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Synthesis of Labelled PNA Oligomers by a Post-synthetic Modification Approach

Beatriz G. de la Torre and Ramon Eritja*

Institut de Biologia Molecular de Barcelona, C.S.I.C., Jordi Girona 18–26, E-08034 Barcelona, Spain

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Abstract—The preparation of *t*-butoxycarbonyl (Boc)-protected O^4-(*o*-nitrophenyl) thymine peptide nucleic acid (PNA) monomer is described. This PNA monomer was incorporated into PNA oligomer sequences. The post-synthetic modification of the oligomers to yield fluorescently-labelled PNA oligomers was studied before and after the removal of the protecting groups. In both cases, the desired fluorescently-labelled PNA oligomer was obtained in good yields.

Peptide nucleic acids (PNA) are oligonucleotide analogues in which the negatively charged sugar-phosphate backbone has been replaced by a neutral backbone of *N*-(2-aminoethyl)glycine units with the common nucleic acid bases attached via a carbonyl methylene linker.[1] PNA oligomers recognize and bind to a specific DNA or RNA strand with high affinity and selectivity.[2] Their chemical stability and their resistance to nucleases and proteases make PNA oligomers good candidates as therapeutic agents, diagnostic tools and probes in molecular biology. For most of these applications, the functionalisation and labelling of PNA are necessary.[3]

Labelled PNA can be prepared by modification of the N-terminal amino group.[4,5] This is the most common approach but the reactive amino group can only be used for the introduction of one modification. Also trifunctional amino acids such as cysteine or lysine or nucleobases carrying hexylamino groups have been introduced at various positions on the PNA oligomers to generate amino or thiol groups, which were further reacted with fluorescent labels.[6–8] Moreover, the introduction of labels by using modified PNA monomers has been described.[9,10] This method requires the previous synthesis of one monomer for each label. To avoid the need to synthesise a large number of different monomers, the reaction of fluorescent compounds to amino groups after the assembly of the oligomer was preferred.[6–8] In the present report we describe the synthesis of a novel PNA monomer (T^{NP}, Scheme 1) carrying an *o*-nitrophenyl group at position 4 of thymine. This group reacts with amines to generate 5-methyl-C derivatives (Scheme 2). When this reaction is performed with fluorescent compounds carrying amino groups, PNA oligomers bearing fluorescent compounds may be obtained. Previously, this post-synthetic method was assayed together with PNA monomers carrying base-labile protecting groups.[11] Unfortunately, the conditions used during the labelling reaction and removal of protecting groups (aqueous solutions of amines at 50 °C) were detrimental to the PNA integrity.[11] To avoid this problem we used *t*-butoxycarbonyl (Boc)-protected PNA monomers carrying acid-labile (benzyloxycarbonyl, Z) protecting groups for the nucleobases. The use of acid conditions for the removal of the protecting groups allows the isolation of the PNA-carrying T^{NP}. In this way, the labelling reaction can be performed in both solid phase and solution.

The preparation of PNA-monomers requires several steps to generate the properly protected *N*-(2-aminoethyl)glycine unit followed by *N*-acylation of the glycine derivative by a carboxymethylated nucleobase.[12,13] We followed this strategy to synthesise *N*-[2-(*t*-butoxycarbonylamino)-ethyl]-*N*-[(O^4-*o*-nitrophenylthymin-1-yl) acetyl] glycine (T^{NP}, Scheme 1). The *N*-(2-aminoethyl)glycine unit was synthesized as the Boc-protected cyanoethyl ester derivative.[14] 1-Carboxymethyl-O^4-(*o*-nitrophenyl) thymine was synthesized as described elsewhere.[11,15] Coupling of the two compounds was

*Corresponding author. Tel.: +34-93-400-6145; fax +34-93-204-5904; e-mail: recgma@cid.csic.es

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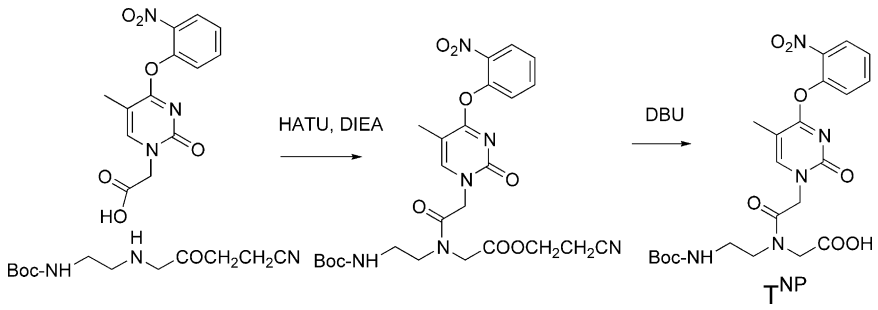
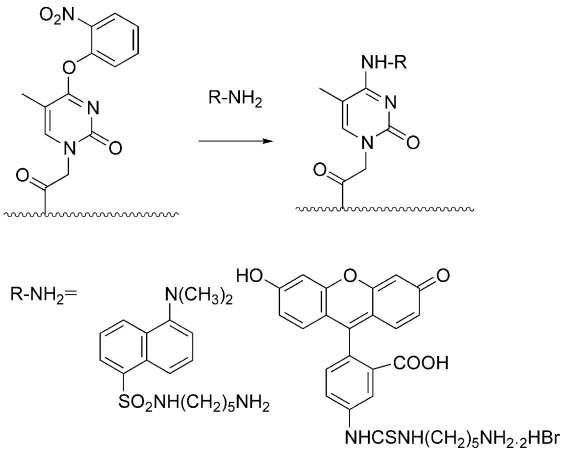


Scheme 1.



Scheme 2.

performed with *N*-[(dimethylamino)-*1H*, 1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) (1 equiv) and *N,N*-diisopropyl-*N*-ethylamine (DIEA) (2 equiv). The choice of the cyanoethyl ester instead of a methyl or ethyl ester was to avoid saponification that could provoke hydrolysis of the *o*-nitrophenyl group by nucleophilic attack, giving the undesired T-PNA monomer. To obtain the carboxylic acid, cyanoethyl ester was removed by β-elimination with a strong and non-nucleophilic base, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). In the presence of 0.5 M DBU in acetonitrile for 5 min at room temperature, loss of the nitrophenyl group was not detected. After silica-gel purification PNA monomer (T^{NP}) was isolated as triethylammonium salt in 70% yield.

PNA oligomer synthesis was carried out manually on a methylbenzhydrylamine (MBHA)-polystyrene support, following the synthesis cycle for the Boc/Z strategy described elsewhere.[16] The coupling efficiency of PNA monomer (T^{NP}) was similar to that of the natural bases obtained from commercial sources (Applied Biosystems). Coupling reaction was monitored by the Kaiser test,[17] and the absence of colour in the solution or resin beads indicated nearly quantitative yields (>95%).

Three PNA oligomer sequences containing monomer (T^{NP}) were synthesized: (A) Ac-T^{NP}TC AAC TCT-NH_2, and (B) Ac-T^{NP} GTG CTC ATG GTG-NH_2. During the assembly of sequences A and B, aliquots of the solid support were taken before and after the addition of T^{NP} monomer. They were deprotected, cleaved, and analysed by reverse-phase HPLC (RP-HPLC). In both cases, the addition of T^{NP} monomer was clearly observed by the formation of a new compound with a higher retention time, which had the expected molecular mass (Table 1). The hydrophobicity of the *o*-nitrophenyl group facilitated the purification of the full-length product.

The post-synthetic modification of PNA in solid phase was studied on sequence A: 10 mg of resin was treated with 75 μL of a solution 0.2 M of Dansyl cadaverine [5-dimethylaminonaphthalene-1-(*N*-(5-aminopentyl))sulfonamide, Molecular Probes Inc.] in *N*-methylpyrrolidone. After overnight incubation at 55 °C, the fluorescent solution was filtered and the resin was washed until no fluorescence was detected. The product was deprotected and cleaved from the resin under standard protocols. The crude was desalted by a Sephadex column (NAP-10) and then analysed and purified by RP-HPLC. The HPLC analysis revealed a major peak which was collected. The product was fluorescent and it had the expected mass, as judged by MALDI-TOF mass spectrometry (Table 1).

The post-synthetic modification of PNA in solution was studied on sequence B. First, supports carrying PNA sequence B was treated with a mixture of trifluoroacetic acid and trifluoromethanesulfonic acid (using dimethylsulfide and *m*-cresol as scavengers) as described.[16] The resulting product was purified by RP-HPLC obtaining the desired PNA oligomer carrying T^{NP} (Table 1). Aliquots of 0.1 mg were treated with dansyl cadaverine

Table 1. PNA oligomers prepared in this work

Sequence[a] (N- > C)	rt (min)[b]	Mass, found	Mass, calcd
TCAACTCT	11.8	2120.5	2119
(A) Ac-T^{NP}TCAACTCT	19.4	2549.0	2506
Ac-M^{DC}TCAACTCT	18.9	2745.7	2746
GTGCTCATGGTG	13.8	3316.4	3315
(B) Ac-T^{NP}GTGCTCATGGTG	19.5	3746.5	3745
Ac-M^{DC}GTGCTCATGGTG	19.1	3943.1	3942
Ac-M^{FC}GTGCTCATGGTG	22.0	4097.9	4098

[a]T^{NP} = *O*⁴-*o*-nitrophenylthymine, M^{DC} = 5-methylcytosine carrying dansylcadaverine (DC), M^{FC} = 5-methylcytosine carrying fluoresceinecadaverine (FC).
[b]HPLC conditions. Column: Nucleosil 120 C_{18}, (250×8 mm), flow rate 3 mL/min, a 25 min gradient from 0% B to 50% B. Solution A: 5% acetonitrile in 0.1% trifluoroacetic acid in water. Solution B: 70% acetonitrile in 0.1% trifluoroacetic acid in water.

or with fluorescein cadaverine [5-(5-aminopentyl)thioureidyl]fluorescein dihydrobromide salt, Molecular Probes Inc.). In both cases a solution of the amine (10 mM) in dimethylformamide containing 1% of DIEA was added to sequence B and allowed to react overnight at 55 °C. After the treatment, the resulting solutions were concentrated to dryness and desalted with a Sephadex G-25 column (NAP-10). The labelled PNA containing fractions were analyzed and purified by RP-HPLC (Table 1). Mass spectrometry confirmed that the desired product corresponded to the major peak on the chromatogram. In both cases, the conversion to the desired compound, estimated by comparison of the areas under the peaks, was higher than 70%.

In conclusion, we have shown that PNA monomer (T^{NP}) is a valuable intermediate for the introduction of fluorescent compounds or other compounds of interest in PNA oligomers, by nucleophilic displacement of the appropriate amino derivative. This method may be used to prepare several derivatives from the same PNA sequence. Also, this method may be used in combination of others[4–10] to introduce multiple labels into the same compound. For example, when monomer (T^{NP}) is placed at any position, the amino terminal group is still free to react with a second label. The introduction of two different fluorescent labels in PNA is of interest for the preparation of molecular beacons[8,18] for diagnosis and biomedical studies. Although PNA monomer (T^{NP}) was incorporated at the N-terminal position it may be introduced at any position on the PNA oligomer as shown elsewhere.[11]

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