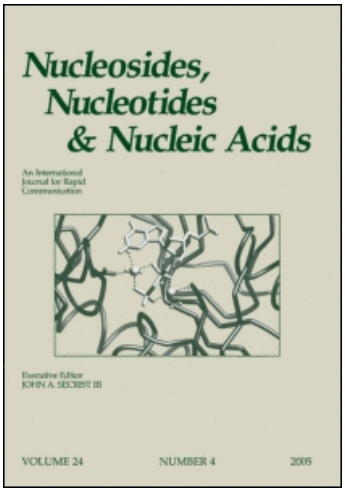

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SOLID SUPPORT POST-CONJUGATION OF AMINO ACIDS AND A PHENANTHROLINE DERIVATIVE TO A CENTRAL POSITION IN PEPTIDE NUCLEIC ACIDS

Jessica Sandbrink, Merita Murtola, and Roger Strömberg □ *Department of Bioscience and Nutrition, Karolinska Institutet, Novum, Huddinge, Sweden*

□ *A solid phase synthesis strategy for post-conjugation of amino acids and a phenanthroline derivative to peptide nucleic acids is described. The peptide nucleic acids, synthesized by 9-fluorenylmethyloxycarbonyl chemistry on TentaGel S Rink Amide resin, have an internally placed unit carrying an amino linker with 4-methyltrityl protection. Methyltrityl removal by mild acidic conditions and conjugation of amino acids or a phenanthroline derivative, via an amide or urea linker, was performed on-resin after completion of the chain assembly. This solid phase methodology resulted in excellent purities of the crude conjugates.*

Keywords PNA conjugate; PNA solid phase synthesis; RNA bulge

As part of our interest in recognition and cleavage of RNA bulges,[1−4] we have synthesized PNAs carrying bulge interacting elements, such as polar and stacking functionalities **3–7**, as well as a phosphate ester cleaving group **8** (in the presence of Zn^{2+}). In the present strategy we use PNA sequences with an internally placed diaminopropionic acid (Dapa) unit, serving as attachment point via its side chain amino group (Scheme 1). This amino "linker" is used to attach amino acids (glycine, arginine, histidine, tryptophan and pyrenylalanine) and a 5-amino-2,9-dimethyl-1,10-phenanthroline derivative via an amide or urea linkage, respectively.

The PNA sequences, *N*′-Ac-GAGT(Dapa)ACCGAGA-Lys-NH_2-C′ and *N*′-Ac-GGGT(Dapa)TCCTTAT-Lys-NH_2-C′, were assembled automatically on a solid support, TentaGel S Rink Amide resin, using FMOC-chemistry.[5] Undesirable N-acetylation of the free amine in peptide coupling reactions, due to remains from preceeding capping steps has been reported for solid phase synthesis of peptide-oligonucleotide conjugates (POCs).[6] This acetylation was almost completely avoided by a piperidine washing step prior to peptide coupling.[6] As post-conjugation of amino acids to the solid support

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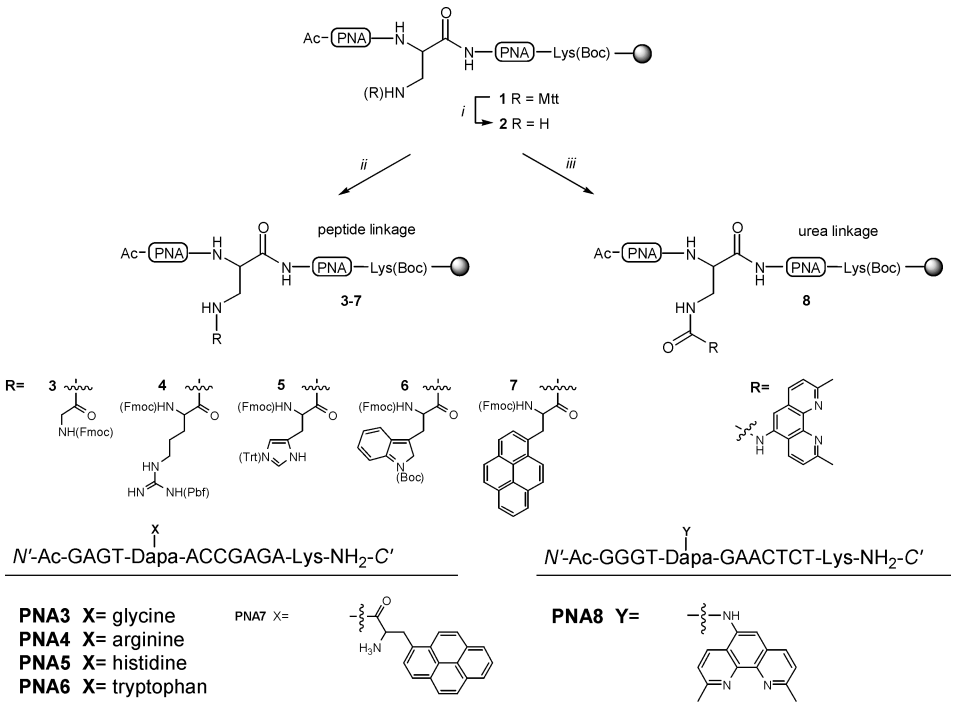

SCHEME 1 Solid phase post-conjugation of amino acids and a phenanthroline derivative to PNA. i) Treatment with 1% TFA/DCM (v/v) for 1 minute, repeated five times; ii) Amino acid, HBTU/HOBt, NMM, 30 minutes. Derivatives conjugated to the PNA sequence *N′*-GAGT(Dapa) ACCGAGALys-*C′*; glyine **3**, arginine **4**, histidine **5**, tryptophan **6** and pyrenylalanine **7**; iii) Phenyl carbamate of 5-amino-2,9-dimethyl-1,10-phenanthroline, NMM, overnight, giving **8**. PNA sequence: *N′*-GGGT(Dapa)TCCTTATLys-*C′*.

bound PNA resembles the situation of the POC synthesis we introduced a similar piperidine washing cycle at the end of the PNA synthesis protocol to consume potential reactive acetyl species remaining from the capping steps during the PNA assembly.

Prior to the post-conjugation, the amino protecting group of the Dapa-linker has to be removed selectively. Two different protecting groups, allyloxycarbonyl (Alloc) and 4-methyltrityl (Mtt), were evaluated for this purpose. In our hands, attempts to use Alloc as orthogonal protection caused problems in removal of the protection, using $PdCl_2(PPh_3)_2$, AcOH and Bu_3SnH as cleaving cocktail,[7] as well as difficulties in getting the subsequent conjugation reaction to work. The post-conjugation resulted in very low yields or no product at all, possibly due to remains from the Alloc cleaving cocktail on the support. The highly acid-labile methyltrityl (Mtt) group, on the other hand, was readily removed by mild acidic treatment without noticeable cleavage of the PNA from solid support. This was achieved by subjecting the solid-supported PNA to 1% (v/v) triflouroacetic acid (TFA) in dichloromethane (DCM)[8] (Scheme 1). A neutralization step is omitted

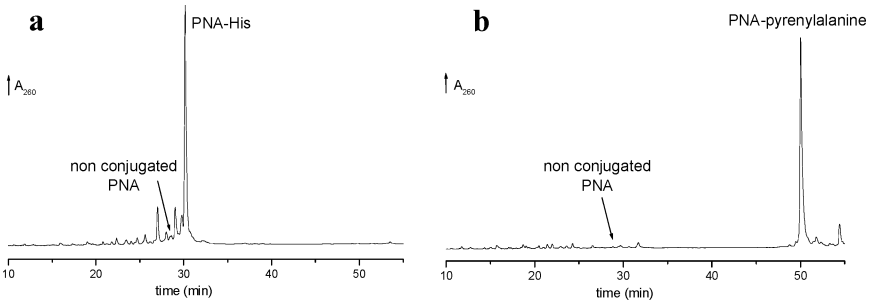

FIGURE 1 RP HPLC profiles of crude PNA-amino acid conjugates. a) PNA-histidine conjugate **5**; b) PNA-pyrenylalanine conjugate **7**.

and the free amine is left protonated after the Mtt removal in order to minimize potential N-acyl transfer (not observed under the conditions used) from the carboxymethyl nucleobase side-chain, five atoms further away. The amine is in situ neutralized in the subsequent conjugation reaction by adding an excess of base. All amino acids were conjugated to the PNA via an amide linkage, using HBTU/HOBt as condensation agents. To minimize racemization of the activated amino acids the relatively weak base *N*-methyl morpholine[9] (NMM) was used in coupling of all amino acids, except for glycine. Conjugation of the catalytic group was performed by reaction with the phenyl carbamate of 5-amino-2,9-dimethyl-1,10-phenanthroline,[4] to the PNA giving conjugate **8**.

Prior to cleavage of the PNA conjugates from solid support the N^{α}-Fmoc protections of all amino acids were removed by 20% piperidine in DMF. The PNA conjugates were cleaved from support by a mixture of TFA, triisopropylsilane (TIS) and water, thus yielding PNA *C*′-carboxamides. The peptide and urea coupling reactions were quantitative as judged from analysis by reversed phase HPLC (Figure 1). The PNA conjugates were purified by reversed phase HPLC and the authenticity of the conjugates were confirmed by mass analysis.

An efficient synthesis strategy for rapid solid phase preparation of PNA-amino acid and PNA-phenanthroline conjugates has been developed. The amino acids and the catalytic group are post-conjugated to the solid support bound PNA in a virtually quantitative yield, by reacting the free amino group of the linker with the activated amino acid unit or with the phenyl carbamate of the amino phenanthroline derivative. This synthetic methodology was designed to give access to a large diversity of amide or urea linked PNA conjugates. We are currently studying the interaction of PNAs **3–7** with target oligonucleotides as well as the cleavage of RNA by the 5-amino-2,9-dimethyl-1,10-phenanthroline PNA conjugate **8,** which will be reported later.

EXPERIMENTAL PROCEDURES

Post-Conjugation of Amino Acids to PNA

Mtt was cleaved off by subjecting the solid support bound PNA (**1**) (1 μmol) to 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) (80 μl) for 5 × 1 minute, whereupon the support was washed with DCM and NMP. Amino acid (0.20 mmol, 0.40 M), HBTU (0.19 mmol) and HOBt (0.19 mmol) was dissolved in NMP (442 μl). Base (0.53 mmol) DIEA (used for coupling of glycine) and NMM (used for coupling of all other amino acids) was added and the amino acid was pre-activated for 6 minutes by vortexing. The activated amino acid (0.1 mmol, 250 ul) was added to the support bound PNA **2** and the reaction mixture was vortexed for 30 minutes. The support was filtered and washed with NMP and DCM. The N^{α}-Fmoc was cleaved off by 20% piperidine in DMF (250 μl) 2 × 10 minutes and the support was washed with DMF and DCM. The PNA conjugate was cleaved from support by TFA/TIS/water (95/2.5/2.5) (500 μl) for 3 hours, freeze dried and purified by RP HPLC on a Phenomenex Jupiter (5 μm 250 × 4.6 mm) column at 60°C, 1 ml/minute. Four different linear gradients of 0.1% aq. TFA (eluent A) and 0.1% TFA in MeCN 50% (eluent B) were used. Gradient 1 (**PNA 3** and **5**): 0–30% B for 50 minutes. Gradient 2 (**PNA 4**): 0–16% B in 40 minutes. Gradient 3 (**PNA 6**): 7–20% B in 50 minutes. Gradient 4 (**PNA 7**): 10–35% B in 45 minutes. **PNA3**: M_{obs} 3365.5 M_{calc} 3366.3**, PNA4**: M_{obs} 3464.5 M_{calc} 3465.4, **PNA5**: M_{obs} 3445.8 M_{calc} 3444.4, **PNA6**: M_{obs} 3495.0, M_{calc} 3495.5, **PNA7**: M_{obs} 3580.0, M_{calc} 3581.0.

Post-Conjugation of 5-Amino-2,9-Dimethyl-1,10-Phenanthroline to PNA

Removal of Mtt from **1** was performed as above. To the solid support bound PNA **2** (1.0 μmol) was then added 5-amino-2,9-dimethyl-1,10-phenanthroline carbamate (5 μmol), NMM (20 μmol) and NMP (17 μl). The reaction was left over night and the PNA conjugate **8** was cleaved from solid support, using the same reagent as above, freeze dried and purified by RP HPLC on a Phenomenex Jupiter Proteo (5 μm 250 × 10.0 mm) column at 60°C, 3 ml/minute. A linear gradient of 10% B for 20 minutes, then 10–37% B for 20 minutes was used. **PNA8**: M_{obs} 3506.5, M_{calc} 3505.5.

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