Pergamon

Tetrahedron Letters 39 (1998) 7255–7258

TETRAHEDRON LETTERS

PNA Solubility Enhancers

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Received 28 April 1998; revised 16 July 1998; accepted 21 July 1998

Fmoc-protected hydrophilic amino acids were prepared and incorporated into peptide nucleic acid (PNA). N- and C-terminal modification of PNA did not affect its ability to hybridize to complementary nucleic acid targets as determined by dot blot, PNA-FISH and Tm experiments. The compounds allowed us to prepare several probes which were previously impossible to make, purify or use due to limited aqueous solubility or self-aggregation. © 1998 Elsevier Science Ltd. All rights reserved.

Peptide nucleic acid (PNA) is a non-natural polyamide which binds to RNA and DNA with a high degree of sequence specificity to form hybrids which are more thermodynamically stable than corresponding nucleic acid/nucleic acid duplexes.[1-3] Unlike traditional nucleic acid hybridization, binding of PNA to nucleic acid and the stability of the resulting complex is fairly independent of ionic strength.[3-4] Thus, PNA probe assays may be performed under "low salt" conditions whereby the inter- and intramolecular base pairing within the DNA or RNA target is destablized. As a result, PNAs can be used to access targets in dsDNA[5] and to detect sequences in highly structured regions of viral or ribosomal RNA.[6] The PNA backbone is also more chemically stable than is its sugar-phosphodiester counterpart. Furthermore, PNA is not known to be a substrate for any enzymes which degrade peptides or nucleic acids.[7]

Because it possesses these desirable properties, PNA is being investigated as an alternative to traditional nucleic acid probes in hybridization assays. However, a practical limitation which prevents widespread acceptance of PNA in diagnostic and research applications is its limited aqueous solubility and tendency toward self-aggregation. Generally, the solubility of PNA decreases as the polymer length increases.[8] Furthermore, the synthesis, purification and use of purine-rich PNA oligomers is known to be especially problematic.[8,9] Attempts to improve the solubility of PNA oligomers have included terminal modification with charged amino acids such as L-lysine[1] as well as modification of the aminoethylglycyl backbone.[10,11] These modifications, however, typically introduce reactive groups and/or chiral centers into the otherwise achiral and unreactive PNA structure.

In our efforts to develop probe-based diagnostic assays, the solubility and self-aggregation properties of PNA limited our ability to obtain many desired probes. This encouraged us to explore whether it would be possible to develop solubility enhancers which could be incorporated into PNA using our standard Fmoc-synthesis methodologies. Criteria considered during the design of potential enhancers included; lack of chirality, ease of synthesis, cost, and the exclusion of potentially reactive (i.e., nucleophilic) groups which could complicate probe labeling and immobilization. Ultimately, we opted to prepare N-(2-aminoethyl)-glycine (aeg) amino acids wherein the nucleobases of the standard PNA monomers were replaced with either neutral (**3**) or positively charged (**7**) hydrophilic moieties.

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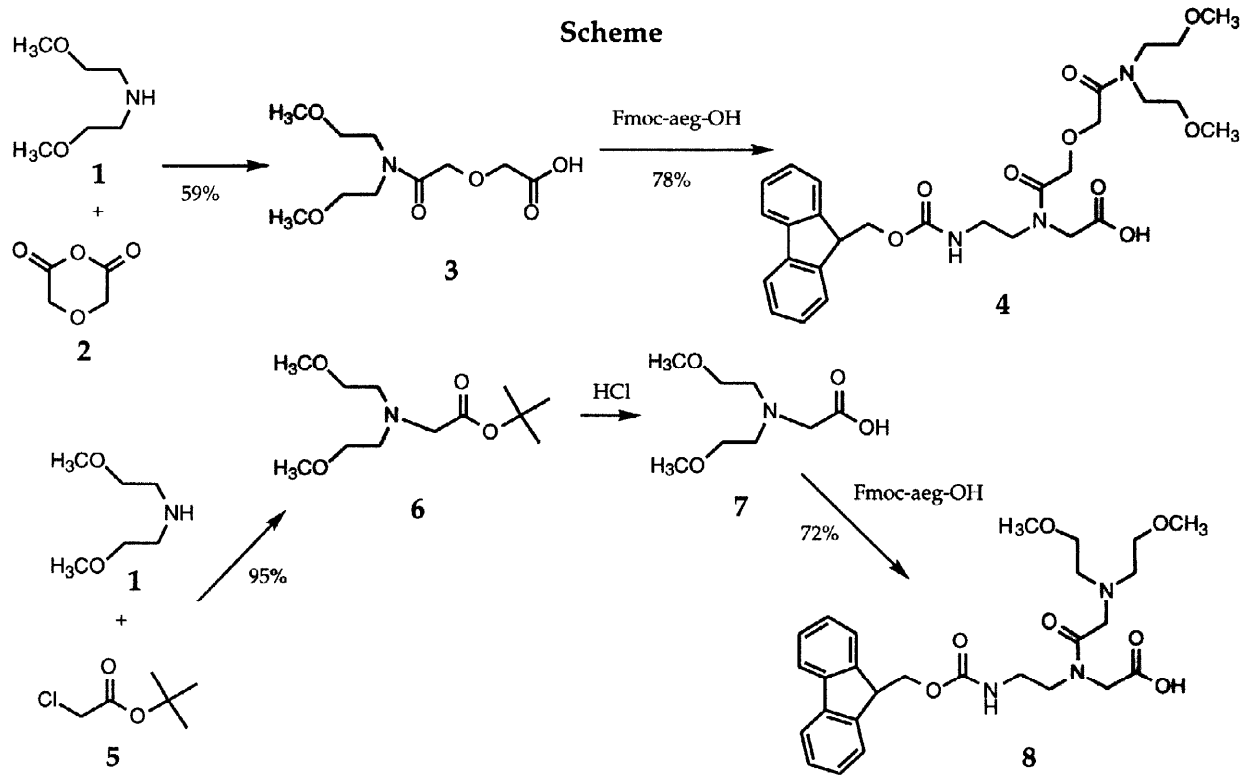
PII: S0040-4039(98)01581-0

As shown in the Scheme, synthesis of Fmoc-protected solubility enhancers 4 and 8 was accomplished by preparation of branched carboxylic acid derivatives 3 and 7.[12-14] The branched acids were then activated, by formation of their pivalic acid mixed anhydrides, for condensation with Fmoc-aeg-OH.[15] Thus, compounds 4 and 8 were obtained in high overall yield in two or three steps, respectively, from readily available inexpensive starting materials. Using commercially available equipment, monomers 4 and 8 were easily incorporated at any desired sequence position during PNA probe synthesis.

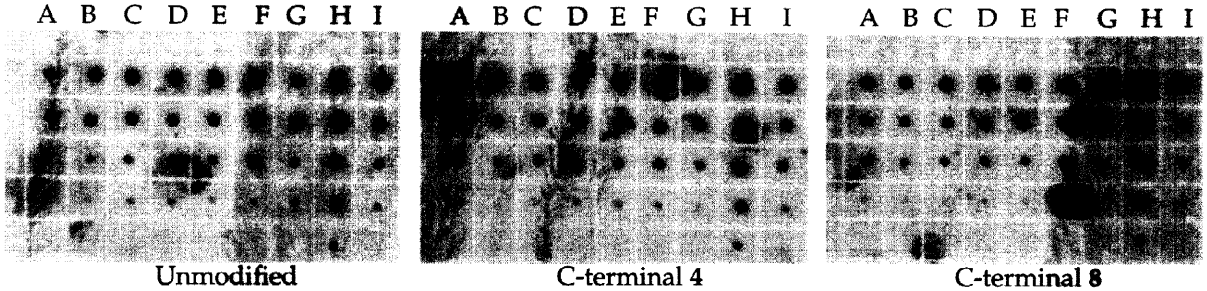
Scheme



Initial experiments were designed to assess whether the enhancers had any effect on PNA hybridization efficiency and signal. First, we modified the well behaved and well characterized probe, Flu-OO-CTG-CCT-CCC-GTA-GGA-NH_2 (Uni-Flu), wherein Flu and O represent fluorescein and the linker 8-amino-3,6-dioxaoctanoic acid, respectively. The probe hybridizes to a highly conserved bacterial rRNA sequence. Modified versions of the probe, having a single C-terminal subunit of 4 or 8, were prepared and the properties of the modified and unmodified probes were then compared in dot blot and fluorescence *in-situ* hybridization (FISH) assays.

For the dot blot assays, serial dilutions of nine bacterial rRNAs were spotted on three nylon membrane filters in the order indicated; **A** *Pseudomonas fluorescens*, **B** *Pseudomonas aeruginosa*, **C** *Pseudomonas cepacia*, **D** *Pseudomonas putida*, **E** *Escherichia coli*, **F** *Bacillus subtilis*, **G** *Staphylococcus epidermidis*, **H** *Staphylococcus aureus*, and **I** *Salmonella typhimurium*. Each membrane was then treated with one of the three fluorescein labeled PNA probes. Probe binding was visualized by contacting the membrane with an anti-fluorescein-antibody-alkaline phosphatase conjugate which was used to generate

chemiluminescent signal that was detected by exposure to X-ray film. As seen below, there was no detectable performance difference between the unmodified and modified PNA.



Unmodified C-terminal **4** C-terminal **8**

Similarly, fixed E. coli cells were treated with the three PNAs in a typical PNA-FISH experiment.[16] Again, no performance differences were observed between the unmodified and modified PNAs (data not shown).

Finally, thermodynamic stability of PNA/DNA duplexes was examined using thermal melting analysis (Tm). The Uni-Flu probe, as well as modified versions of the probe having a single C-terminal subunit of **4**, **8** or L-lysine were examined and found to have identical T_m's (81°C+/- 1°C)[19] thereby demonstrating that terminal modification had little or no influence on hybridization.

Compounds **4** and **8** where then used to overcome solubility problems previously encountered with several PNAs. For example, the precursor free amine of the cyanine-3 (Cy3) labeled telomere probe Cy3-OO-TTA-GGG-TTA-GGG-TTA-GGG-NH_2[17] was found to be virtually insoluble in aqueous HEPES buffer at pH 8.5, under conditions necessary to label the probe with Cy3 N-hydroxysuccinimide ester.[18] However, the modified PNA, H-O-**4**-TTA-GGG-TTA-GGG-TTA-GGG-**44**-NH_2, was freely soluble and was labeled with ease.

Table

PNA	PNA Sequence	Purity	Purine/(Purine + Pyrimidine)
1	Flu-O-**4**-GGT-GGT-GGT-GGT-GGT-**4**-NH_2	> 90%	0.667
2	Flu-O-**4**-AGA-AGA-AGA-AGA-AGA-**4**-NH_2	> 90%	1.00
3	Flu-O-**4**-GAG-GAG-GAG-GAG-GAG-**4**-NH_2	> 80%	1.00
4	Flu-OO-**4**-AAA-AAA-GAG-**8**-NH_2	> 98%	1.00
5	Flu-O-**44**-GGG-GGG-G-**44**-NH_2	> 95%	1.00
6	Flu-O-**44**-GGG-GGG-GGG-GG-**44**-NH_2	> 80%	1.00
7	Flu-OO-**4**-AAA-AAA-AAA-AAA-AAA-**4**-NH_2	> 99%	1.00
8	Ac-**8**-TCG-GGC-ATG-AAC-OO-TCT-TT-NH_2	> 98%	0.389

Difficulties with other PNAs were likewise overcome through modification with **4** and **8**. The Table lists homopurine and purine-rich modified PNAs that were successfully prepared by incorporation of the solubility enhancers. As a matter of course, we now routinely modify our PNAs in this fashion. In rare cases where flanking with **4** or **8** fails to provide adequate solubility, double modifications at one or both termini can be made. See, for example, entries 5 and 6 of the Table. We have observed no difference in the performance of **4** and **8** and thus prefer to use the neutral and more easily prepared compound **4**.

Although initially designed to improve the solubility of PNA, we anticipate these compounds, or closely related analogs thereof, will be useful for improving the aqueous solubility of hydrophobic peptides, nucleic acid minics, nucleic acid analogues, other polymers, surfaces and resins.

Acknowledgment:

The authors wish to thank Dr. Kim Adelhorst and Dr. Kenneth Petersen of DAKO A/S, Copenhagen, Denmark for their assistance in performing the T_m analysis.

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12. Diglycolic anhydride (500 mmol) in 800 mL of dichloromethane (DCM) was added dropwise with stirring to 1.1 mole of bis(2-methoxyethyl)amine. The mixture was stirred for 2 hours and then 280 mL of 6N HCl was added dropwise. The contents were transferred to a separatory funnel and the product obtained by evaporation of the organic layer. 73.8 g (296 mmole; 59 % yield).
13. Bis(2-methoxyethyl)amine (1.1 mole) was added dropwise to 500 mmol of *tert*-butyl chloroacetate. After stirring for three days, 250 mL of DCM, 200 mL of water and 300 mmol of solid K_2CO_3 was added. After complete mixing, the layers were separated and the product isolated from the organic layer to provide 66.3 g of a thin yellow oil. The crude product was Kugelrohr distilled at 60 °C (200-500 μM Hg) to yield 58.9 g of a clear colorless oil (238 mmol; mmol; 95%).
14. To purified N,N'-(2-methoxyethyl)-glycine-*tert*-butyl ester was slowly added 12.1 mL of 12N hydrochloric acid. The reaction was stirred overnight and byproducts (e.g. water, HCl, isobutylene) were removed by vacuum evaporation. A 4.4 g portion of the crude product was Kugelrohr distilled at 135-155 °C (100 - 200 μM Hg with rapidly dropping pressure after distillation began). Yield, 4.2 g.
15. General procedure for preparation of Fmoc amino acids; Fmoc-aeg-OH (1 mmol) was added to 6 mL of water, 3 mL of acetone, 2 mmol of $NaHCO_3$ and 1 mmol of K_2CO_3. The mixture was stirred until all the Fmoc-aeg-OH had dissolved. In a separate flask, 1.15 mmol of **3** or **7** was dissolved in 1.15 mL of dry acetonitrile and 2.2 mmol of N-methylmorpholine and 1.25 mmol of trimethylacetyl chloride were added. This solution was stirred for 30 minute at room temperature and then added dropwise to the Fmoc-aeg-OH solution prepared as described above. The reaction was acidified (pH < 3 for **4**, pH ~ 8 for **8**), and the crude product was isolated by extraction with ethyl acetate (DCM for **8**). Excess pivalic acid was removed by column chromatography using a C18 stationary phase.
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18. HPLC-purified amine containing PNA was dissolved in DMF/water (1/1, v/v) at a concentration of 330pmol/μL. From this stock, approximately 30 nmole of PNA was combined with 125 μL 0.1 M HEPES (pH 8.5), and enough DMF/water (1/1, v/v) to bring the total volume to 250 μL. This solution was thoroughly mixed and then added to a tube of Cy3 dye (Amersham) according to the manufacturers instructions.
19. Buffer: 100 mM NaCl, 20 mM Sodium Phosphate (pH 7.0); 1:1 PNA:DNA each at 5.1 μmol/L; Perkin-Elmer Lambda 2S fitted with 6 cell holder running Winlab 2.0 and Templab 1.0 software.