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The challenge of peptide nucleic acid synthesis

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Peptide nucleic acids (PNAs) are an important class of DNA/RNA mimics that can hybridize complementary chains of nucleic acids with high affinity and specificity. Because of this property and their metabolic stability, PNAs have broad potential applications in different fields. Consisting of a neutral polyamide backbone, PNAs are prepared following the method used for peptide synthesis. In this regard, they are prepared by the sequential coupling of the protected monomers on a solid support using a similar approach to solid-phase peptide synthesis (SPPS). However, PNA synthesis is a little more challenging due to issues of the difficulty on the preparation of monomers and their solubility. Furthermore, the PNA elongation is jeopardized by intra/inter chain aggregation and side reactions. These hurdles can be overcome using different protecting group strategies on the PNA monomer, which also dictate the approach followed to prepare the oligomers. Herein, the main synthetic strategies driven by the protecting group scheme are discussed. However, there is still ample scope for further enhancement of the overall process.

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1. Introduction

In the last two decades, interest in oligonucleotides as therapeutic drugs has grown due to their demonstrated capacity to modulate the expression of certain genes and provide treatments for serious genetic disorders. Between 2016 and 2021, the United States Food and Drug Administration (FDA) approved eleven oligonucleotide drugs with a range of applications, some



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Nandhini KP (Nandhini Kalavathi Palanisamy) was born in Coimbatore (India). She obtained her Bachelor of Technology (BTech) degree at Sathyabama University in Tamil Nadu (India) and her Master of Science (MS) at the University of Skövde (Sweden). During that time, she was an Erasmus student at the University of Copenhagen (Denmark) under the supervision of Prof. Paul Robert Hansen. After that, she moved to the University of Kwazulu-Natal (South Africa) for carrying out the PhD under the supervisions of Professors Beatriz G. de la Torre and Fernando Albericio. Her PhD research involves both classic organic chemistry techniques and solid-phase strategies (SPS) for the synthesis of Peptides and Peptide Nucleic Acids (PNA).



Danah Al Shaer

Danah Alshaer is from Jordan, she was born in Kuwait. She obtained her BSc and MSc in chemistry from the University of Jordan. Her Master project was focused on the synthesis of new sequestering agents for some lanthanides. She was Lecturer in the University of Hail (Saudi Arabia) (2009–2017). Then, she joined the groups of Professors Fernando Albericio and Beatriz G de la Torre at the University of KwaZulu-Natal (South Africa) for the PhD. Her research was focused on the solid-phase synthesis and physico-chemical characterization of peptide-based HOPO containing ligands for Fe(III) chelation, as potential bacteriostatic agents. She currently works as a medicinal chemist, drug discovery scientist I, in Evotec Ltd. (Abingdon-United Kingdom).



of which act as short interfering nucleotides and others as gene antisense nucleotides.¹ However, oligonucleotides in natural forms have weak binding affinity and membrane permeability, and they are also readily digestible, thus leading to low bio-availability. Hence, chemical modifications of the nucleobases, phosphodiester backbones, and sugar moieties of antisense oligonucleotides were necessary to improve target affinity, specificity and cellular uptake, decrease toxicity, and reduce manufacturing costs.² To enhance the stability of oligonucleotides against degradation inside cells, several strategies have been exploited for the sugar-phosphate backbone, such as phosphorothioation,^{3,4} phosphoramidation^{5,6} and phosphoromethylation⁷ of the phosphate, 2'-fluorination or methylation of the ribose ring,⁸ and replacement of the whole backbone with a peptidyl-like one to render a peptide nucleic acid (PNA).

PNAs were chemically developed at the Buchardt laboratory in 1991^{9,10} as an *N*-(2-aminoethyl)glycine (AEG) unit that replaces the sugar-phosphodiester backbone of natural oligonucleotides, on which the nucleobases are connected. PNAs were hypothesized to form the genetic molecules in the very early forms of life on earth,^{11,12} a notion also supported by the presence of the aminoethyl glycine molecule in cyanobacteria.¹³ The distances between the nucleobases linked to the AEG backbone are approximately the same as the natural phosphodiester-sugar backbone of DNA/RNA, thus allowing hybridization of their complementary chain in a sequence-specific fashion.^{14–16} Therefore, PNAs are DNA/RNA analogues that are capable of forming hydrogen bonds with DNA/RNA nucleobases, obeying the Watson-Crick or Hoogsteen bonding rules (Fig. 1).^{14,15,17,18} Furthermore, being the PNA backbone non-ionic, unlike DNA/RNA, prevents electrostatic repulsions and the duplex PNA-DNA/RNA shows high thermal stability and is not affected by low ionic strength media. Moreover, PNAs are not susceptible to hydrolytic (enzymatic) cleavage as are not easily recognized by proteases or other enzymes.^{18,19}

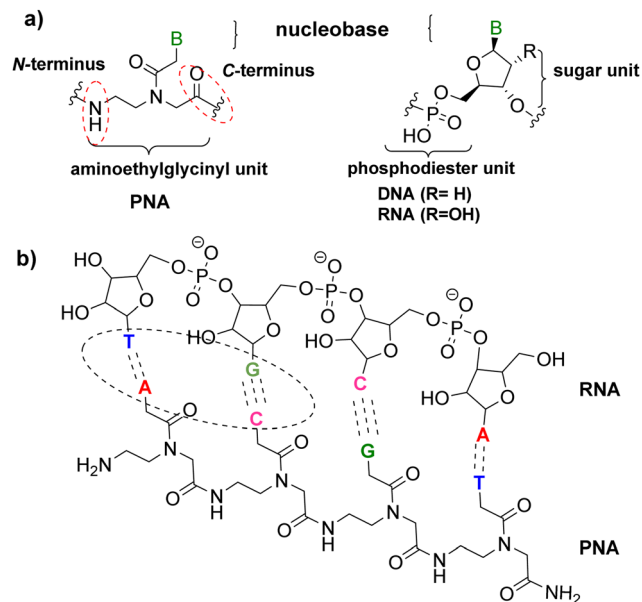
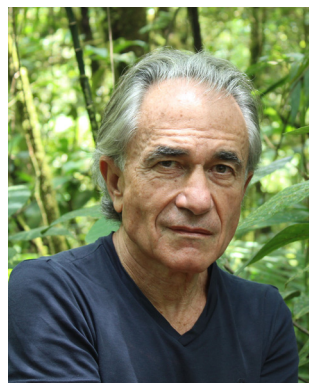


Fig. 1 (a) General structure of PNA vs. DNA or RNA monomers. (b) PNA–RNA duplex via Watson–Crick base pairing.

PNAs show a greater capacity to inhibit reverse transcription than phosphorothioate oligonucleotides.²⁰ Therefore, PNAs attracted attention as antisense and anti-gene agents for drug therapy applications,^{21,22} and more recently in gene editing. Beyond these applications, they have also been developed as biomolecular tools, molecular probes, and biosensors.^{23,24}

The possibility of broadening PNA applications through the development of PNA conjugates with other biomolecules such as peptides and DNA^{25,26} has fuelled the development of various synthetic strategies. Furthermore, the possibility of modifying the classic PNA peptide backbone (the aminoethylglycyl) at the α -, β -, γ -positions, or the use of cyclic backbones,



Fernando Albericio

Fernando Albericio's career has taken him through Europe, the USA, Latin America, and Asia to Africa (presently Research Professor at the University of KwaZulu-Natal (South Africa)). His research covers practically all aspects of peptide synthesis, as well as the synthesis of small molecules with therapeutic activities, especially against tuberculosis, infectious diseases, and cancer. His group is also involved in developing new

strategies for drug delivery. He is involved in the third mission of the University: the transference of knowledge and technology to society. He was co-founder and general director of the Barcelona Science Park and is co-promotor of BioDurban.



Beatriz G. de la Torre

Beatriz G. de la Torre obtained her PhD from the University of Barcelona (Spain). After a dilated career in Spain, she is presently a research Professor at KRISP, College of Health Sciences, University of KwaZulu-Natal (Durban, South Africa). She has been working extensively on glyco-, nucleo-, and lipopeptides. Her scientific interests are focused more on the discovery of new antimicrobial peptides, including those for fighting

tuberculosis, peptide-based vaccines, and peptide-based drug-delivery systems. Lastly, she is also deeply involved in developing GREEN Solid-Phase Peptide Synthesis Strategies.



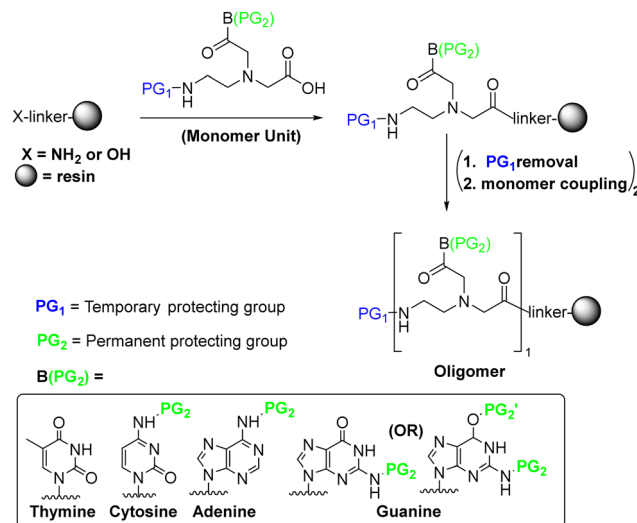
allows tuning the PNA conformations and hybridization capability.^{27,28} The modification on the γ -position is one of the most attractive to be exploited in many applications.^{18,22,29–31} The γ -substituted chiral PNA has shown several advantages compared with AEG–PNA such as minimal self-aggregation, good solubility, and stronger PNA–DNA duplex formation. It has been demonstrated that γ -PNA adopt a well-defined helical conformation left or right-handed depending on the chirality on the γ -stereocenter. The right-handed helix is afforded by the L-enantiomer which hybridizes to DNA/RNA with high affinity and sequence selectivity while the left-handed-helix shows less affinity than the achiral PNA.³² This is the reason why the enantiomeric purity of the γ -monomers is crucial for the synthesis of the oligomers.³³

Although PNAs are closer to DNA/RNA complexes from a structural perspective and in terms of biological application, their synthesis takes advantage of the solid-phase synthesis (SPS) methodology developed for peptides.^{34–37} This review seeks to provide a synthetic overview of the chemical construction of PNA monomers, which involves the preparation of the AEG backbone and the nucleobase acetic acid, and it gives special attention to the protecting scheme used during the preparation of the monomers as the scheme will determine the strategy to be followed for PNA SPS. This review also aims to shed light on possible areas to explore in order to improve current synthetic strategies.

2. Synthesis of PNA at a glance (SPS)

As mentioned above, PNAs are synthesized using the SPS approach, which was initially developed for the synthesis of peptides. In this regard, the strategy is basically the same as that used for peptides. The C-terminal monomer is anchored to a linker through its carboxylic group on a solid support, while the primary amino function of the backbone and the exocyclic amino of the base are conveniently masked through temporary and permanent protecting groups, respectively. The temporary protecting group of the amino function is then removed, followed by the incorporation of the next monomer through an amide bond. At the end of PNA chain elongation, the permanent protecting group is removed from the bases and PNA is cleaved from the resin concomitantly (Scheme 1).

The synthesis of PNA was first achieved more than 30 years ago.⁹ However, as its preparation is not as straightforward as that of peptides, a large number of improvements by the Danish group that initiated the PNA synthesis and by other groups have since followed. In this regard, there are several challenges to overcome before achieving an optimum strategy that makes these molecules appealing from both a chemical and biological perspective. Some of the hurdles to overcome are that the nucleobases of PNA are more difficult to protect than most amino acid side-chains and PNA monomers show poor solubility,³⁸ which is translated into inefficient coupling and the tendency to self-aggregate on resin. These factors limit PNA SPS to relatively short sequences.^{27,39} Thus, the choice of the



Scheme 1 General PNA SPS, showing the temporary (PG_1) and permanent (PG_2) protecting groups on the backbone and nucleobase, respectively, during synthesis.

protection for the backbone amino group and base (PG_1 , PG_2 in Scheme 1) is crucial.

As in solid-phase peptide synthesis (SPPS), the first strategy developed by Buchardt's group was based on *tert*-butoxy-carbonyl (Boc) for the temporary protection of the amino function and benzyloxycarbonyl (Z) for the permanent protection of the exocyclic amino of the nucleobases, while *p*-methyl-benzhydrylamine resin (MBHA) was used for linking the C-terminal monomer to the resin. An acid-labile amino protecting group such as Boc on the AEG backbone is compatible with Z on the nucleobase acetic acid. However, in this strategy, the strong conditions required to cleave PNA from the resin which involves trifluoroacetic acid (TFA)/hydrogen fluoride (HF) or trifluoromethanesulphonic acid (TfMSA), are quite harsh and not available in all research laboratories. Furthermore, the use of HF is banned in many countries. With the idea of using a milder scheme, Coull and co-workers developed the so-called 9-fluorenylmethyloxycarbonyl (Fmoc)/benzhydryloxycarbonyl (Bhoc) strategy.⁴⁰ More recently, Franzky and co-workers demonstrated the efficient synthesis and assembly of PNA following the Fmoc/Boc strategy.⁴¹

The base-labile protecting group Fmoc, which has mild removal conditions (20% piperidine in DMF), was introduced on the AEG backbone. However, the repetitive use of basic conditions to remove the temporary N-terminal protecting group has two major drawbacks. The first is the cyclization of AEG moieties of the backbone where the free amino group attacks the α -carboxyl group (Fig. 2A). This intramolecular reaction is favoured because of the formation of a stable six-membered lactam (ketopiperazine), leading to the loss of the monomer and, therefore, to deletion sequences. When preparing C-terminal carboxylic acid PNAs, this secondary reaction occurs dramatically upon attachment of the first PNA monomer to the functionalized resin.⁴² The initial attachment of a glycine residue for instance, as a spacer to the cleavable linker, for



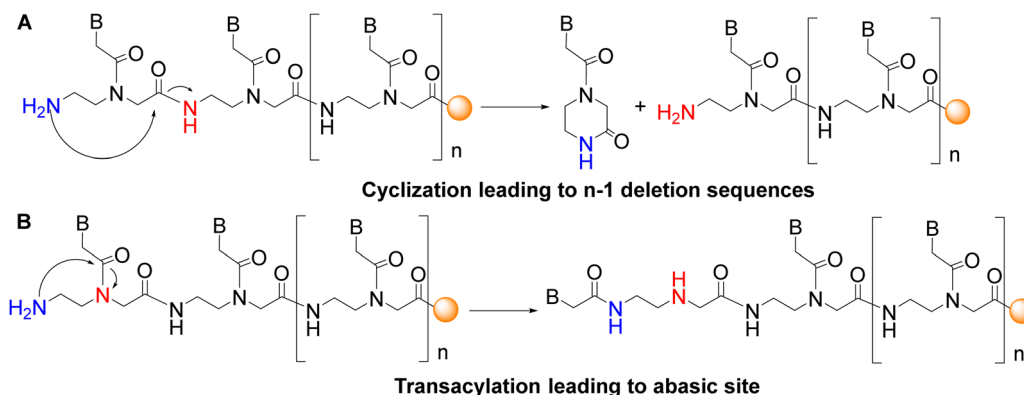


Fig. 2 Major side reactions when using the Fmoc strategy.³⁹

instance, helps prevent resin cleavage during the first deprotection because the six-member ring formation cannot take place. Given that this side reaction occurs mainly under basic conditions, the use of the right protecting group can also help prevent ketopiperazine formation. The second drawback is the transacylation of the nucleobase moiety from the α -amine of glycine to the free amine group of the ethylene diamine moiety (Fig. 2B). In this case, the secondary amine released can be acylated by the next monomer, thus leading to branched PNA. As in ketopiperazine formation, the driving force of the reaction is that it goes through a five-member ring intermediate.

Although PNA oligomers can be synthesized using standard Boc/Z^{15,17,43} and Fmoc/Bhoc⁴² and the corresponding monomers are commercially available, the drawbacks outlined earlier emphasize the need to explore other strategies or enhance current ones. The alternative approaches using different combinations of protecting groups on the PNA monomer are discussed below.

A major problem during the elongation of the PNA sequence is the great tendency to have chain aggregation and therefore the low loading resins have been preferred. Furthermore, the use of more hydrophilic resins as polyethyleneglycol (PEG)-based resins, such as Tenta-Gel⁴⁴ or ChemMatrix⁴⁵ have been also used to reduce the aggregation.

At the end of the synthesis, the cleavage and final deprotection of the permanent protecting groups is also a key for the choice of the strategy to follow. The standard solid supports (resin and linker) used for Boc/Z and Fmoc/Bhoc methodology require strong acid treatment, HF or TFMSA, and TFA. As the AEG-based PNAs does not have sensitive moieties as the peptides do, simple scavengers as H₂O are very frequently used.³⁹ However, when the target molecule is a PNA-peptide hybrid the proper scavengers should be included in the cleavage solution following the recommendations for peptides.⁴⁶ Finally, these harsh cleaving conditions are incompatible with the synthesis of other types of modified PNAs like PNA-DNA chimeras. In that cases, the best supports are the ones commonly used in solid phase oligonucleotide synthesis which are cleavable by ammonia treatment. Thus, the nucleobases have to be protected with protecting groups labile to these conditions

and the temporary amino protecting group preferable removed in mild acidic conditions as is the case of Mmt.^{25,47,48} But, in a more recent study Inagaki *et al.*⁴⁹ demonstrated the compatibility of Fmoc as temporary amino protecting group with Bz protection for the nucleobases due to a significant slower kinetics of the Bz removal compared to Fmoc in presence of piperidine.

3. Elongation of the PNA oligomer

3.1 Solid-phase strategy

The synthesis of PNA should be considered in two steps. The first is the preparation of the protected monomers, which is preferably carried out in solution. The second step involves the elongation of the PNA chain, which is carried out on a solid support. For successful PNA synthesis, as pointed out before, the choice of the amino and base protecting groups is crucial. These groups can be orthogonal or not, thus the deprotection scheme should be carefully designed. Awareness of the possible side reactions and the drawbacks associated with each protection scheme can help identify the most appropriate option.

The concept of orthogonality in SPPS was first demonstrated in 1985 by Barany and Albericio, using three distinct kinds of protecting groups.⁵⁰ In an orthogonal protection scheme, each group is removed independently by a different chemical mechanism and can be removed in any order. The main advantage of an orthogonal protection scheme is that the removal conditions can be optimized to ensure the full deprotection of the chemical function without altering the rest. The Fmoc/Bhoc scheme is a good example of orthogonality because these groups are removed by base and acid, respectively. Orthogonality is desirable but not paramount for efficient synthesis.⁵¹

The concept of compatibility applied to a protection scheme is when two protecting groups are removed from the same molecule using the same kind of chemical reagent but at different concentrations, and then the removal of the first one does not damage the integrity of the second. However, the order of removal cannot be inverted as in the case of orthogonality.⁵¹

Table 1 shows the protecting groups explored in PNA SPS, classified on the basis of the chemical mechanism involved in



Table 1 Protecting groups explored on PNA monomers

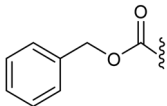
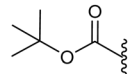
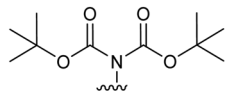
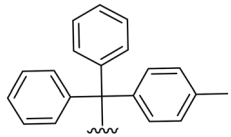
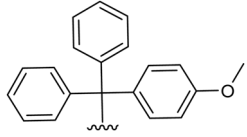
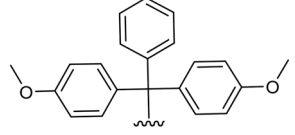
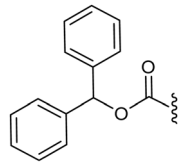
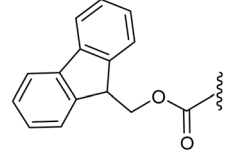
Entry	Name and structure	Lability	PG ₁ /PG ₂	Removal protocol	Ref.
1	 Benzyloxycarbonyl (Z)	Acid- and reduction-labile	PG ₂	(1) HF (2.5% thioanisole) for 1 h @ 0 °C (2) 0.5 ml anisole for 2–12 h, then cooled in N ₂ bath, followed by HF @ 0 °C for 30–45 min (3) 36% solution of dry HBr in AcOH for 15 min to 1 h @ RT (4) Refluxing anhydrous TFA for 30 min (5) Refluxing 18.5 mol% thioanisole in TFA for 3 h @ 25 °C (6) Catalytic hydrogenation (7) 1 M BBr ₃ in DCM for 1 h @ –10 °C and for 2 h @ 25 °C	42,43 and 52–56
2	 tert-Butyloxycarbonyl (Boc)	Acid-labile	PG ₁ and PG ₂	(1) 50% TFA in DCM (1 × 2 min and 1 × 30 min) (2) TFA/ <i>m</i> -cresol (95 : 5) for 4 min (3) TFA/DCM (1 : 1) for 1 h	43,57 and 58
3	 bis- <i>N</i> -tert-Butyloxycarbonyl (bis- <i>N</i> -Boc)	Acid-labile	PG ₂	(1) 50% TFA in DCM (1 × 2 min and 1 × 30 min)	58–61
4	 Monomethyltrityl (Mtt)	Acid-labile	PG ₁	(1) 1% TFA in DCM (5 min) or 10 min in neat hexafluoroisopropanol (HFIP)	62
5	 Monomethoxytrityl (Mmt)	Acid-labile	PG ₁ and PG ₂	(1) 3% trichloroacetic acid in methylene chloride (3 min) (2) 1% TFA in DCM (5–10 min) with HFIP	25,63 and 64
6	 Dimethoxytrityl (DMT)	Acid-labile	PG ₁	(1) Successive 30 s treatments with TFA/MeOH/DCM (1 : 2 : 97)	65
7	 Benzhydryloxycarbonyl (Bhoc)	Acid-labile	PG ₂	(1) TFA/ <i>m</i> -cresol (4 : 1) for 90 min	40 and 66
8	 9-Fluorenylmethoxycarbonyl (Fmoc)	Base-labile	PG ₁	(1) 20% piperidine in H ₂ O @ 0 °C for 30–45 min (2) 20% piperidine in DMF (1 × 2 min, 1 × 8 min)	42,57 and 67



Table 1 (continued)

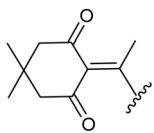
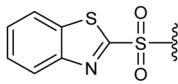
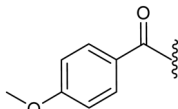

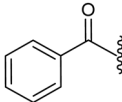
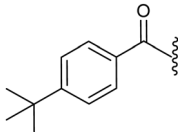
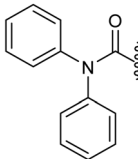
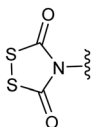
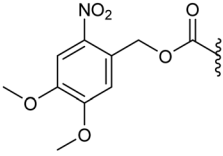
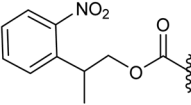
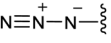
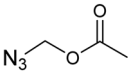
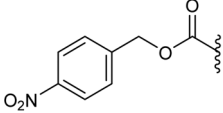
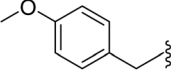

Entry	Name and structure	Lability	PG ₁ /PG ₂	Removal protocol	Ref.
9	 (1-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)-3-ethyl) (Dde)	Base-labile	PG ₁	(1) 2% hydrazine in DMF (2) 20% NH ₂ OH·HCl/imidazole (1/0.75 equiv.) in NMP/DMF (5/1)	26,68 and 69
10	 Benzothiazole-2-sulfonyl (Bts)	Base-labile	PG ₁	(1) 0.8 M 4-methoxybenzenethiol and 0.4 M DIEA in DMF @ 40 °C for 10 min (2) 3.89 mol% Al in crystals of HgCl ₂ ; under THF–H ₂ O overnight @ RT (3) 69.7 mol% Zn powder in AcOH for 14 h @ RT (4) 50% aq. H ₃ PO ₂ for 2 h with substrate under refluxing THF (5) 2–3 equiv. thiophenol in 3 equiv. K ₂ CO ₃ for 3–5 h @ RT (6) 5 equiv. of NaBH ₄ in EtOH for 1–3 h @ RT (7) 20 equiv. PS-thiophenoxide resin in THF–EtOH (1 : 1) containing substrate; shaking for 2 h @ RT	56 and 70–74
11	 Anisoyl	Base-labile	PG ₂	(1) Ammonia (32%) or methylamine (40%) @ 60 °C for 20 h (2) 28% ammonia @ RT for 5 h (3) Conc. aq. ammonia solution @ 50 °C for 6 h	25,63,75 and 76
12	 Isobutyryl	Base-labile	PG ₂	(1) Ammonia (32%) or methylamine (40%) @ 60 °C for 20 h (2) 28% ammonia @ RT for 5 h (3) Conc. aq. ammonia solution @ 50 °C for 6 h	25,63,75 and 76
13	 Benzoyl	Base-labile	PG ₂	(1) Ammonia (32%) or methylamine (40%) @ 60 °C for 20 h (2) 28% ammonia @ RT for 5 h (3) Conc. aq. ammonia solution @ 50 °C for 6 h	25,63,75 and 76
14	 4-tert-Butyl benzoyl	Base-labile	PG ₂	(1) Ammonia (32%) or methylamine (40%) @ 60 °C for 20 h (2) 28% ammonia @ RT for 5 h (3) Conc. aq. ammonia solution @ 50 °C for 6 h	25,63,75 and 76
15	 N-Diphenyl carbamoyl	Base-labile	PG ₂	(1) Ammonia (32%) or methylamine (40%) @ 60 °C for 20 h (2) 28% ammonia @ RT for 5 h (3) Conc. aq. ammonia solution @ 50 °C for 6 h	25,63,75 and 76
16	 Dithiasuccinoyl (Dts)	Reduction-labile	PG ₁	(1) Dithiothreitol (DTT) (0.5 M) in AcOH (1 × 2 min, 1 × 8 min)	77



Table 1 (continued)

Entry	Name and structure	Lability	PG ₁ /PG ₂	Removal protocol	Ref.
17	 Nitroveratryloxycarbonyl (NVOC)	Photo-labile	PG ₁	(1) Photolysis at wavelengths > 300 nm; additives: N ₂ H ₄ , NH ₂ OH·HCl, or semicarbazide·HCl (several hours)	56 and 78
18	 2-(2 nitrophenyl)propyloxy carbonyl (NPPOC)	Photo-labile	PG ₁	(1) Photolysis at wavelengths > 300 nm	79
19	 Azide	Reduction-labile	PG ₁	(1) 1 M THF solution of trimethyl phosphine and tributyl phosphine @ 23 °C for 5 min	66
20	 Azidomethoxycarbonyl (Azoc)	Reduction-labile	PG ₁	(1) 1 M trimethyl phosphine in THF/H ₂ O (95 : 5) for 5 min	80
21	 p-Nitrobenzyloxycarbonyl (pNZ)	Reduction-labile	PG ₁	(1) 3 M SnCl ₂ in 20 mM HCl in DMF @ 60 °C for 15 min (2) Catalytic hydrogenation	61
22	 p-Methoxybenzyl (PMB)^a	Acid-labile	PG ₂	(1) 0.6 M AlCl ₃ in anisole @ RT for 30 min, followed by alkaline aqueous workup	65
23	 Allyl (All)^b	Acid-labile	PG ₂	(1) Removed in cleavage conditions: TFA/TIS/ <i>m</i> -cresol/H ₂ O 85 : 5 : 5 : 5	81

^a Used as protecting group for N³-T to prevent alkylated under Mitsunobu conditions. ^b Used as protecting group for O⁴-T to prevent aggregation during PNA synthesis.

the removal, indistinctly of whether the function to be removed is temporary [amino function (N-terminal)] or permanent (nucleobase).

As mentioned above, Boc/Z and Fmoc/Bhoc are the two protection schemes most widely used for PNA synthesis; however, their drawbacks have led to the exploration of alternatives. The Fmoc group has also been studied with other permanent protecting groups for nucleobases, such as Z, Mmt, Acyl, Boc, and bis-*N*-Boc^{42,57–60,67,76,82–84}. Alternatively, other temporary protecting groups, such as Mmt, Dts, Dde, Bts, azide, Nvoc, and pNZ, for the amino-terminal of the AEG backbone have been tested.

The Mmt/Boc strategy was established because of the convenience of using only TFA to remove both protecting groups. This is an example of protecting group compatibility. Mmt and

Boc are compatible because the diluted TFA solution used to remove the former leaves the latter unaltered.⁶² The combination of Mmt for the backbone amino group and acyl protecting for the exocyclic amino group of the bases allowed the synthesis of PNA–DNA conjugates in SPS, thereby minimizing the risk of depurination.^{25,63}

The use of Dts as a temporary protecting group at the N-terminal of the PNA monomers showed the same performance for the PNA synthesis compared to the use of Boc-monomers. However, using thiolytic reagents under mild acidic conditions to remove the Dts moiety and a shorter time than the usually described for the Boc strategy for the neutralization steps helped to minimize the typical side reactions explained above.⁷⁷

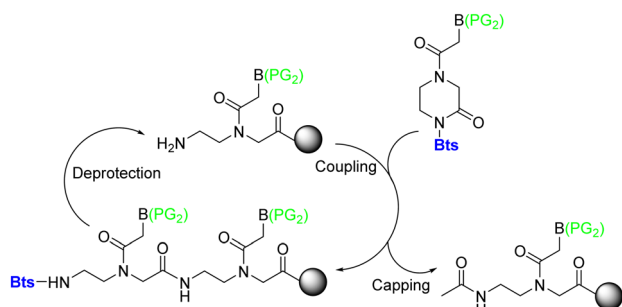
Along with preventing side reactions during PNA SPS, the need to prepare peptide–PNA conjugates to enhance, for



instance, cellular uptake has driven the development of new protecting groups for PNA synthesis. In this regard, Bradley's lab developed the use of Dde as a temporary protecting group. In the search for N-terminal protecting groups for PNA monomers that are compatible/orthogonal with the standard Fmoc-amino acids and their side chain protection, they first explored the use of the Alloc group, which is removed under neutral conditions using Pd(0) catalysis. However, this protecting group did not render satisfactory results for chain elongation. Therefore, the Dde group emerged as the alternative since it is stable under Fmoc removal conditions. Nevertheless, the most used conditions to remove Dde, namely hydrazine solution, cause partial deprotection of the Fmoc group, hence Dde removal was tested using hydroxylamine, in which the Fmoc group showed full stability. Thus, the synthesis of Dde/Mmt protected PNA monomers was successful as well as their use for PNA oligomers and PNA/peptide conjugates.^{26,68,69}

Lee *et al.*⁷⁴ reported a novel type of cyclic PNA monomer where the Bts group was used as both protecting and self-activating group for the effective preparation of PNA oligomers. The amino group of the backbone carrying the Bts group cyclizes through the carboxyl group, giving a piperazinone, in which the carbonyl group is activated and prone to attack by nucleophiles such as the amine of PNAs (Scheme 2). The Bts monomers were synthesized in combination with the Bhoc protecting group for the exocyclic amino group of the bases. The removal of Bts in each cycle of the oligomerization was performed by means of thiols in the presence of base. The application of these monomers in the synthesis of a 15-mer PNA demonstrated their high performance. Although Bts cyclic monomers could be ideal for the large-scale SPS of PNA oligomers, the use of malodorous thiols is a huge limitation for their general implementation.

NVOC has also been used as protecting group for the amino function of the PNA backbone in combination with anisoyl protection for adenine and cytosine, and isobutyryl for guanine.⁷⁸ The advantage of NVOC lies in its photolytic cleavage by irradiation at > 300 nm, thus circumventing the need for commonly used deprotection reagents such as TFA or piperidine. The smooth deprotection conditions make these monomers suitable for photolithographic methods for the synthesis of PNA microarrays and PNA-DNA chimeras.⁸⁵ Another photolabile group, namely NPPOC, has also been developed for the same application.⁷⁹



Scheme 2 PNA synthesis using Bts-protected/self-activated monomers.

Another strategy developed involves the use of the azide group to mask the N-terminal amine of the AEG backbone.⁶⁶ The azide group was made to react with three phosphines: Me₃P, Bu₃P, and Ph₃P.

Only the two alkyl phosphines rendered the iminophosphorane derivative rapidly. However, the hydrolysis to amine was slow in both cases. This problem was circumvented using the pre-activated *N*-hydroxybenzotriazole ester of the incoming monomer, although it was successful only in the case of the Me₃P derivative. Along the same line, the protecting group Azoc was developed.⁸⁰ In this case, full removal of the protecting group was achieved with a solution of Me₃P in 5 min. Azoc is compatible with Fmoc, Alloc, and other protecting groups used in peptide synthesis. The Boc group was chosen to protect the exocyclic amino group of the bases for azide and Boc for Azoc.

Although protecting groups such as Mmt, Dde, and Azide were successfully used to synthesize PNA monomers and oligomers and they avoided many of the disadvantages of protecting groups such as Fmoc and Dts and Bts, their scale-up was problematic due to the need for expensive starting material. With these considerations in mind, Huang *et al.*⁶¹ explored the use of *p*NZ as protecting group for the AEG backbone. This protecting group had already demonstrated effectiveness in peptide synthesis and can be produced cost-effectively from the non-expensive *p*NZ chloride at gram scale. The *p*NZ group is removed by nitro-reducing methods, the use of SnCl₂ under acidic catalysis being described as preferable. Thus, this protecting group is orthogonal with Fmoc, Boc, and Alloc, which are all widely used in peptide chemistry. Nevertheless, because of the higher acidic lability of the Bhoc group, which is frequently used as protecting group of the bases in PNA monomers, the authors of this work preferred the combination with bis-*N*-Boc protection. Previous reports have compared bis-*N*-Boc protection with that afforded by Bhoc and Boc.^{59,60} The bis-*N*-Boc group offered higher stability than Bhoc and the same performance as Boc. However, as bis-*N*-Boc reduces one hydrogen-bond donor in the monomer, it is easier to solubilize in organic solvents.

Finally, regarding the protection of the exocyclic amino group of the nucleobases, attention has also been given to acyl groups such as anisoyl, benzoyl, isobutanoyl, *t*-butyl benzoyl, and isobutyryl, which were already established in oligonucleotide synthesis.⁷⁶

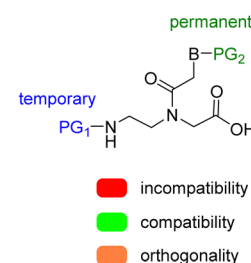
Table 2 shows a summary of protecting groups explored for PNA synthesis. Their combination as protecting groups for the amino backbone and exocyclic bases are highlighted in different colours on the basis of incompatibility, compatibility, and orthogonality. As can be appreciated from the table, there is still room to explore several combinations of protecting groups to improve PNA synthesis.

PNA chain elongation is achieved mainly monomer by monomer. However, from a structural perspective, given that the repetitive units consist of three moieties, namely glycine, ethylenediamine, and acyl-nucleobase, a subunit approach has also been used for oligomer elongation. This sub-monomeric approach can comprise two- or three-component assemblies.^{86,87}



Table 2 Protecting groups in PNA synthesis

Temporary PG ₁	Permanent PG ₂					
	Z	Bhoc	Boc	Bis-N-Boc	Mmt	Acyl
Boc	16-18 ^a					51
Fmoc	33	31	57, 58	38-40	47	52
Mmt			42			24, 43
Dts	53					
Dde					48	
Bts		50				
Azide		45				
NVOC						54
pNZ				41		

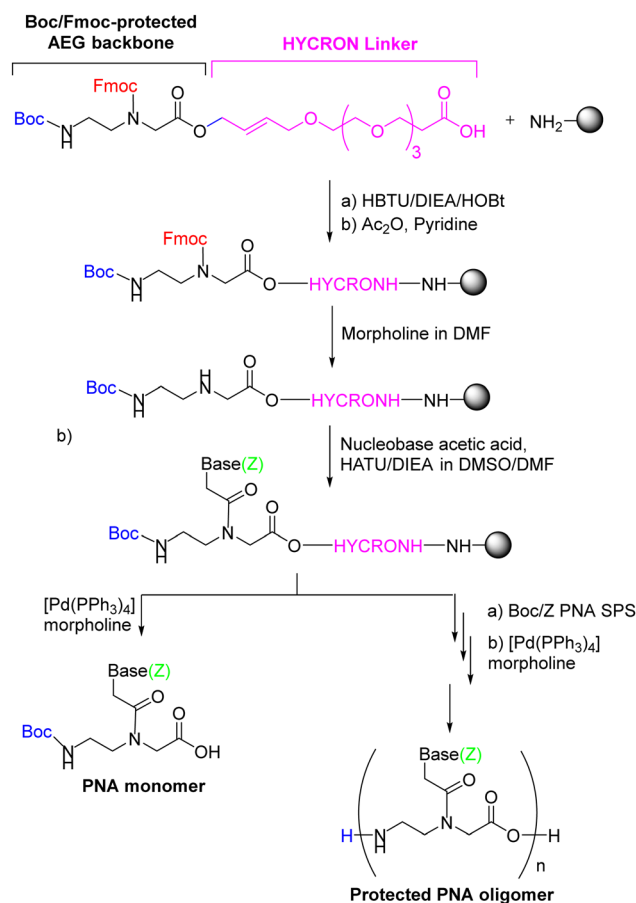


^a Numbers correspond to references.

The first synthetic attempt at the two-component strategy was made by Seitz's group⁸⁶ using the HYCRON linker (hydroxycrotyl-oligoethylene glycol-*n*-alkanoyl), an allylic anchor that contains a mini-chain of polyethylene glycol (PEG) to facilitate cleavage of the oligomer from the resin, a process achieved with Pd(0) catalysis.⁸⁸ This linker offers a complete orthogonal protection scheme since both acid- and base-labile protecting groups can be used as temporary N-protection. The sub-monomeric strategy requires an extra temporary protecting group for the secondary amine of the AEG moiety. In the mentioned work, the N-terminal was protected with Boc, while Fmoc was used to protect the secondary amine of the backbone. Once the first component is attached to the resin as a preformed linker, which contains its own linker and the first AEG backbone moiety, the Fmoc group is removed, and the nucleobase acetic acid (second component) with the base protected with Z is introduced. At this point, the protected monomer can be obtained from the resin by Pd(0)-catalyzed cleavage, or Boc/Z monomers are incorporated until the oligomer has been completed (Scheme 3). Although only the first monomer was built on solid phase, the authors of that study found that the yield of the resulting PNA oligomer was markedly increased. The convenience of obtaining the protected oligomer lies in the possibility of introducing modifications to the C-terminus of the oligomer.

Following the two-subunit monomeric approach, Condom, Patino, and co-workers⁸⁹ elongated first the PNA backbone using Boc as temporary protecting group and a variety of orthogonal protecting groups for the secondary amine, each one for each kind of nucleobase. Once the backbone had been elongated, the selective removal of each protecting group, followed by the incorporation of the corresponding nucleobase acetic acid derivative, led to the target sequence (Scheme 4).

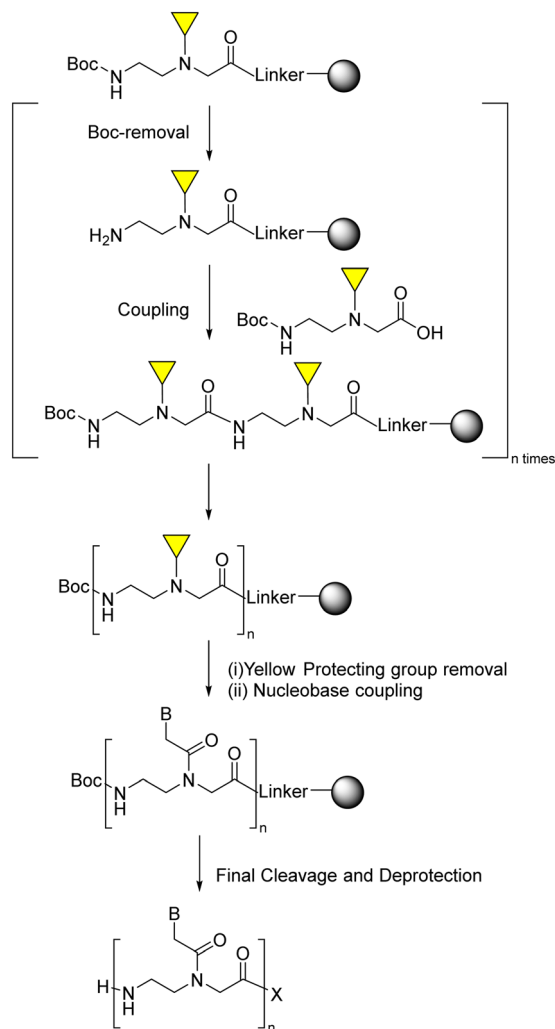
A further step forward in the field was the strategy to construct the PNA monomer entirely in solid phase from three components.



Scheme 3 The first PNA monomer-HYCRON conjugate built from scratch to obtain a high-yield end product (oligomer).

Richter and Zuckermann⁸⁷ were the pioneers in the three-component strategy, which sought to circumvent the solubility issues associated with the protected PNA monomers.

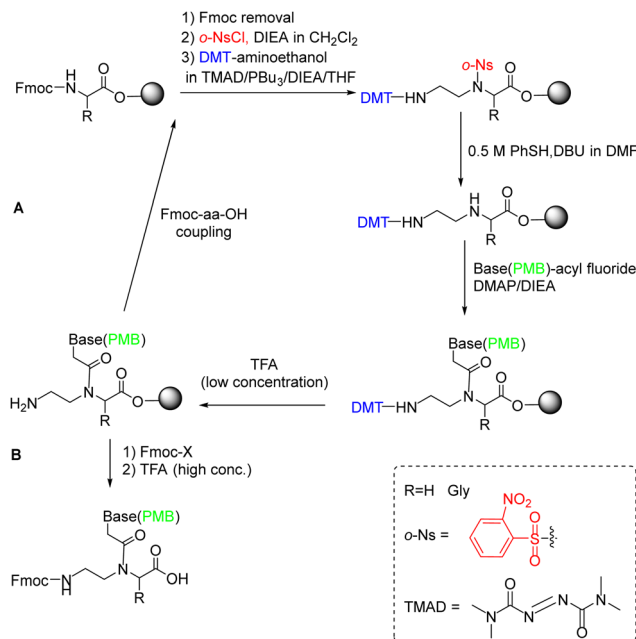




Scheme 4 Sub-monomer PNA SPS through two subunit approach: secondary amine protected Boc-PNA backbone and nucleobase acetic acid derivatives.

Using bromoacetic acid, Moz-ethylenediamine (Moz = 4-methoxybenzyloxycarbonyl), and 1-carboxymethyl-thymine as subunits, they were able to synthesize successfully an 8-mer thymine oligomer.

Later, in the work developed by Viirre and Hudson,^{65,90} the synthesis was started by incorporating Fmoc-Gly-OH (or another amino acid) onto Wang resin, which allows the detachment of the products by treatment with a high concentration of TFA. Then, after removal of the Fmoc group, the amino terminus was reprotected in the form of *o*-nitrobenzenesulphonamide (*o*-Ns), with a double function: masking the amine nucleophilicity at the same time that activating it for participating in a Fukuyama-Mitsunobu reaction. Thereby the formation of the AEG backbone bearing the primary amino group protected with dimethoxytrityl (DMT) was facilitated (Scheme 5). In the next step, the secondary amine was deprotected by thiolysis of the *o*-NS group and made reacting with the nucleobase derivatized as acid fluoride and protected with the *p*-methoxybenzyl (PMB) group. At this point, DMT can be removed using a low



Scheme 5 Sub-monomer PNA SPS using the Fukuyama-Mitsunobu reaction. (A) PNA oligomer synthesis and (B) Protected PNA monomer synthesis.

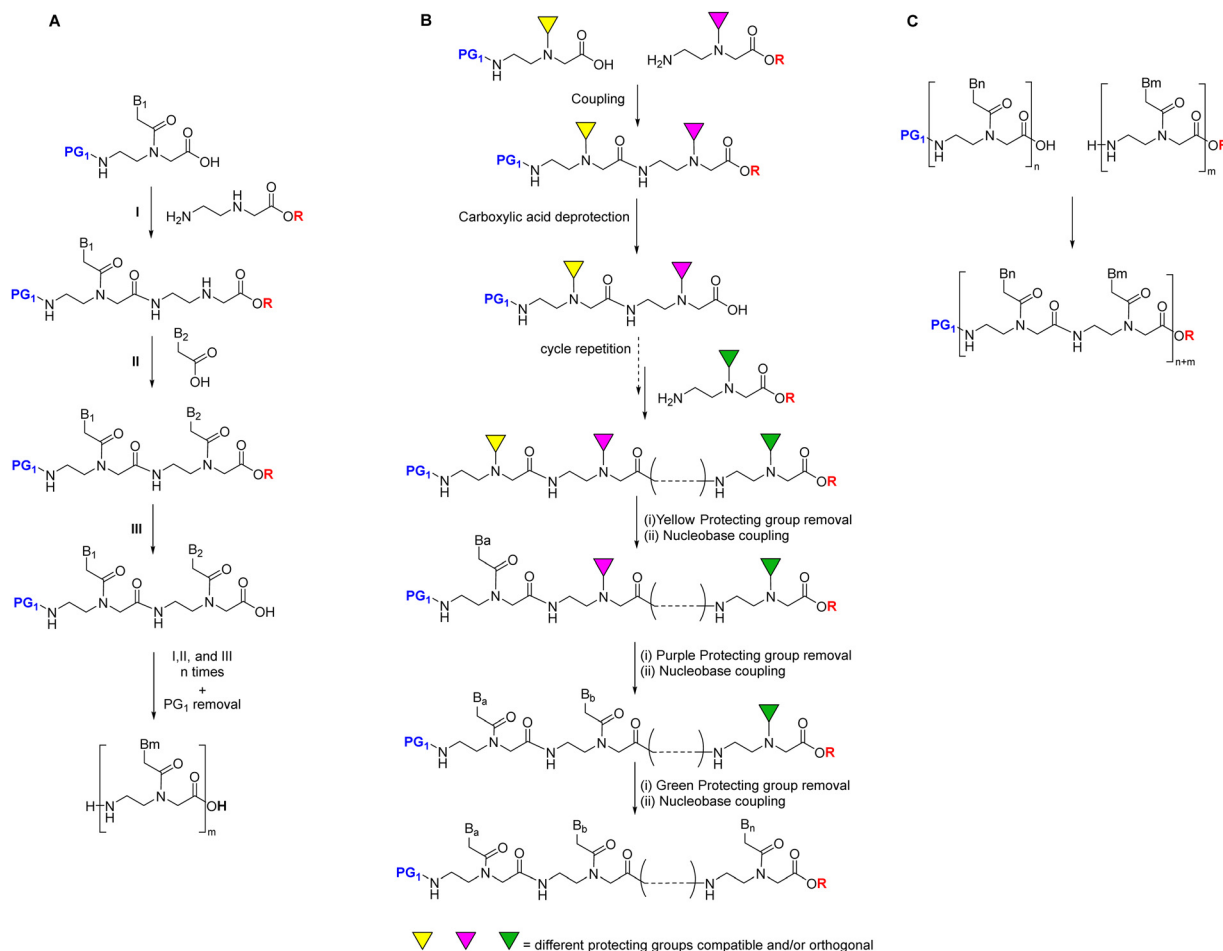
concentration of TFA, and the cycle from the Fmoc-amino acid coupling can be reinitiated and repeated to obtain the oligomer (Scheme 5, route A), or the amino group could be protected again with Fmoc followed by cleavage from the resin using a high concentration of TFA obtaining the protected PNA monomers for further use in PNA SPS (Scheme 5, route B).

The advantage of the three-component approach is that it allows the introduction of other amino acid residues instead of Gly in a straightforward manner. Such modifications introduce variability into the oligomer as chirality, charges that improve solubility, and functionalities to allow conjugations without modifying the binding to nucleic acids—all properties that are highly desirable in many PNA oligomers.

3.2 Solution-phase strategy

Although solid phase is the mode of choice for PNA synthesis, as occurs with peptides, several interesting strategies have been developed for the preparation of these molecules in solution. The group of Condom, Patino, and co-workers⁹¹ proposed the following three strategies for PNA synthesis in solution following an N → C chain elongation (the solid-phase mode is C → N): (i) the carboxylic acid of one protected monomer is reacted with the primary amino group of an AEG ester. Once this AEG ester has been incorporated, the nucleobase acetic acid derivative is attached in, and the ester hydrolysed. Then, the next AEG ester is incorporated and the cycle repeated until the full-length oligomer is built (Scheme 6A); (ii) based on a fully protected polyamide backbone (discussed above), where the whole backbone is built first, with all the secondary amines protected with different compatible amino protecting groups that are sequentially removed for the incorporation on each one the





Scheme 6 PNA synthesis in solution. (A) N → C chain elongation; (B) fully protected PNA backbone, followed by removal of protecting group sequentially for nucleobase acetic acid addition and (C) condensation between PNA di- or trimers after the removal of protecting groups from N- and C-fragments.

corresponding nucleobase acetic acid derivatives to finally give the desired PNA (Scheme 6B); (iii) shorter fragments, di- or trimers, are prepared by one of the previously mentioned strategies and the condensation takes place after hydrolysis of the ester of the N-fragment and the removal of the protecting group from the C-fragment (Scheme 6C).

These solution strategies are recommended only for the synthesis of PNAs with short sequences. The second strategy was successfully applied independently by the groups of Condom and van Boom for the preparation of cyclic PNAs consisting of two to six monomer units.^{92–96}

Recently, Periyalagan and Hong described a variation of the third strategy for the preparation of trimers, where the carboxylic group to react is activated in the form of pentafluorophenyl ester and the incoming monomer has both the primary amine and the carboxylic group free. The trimers were used for the synthesis of PNAs in solution and solid phase, demonstrating great performance for solution synthesis up to 12-mer.⁹⁷

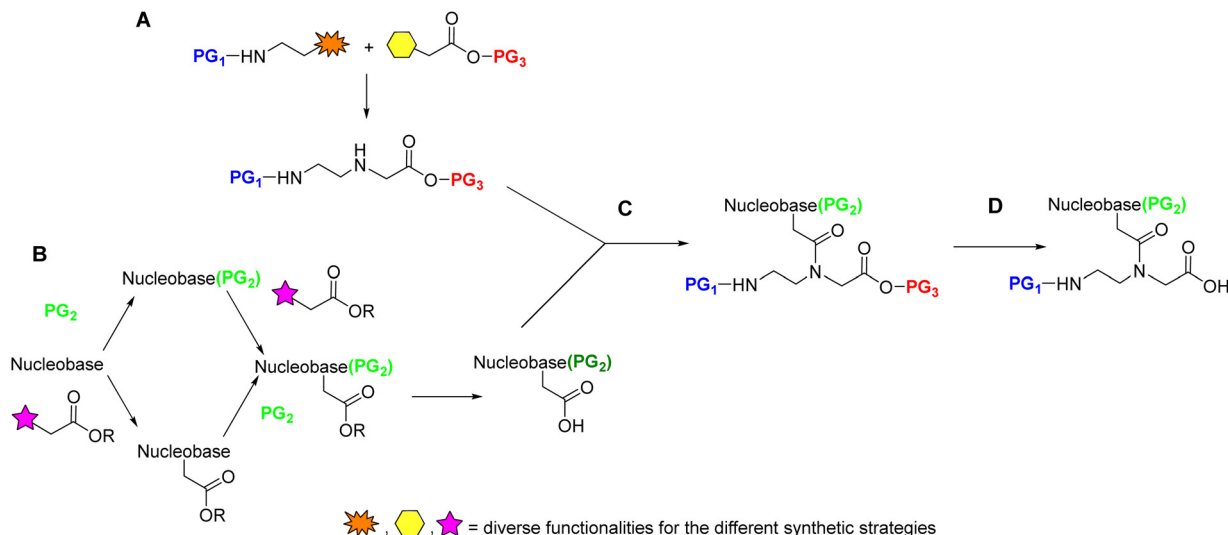
Finally, Chiba and co-workers,⁹⁸ who have extensively developed tag-assisted liquid phase peptide synthesis (LPPS),⁹⁹ used this methodology to prepare PNAs. This LPPS approach is

based on SPPS, where the solid support is substituted by a hydrophobic soluble linker, making the growing PNA soluble in solvents such as THF. After the incorporation of each monomer, PNA is collected by precipitation by adding a polar solvent such as acetonitrile (ACN).

4. Synthesis of PNA monomers

Although the preparation of the monomers in solid phase, like the examples shown in Schemes 3 and 4, can be appealing from the perspective of efficiency as it circumvents a tiresome workup and chromatographic purification after each step, most monomers have been prepared in solution. Most syntheses of PNA monomers reported in the literature start with the separate preparation of the AEG backbone with protected amino and carboxylic acid ends (Scheme 7A) and the protected nucleobase carboxylic acid derivative (Scheme 7B). The protected nucleobases are then introduced through an acylation reaction of the α -amine of the glycine moiety in the backbone (Scheme 7C). Finally, the protection of the carboxylic group of





Scheme 7 Schematic representation of the subunit synthesis of PNA monomers.

the glycine moiety is removed (Scheme 7D). To select the temporary and permanent protecting groups (PG_1 and PG_2), two points should be taken into consideration. The first is that the route through which the AEG backbone is prepared should be suitable for an efficient amino temporary protecting group PG_1 . The second point is that the protecting group of the C-terminus of the monomer (PG_3) should be removable under conditions that do not affect the amino/base protecting groups PG_1 or PG_2 , respectively.

4.1 Synthesis of the AEG backbone

The AEG backbone can be synthesized by distinct pathways:³⁹ (i) alkylation;⁷⁵ (ii) the Fukuyama–Mitsunobu¹⁰⁰ reaction; and (iii) reductive amination,¹⁰¹ which are discussed below.

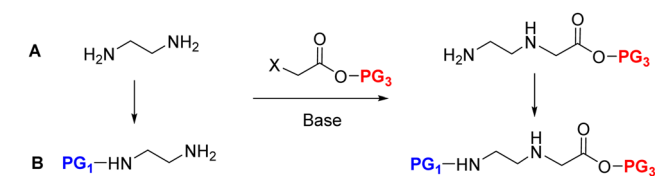
Alkylation. The strategy involves the reaction of ethylenediamine (EDA) or its mono *N*-protected derivative (PG-EDA) with an α -C halo-substituted acetic acid derivative in the presence of a base (Scheme 8). PG-EDA was initially synthesized using di-*tert*-butyl dicarbonate, giving excellent purity.¹⁰² The synthesized PG-EDA then underwent alkylation through the use of *tert*-butyl chloroacetate in the presence of triethylamine (TEA) and a catalytic amount of KI. However, the yield obtained was only 41%.²⁵ Here, the catalytic KI was believed to be used for the *in situ* generation of alkyl iodide, from other alkyl halides (chloride in this case) to improve the yield of the alkylation reaction, as mentioned in the previous literature.^{103–105} Another strategy was the use of methyl bromoacetate in the presence of

TEA for the alkylation of PG-EDA, giving rise to moderate yields of the desired PNA backbone.^{62,75} As expected, the bromo derivative of haloacetic acid gives a higher yield during the nucleophilic substitution reaction because of its leaving group ability compared to that of the chloroacetic acid derivative.^{25,62,75}

The obtention of the dialkylated amine as a by-product, along with unreacted amine, has been reported when using PG-EDA in the presence of base as starting material. Instead, a high excess of the unprotected EDA (9-fold excess) can substitute the use of a base and limit the likelihood of over-alkylation, affording the free N-terminal backbone with high yield.^{26,42,106} However, the overall yield was dramatically reduced after synthesis completion when attempting to achieve the desired PNA backbone with N-terminal protecting groups such as Fmoc, Dde, and Mmt.^{25,62,58,107}

Advances made in PNA backbone synthesis have brought about the development of protocols with distinct protecting group schemes and synthetic methodologies. However, initially little attention was paid to the Fmoc strategy for PNA synthesis due to both costly materials and challenging synthetic routes rendering low yields. Although later on, the Fmoc strategy was probably the main applied approach, there are not many reports in the literature describing their synthesis.^{42,59,60}

Thomson and co-workers⁴² efficiently synthesized the backbone Fmoc-AEG-*Ot*Bu starting from A, and PG_3 was *t*Bu (Scheme 8). For the obtention of the final monomer, Z was used as protecting group for the nucleobases. However, although the chain elongation to synthesize PNA oligomers could be run under smooth conditions, the final deprotection required treatment with HF. Based on this approach, Feagin and co-workers¹⁰⁷ attempted the same strategy but substituting *t*-butyl ester by a benzyl ester, which has a significant advantage over the methyl/ethyl ester in that it can be removed by catalytic hydrogenation avoiding the basic treatment. Nevertheless, the reaction led to ketopiperazine and benzyl alcohol (Scheme 9). Thus, the less bulky nature of the benzyl ester favoured the

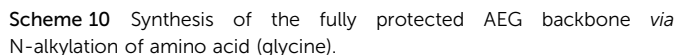


Scheme 8 Protected PNA backbone obtained by alkylation reaction.

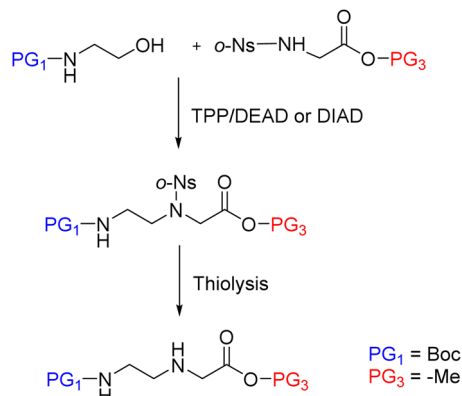




Malamgari *et al.* adopted the Fukuyama N-alkylation reaction for AEG synthesis. Unlike the aforementioned approach where the EDA was alkylated by haloacetate derivatives, here the α -amino group of the Gly ester was alkylated by *N*-Boc-bromoethylamine.¹⁰⁹ Thus, the glycine ester was first N-protected by *o*-nosyl chloride, followed by the alkylation reaction with the protected amine alkyl halide in the presence of DBU to obtain the fully protected AEG backbone. The *o*-nosyl protection was essential to prevent an over-alkylated side-product.



Reductive amination. An alternative strategy for synthesizing the PNA backbone is reductive amination, which aims to alkylate the primary amine in a more controlled manner by



Scheme 11 PNA backbone synthesis by Fukuyama–Mitsunobu reaction.

forming imine as an intermediate, thereby avoiding over-alkylation and improving the yield of the desired product.¹¹³ Two approaches can be used for synthesizing the PNA backbone *via* reductive amination. The first route involves the reaction between protected-aminoacetaldehyde (PG₁-Aa) and glycine ester (Scheme 12A). Thus, the obtention of the aminoacetaldehyde derivative is key. The synthesis of PG₁-Aa was previously reported through various procedures, such as Swern oxidation from alcohols,^{114–118} ozonolysis,^{119,120} and oxidation with pyridinium chlorochromate (Corey's reagent).¹²¹ However, all these procedures are subject to issues during the isolation, purification, and storage of these compounds due to their high instability.^{122–124} However periodate oxidation (KIO₄, NaIO₄) of protected 3-amino-1,2-propanediol,^{47,122} and protected aminoethanol by means of 2-iodoxybenzoic acid (IBX)⁶⁰ led to the PG₁-Aa compound in good yield, and purification was not required (Scheme 12B). Another successful approach that does not involve an oxidation step was proposed by Falkiewicz *et al.*¹²⁵ The N-protected glycine unit was amidated with *N,O*-dimethylhydroxylamine by means of a coupling reagent in the presence of a base (TBTU/TEA). The Weinreb amide obtained was then reduced using LiAlH₄ to yield PG₁-Aa (Scheme 12C).

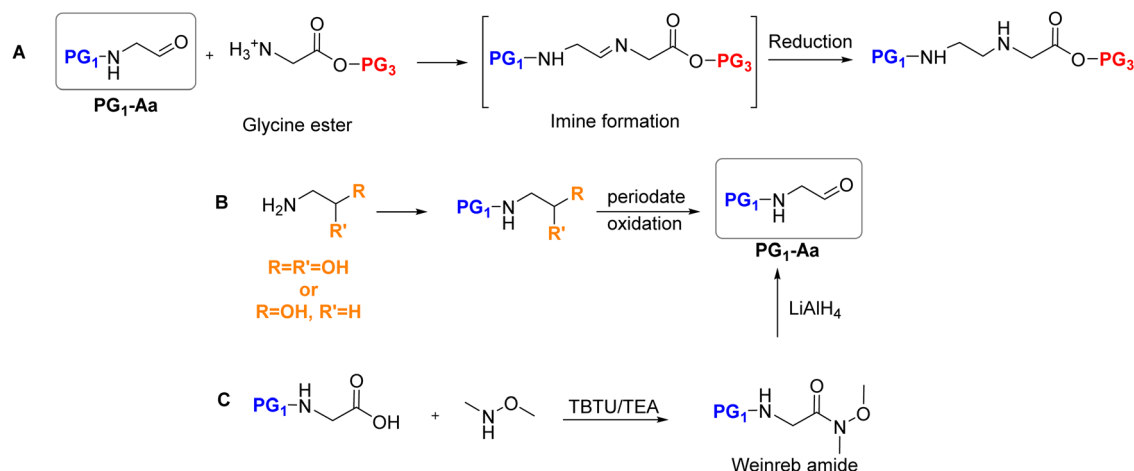
The Weinreb approach can be considered the best method of all as it gave an excellent yield not only when Gly was used but also for other amino acids.¹²⁵

The second critical step in this process is the reduction of the imine intermediates to form the final protected backbone. The final yields of PG₁-AEG obtained in various studies depended on the reducing reagent used, the N-protecting group, and the ester. The trend observed is that reduction using 10% Pd/C under H₂ gave acceptable yields. However, more potent reducing agents like NaBH₄ led to lower yields and are therefore not suitable reagents for this purpose. In the case of using NaCNBH₃ as a reductant, the moderate yields obtained were improved when the reaction was performed in the presence of AcOH.¹²⁵ Although, it has not been reported for AEG backbone, Volpi *et al.*¹²⁶ performed the reductive amination by transfer hydrogenation promoted by an Ir(III) catalyst¹²⁷ in the presence of a mixture of formic acid and DIEA. The results were successful, reacting the (PG₁-Aa) and D-Lys(Boc)-OH or DArg(Pbf)-OH. In this work the carboxylic acid did not require protection since it was used in a two-subunit monomeric approach.

4.2 Synthesis of protected nucleobase acetic acid derivatives

In this section, the four DNA canonical bases (T, C, A, and G) will be discussed along with the pseudo-complementary nucleobases (pcNB) 2-thiouracil (^sU) and 2,6-diaminopurine (D).¹²⁸ However, many other modified bases have been used as part of PNA monomers.^{129,130}

Prior to assembly onto the AEG backbone, the nucleobases have to undergo two modifications. One is common to all of them, namely the need to carry a methyl carboxylic acid, which will act as a linker between the nucleobase and the AEG backbone. This group is placed on the endocyclic N¹ in the case of thymine and cytosine and on N⁹ in the case of adenine and guanine. The other modification is the protection of the exocyclic amine group of cytosine (N⁴), adenine (N⁶), and guanine (N²). While thymine does not require protection (Fig. 3). However, the protocols described for the protection



Scheme 12 (A) Synthesis of the fully protected AEG backbone by reductive amination; (B) and (C) synthesis of the key aldehyde intermediate.



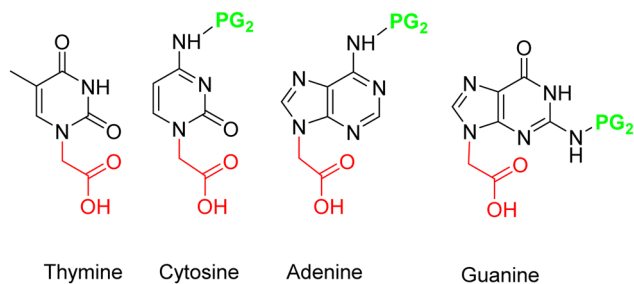


Fig. 3 Protected exocyclic amino group on the nucleobase acetic acids.

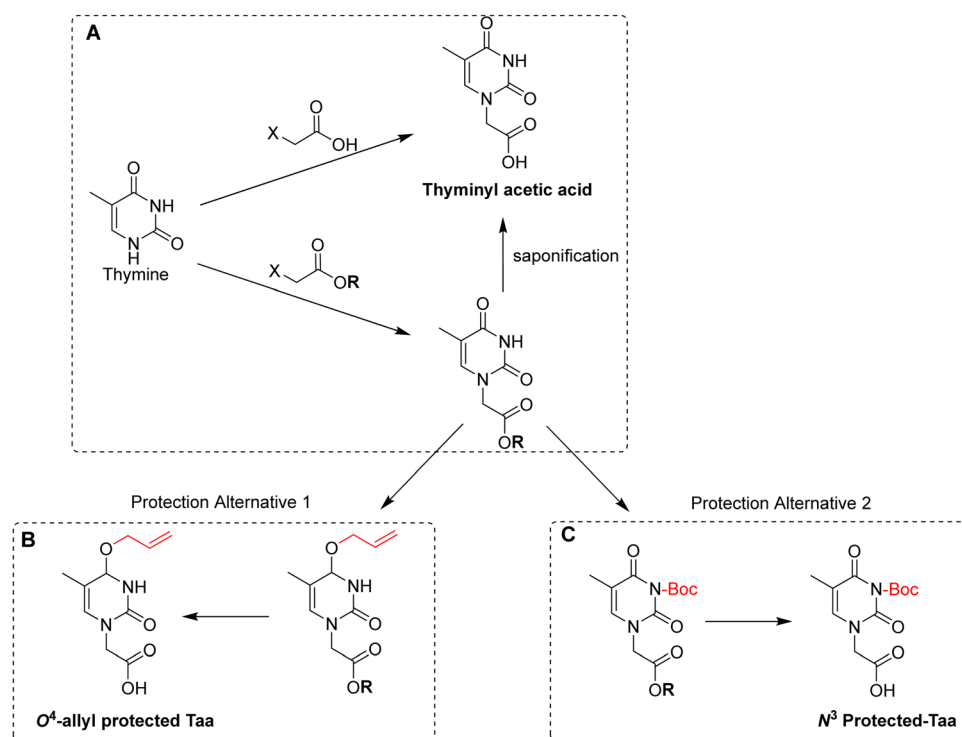
of the exocyclic amino group are not common for the three nucleobases. The following discussion about the synthesis of the building blocks to be attached to the backbone is divided into three sections: (i) derivatization of thymine; (ii) derivatization of cytosine and adenine; and (iii) derivatization of guanine.

Synthesis of thyminylic acid (Taa). The synthesis of Taa can be achieved directly by N-alkylation using haloacetic acid or by a two-step process using haloacetate esters, followed by their saponification (Scheme 13A). The initial studies published, as well as later ones, followed the second route using methyl or ethyl bromoacetate as alkylating agent.^{9,16,26,76,122,131} However, the overall yields achieved were moderate (50 to 65%). Thomson *et al.*⁴² used the protocol described by Jones *et al.*¹³² by means of chloroacetic acid, which gave a 71% yield. Nevertheless, the most extended acid used was the Br derivative introduced by Kosynkina *et al.*¹³³ and followed by other researchers^{25,47,63,78} since the yield increased to 80%. Of note,

Taa is commercially available and, hence, in many studies, its synthesis is not described.

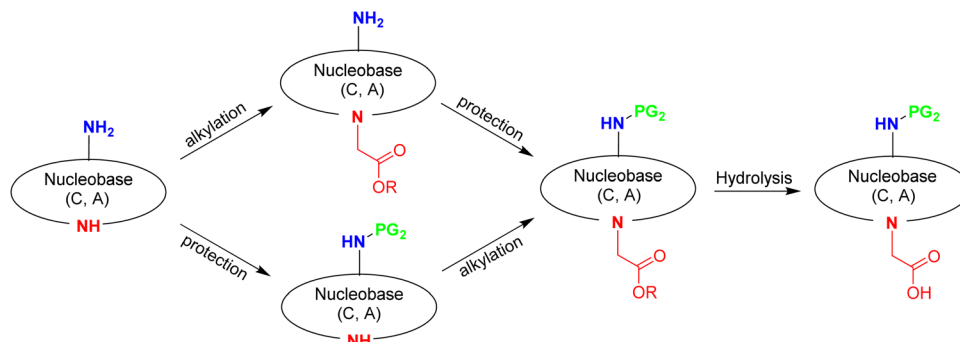
Although thymine does not require further protection, owing to on-resin aggregation during the synthesis of poly-T PNAs, the use of 'protected thymine' was found not only to solve this issue but also to improve coupling efficiency. One of the alternatives to protect thymine adopted the notion from *O*⁴-allyl protection of thymidine applied successfully in oligonucleotide synthesis¹³⁴ to prepare *O*⁴-allyl-protected Taa for PNA monomer synthesis.⁸¹ *O*⁴-allyl-protection is achieved by activating the *O*⁴-carbonyl oxygen of Taa using 2-mesitylene-sulfonyl chloride, followed by treatment with allyl alcohol to give the desired product in high yield (Scheme 13B).⁸¹ The second alternative consists of introducing a Boc protecting group on *N*³-position. In this synthesis, the benzyl ester of the bromoacetic acid was used for the alkylation. Thus after the introduction of the Boc group, the product was subjected to catalytic hydrogenation to give the desired product in quantitative yield (Scheme 13C).⁸³

Synthesis of protected exocyclic amino cytosinyl/adeninyl acetic acid (Caa/Aaa). As stated earlier, cytosine and adenine require the protection of their exocyclic amine and also have to be derivatized with a carboxylic acid in their endocyclic amino group. The sequence of reactions to achieve these derivatives could be alkylation followed by protection, or protection followed by alkylation. Contrary to the thymine derivative, the alkylating agent is always a bromoacetate ester, and therefore a final hydrolysis step is needed (Scheme 14). There is no consensus about the most convenient route in terms of efficiency



Scheme 13 (A) Two basic routes for the synthesis of thyminylic acid; (B) synthesis of *O*⁴-allyl-protected thyminylic acid and (C) synthesis of *N*³-Boc-protected thyminylic acid.





Scheme 14 Two basic routes followed in the synthesis of protected exocyclic amino cytosinyl and adeninyl acetic acid.

and final yield, and some authors even follow opposite routes for Caa and Aaa synthesis.^{26,43,47}

The most relevant synthesis described in the literature for Caa and Aaa derivatives can be found in Tables 4 and 5.

It is important to highlight that adenine is more complex than cytosine because it has more reactive sites. For instance, carbamate formation can occur at both N^1 and N^6 although the N^1 carbamate is hydrolyzed during the work-up. When N^6 of adenine is protected, the alkylation can take place at N^7 and N^9 , but the product can be purified by crystallization.⁴²

The initial reports of synthetic procedures were devoted to the use of Z as protecting group (Table 3, #1; Table 4, #1). The laboratory of Nielsen-Buchardt carried out the protection and then the alkylation for Caa¹⁷ and then the opposite, alkylation–protection for Aaa.¹⁵ In a later study, the same group found that the use of benzyl chloroformate reagent gave some problems for Aaa, but the more sophisticated Rappoport's reagent ($\text{BzIOCOImEt}^+ \text{BF}_4^-$) rendered better results.⁴³ However, Thomson *et al.*,⁴² using a protection–alkylation route, found that Rappoport's reagent, which in their opinion was tedious to prepare, gave a mixture of difficult purification. Thus, in this case, benzyl chloroformate was preferred.

Benzoyl^{47,75,135} (Table 3, #3; Table 4, #2) and anisoyl (Table 3, #5; Table 4, #3)^{25,63} were proposed as acyl protecting

group for both cytosine and adenine. Nevertheless, for the synthesis of Caa, the *t*-butylbenzoyl group has also been described. The introduction of this protecting group aimed to avoid the precipitation of the final monomer during its incorporation into a PNA sequence, as observed in the case of being protected by the benzoyl and anisoyl groups (Table 3, #2).^{63,76}

The use of TFA labile protecting groups such as Mmt (Table 3, #4; Table 4, #4), Boc (Table 3, #7; Table 4, #6), Bhoc (Table 3, #6; Table 4, #5), Cl-Bhoc (Table 3, #8; Table 4, #7), and Bis-*N*-Boc (Table 3, #9; Table 4, #8) had been gradually implemented due to: (i) the search of protecting groups friendlier to be removed; (ii) improved solubility of the final monomer; and (iii) compatibility with protecting groups of the amino of the AEG backbone removable in mild conditions. Mmt has been used to protect the nucleobases in combination with Fmoc⁶⁷ and Dde²⁶ as AEG protecting groups. In these two studies, the Caa derivative was synthesized similarly (protection–alkylation), but Aaa was prepared using both protection–alkylation⁶⁷ and alkylation–protection strategies.²⁶ Bhoc to protect nucleobases is the most widely used approach when Fmoc is the protecting group at the N-terminal of the AEG backbone since these monomers are commercially available. However, few studies describe the synthesis of these monomers. The Bhoc protecting group is preferred over Boc because it confers

Table 3 Protecting groups used in the synthesis of Caa

#	PG ₂	Reagent	Ester	Route ^a	Yield%	Ref.
1	Z	Benzyl chloroformate, pyridine Benzyl chloroformate, DMAP	Methyl <i>t</i> -Butyl	P + A P + A	56/40 ^b 43/59	16 and 43 42
2	<i>t</i> -Butyl benzoyl	<i>t</i> -Butyl benzoyl chloride, TEA	Methyl Ethyl	P + A P + A	61/36 46/63	63 76
3	Benzoyl	Benzoyl chloride, pyridine	Benzyl Methyl	A + P P + A	79/56 62/45	135 47
4	Mmt	Mmt-Cl, <i>N</i> -ethylmorpholine (NEM)	Methyl	P + A	42/66 45/66	67 26
5	An	Anisoyl chloride, pyridine	Methyl	P + A	93/48 81/82	76 and 78
6	Bhoc	Benzhydrol, CDI	Benzyl	A + P	91/71	66
7	Boc	<i>t</i> -Butanol, CDI	Benzyl	A + P	92/65	80
8	Cl-Bhoc	4,4'-Dichlorobenzhydrol, CDI	Benzyl	A + P	92/76	80
9	bis- <i>N</i> -Boc	(Boc) ₂ O, TEA, DMAP (cat.) (a) (Boc) ₂ O, DMAP (b) NaHCO ₃	Ethyl methyl	A + P P + A	—/83 97/69–78	59 58, 60 and 61

^a P = protection, A = alkylation. ^b Yields correspond to ref. 43.



Table 4 Protecting groups used in the synthesis of Aaa

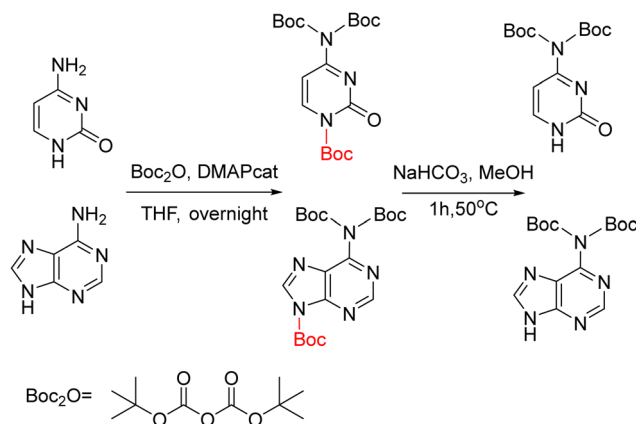
#	PG ₂	Reagent	Ester	Route ^a	Yield %	Ref.
1	Z	PhCH ₂ OCOImEt ⁺ BF ₄ [−] (Rapoport's reagent)	Ethyl	A + P	73/63 ^b	16 and 43
		Benzyl chloroformate, NaH	<i>t</i> -Butyl	P + A	44/53	42
2	Benzoyl	Benzoyl chloride, pyridine	Methyl	A + P	75/46	47
			Ethyl		44/35	75
3	An	Anisoyl chloride, TEA, or pyridine	Methyl	P + A	82/37	63
			Methyl		74/37	76
			<i>t</i> -Butyl		74/54	
			Ethyl	A + P	64/70	
4	Mmt	Mmt-Cl, pyridine -NEM, or NaOH	Methyl	P + A	77/52	67
			Ethyl	A + P	70/78	26
5	Bhoc	Benzhydrol, CDI	Benzyl	A + P	72/76	66
			Ethyl		68/69	31
6	Boc	<i>t</i> -Butanol, CDI	Benzyl	A + P	84/47	80
7	Cl-Bhoc	4,4'-Dichlorobenzhydrol, CDI	Benzyl	A + P	84/73	80
8	bis- <i>N</i> -Boc	(Boc) ₂ O, TEA, DMAP (cat.)	Ethyl	A + P	—/61	59
		(a) (Boc) ₂ O, DMAP	Benzyl	P + A	95/70	58 and 60
		(b) NaHCO ₃	Methyl		55 (overall)	61

^a P = protection, A = alkylation. ^b Yields correspond to ref. 43.

greater solubility to the final monomer, and it shows higher lability to TFA (1% of TFA).⁶⁶ Like Boc, Bhoc can be removed at the same time as the PNA chain is cleaved from the solid support. Given its high acid lability, Bhoc was not a good candidate as nucleobase protector when the synthesis of protected PNA fragments for convergent strategy was the target. To increase acid stability, Cl-Bhoc was studied. However, it was still found to be too labile. In this case, the best alternative was found to be Boc.⁸⁰

The bis-*N*-Boc group (Table 3, #9; Table 4, #8) was first described for adenine protection¹³⁶ and later as an intermediate in the synthetic route of Boc-protected purines.¹³⁷ The implementation of this protecting group in the synthesis of PNA monomers, independently carried out by Wojciechowski and Hudson⁵⁹ and Porcheddu *et al.*,⁶⁰ was driven by the high cost associated with the preparation of the Bhoc derivative. Furthermore, the introduction of Bhoc requires CDI or triphosgene, and Bhoc is not stable to catalytic hydrogenation (H₂/Pd) or even mild acidolysis.⁵⁹ The bis-*N*-Boc group has a double advantage because, on the one hand, it improves the solubility of the final monomer and, on the other hand, the absence of H, at position 6 avoids the interchain aggregation, thereby facilitating elongation of the PNA chain, similar to the role of Pro in peptide chemistry.¹³⁸ While Wojciechowski and Hudson carried out first the alkylation and then the protection, Porcheddu *et al.* did the opposite. In both cases, the bis-*N*-Boc group was introduced using readily affordable reagents such as Boc₂O on a large scale without significant purification of intermediates. Although performing the alkylation first prevents Boc introduction in other positions of the nucleobase, the strategy of Porcheddu *et al.* is more convenient in terms of yields and purity of the final products and has been adopted by other groups.^{58,61} In this strategy, the extra Boc introduced in position 1 in the case of cytosine and 9 in case of adenine, is easily removed by a short treatment with a NaHCO₃ solution (Scheme 15).

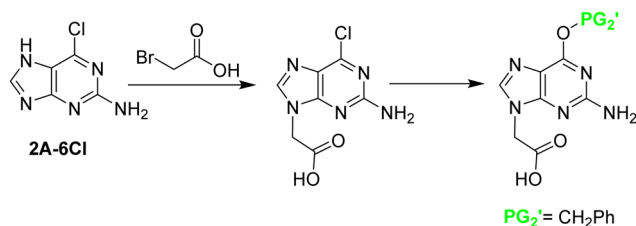
Synthesis of protected exocyclic amino guanyl acetic acid (Gaa). The preparation of Gaa is the most challenging among

Scheme 15 The synthesis of bis-*N*-Boc cytosine and adenine.

the bases because it is a polyfunctional molecule bearing different reactive points. The first problem to tackle is the poor solubility that guanine shows in a large number of solvents. On the other hand, direct alkylation of guanine is not regio-selective and it renders a mixture of alkyl derivatives of difficult purification. For this reason, derivatives of guanine, such as the 2-amino 6-chloropurine (2A-6Cl), are used as starting material for the preparation of Gaa.

2A-6Cl changes the reactivity of the molecule and renders alkylation at position 9 as the main product, along with alkylation at position 7. In the first route described by the Nielsen-Buchardt laboratory, 2A-6Cl was directly alkylated using bromoacetic acid, followed by the Cl substitution by a benzyloxy protecting group. The amino group at position 2 was not protected because of its low reactivity (Scheme 16). The overall yield obtained was 23%. However, this derivative presented two further problems; the benzyloxy group was not stable to the TFA treatment used to remove the Boc group, which was protecting the backbone in the final monomer, and the free amino at position 2 was partially acetylated during the capping step in the chain elongation process.⁴³

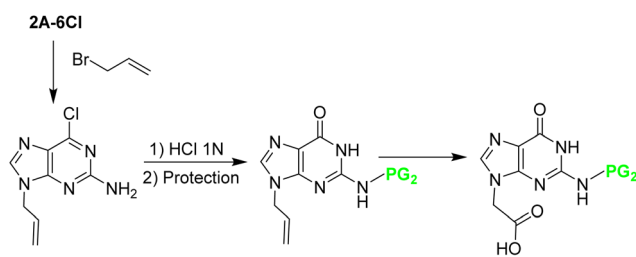




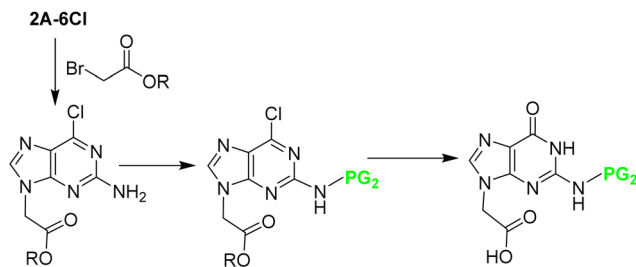
Scheme 16 Synthesis of O^6 protected Gaa from 2A-6Cl: N^9 alkylation and 6-Cl replacement by 6-O- PG_2 .

Later, Thompson *et al.*⁴² developed an alternative strategy to circumvent some of the problems previously mentioned. First, 2A-6Cl was alkylated with allyl bromide, giving a ratio of 3 : 1 between regioisomers at positions 9 and 7, which were easily separated. The Cl derivative was then converted into the guanidine one by refluxing with HCl, and the amino at position 2 was protected with the Z group. Finally, the allyl group that was masking the carboxylic acid, was submitted to ozonolysis followed by oxidation leading to Gaa (Scheme 17). All steps except the allylation, which requires purification, were performed with relatively good yields. The target product was obtained with an overall yield of 40%.

Although the synthetic route described before gives an acceptable overall yield, it was not generally implemented by other research groups. Instead, much attention was given to the procedure starting from 2A-6Cl, in which it is first alkylated, followed by the 2-amino protection, and, finally, hydrolysis of the ester with the concomitant replacement of the 6-Cl by 6-OC (Scheme 18). This methodology has been used to synthesize Gaa protected with Mmt,^{26,67} Bhoc,⁶⁶ Cl-Bhoc, Boc⁸⁰ and



Scheme 17 Synthesis of N^2 protected Gaa from 2A-6Cl: N^9 allylation; conversion of 6-Cl to the O derivative; N^2 protection; oxidation of the N^9 alkene to N^9 acid.



Scheme 18 Synthesis of N^2 protected Gaa from 2A-6Cl: N^9 alkylation; N^2 protection; conversion of the 6-Cl to O derivative.

iso-butyryl (iBu),¹³⁹ achieving moderate yields for the first two mentioned protecting groups (30–40%), but a good yield for the rest (around 70%).

By a similar route, a Gaa derivative with an extra protection at the O6 position was obtained. After the alkylation with the benzyl bromoacetate, 6-Cl was replaced by 6-OBzl. This reaction took place with concomitant removal of the benzyl ester, the free acid was again esterified in form of ethyl ester, and position 2 was protected in the form of iBu⁷⁵ or as bis-*N*-Boc.⁵⁹ Although these routes involved more steps, the overall yield was approximately 40% in both cases. The advantage of these derivatives was their solubility and minimization of the inter-chain aggregation caused by hydrogen bonds during the PNA chain elongation.

Porcheddu *et al.*⁶⁰ attempted to directly protect guanine in a similar way as they did with adenine using Boc₂O. In addition to bis-*N*-Boc in the amino at position 2, the Boc group also introduced at positions 1 and 9. However, in contrast to what happened with Aaa, in this case, the extra Boc groups could not be removed. But, when 2A-6Cl was used as starting material, the introduction of Boc at position 1 did not take place and the one at position 9 could be easily removed, as it was in the case of C and A (Scheme 15). After that, alkylation, followed by the substitution of the 6Cl by O, was carried out. That was done in two steps, the first by reaction with trimethylamine to give the ammonium salt and finally hydrolysis with NaOH. The overall yield was 50%. Similarly, Huan *et al.*⁶¹ followed the same synthetic scheme using the 6-OBzl guanine instead of 2A-6Cl as starting material. The benzyloxy moiety present in the final PNA monomer favours its solubility in organic solvents.

As mentioned earlier, the direct alkylation of the guanine is not a convenient starting point, but the synthesis of Gaa starting by acylation of the amine group at position 2 of guanine is possible. However, modification of position 2 alone is not enough to achieve regioselective alkylation thus, this step requires a further purification.⁶³ Alternatively, the incorporation of the diphenylcarbamoyl derivative at position 6 was shown to improve alkylation regioselectivity in the case of glycosidation.¹⁴⁰ In the case of alkylation, this substitution also enhanced the regioselectivity of the alkylation at position 9,^{25,76} but this improvement was found to be dependent on the alkylating reagent.⁷⁶

Synthesis of protected psNB acetic acid (^SUaa and Daa). The so-called pseudo-complementary peptide nucleic acid (pcPNA) is described as the PNA in which thymine and adenine are replaced by the modified nucleobases 2-thiouracil (^SU) and 2,6-diaminopurine (D) respectively. The main characteristic of this pair is that are destabilized when hybridizes among them but have a stronger binding with the respective adenine and thymine (Fig. 4A).^{141,142} Thus, double-strand pcPNA have the ability of effect double duplex invasion of DNA sites that induce critical changes in the biological and the physicochemical properties of the DNA (Fig. 4B).^{128,143}

Although pcPNAs were very promising, their use has not been extended as expected because it is not easy to get the monomers from commercial sources. Initially, U and D



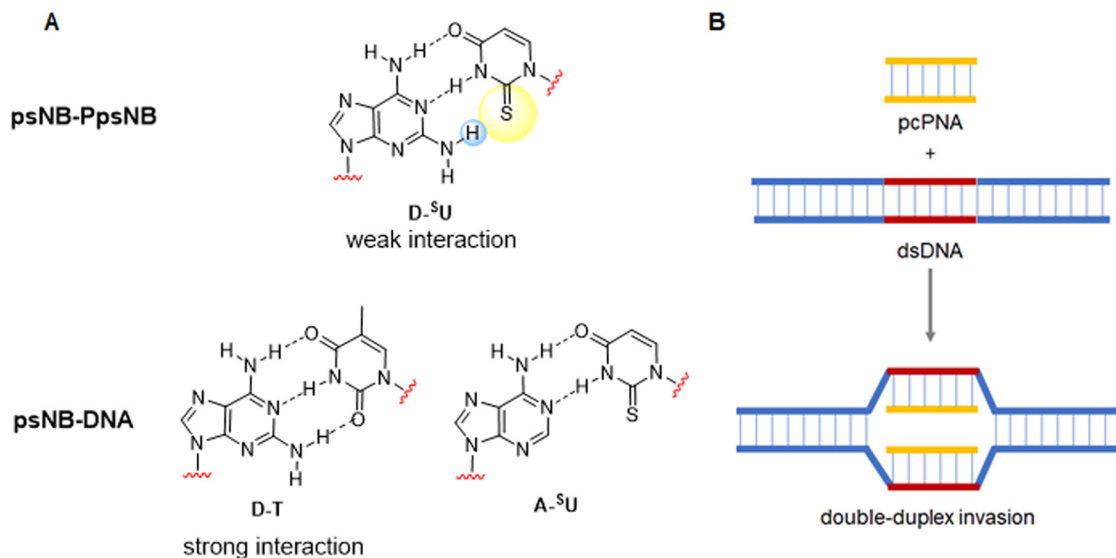


Fig. 4 (A) Pattern of H-bonding between D-SU, D-T and A-SUs. (B) Double-duplex invasion of pcPNA into double-stranded DNA.

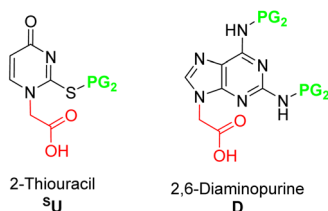
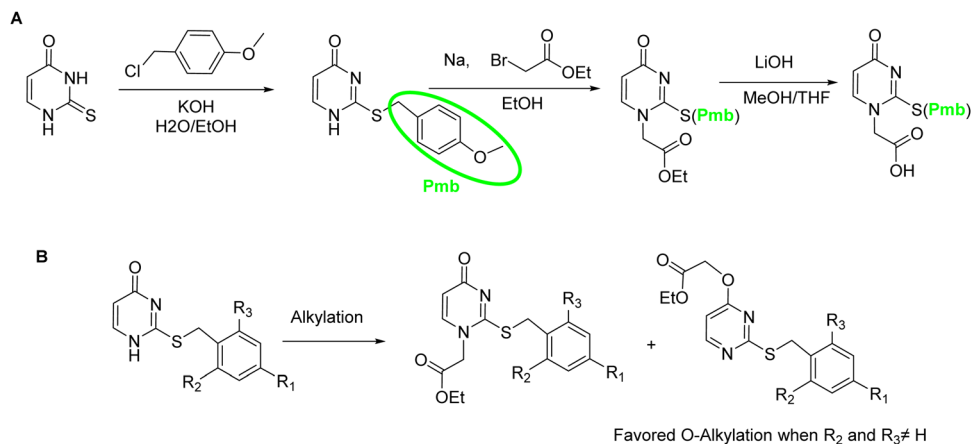


Fig. 5 Protected psNB acetic acid.

monomers were only described for Boc/Z strategy^{128,144,145} but later, these monomers were also protected in a compatible Fmoc/Bhoc way.^{84,146} Thus, like the canonical nucleobases, ^SU and D need to be protected and derivatized with a methyl carboxylic acid group (Fig. 5).

The sulfur atom on ^SU must be protected to avoid alkylation thus, when working with this nucleobase, the sequence of protection followed by alkylation is mandatory (Scheme 19A).

The main protecting group used is *p*-methoxybenzyl (Pmb). This group was thought to be only compatible with a Boc-protected backbone because its high acidic stability. For that reason, SU monomers compatible with Fmoc chemistry were not available for a long time. It was not until 2017 that Sugiyama *et al.*⁸⁴ introduced the 2-methyl-4-methoxybenzyl as protecting group. The additional methyl group at position 2 of the phenyl ring conferred higher acidic lability and enabled the removal under the cleavage conditions used at the end of the Fmoc strategy. Interestingly, the ring electron enrichment due to the methyl substituent provoked the competition of O-alkylation with the desired at position *N*¹. This secondary reaction was not observed when ^SU was protected with Pmb. In a more recently study, Hudson *et al.*¹⁴⁶ examined the need for SU protection. This study explored the use of protecting groups for 2-thiouracil such as trimethoxybenzyl, 2,4-dimethoxybenzyl, and 2-methoxybenzyl. Their synthesis and acidic lability were tested and compared with 2-methyl-4-methoxybenzyl, and



Scheme 19 (A) Synthesis of s-protected ^SUaa. (B) O-Alkylation of protected ^SU.



4-methoxybenzyl. The first observation was that the more electron-donating substituent on the ring, the more favored O alkylation *vs.* N^1 . Thus, the trimethoxybenzyl group resulted not useful. For the other mentioned groups the amount of O alkylation was variable (Scheme 19B). However, the most unexpected finding was that acidolysis of 4-methoxybenzyl-protected thiouracil occurred rapidly under 95% TFA treatment, and by using 2% TFA, 1% TES in CHCl_3 in 2 h the deprotection was almost complete. The conclusion of this observation was that the Pmb group would be removed at the same time that Boc, thus the S-protection should be not necessary for the chain elongation. This was demonstrated by synthesizing one PNA oligomer introducing the ^5U monomer unprotected.

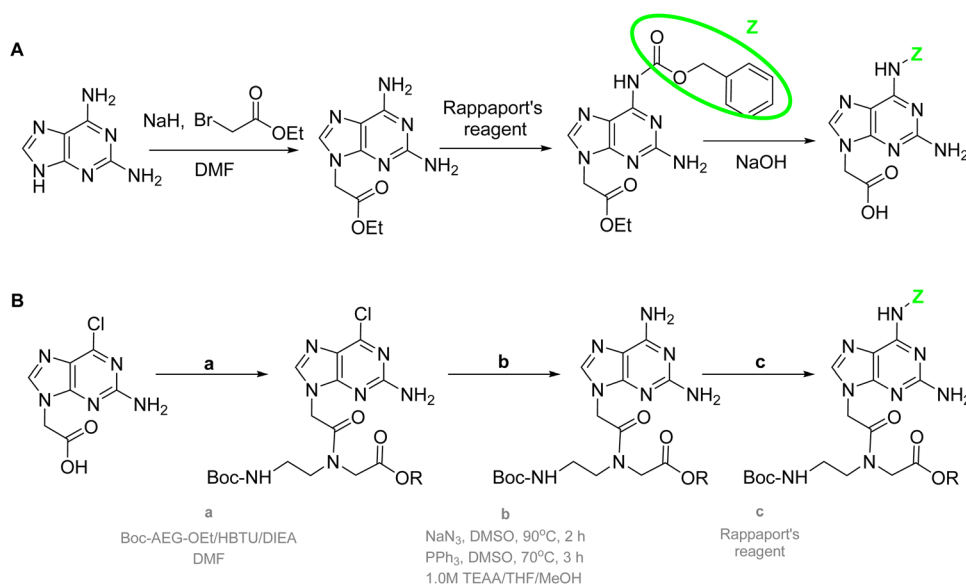
Initially, for the synthesis of the Daa the preferred reaction sequence was alkylation followed by protection as in the case of Aaa (Scheme 20).^{144,145} However, it is remarkable that only the amino group at position 6 was protected because the lower reactivity of the amino group at position 2. Thus, when the D-PNA monomer is used for PNAs synthesis, once the first unit is incorporated into the sequence, the capping step should be omitted. This protocol was the only available for long time, but in 2013, Ackermann *et al.*¹⁴⁷ proposed a new synthetic protocol (Scheme 20B). The new synthetic scheme instead of using D as starting material, used 2A-6Cl as in the case of G. The alkylation

of this nucleobase was well known as described in the previous section, but the innovative step of this synthetic route was to perform first the condensation between the nucleobase and the protected AEG backbone and then conversion of Cl into NH_2 followed by protection of it.

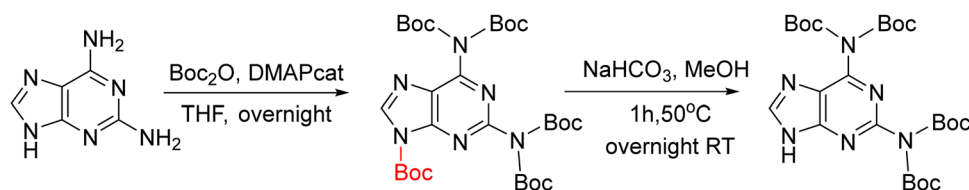
The D protection compatible with Fmoc strategy was developed similarly to protect adenine using bisBoc. Like in the case of adenine, in this strategy an extra Boc group is introduced in position 9 that is easily removed by a treatment with a NaHCO_3 solution (Scheme 21).^{83,84,148} The alkylation had been described using the benzyl ester of bromoacetic acid followed by catalytic hydrogenation⁸³ or ethyl ester of bromoacetic acid followed by NaOH hydrolysis.^{84,148}

4.3 Assembly of the two subunits onto the final monomer

Once the two subunits have been synthesized, they have to be linked through an amide bond. For this purpose, a great arsenal of coupling cocktails has been used and summarized in Table 5. The analysis of the data shows that the amide bond formation between the two moieties is not a particularly demanding reaction since the yield values are moderate to good regardless of whether mild derivatives such as *N*-hydroxy-succinimide (OSu) or pentafluorophenyl (Pfp) mediate the coupling, or stronger ones, such as derivatives of 1-hydroxy-7-azabenzotriazole (HOAt) and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine



Scheme 20 (A) Synthesis of protected Daa. (B) Alternative route by condensing the nucleobase and the protected backbone before the protection.



Scheme 21 The synthesis of bis-*N*-Boc protected D.



Table 5 Coupling conditions used for the synthesis of esters of PNA monomers

#	PG ₁	PG ₂	Coupling conditions ^a	Yield	Ref.
1	Boc	—	PfpOH/DCC (Taa)	Not reported	16
		—	HBTU/DIEA (Taa)	69–75%	109
		—	EDC·HCl/DhbtOH (Taa)	High yield	100
		Z	DhbtOH/DCC (Caa)	Not reported	17
		Z	DhbtOH/DCC (Aaa) and PyBroP(Gaa)	99% for T and C, 63% for G, and 23% for A	15 and 43
		Acyl	DhbtOH/DCC (Aaa, Caa) and PyBroP (Gaa)	65–67%	75
2	Fmoc	Z	EDC·HCl (Taa, Caa) and BOP/HOBt (Aaa, Gaa)	55–80%	42
		Mmt	TOTU/DIEA	52–78%	67
		Acyl	HBTU/HOBt/DIEA	40–85%	76
		Boc	HOBt/DCC	70–85%	57
		Boc	BOP/HATU	Not reported	82
		Boc	EDC·HCl/HOBt	77%	83
		bis- <i>N</i> -boc	DCC/NHS(Aaa), EDC·HCl (Caa) and EDC·HCl/HOBt (Gaa)	High yields	59
		bis- <i>N</i> -boc	IBC/NMM (Aaa) and Piv-Cl/NMM (Caa, Gaa)	85%	60
		bis- <i>N</i> -boc	EDC·HCl	70–75%	58
3	Mmt	—	PfpOH/TEA (Taa)	70%	149
		Acyl	DIC/HOObt/NEM	> 80% for T, A, C and 49% for G	63
		Acyl	50% propylphosphonic anhydride in EtOAc/DIEA (Taa) or TEA (Caa) and TOTU/DIEA (Aaa, Gaa)	64–91%	25
		Boc	HATU/2,6-lutidine/DIEA	85–95%	62
4	Azide	Bhoc	EDCl/4-DMAP (Taa), Piv-Cl/NMM (Aaa, Caa) and TOTU/DIEA (Gaa)	95% for C and 76–80% for A, T, G	66
5	Bts	Bhoc	DCC/HOBt	68–82%	74
6	Dde	Mmt	DCC/HOBt/NMM (Taa) and PyBroP/DIEA (Caa, Aaa, Gaa)	75% for C, and < 50% for A, C, G	68
			50% propylphosphonic anhydride in DMF/NEM (Taa) and PyBroP/DIEA (Aaa, Caa, Gaa)	77% for T and < 50% for A, C & G	26
7	NVOC	Acyl	50% propylphosphonic anhydride in EtOAc/DIEA (Taa, Caa) and BOP/HOBt/DIEA (Aaa, Gaa)	98% for T, 87% for C, A, and 81% for G	78
8	<i>p</i> NZ	Bis- <i>N</i> -boc	DIC/HOAt	82% for T and 50–64% for G, A, C	61

^a BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DCC = *N,N'*-dicyclohexylcarbodiimide; DIC = *N,N'*-diisopropylcarbodiimide; EDC·HCl = 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; HBTU = *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt = 1-hydroxy-7-azabenzotriazole; HOBt = 1-hydroxybenzotriazole; PyBroP = bromotri(pyrrolidino)phosphonium hexafluorophosphate; TOTU = *O*-[cyano(ethoxycarbonyl)methyleneamino]-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; DhbtOH = 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; IBC = isobutylchloroformate; Piv-Cl = pivaloyl chloride; NMM = *N*-methylmorpholine; NEM = *N*-ethylmorpholine; HOObt = 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazin and NHS = *N*-hydroxysuccinimide.

(DhbtOH), do. However, the protected nucleobase acetic acid derivative to be introduced seems to be the main factor affecting the yields, being in general higher in the case of pyrimidines than purines.

Upon completion of monomer assembly, the C-terminal protection of the backbone (PG₃, Scheme 3) is removed (Table 6) and the monomer is then ready to be used for PNA SPS. The removal of PG₃ should be compatible with the N-terminal and nucleobase protection. Thus, *t*Bu esters failed to be used with Boc, thus making this protection scheme unsuitable for the purpose.^{58,150} Allyl is totally compatible with Fmoc/bis-*N*-Boc PNA monomers,⁵⁹ but its use requires Pd(0) catalyst [Pd(Ph₃P)₄], whose is moisture sensitive, hence is more complex to handle in non-organic chemistry labs. In this context, simple methyl and ethyl esters are the most used as PG₃. The removal of these esters in the presence of the Fmoc group is very often accompanied by the removal of Fmoc, which could be re-introduced *in situ* in the same reaction pot.^{58,67}

4.4 Alternative methods of PNA monomer synthesis

As described in the previous sections, the chemistry associated with the preparation of PNA monomers and then the PNAs themselves is complex. Such complexity has fuelled research into alternative methods for the preparation of these intriguing compounds. Herein, the two most representative alternative methods are discussed. The first comprises N-alkylation of the nucleobase by the PG₁-AEG-PG₃ backbone which was derivatized with a chloroacetyl moiety at the secondary amine. The second one involves a Multi-Component Reaction (MCR) approach, where four components are used in 'one pot' to synthesize the PNA monomer.¹⁵¹ MCRs are considered to be green reactions due to the excellent atom economy associated with them.

Synthesis by nucleobase alkylation with *N*-chloroacetyl-derivative of the protected AEG backbone. In 1995, Meltzer *et al.*¹⁵² described an alternative approach for synthesizing the PNA monomer using, *a priori*, a simple and direct strategy.



Table 6 Removal conditions used on the C-terminal PNA monomeric esters

S. no.	C-Terminal esters on PNA monomer	Ester removal conditions		Yield ^a	Ref.
1	Methyl ester	NaOH	THF/H ₂ O	Excellent	60
			MeOH	High	100
		LiOH	Dioxane/H ₂ O	High	63, 67 and 149
			Dioxane/H ₂ O	High	62
		1 M KOH in H ₂ O	THF/H ₂ O	Moderate	15, 43 and 75
			1 M Cs ₂ CO ₃ in MeOH/H ₂ O	Moderate	75
2	Ethyl ester	LiOH	THF	High	58
			THF/H ₂ O	High	17, 61 and 74
			NaOH/EtOH/H ₂ O	Moderate	15
3	<i>t</i> Bu ester	HCl	1,4-Dioxane	Moderate	42
		TFA	DCM/TES	High	25 and 78
		DCM/1,3-dimethoxybenzene		Moderate	76
4	Benzyl ester	H ₂ , Pd/C		Quantitative	59, 82 and 83
		NaOH/dioxane/H ₂ O		Excellent	66
5	Cyanoethyl ester	0.5 M DBU in acetonitrile			64
6	Allyl ester	Pd(pph ₃) ₄ , NMM in DCM		High	59

^a Quantitative = close to 100%, excellent = >90%, high = >80%, moderate = 60–70%, and low = 50%.

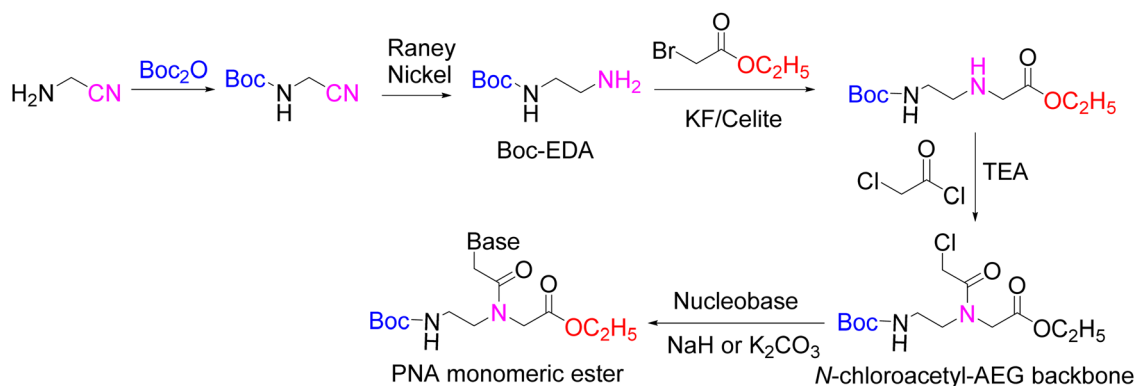
In this study, aminoacetonitrile was used as starting material for the synthesis of the backbone, which was protected with Boc. In this way, the use of ethylenediamine, which can undergo a bis-protection,¹⁰² was avoided. Then, by catalytic hydrogenation using Ni-RANEY[®] as catalyst the nitrile was converted to amino group which was alkylated with ethyl bromoacetate. Finally, acylation at the secondary amine was carried out by dropwise addition of chloroacetyl chloride giving the target product (Scheme 22).

Once the backbone is ready, direct N-alkylation is performed with three unprotected nucleobases, T, C, and A, in the presence of NaH/DMF, giving moderate yields. However, 2A-6Cl, used as G precursor, was alkylated in the presence of K₂CO₃ in DMF with 95% yield (Scheme 22).¹⁵² The ester hydrolysis were carried with NaOH, giving variable results, the worse being for G, where, in addition to the hydrolysis, the conversion of the Cl to O derivative occurred. Years later, a similar strategy was also described for T, using K₂CO₃ in DMF

and giving an excellent yield.¹⁵³ In this second example, Boc-ethylenediamine was used as the starting product.

These monomers with unprotected bases cannot be used for the synthesis of PNA, and although the authors claim that this strategy is also compatible for the preparation of the monomers with protected bases,¹⁵² there is no report in the literature showing the applicability of this method.

Multi-component reaction (MCR) for PNA monomer synthesis. Ugi four-component MCR (Ugi-4C) is a very useful strategy used in synthetic organic chemistry for the preparation of complex bis-amides in a one-pot reaction.¹⁵¹ As PNAs are structurally bis-amides, several groups have studied the use of this green Ugi-4C reaction for the preparation of PNA monomers and eventually for the elongation of the PNA chain.^{154–156} The four components needed for building the PNA monomers are: (i) alkyl amine; (ii) oxo (ketone/aldehyde); (iii) nucleobase acetic acid and (iv) N-protected 2-isocyanoethan-1-amine. Following this method, the preparation of the AEG backbone



Scheme 22 Synthesis of PNA monomeric through *N*-chloroacetyl-AEG backbone.



would require the concourse of formaldehyde ($R_1=R_2=H$) as the oxo component. As formaldehyde is not very friendly for use in the Ugi-4C,¹⁵⁷ most of these bis-amide monomers contain chiral centres. Of all the work described in the literature, Xu *et al.*¹³¹ has been the only group to describe a dimer.¹³¹

5. Conclusions and perspectives

PNA has gained special interest over the last three decades as nucleic acid analogues. While their synthesis resembles the solid-phase synthesis strategies used to prepare peptides, the process is rather more tedious and greatly depends on the choice of a suitable orthogonal/compatible permanent/temporary pair of protecting groups for the nucleobase and backbone, respectively. The suitability of different protecting groups on PNA not only makes the synthesis of this compound convenient but also could have an important impact into the development of PNA-peptide conjugates and PNA-DNA chimeras in SPS. The distinct strategies used to synthesize PNA have advantages and disadvantages. Thus, for example the use of Boc for the N-terminal AEG backbone is subjected to less side-reactions than when Fmoc is used. On the other hand, the use of Boc could require protecting groups for the nucleobases more difficult to be removed. Therefore, there is still considerable scope to explore other closely related options, as shown in Table 2. Regarding solid supports, the conventional polystyrene is the most used, although the use of PEG-based resins such as ChemMatrix and Tenta-Gel use to circumvent some of the synthetic problems associated with the interchain aggregation.

Although this review focuses only on the chemistry of the original PNA backbone (AEG backbone) and its monomer preparation, it also helps to understand ongoing research into the chemical modifications of the AEG backbone and nucleobases themselves to improve the properties of the monomers during synthesis. Since PNA is similar to DNA from a structural perspective, modified PNA is likely to drive further research into its capacity as a potential drug candidate in the near future.

Author contributions

All authors have contributed to the preparation of the manuscript and agreed with the final version.

Conflicts of interest

There are no conflicts to declare.

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