presumably arises from a slight reduction in the entropy loss on triple-helix formation. The T_m value of the hybrid formed with H-tctcttt-(*eg*-1)$_3$-tttctct-Lys-NH_2 and 5′-d(CGCAGAGAAACGC) is 49 °C, where (*eg*-1) is the linker 8-amino-3,6-dioxaoctanoic acid. The corresponding triple helix formed by H-tctcttt-Lys-NH_2 and H-tttctct-Lys-NH_2 with the same DNA sequence melts at 45 °C. The hysteresis in the UV–melting curve, which is always observed for $(PNA)_2 \cdot DNA$ triple helices, is in this case largely suppressed, amounting to only 2–3 K. The high local concentration of the third strand presumably leads to much more rapid triple helix formation.[62] Sequence discrimination in bis(PNAs) is extremely high. Base mismatches result in a reduction of the melting temperature of, in some cases, over 30 K, which Egholm et al.[62] interpreted as evidence for the double-recognition process (by both PNA strands). The pH-dependence of bis(PNA) triple helix formation could be eliminated by the incorporation of pseudoisocytosine in place of cytosine in the Hoogsteen strand, but was unaffected by the same substitution in the Watson–Crick strand,[62] which confirms the proposed mode of binding (Figure 21). Variation of the linker molecule indicates that bis(PNAs) with a positively charged lysine–aminohexyl linker bind considerably better than those with a neutral (*eg*-1) linker (see above),[83] possibly as a consequence of electrostatic interactions with the negatively charged DNA. Yet another example of a bis(PNA) is the PNA/DNA chimera (*pseudo*-5′)-ctcttcTTTTtcttctc-(*pseudo*-3′) shown in Figure 22b. This molecule is so designed that the amino terminal PNA part binds through a Watson–Crick base pairing to a complementary hexamer 3′-GA-GAAG-5′, while the DNA part serves as a loop to allow the carboxy terminal PNA part to fold back and bind through Hoogsteen base-pairing.[81] The resulting triple helix formed under acidic conditions melts at 65 °C, while the DNA analogue melts at a mere 27 °C.

The binding behaviour of alternating PNA–PHONA oligomers is similar to that of PNAs. An alternating thymine 12-mer of this type binds to $d(A)_{12}$ to form a triple helix with a melting temperature of 62.6 °C.[39b] This is somewhat lower than that of the corresponding $(PNA)_2 \cdot DNA$ triple helix and can be explained by the charge repulsion between the phosphate and phosphonate groups, but is nonetheless much higher than that of the corresponding DNA triple helix. Very little is known about the binding properties of pure PHONAs.[39a] Although a PHONA-$(t)_{10}$/$(dA)_{10}$ complex has a similar T_m value to the corresponding $(T)_{10} \cdot (dA)_{10}$ duplex, it probably also forms a triple-helical structure.

PNA oligomers that contain a monomer unit with a backbone extended by one methylene group, or with a lengthened linker to the nucleobase (Figure 23), maintain the basic ability to form triple helices. Although this modification is combined with a considerable drop (approximately 13–18 K) in triple-helix stability,[41, 42] analysis of the sequence-specificity of binding nevertheless indicates that the nucleobases of these units apparently contribute to binding: base-pair mismatches result in a T_m drop of 6–21 K. The incorporation of *pa*–PNA units (Figure 23) results in the smallest loss of triple-helix stability in this series. Homo-



n	m	q	Backbone / Linker	Abbreviation
1	2	1	*N*-(2-aminoethyl)-β-alanine	β-PNA
2	1	1	*N*-(3-aminopropyl)glycine	*apg*-PNA
1	1	2	propionic acid	*pa*-PNA

Figure 23. PNA units with extended backbones or extended spacing of the nucleobase from the backbone.

oligomers of *pa*–PNA monomers bind to complementary DNA, although with a very low melting temperature. Homo-oligomers of β-PNA and *apg*-PNA monomers do not bind to complementary DNA. This is to be expected as only *pa*–PNA building blocks maintain the correct nucleobase spacing in the corresponding oligomers, whereas the incorporation of several β-PNA or *apg*-PNA building blocks forces the nucleobase spacing "out-of-phase" relative to natural nucleic acids.

A $(t)_{10}$ homo-oligomer of *orn*–PNA, a PNA analogue based on the amino acid ornithine, does not bind to complementary DNA. It does bind, however, to the oligoribonucleotide $r(A)_{10}$ to form a triple helix, $(orn\text{-}PNA)_2\cdot$RNA, with a T_m of 21 °C.[48] The replacement of the acetyl linker that connects the nucleobase to the Aeg backbone, by an ethylene group also compromises the stability of $(PNA)_2\cdot$DNA triple helices (and PNA·DNA duplexes), with a reduction in T_m of approximately 17 K.[43] The substitution of a t–t unit by the heterodimer shown in Figure 11 (Structure **G**) has little effect on the triple helix binding properties of PNAs.[45]

4.3.3. Strand Displacement

The propensity of homopyrimidine-PNAs to form $(PNA)_2\cdot$DNA triple helices is so strong that under certain conditions they can bind as a triple helix to one strand of double-stranded DNA (ds-DNA), while the second DNA strand is displaced[6, 15, 90, 99–101] and forms a single-stranded loop structure (P loop[90]). This loop structure is equivalent to the D-loop structure (displacement loop) formed under RecA-protein-mediated recombination. Prerequisites for strand displacement by PNA are: a DNA duplex that is not too stable, such as in A–T-rich regions, and, most importantly, a low salt concentration (< 50 mM NaCl).[15, 101]

Even in their original work on PNAs, Nielsen et al.[6] applied various techniques to demonstrate strand displacement between a PNA H-$(t)_{10}$-Lys-NH_2 and a DNA fragment that contained the $(dA)_{10}\cdot(T)_{10}$ target sequence: photofootprinting experiments with an acridine derivative showed protection of $(dA)_{10}$, whereas $(T)_{10}$ was not protected. In addition, incubation with staphylococcal nuclease resulted in increased cleavage of $(T)_{10}$, whereas the cleavage of $(dA)_{10}$ was unaffected. The $(T)_{10}\cdot P$-loop structure can also be cleaved by the single-strand-specific reagents $KMnO_4$ and nuclease S1. In another experiment the displaced single strand was even recognized by RNA-polymerase, such that the PNA-(ds-DNA) complex was an efficient transcription promoter for RNA-polymerases.[102] Evidence for the formation of a triple helix came from treatment of the complex (PNA: H-ttcttctttt-Lys-NH_2; ds-DNA: 5′-AAGAAGAAAA·3′-TTCTTCTTTT) with dimethyl sulfate. In this experiment the N^7 residues of the guanines were not methylated because of their involvement in Hoogsteen base pairs with a further PNA strand.[99] The pH dependence of strand displacement (SD) ($SD_{pH5.5} > SD_{pH6.5} > SD_{pH7.5}$) is also good evidence for triple helix formation,[99] as is the binding of two streptavidin molecules per complex when biotinylated PNAs are used.[103] This last experiment was carried out using electron microscopy, which allowed direct visualization of strand displacement.[103] Strand displacement is, as already discussed for triple-helix formation, largely independent of the orientation (parallel or antiparallel) of the pyrimidine PNA strand in relation to the purine strand of the complementary DNA.[93] An especially interesting aspect of strand displacement is its dependence on salt concentration. In a detailed kinetic study, Wittung et al.[100] showed that the activation energy required for strand displacement increases considerably with increasing salt concentration (Table 4). This is clearly a kinetic,

Table 4. Activation energies E_a for the binding of PNA to a poly(dA)·poly(dT) by strand replacement.[100]

PNA[a]	*c*(NaCl) [mM]	E_a [kJ mol^{-1}]
t_{10}	50	58.4 ± 3
t_{10}	150	68.8 ± 4
t_5ct_4	50	79 ± 4

[a] Carboxy terminal lysine amide.

rather than a thermodynamic effect: whereas strand displacement no longer occurs at salt concentrations as low as 80–100 mM,[101] the PNA complex can be preformed without difficulty at lower salt concentrations and then resists even higher salt concentrations of up to 500 mM.[15]

Strand displacement is highly sequence specific. In the example shown in Table 4 for a 10-mer, the activation energy increases by 20 kJ mol^{-1} for one base mismatch,[100] and strand displacement is no longer observed at 20 °C when two mismatches are present. Nielsen et al.[101] reached the same conclusion in studies of the inhibition of the cleavage of double-stranded DNA by restriction enzymes. The cleavage was completely inhibited by the complementary PNA oligomer; was reduced in the presence of a single mismatch; and was not inhibited at all when two mismatches were present. This was in spite of a reduction in the T_m value from 73 to 59 °C and 46 °C for a single and double mismatch, respectively, which, in an assay carried out at 37 °C, should still have allowed complex formation. This can be explained by the slow kinetics of strand displacement (60 to 100 times slower) when a base mismatch is present.[90, 100] The sequence specificity in this case can be described as being kinetically controlled.

Peffer et al.[93] used a transcription assay to show that although strand displacement normally occurs very rapidly (50% in 4 min), the dissociation of PNAs from these complexes is very slow (around 50% in 5–20 h). Detailed kinetic studies of strand displacement have been carried out by Demidov et al.[90] with gel shift and nuclease S1 assays, and by Wittung et al.[100] with CD spectroscopy, which allows real-time observation of strand displacement. The striking result from these studies is that the reaction kinetics are approximately second or third order with respect to the PNA

concentration, which suggests that two or more PNA molecules are involved in the rate-determining step. The activation energy of the reaction is around that required for the opening of several base pairs in double-stranded DNA. The salt concentration dependence also indicates that this is an essential part of the "activated state." Furthermore, at high PNA concentrations an intermediate can be observed at the beginning of the reaction, which may be a complex that is locally overloaded with PNA. On the basis of these results several suggestions for reaction pathways and intermediates were made, however none could fully account for all the experimental findings.[100] One variant is reversible strand displacement by formation of a PNA·DNA double helix of the Watson–Crick type, followed by the very rapid and irreversible formation of a triple helix to give the stable product[90, 100] (see Section 5.3.2 and Figure 26).

4.3.4. Binding of PNA to PNA

PNAs form extremely stable duplexes with complementary PNA sequences.[104] For example, the duplex formed by the PNA decamer H-gtagatcact-(L)-Lys-NH_2 and the complementary antiparallel sequence H-agtgatctac-(L)-Lys-NH_2 melts at 67 °C. The corresponding antiparallel DNA·PNA duplex melts at 51 °C and the DNA·DNA duplex at 33.5 °C. The antiparallel strand orientation is characteristically more stable. The corresponding parallel duplex melts at 45.5 °C, which, although 20 °C lower, is still considerably higher than the T_m of the corresponding DNA·DNA duplex. The kinetics of PNA·PNA duplex formation has been investigated by Wittung et al.[104] with CD spectroscopy. Carboxy terminal L- and D-lysine amides induce the formation of helices with opposite chirality. The spectrum of the duplex with L-lysine amide is comparable to that of the DNA·DNA double helix. A CD spectrum could not be obtained in the absence of an amino acid, presumably because of the formation of a racemate of duplexes with opposite helicity. Duplex formation occurs in seconds, and is followed by a reorganization process that is ascribed to inversion of the duplex to give the favored chiral form. Furthermore, the existence of PNA·PNA·PNA triple-helical structures has been proposed based on studies by CD spectroscopy.[54]

It should be mentioned that other self-pairing PNA derivatives exist, which are not based on the aminoethyl glycine backbone, for example the alternating alanyl/homoalanyl oligomers.[105, 106] Obviously, the nucleobase spacing in these artificial polyamide nucleic acid analogues is different from that in natural nucleic acids.

4.4. The Structure of PNA/DNA and PNA/RNA Complexes

4.4.1. The Structure of PNA·DNA Duplexes

Reasonably detailed structural information has been obtained from the NMR spectroscopic study of two antiparallel PNA·DNA duplexes (8-mer and 10-mer).[107] The DNA strand is in a conformation similar to the *B* form, with a glycosidic anti-conformation, and the deoxyribose in the C2′-endo form. The chemical shift of the imino protons of the PNA nucleobases is shifted slightly upfield relative to those of the DNA, and the NOESY cross peaks indicate Watson–Crick base-pairing.

A more recent NMR study[108] showed that an octameric antiparallel PNA·DNA duplex contained elements of both *A*-form (lateral positioning of the base pairs) and *B*-form DNA (backbone curvature, base pair inclination, helical rise). The right-handed helix contains approximately 13 base-pairs per turn compared to 10 base pairs for B-form DNA. The major groove is widened, and the minor groove is correspondingly shallow and narrow. The base stacking varies considerably within the sequence. The primary amide bonds of the PNA backbone are, without exception, in the *trans* conformation. The carbonyl oxygen atoms of the backbone–nucleobase linker point towards the carboxy terminus of the PNA strand, while the amide carbonyl groups of the backbone are, with a few exceptions, directed towards the solvent. As with PNA·RNA duplexes (see Section 4.4.2) there is no evidence for the formation of hydrogen bonds between the amide and carbonyl groups in the backbone.

The CD spectra of antiparallel PNA·DNA duplexes are similar to DNA·DNA spectra and indicate the formation of a right-handed helix.[10, 107] The CD spectra of parallel PNA·DNA duplexes, however, indicate a structure that differs considerably from that of either the *B* and *A* form.[10] Although crystals of a 10-mer PNA·DNA duplex have been obtained,[109] no results from the X-ray structure analysis were obtained because of insufficient resolution. The reduced affinity of minor groove binders and the inability of intercalators to bind to PNA·DNA duplexes[110] make it difficult to draw conclusions concerning structural changes, as these results are additionally complicated by the "missing" charges on the backbone.

4.4.2. The Structure of PNA·RNA Duplexes

Detailed structural information for PNA·RNA duplexes has also been obtained from a ^1H NMR spectroscopic study.[111] In this case the backbone of the PNA strand was enriched to greater than 98% with ^{13}C and ^{15}N to simplify the assignment of the ^1H signals. All bases form Watson–Crick base pairs, the glycosidic torsion angle in the RNA strand indicates an anti-conformation, and the ribose sugars are in the 3′-*endo* form. The RNA strand thus resembles an *A*-form structure. The tertiary amide bonds are all in the *cis* conformation, and the secondary amide protons of the backbone do not participate in any form of hydrogen bonding, which contradicts predictions from molecular modelling.[112, 113] NMR spectroscopy has also ruled out the existence of such hydrogen bonds in single-stranded PNAs.[114] The carbonyl group of the tertiary amide in the PNA backbone is isosteric to the C2′-hydroxyl group, which increases the solvent contact of the carbonyl oxygen atom.

The CD spectra of antiparallel PNA·RNA duplexes are also in agreement with this structure, and indicate the formation of a right-handed helix with a geometry similar to the *A* or *B* form.[10]

4.4.3. The Structure of $(PNA)_2 \cdot DNA$ Triple Helices

Important information on the structure of $(PNA)_2 \cdot DNA$ triple helices was obtained from a 1995 publication by Betts et al.,[64] who carried out an X-ray crystal structure analysis of the complex formed by a bis(PNA) and its complementary antiparallel DNA (see Figure 22). The nucleobases of the PNA strand bind to the DNA by Watson–Crick and Hoogsteen base pairing. The structure is different, however, from both *A*-form and *B*-form DNA, and forms a "*P* helix" with 16 bases per turn. The DNA phosphate groups are hydrogen bonded to the PNA backbone amide protons of the Hoogsteen strand. These hydrogen bonds, together with additional van der Waals contacts and the lack of electrostatic repulsion, are the main factors responsible for the enormous stability of the triple helix. The helix is considerably widened, with an average base displacement of 6.8 Å, compared to 4.5 Å in *A*-form DNA. The deoxyribose of the DNA strand is, as in *A*-form DNA, in the C3′-*endo* conformation. The bases, however, lie almost perpendicular to the helix axis, which is characteristic of *B*-form DNA. The Watson–Crick PNA–DNA interaction is further stabilized by hydrogen bonding to the solvent in the minor groove.

The crystal structure is in agreement with the CD spectra of $(PNA)_2 \cdot DNA$ triple helices measured in solution.[115] These indicate the presence of a right-handed helix and a geometry similar to that of the pure DNA triple helix.

The first evidence for the structure of *P* loops (see Section 4.3.3), which are formed on strand displacement in double-stranded DNA by bis(PNA) to produce a $(PNA)_2 \cdot$ DNA triple helix,[116] are also of interest. The introduction of a DNA-cleaving group (Gly-Gly-His, Ni^{2+}-cleavage) onto the 5′- or 3′-end of the bis(PNA) results in cleavage of not only the hybridizing DNA strand, but also of the *P* loop at specific positions. This is evidence for a close spatial arrangement of all four strands, and perhaps even for the existence of a quadruple-stranded bis(PNA) $\cdot$ $(DNA)_2$ complex.

4.4.4. The Structure of PNA · PNA Duplexes

In a recent article Rasmussen and Sandholm[65] reported the X-ray crystal structure analysis of a self-complementary PNA · PNA duplex (H-cgtacg-NH_2). This duplex exists as both right- and left-handed helices, which are stacked coaxially and alternately in the crystal, and thus form a continuous pseudohelix. As expected the base-pairing is of Watson–Crick-type, whereby the bases lie almost perpendicular to the helix axis, with a propeller twist of only about 5–9°. The base pairs are displaced by 8.3 Å relative to the helix axis, which gives a wide helix (28 Å) with 18 bases per turn (compare with the 11 and 10 bases per turn in A- and B-form DNA, respectively). Since both strands lie rather close to each other the helix has a very wide and deep major groove, and a narrow and shallow minor groove. The amide groups of the backbone are in the *trans* conformation and the carbonyl groups of the linkers point towards the carboxy terminus. Thus, the structure bears a strong similarity to the *P* form of $(PNA)_2 \cdot DNA$ helices described above.[64]

5. Biological Properties of PNAs and Chimeras

5.1. Stability in Biological Systems

The use of PNAs as antisense or antigene therapeutics requires that they show sufficiently high biological stability in serum and in cells. While unmodified oligonucleotides are digested relatively rapidly by nucleases in serum,[1] the peptide-like structure of PNAs makes their potential degradation by peptidases or proteases a possibility. However, PNAs have a remarkably high biostability in both human serum and in cell extracts.[117] For example, after a two hour incubation of the homopolymer H-$(t)_{10}$-Lys-NH_2 in human serum, or in cytoplasmic, or nuclear fractions of mouse tumour cells no significant degradation could be detected by HPLC. The PNA homopolymer was also stable to proteinase K and porcine intestinal mucosa peptidase, whereas the control peptide ACTH(4–10) was degraded rapidly under the same conditions.[117]

Figure 24 shows the stability of the PNA oligomer H-$(t)_8$-Lys-NH_2 compared to the unmodified oligonucleotide $(T)_8$ on incubation with fresh guinea pig serum. The HPLC analysis

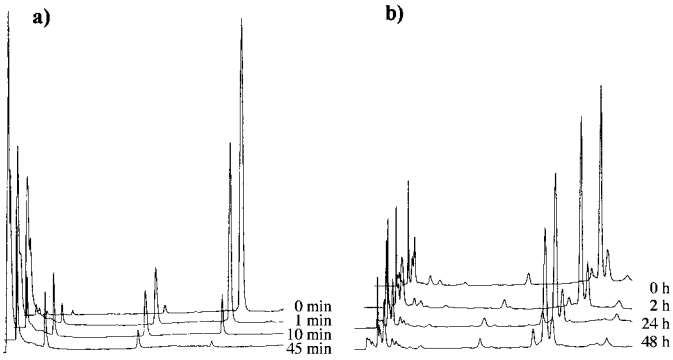


Figure 24. Comparison of the stability of unmodified DNA and PNA in fresh guinea pig serum: a) Ion exchange HPLC after incubation of $(T)_8$; b) reversed-phase HPLC after incubation of H-$(t)_8$-Lys-NH_2.

shows that the natural oligonucleotide has a very short half-life of only a few minutes, whereas around 50% of the PNA is still intact after two days. In contrast to the oligonucleotide, where distinct cleavage intermediates can be observed, the PNA oligomer produces no detectable degradation intermediates. It is thus probable that the disappearance of the PNA from the guinea pig serum is not because of its degradation, but rather that its detection is concealed by slow association with components of the serum.

DNA/PNA chimeras in which the PNA part is connected to the 3′-end of the DNA part[81] are approximately 50 times more stable in foetal calf serum than the corresponding unmodified oligonucleotides. PNA/DNA chimeras with only one PNA unit at the 5′- and 3′-ends are still 25 times more stable in human serum than the corresponding unmodified oligodeoxynucleotides.[68] The explanation for this behaviour is that the major nuclease activity in serum is caused by 3′-exonucleases. However, oligonucleotides are additionally subject to slow degradation by endonucleases, which cleave predominantly at pyrimidine nucleotides.[118, 119] Therefore, mod-

ification of the DNA part of the PNA/DNA chimera, by for example the introduction of phosphorothioate internucleoside bridges, can contribute considerably to its stabilization.

5.2. Cellular Uptake of PNAs and Chimeras

The cellular uptake of PNAs is extremely low.[11, 13, 120] It is thus not surprising that so far there are no reports of antisense activity of pure PNAs in cell culture without the use of techniques that help to bypass the membrane barrier. The antisense and antigene activity of PNAs has been studied by direct intracellular microinjection of the PNAs.[96, 97, 121] Alternatively, permeabilization of the cell membrane by lysolecitin[122] or detergents like Tween 20[123] allows the entry of PNAs into cells. In the case of transient transfection assays the PNA can be transfected in the form of a plasmid/PNA complex by using LipofectAmine in the absence of serum.[124] Covalent attachment of PNA to peptides that bind selectively to cell surface receptors can increase their cellular uptake into specific cell types.[125]

There are two reports which contradict these and our own studies and show that PNAs are taken up in rat embryo fibroblasts[121] and human myoblasts.[126] However, PNAs were applied at the high concentration (20 μM) in these studies. A punctate intracellular distribution was observed, which suggests a localization of the PNA in vesicles. However, neither of these reports demonstrated biological activity of the PNAs on incubation with these cells.

DNA/PNA chimeras, on the other hand, are taken up by Vero cells or NIH3T3 cells even at the low extracellular concentration (1 μM). The degree and kinetics of uptake are similar to those observed for an oligonucleotide of the same sequence.[81] The intracellular distribution of fluorescently labelled DNA/PNA chimeras is also similar to that of pure oligonucleotides. In our studies, pure PNAs, such as H-$(t)_8$-Lys-NH_2, could only be detected in cells at high concentrations (> 50 μM), where cytotoxic effects could also be observed.

5.3. Inhibition of Gene Expression

5.3.1. Inhibition of Translation

The sequence-specific inhibition of replication, transcription, and translation by PNAs could potentially be exploited for therapeutic applications (Figure 25). Although in the meantime numerous publications have appeared on translation inhibition by antisense oligonucleotides, there are remarkably few studies of PNA oligomers as antisense agents. Most reports so far have been on translation inhibition by PNAs in cell-free systems. For instance, the in vitro translation of an SV40-T-antigen-mRNA containing a sequence complementary to H-$(t)_3ac(t)_2c(t)_2$ could be inhibited sequence specifically at a PNA concentration of 1 to 2 μM.[97] The mechanism of inhibition was very probably steric blocking and not an RNase H-mediated mRNA degradation (see Section 5.6.1). A specific reduction in SV40-T-antigen expression was obtained at an intracellular PNA concentration of 1 μM by microinjection of PNA into cells. The expression of the *β-gal* control was not affected under these conditions. The therapeutic window was very narrow, however, as at a PNA concentration of only 5 μM, unspecific inhibition of *β-gal* expression was found, whereas at PNA concentrations lower than 0.5 μM no translation inhibition was detectable.[96] Interestingly, the antisense activity of PNAs after microinjection is less than that of the analogous propynyl–pyrimidine phosphorothioate sequences. Possible reasons are the slower kinetics of association of PNAs with the complementary RNA, or the inability of PNAs to stimulate RNase H (see Section 5.6.1). PNAs in these studies offer the advantage over other steric blockers, such as 2′-O-alkyl oligoribonucleotides, that their antisense activity is not limited to sequences targeted to the 5′-untranslated region.[96] It must be borne in mind, however, that homopyrimidine PNAs, such as H-$(t)_5(ct)_5$, were used in these studies, which in our experience tend to form partial triple-helical structures even under neutral conditions.

In vitro translation inhibition is also possible with PNAs containing all four bases at concentrations as low as 100 nM.[127] An 80% inhibition in the translation of PML/RAR (promyelocytic leukemia/retinoic acid receptor-α) was achieved with a PNA pentadecamer targeted against the translational start region at a concentration of 200 nM. A 40-fold higher concentration of an unmodified oligonucleotide was necessary to reach the same level of inhibition. However, the use of PNA concentrations of greater than 300 nM again led to unspecific inhibition of translation. A different PNA oligomer targeted against the PML/RAR fusion region was inactive under the same conditions. This is in agreement with the results of Knudsen and Nielsen,[98] which show that PNAs targeted against the 5′-proximal region to the AUG start codon can cause specific inhibition, whereas PNAs targeted to the coding region are inactive.

The behavior of triple helix forming PNAs is different, as is that of bis(PNAs) or clamp-PNAs which form at least partial triple-helical structures. Such $(PNA)_2 \cdot$ RNA triple-helix forming derivatives cause a stop in translation even when they are targeted against the coding region of mRNA. This can be confirmed by the detection of a truncated protein.[98] However, since the efficiency of translation inhibition does not correlate simply to the binding affinity, sequence optimization has to be carried out for each new target.

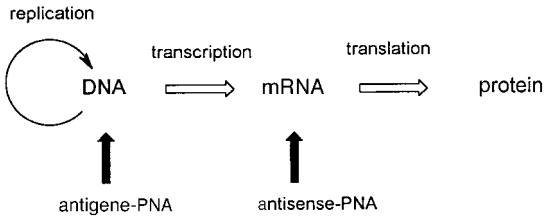


Figure 25. Potential applications of PNAs in therapeutics: inhibition of replication and transcription of DNA by antigene PNAs, and inhibition of the translation of mRNA by antisense PNAs.

5.3.2. Inhibition of Transcription

Transcription inhibition by PNAs can occur either by triple-helix formation, or by a strand invasion or strand displace-

ment process (Figure 26). Strand invasion, in which the PNA displaces one DNA strand in a DNA duplex, is especially attractive as the PNA · DNA duplex formed initially obeys the Watson – Crick base pairing rules. Consequently all four bases

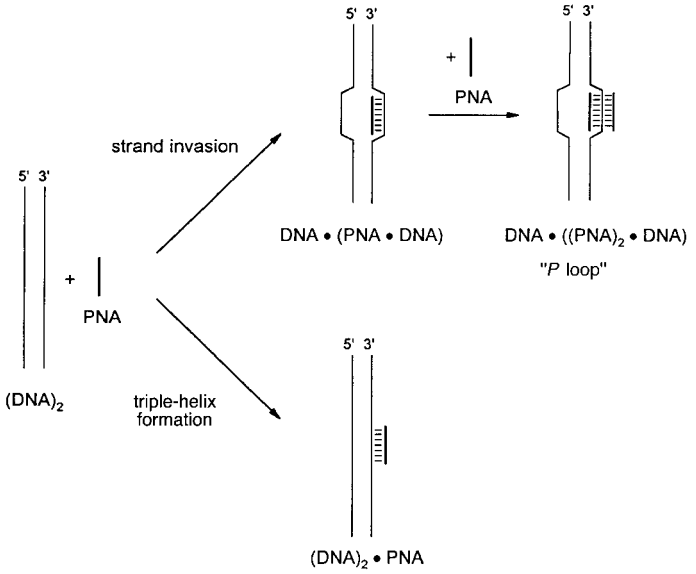


Figure 26. Strand invasion and triple-helix formation by PNAs.

in the double-stranded DNA can, in principle, be recognized. The locally formed PNA · DNA duplex can then, when appropriate, bind to a further PNA molecule to form a local $(PNA)_2 \cdot DNA$ triple helix. In contrast, direct formation of $(DNA)_2 \cdot PNA$ triple helices is at present only efficient for homopurine · homopyrimidine double strands and C-rich homopyrimidine PNAs.[128] For example, the thymine of a T · A base pair in a DNA duplex cannot be specifically recognized on triple-helix formation by any of the four natural bases. The manner in which PNAs interact with double-stranded DNA receptors, is therefore highly dependent upon the base composition of the sequence employed.

The original studies of strand invasion were carried out on homopyrimidine PNAs at low salt concentrations.[12, 97, 129] The binding of a homopyrimidine PNA oligomer to the homopurine sequence of the template strand results in an efficient blockade of transcription (Figure 27a). The binding of the PNA oligomer to the nontemplate strand, however, has little effect on the RNA polymerase reaction in vitro. The length of the transcript formed depends upon both the sequence and the polymerase employed. A high thymine content in the pyrimidine PNA gives shorter transcripts, which is equivalent to more efficient blocking of the RNA polymerase. This may be a consequence of retarded protonation of the cytosine, or with the tendency of thymine-rich PNAs to form at least partial triple-helical structures. The displacement of the nontemplate strand by homopyrimidine PNA, with the subsequent formation of a P loop,[90] which is stabilized by a $(PNA)_2 \cdot DNA$ complex, can be demonstrated by DNase footprinting.[130] This displacement leads to efficient inhibition of T7-DNA-polymerase in cell-free systems. However, this inhibition could not be reproduced in living cells, as the

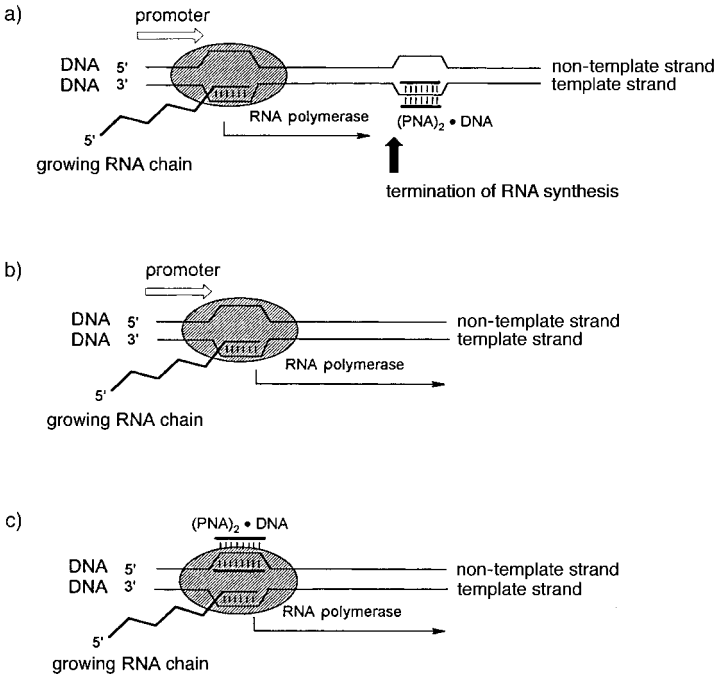


Figure 27. a) Inhibition of transcription by PNAs through strand invasion and triple helix formation on the single strand; b) open promoter complex; c) activation of transcription by PNAs.

$(PNA)_2 \cdot DNA$ complex is probably not formed at physiological salt concentrations.

We know of only one study so far in which PNAs that contain all four bases inhibited transcription in a cell-based assay successfully.[122] The PNAs, targeted against CAG triplet repeats, were originally used for the selective isolation of transcriptionally active chromatin fragments by strand displacement.[131] The cells had to be permeabilized by treatment with lysolecitin before a sequence specific and dose dependent inhibition of the transcription of the genes for the androgen receptor (AR) and the TATA binding protein (TBP) could be obtained. The transcription of the c-myc gene was not inhibited by the AR/TBP-specific PNAs, and conversely a c-myc-specific PNA oligomer inhibited c-myc transcription, but not that of AR/TBP.

The main obstacle to therapeutic use of the strand displacement principle is the stability of the natural DNA double strand, especially GC-rich sequences, under physiological salt conditions (see Section 4.3.3). A possible explanation for the surprisingly positive findings of Boffa et al.[122] may be that the PNA oligomer was targeted against a transcriptionally active DNA fragment. This supposition is supported by the dramatically increased kinetics of strand invasion during the active transcription process.[132]

5.4. Activation of Transcription

The initiation of transcription of double stranded DNA into RNA by RNA polymerases/transcription factors involves the formation of an "open" complex, in which about 12 base pairs are exposed as a single strand such that they can base-pair with the synthesized RNA (Figure 27b). This open complex is

equivalent to the P-loop structure[90] formed when two PNA strands form a triple helix with the nontemplate strand. In fact, a DNA · ((PNA)$_2$ · DNA) complex is recognized by RNA polymerases, and RNA transcription is initiated at the P loop (Figure 27c).[102] It is thus possible to view such PNA target structures as artificial promoters, in which an appropriate PNA oligomer can act, in a broader sense, as a transcription factor to control transcription.

5.5. Inhibition of Replication

The elongation of DNA primers by DNA polymerases can be inhibited by PNAs in cell free systems (see Section 5.6.3). Consequently the inhibition of DNA replication by PNAs should be possible if the DNA duplex is susceptible to strand invasion under physiological salt conditions, or if the DNA is single-stranded during the replication process. In fact, in the case of extrachromosomal mitochondrial DNA, which is largely single-stranded during replication, efficient inhibition of replication by PNAs is possible.[126]

5.6. Interactions with Enzymes

5.6.1. Ribonuclease H (RNase H)

RNA becomes a substrate for cleavage by the ubiquitous intracellular enzyme RNase H when it forms a double strand with unmodified oligodeoxyribonucleotides. The ability of oligonucleotides to stimulate RNA cleavage by RNase H is largely dependent upon their chemical structure. It is well known that antisense oligonucleotides with the ability to activate RNase H (phosphodiester and phosphorothioate oligonucleotides) are generally much better antisense inhibitors than those without this ability (e.g. methylphosphonates, 2′-O-alkyl oligoribonucleotides, α-oligonucleotides, and hexitol nucleic acids). PNAs also belong to the group of nucleic acid analogues that do not stimulate RNase H on duplex formation with RNA. It is thus hardly surprising that, in spite of their excellent binding properties, PNAs have been reported to be less effective as antisense agents than the RNase H-stimulating propynyl pyrimidine phosphorothioates.[96]

We have found, however, that DNA/PNA chimeras are able to stimulate RNA cleavage by RNase H on formation of a chimera · RNA double strand. RNA cleavage occurs at the ribonucleotides which base-pair with the DNA part of the chimera (Figure 28). The cleavage is sequence specific, as random sequence DNA/PNA chimeras do not cleave the RNA under the same conditions.[133]

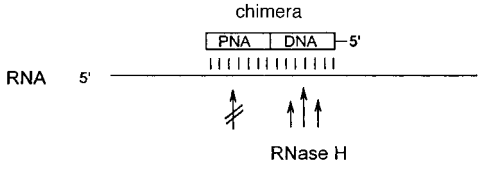


Figure 28. Schematic diagram of RNase H-mediated cleavage of RNA in a duplex with a PNA/DNA chimera.

5.6.2. Telomerase

Telomerase is a ribonucleoprotein composed of a protein component with DNA polymerase activity, and an RNA component that acts as a primer binding site. Human telomerase synthesizes long (TTAGGG)$_n$ repeats at the 3′-end of DNA strands. PNA sequences that are complementary to the RNA-primer binding site inhibit telomerase activity better than the corresponding phosphorothioate oligonucleotides because of their higher binding affinity.[123] IC_{50} values as low as 1 nM were measured for human telomerase inhibition by PNAs.[134] The activity is dependent upon the binding site and base composition of the PNA oligomer.

5.6.3. DNA Polymerases and Reverse Transcriptases

PNAs do not normally interact directly with DNA polymerases and reverse transcriptases. They can, however, terminate the elongation of oligonucleotide primers by binding to the template strand, or even compete directly with the oligonucleotide primer for binding to the template. For example, primer extension catalysed by *Taq*-polymerase can be terminated by using the PNA H-(t)$_{10}$ that binds to a (dA)$_{10}$ sequence in the template.[12] Similarly, primer extension by MMLV reverse transcriptase can be inhibited by an appropriate PNA oligomer.[97]

PNAs themselves cannot act as primers in polymerase reactions because of their structure. However, we recently demonstrated[16] that uncharged PNA primers with only a single 5′-amino-2′,5′-dideoxynucleoside at the *C*-terminus (Figure 29) are recognized by a variety of polymerases, such

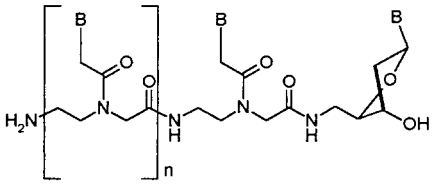


Figure 29. Structure of a PNA primer, which contains only a 5′-amino-2′,5′-dideoxynucleoside at the carboxy terminus.[16]

as the Klenow fragment of DNA polymerase I (*Escherichia coli*) and *Vent*DNA-polymerase (*Thermococcus litoralis*). This observation is made even more remarkable by crystallographic and biochemical investigations that suggest that the polymerases bind to the negative charges of the phosphate backbone through conserved amino acids in their sequences. A linear amplification is possible with the use of thermostable polymerases and an excess of PNA – DNA primer.[16] Wenninger and Seliger found that DNA templates with a PNA unit in the middle are accepted, in principle, as polymerization templates by the Klenow fragment of DNA-polymerase I. However, a partial termination of polymerization in the neighborhood of the modification takes place, which is dependent upon the structure of the incorporated PNA unit.[135] Interestingly the chemical polymerization of both PNA units and ribonucleotides on PNA templates is possible.[18] It is also worth mentioning that PNA-analogue triphosphates can be incorporated by DNA polymerases and lead to chain termination.[136] They may, therefore, be of interest principally for use in Sanger sequencing.

6. Potential Applications

6.1. Therapeutics

Potential therapeutic applications for PNAs arise from their biological properties described in Section 5. The use of PNAs for translation inhibition (antisense agents), transcription and replication modulation (antigene agents), and ribonucleoprotein interactions (inhibitors of enzymes such as telomerase) are, in principle, potential therapeutic strategies. Although the results with PNAs in cell-free systems are very encouraging, their poor cellular uptake is, at present, the main obstacle to their effective therapeutic use. The inability of antisense PNAs[96–98, 121, 137] to stimulate cellular RNase H is a further limitation.

The most promising candidates for antisense PNA constructs are, at present, the DNA/PNA chimeras, as they are taken-up by cells to a similar extent as oligonucleotides, bind to complementary RNA exclusively in the antiparallel orientation, and stimulate the sequence specific cleavage of RNA by RNase H on duplex formation.[81, 133] In addition, they have improved aqueous solubility relative to pure PNAs because of their partially negatively charged structure. The DNA part of these chimeras can be stabilized against endonuclease degradation by, for example, the introduction of phosphorothioate internucleotide bridges.[1] Preliminary investigations in cell culture indicate that three to five nucleotides in DNA/PNA chimeras are sufficient to generate antisense effects that are superior in terms of efficacy and specificity to those generated by uniformly modified phosphorothioate oligonucleotides.

In theory, antigene agents (strand invaders and triple-helix formers) should be extremely useful in therapeutic applications as a consequence of the high dose effect (one or two copies of the DNA target per cell). The first studies aimed at the use of PNA strand invasion for therapeutic purposes are: the specific inhibition of interaction of the transcription factor NF-κB with an IL2-Rα-binding site by PNA strand invasion;[124] the invasion of CAG triplet repeats resulting in inhibition of the transcription of the androgen receptor in human prostate cancer cells;[122] and transcription inhibition of a PML/RAR hybrid gene that is involved in acute promyelocytic leukemia.[127] Although PNAs associate with double-stranded DNA more than 500 times faster than normal oligonucleotides,[138] the strand invasion process in vitro is only really efficient at salt concentrations of less than 50 mM,[129] whereas the physiological salt concentration is greater than 100 mM. This should not, however, discourage investigation of this process at the cellular level, since under in vivo conditions additional effects, such as binding stabilization by cellular proteins, may play a role. For example, Boffa et al.[122] were able to inhibit specifically the transcription of the androgen receptor and TATA binding protein genes, as well as the c-myc gene in lysolecitin permeabilized cells with PNAs. It should be mentioned here that although the antigene PNA was targeted against the sense strand of the DNA, it inhibited transcription from both strands of the DNA template equally well. This contradicts the results of studies in cell-free systems, and may result, for example, from the RNA polymerase II elongation complex in vivo being much larger than the pol II complex in reconstituted systems, or to the additional influence exerted by the nucleosomal structures of the chromatin template used.[122] Strand invasion by PNAs also appears to be favored by an active transcription process ("suicide transcription").[132]

The reverse transcription of the *gag* gene of HIV-1 can be inhibited by PNAs in vitro.[139] In this case the inhibition obtained with a bis(PNA) oligomer, which recognizes the 10 nucleobase RNA target through 20 PNA nucleobases, is more efficient than that obtained with the corresponding antisense PNA decamer. Nevertheless, the reverse transcription can be completely inhibited by a pentadecameric antisense PNA at a molar ratio of 10:1 (PNA/RNA), whereby no RNase H cleavage of the RNA is detected.

The inhibition of the replication of mutant human mitochondrial DNA (*mt*DNA) by PNAs[126] is a potential approach to the treatment of patients with diseases caused by heteroplasmy of the *mt*DNA (see Section 5.5). In heteroplasmy the mutated and wild-type DNA are both present in the same cells. It has been shown that the use of PNAs under physiological conditions can inhibit the replication of the mutated mtDNA without affecting the wild-type *mt*DNA. The potential use of PNAs to inhibit human telomerase,[123] which is found in approximately 90% of all human tumours, has already been discussed (see Section 5.6.2). Finally it should be pointed out that PNAs can also be used to stimulate transcription.[102] This principle (see Section 5.4) should also be therapeutically applicable in place of a protein substitution therapy, provided that the appropriate gene, which is not, or only weakly, expressed, is not defective in the coding region.

6.2. Diagnostics

The following properties of PNAs can be exploited in the use of PNAs in DNA diagnostics: 1) PNAs have better sequence discrimination, and form more stable hybrids than oligonucleotides (see Section 4.3.1). 2) In contrast to oligonucleotides, PNAs can cause strand invasion in double-stranded DNA. 3) PNAs alter the electrophoretic mobility of complementary nucleic acids considerably on binding on account of their neutral character. It is thus advantageous to use PNAs for screening for genetic mutations, such as the detection of the CFTR gene in cystic fibrosis.[140] The procedure is based on the ability of PNA·DNA hybrids to form stable complexes at low ionic strengths and increased temperature (70 °C). These complexes can be detected rapidly by capillary electrophoresis. As an alternative to the Southern hybridization technique, labeled PNAs can be used as probes in pre-gel hybridization experiments.[141] Since PNA binding, in contrast to DNA binding, is largely independent of the ionic strength, it is possible to dissociate double-stranded DNA at low salt concentrations and hybridize it with PNA before gel electrophoresis.

The "PCR clamping" method for the detection of point mutations[142] is based on the ability of PNAs to bind more strongly to complementary nucleic acids than oligonucleotides, combined with their inability to function as primers in

the polymerase chain reaction (PCR). Targeting of the PNA oligomer, even partially, against the primer binding site can block the formation of the PCR product. Skilful choice of the length of the primer can even allow discrimination of alleles, which differ only in one base pair. Discrimination of point mutations in the Ki-*ras* gene has been carried out by PCR clamping.[143] A PNA oligomer complementary to wild-type Ki-*ras* DNA strongly inhibits amplification of the wild-type gene, relative to the mutated gene, to which it binds with lower affinity (Figure 30).

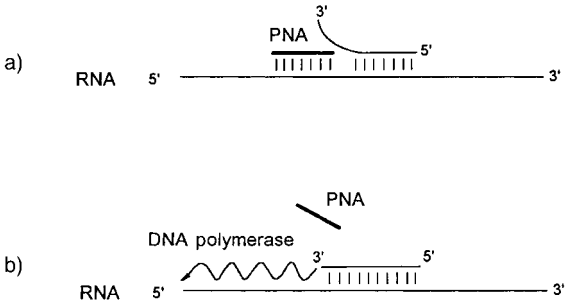


Figure 30. Schematic diagram of the "PCR clamping" technique: a) Blockade of the PCR reaction by strong binding of the PNA to the wild-type gene; b) positive PCR caused by weaker PNA binding to the mutated gene.

Demers et al. have used PNAs to suppress the preferential amplification of small allelic PCR products during copying of VNTR loci (variable number of tandem repeats), which leads to false genotypic patterns.[144] mRNA can be detected rapidly and with high sensitivity in paraffin slices with PNAs in an in situ hybridization technique.[145] The quantitation of telomeric repeats is also possible with PNAs.[146] The use of PNAs immobilized on surfaces as sequence specific DNA biosensors,[147–149] whereby hybridization can be detected electrochemically,[147] is especially interesting. However, the detection of PNA · DNA hybrids is also possible by using antibody Fab fragments.[150]

6.3. PNAs as Tools in Biotechnology

In addition to the applications for PNAs already described, such as PCR clamping and strand displacement, they can also be used for the modulation of enzymatic cleavage. For example, after strand displacement by PNA in a DNA duplex, the displaced DNA strand can be selectively cleaved by nuclease S1.[151] The single-strand-specific nuclease S1 can, in combination with two PNAs, be transformed into an "artificial restriction enzyme" that cleaves both DNA strands, and whose recognition sequence is determined by the PNA sequences employed (Figure 31). Conversely, PNAs can also be used to block DNA cleavage by restriction enzymes.[101] Furthermore, PNAs can be used to prevent the methylation of DNA sequence-specifically. On dissociation of the PNA · DNA complex only the unmethylated recognition sequence can be cleaved by the appropriate methylase-sensitive restriction enzymes.[152, 153] This principle of "rare genome-cutters" has been exploited in a similar way with triple-helix forming oligonucleotides.[154] PNAs can be used as facilitators to increase ribozyme activity in long RNA substrates that are only inadequately cleaved by Hammerhead ribozymes.[155] Modified PNAs are also useful for the sequence-specific purification of nucleic acids,[84] and for the isolation of active genes.[131] Finally it should be mentioned that, similar to oligonucleotides, PNAs can be used to generate chemical compound libraries,[156] from which, analogously to the aptamer principle,[157] molecules with defined properties can be selected.

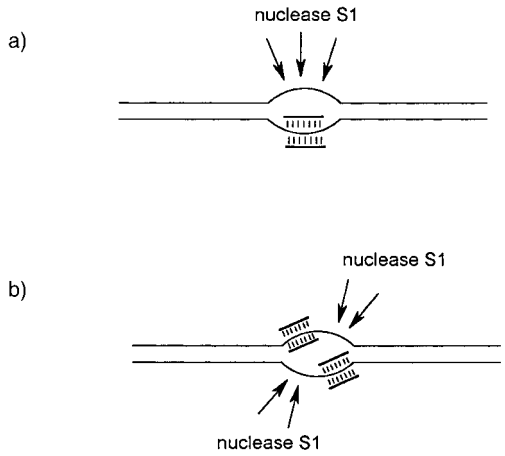


Figure 31. Artificial restriction enzymes: a) Single-strand cleavage by PNA; b) double-strand cleavage by double PNA clamping.

7. Conclusions and Future Prospects

In the last several years, efficient methods for the synthesis of both PNAs and PNA/DNA chimeras have been developed. The initial enthusiasm for this substance class, because of high binding-affinity and specificity, has, in practice, been somewhat dampened by their relatively poor solubility and the tendency for self-aggregation. However, targeted structural modifications, such as the introduction of positive or negative charges, can suppress these problems, so that, at least for in vitro experiments, PNAs are highly valuable. In specialized applications, such as PCR clamping and strand invasion, PNAs have clear advantages over their natural counterparts, and extend the arsenal of biotechnological and diagnostic tools.

The therapeutic utilization of PNAs as antisene agents is, given their nuclease stability, largely dependent upon the intracellular availability of the oligomers. Although the uptake of pure PNAs by cells in culture is poor, the use of PNA/DNA chimeras to inhibit gene expression in cell culture is possible. Unfortunately no data on the pharmacology and toxicity of PNA derivatives has yet been published. Information on this aspect of PNAs is expected to emerge in the next years, especially when the considerable growth in the number of publications in the PNA area is considered. At present, around fifteen first-generation oligonucleotides, phosphorothioates are in clinical development, many with promising results.[158, 159] In 1997 the first of the second-generation oligonucleotides, chemical chimeras of oligodeoxynucleotide

phosphorothioates and 2′-O-substituted oligoribonucleotides, was brought into clinical development. The investigations of the next years will show if PNA-based oligomers also have the potential to become a new generation of drugs. However, even if the therapeutic development of PNAs should fail, PNAs will remain valuable tools for the analysis of the function of new genes which emerge from the sequencing of the human genome ("functional genomics").

It is apparent that the combination of PNA with natural nucleic acid structures in the form of chimeras leads to extraordinarily interesting molecules, which can be recognized by certain enzymes, such as DNA polymerases or RNase H. In addition to their potential diagnostic and therapeutic applications, these PNA/DNA chimeras are of particular interest in mechanistic studies of biological processes.

PNA is also an extraordinarily interesting molecule from a structural point of view. Numerous studies of the structure of PNA complexes with itself and with natural nucleic acids have been published already. Not only do these studies improve our understanding of PNA structure, but they also supply new information on the structural and biological properties of DNA and RNA themselves. Consequently, studies on PNAs and PNA/DNA chimeras should provide valuable insights into the possibility of the evolution of alternative forms of life, and on potential transitions between different genetic systems. This in turn may give rise to new ideas on so far unexplored methods of storing and transmitting genetic information.

Appendix: List of Abbreviations

Aeg	*N*-(2-aminoethyl)glycyl
An	anisoyl
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BOP	benzotriazolyl-1-oxytris(dimethylamino)phosphonium hexafluorophosphate
t-BuBz	4-*tert*-butylbenzoyl
Bz	benzoyl
Cbz	benzyloxycarbonyl
CPG	controlled pore glass
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	dicyclohexylcarbodiimide
DIC	*N,N*′-diisopropylcarbodiimide
DIEA	ethyl diisopropylamine
Dmt	bis(4-methoxyphenyl)phenylmethyl
Dpc	diphenylcarbamoyl
Dts	dithiasuccinyl
Fmoc(ONSu)	9-fluorenylmethyloxycarbonyl (*N*-hydroxysuccimide)
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HOObt	3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine
MBHA	(4-methylbenzhydryl)amine
iBu	isobutyry
Mmt	4-methoxyphenyldiphenylmethyl
NEM	*N*-ethylmorpholine
NMP	*N*-methylpyrrolidone
NPE	(4-nitrophenyl)ethyl
Pfp	pentafluorophenyl
PG	protecting group
PyBOP	benzotriazolyl-1-oxy-trispyrrolidinophosphonium hexafluorophosphate
PyBrop	bromo tris(pyrrolidino)phosphonium hexafluorophosphate
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulfonic acid
THF	tetrahydrofuran
TOTU	*O*-[(cyano(ethoxycarbonyl)methylene)amino]-1,1,3,3-tetramethyluronium tetrafluoroborate

Received: July 16, 1997
Revised version: October 20, 1997 [A 243 IE]
German version: *Angew. Chem.* 1998, *110*, 2954 – 2983

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