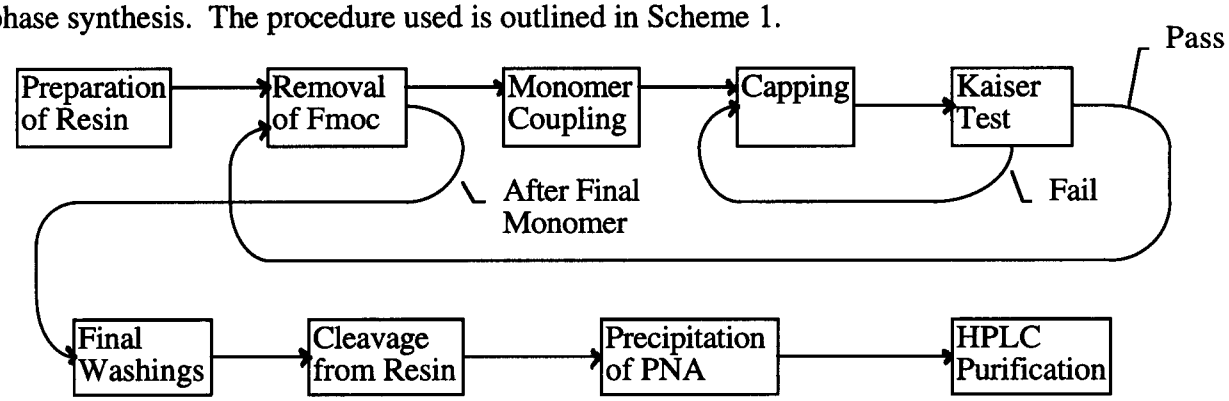
Clancy, Sean O.

27 January 1998

Protocol for PNA Synthesis

I. Introduction

The synthesis of peptide nucleic acids (PNAs) is performed by means of solid-phase synthesis. The procedure used is outlined in Scheme 1.



Scheme 1. Procedure for PNA Synthesis.

The Fmoc-PAL-PEG-PS (Fmoc-peptide amide linker-polyethylene glycol-polystyrene) resin is washed and swollen. The resin and all PNA monomers have an Fmoc (9-fluorenylmethoxycarbonyl; Figure 1e) group attached to the amino ends. This Fmoc protecting group is removed immediately prior to the addition of a monomer and at the end of the synthesis. The first PNA monomer forms an amide bond with the resin and the subsequent PNA monomers form the amide bond with the growing chain's terminal amino group. Any free amino ends, which fail to react with a monomer, must be capped with an acetyl group to prevent undesired PNA sequences to form, or other reactions to occur. The Kaiser Test detects if any amino groups are still present. If the Kaiser Test is negative, then the next Fmoc group may be removed. The Adenine, Cytosine, and Guanine monomers have Bhoc (benzhydryloxycarbonyl; Figure 1f) groups protecting the primary amines on the bases. After the desired PNA sequence is synthesized, the PNA is cleaved from the resin during which the remaining Bhoc protecting groups are also removed. The PNA is then precipitated, and soon afterwards purified. The synthesis takes place in the

fritted reaction vessel, shown in Figure 2, so that the reaction mixture may be stirred by nitrogen bubbling as well as shaking of the vessel on a mechanical shaker. The explicit procedure for the Fmoc PNA synthesis cycle is as follows.

II. Procedure

A. Preparation of Resin

300 mg (50 μmol of the available amino groups) of the Fmoc-PAL-PEG-PS resin is placed in the reaction vessel, and washed four times with 8 mL dichloromethane (DCM). The vessel and resin are then dried over phosphorus pentoxide (P_2O_5) under high vacuum for one hour or overnight. The resin is swelled with 7 mL 1-Methyl-2-pyrrolidinone (NMP), and bubbled/shaken for ten minutes and then the solvent was drained by vacuum.

B. Removal of Fmoc

The Fmoc group is removed from the Fmoc-PAL-PEG-PS resin by adding 4 mL 20% piperidine/NMP (Pip/NMP) and bubbled/shaken for ten minutes and then the solvent was drained by vacuum. The resin is washed twice with N,N-dimethylformamide (DMF), with one minute elapsing per washing. Another 4 mL 20% Pip/NMP is added and bubbled/shaken for 10 minutes and drained. The resin is washed five times with 4 mL of a 1:1 mixture of DCM and DMF. The resin is now ready for the addition of the first PNA monomer.

C. Monomer Coupling

Before a monomer is added to the resin, the carboxyl group of the monomer is "activated" using *O*- (7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), Diisopropylethylamine (DIPEA), and 2,6-Lutidine. The optimized amounts of each were found to be 200 μmol (four equivalents) to the 300 mg of resin as indicated in Table 1. The structures of the PNA monomers can be seen in Figures 1a through 1d.

Activated Monomer Ingredients	*Amount*
Fmoc-A(Bhoc)-OH (mg)	157
Fmoc-C(Bhoc)-OH (mg)	152
Fmoc-G(Bhoc)-OH (mg)	160
Fmoc-T-OH (mg)	109
HATU (mg)	82
DIPEA (μL)	38
2,6-Lutidine (μL)	25

Table 1. Materials used to make the activated monomer complexes.

The PNA monomer and HATU are placed into an Eppendorf vial and 1.4 mL NMP is added, and resulting mixture vortexed until both have dissolved. Then DIPEA and 2,6-Lutidine are added, and the vial is shaken again. The solution is transferred to the reaction vessel and bubbled/shaken for twenty-five minutes. The resin is then washed five times with 4 mL 1:1 DCM/DMF each time.

D. Capping

6 mL of the "Capping" solution, prepared by mixing 45 mL DMF, 3 mL 2,6-Lutidine, and 2.5 mL Acetic anhydride, is added to the resin and bubbled/shaken for ten minutes. This solution acetylates and blocks any amino ends that may not have reacted with the monomer, thereby forbidding later additions to the amino ends. This capping reduces the chances of PNA sequences of varying lengths from being produced, and also increases the purity and quantity of the final product. After the capping step, the resin is washed five times with 4 mL DMF aliquots.

E. Kaiser Test

After each capping, the Kaiser Test may be performed to check for free amino ends. A few beads are removed from the reaction vessel and placed in a small borosilicate glass test tube. Two drops of 76% w/w Phenol/Ethanol is added to the tube, followed by four drops of 0.0002 M Potassium cyanide/Pyridine, and two drops of 0.28 M Ninhydrin/Ethanol. The tube is rotated to mix the solution, and then heated in a heating block set to 100 °C for five minutes. The tube is removed, and the color of the beads are observed. A yellow color indicates the absence of free amino ends. If a purple/blue color is observed, fresh capping solution is made up and the capping step is repeated. Experience has shown that with fresh capping solution, the Kaiser Test need not be performed after every step, but is done after every third monomer addition.

F. Repetition of Cycle

At this point there is a PNA monomer attached to the resin. In order to add another monomer, the Fmoc group must be removed from the monomer. Thus, the PNA synthesis cycle is repeated with the removal of Fmoc, followed by coupling, capping, and again removal of the Fmoc.

G. Final Monomer Washings

After the final monomer is added, the Fmoc is removed, and washed with a 1:1 mixture of DCM/DMF as above. The resin is then washed sequentially five times with 4 mL DCM, and five times with 4 mL Methanol. The resin and the reaction vessel are finally dried overnight in a lyophilizer.

H. Cleavage of PNA from Resin

6 mL of the "Cleaving" solution, made with 1.2 mL of freshly distilled m-cresol, and 4.8 mL trifluoroacetic acid (TFA), is added to the reaction vessel and bubbled/shaken for two hours. The rate of nitrogen for bubbling must be slow enough not to quickly evaporate the TFA over the two hours. TFA may be occasionally added to maintain the volume of the "Cleaving" solution. After two hours, the "Cleaving" solution is drained

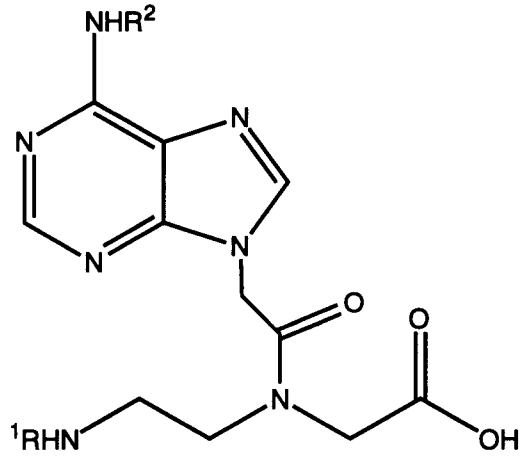
into a 125 mL side-arm filtering flask. The resin is washed two times with 5 mL TFA, and the washings drained into the flask. Nitrogen is blown into the flask at a quick rate to evaporate all of the TFA. A small Dewar flask filled with water slightly above room temperature, is placed around the filtering flask to provide a heat sink for the evaporation of the TFA.

I. Precipitation of PNA

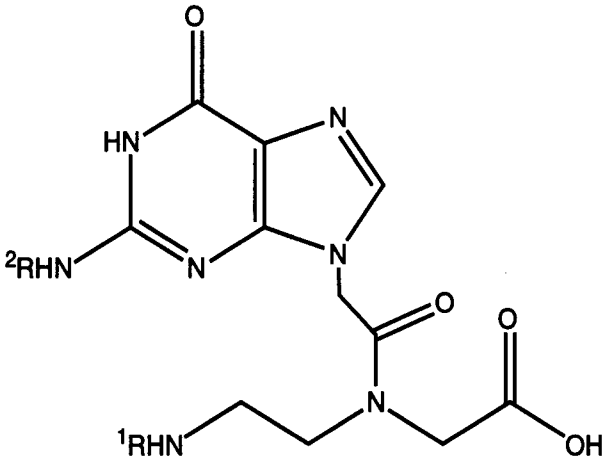
45 mL of freshly distilled ether, chilled to -20 °C, is added while swirling, to the filtering flask to precipitate the PNA. If the precipitate does not appear very granular, the flask may be placed in a -20 °C freezer for thirty minutes. The precipitate is then centrifuged at 3000 rpm for three minutes. After decanting, the step is repeated two more times. The precipitate is finally dried under high vacuum for fifteen minutes.

J. Purification and Storage

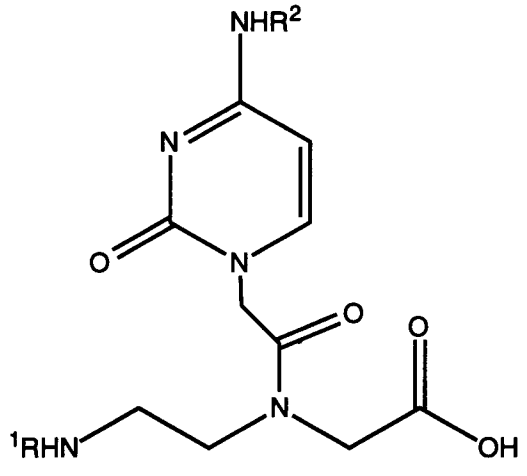
The PNA is then purified by reverse phase HPLC, on a Vydac (2.2 cm x 25 cm) C_8 column, with a gradient elution of 5% A to 100% B in 100 min (solvent A = 0.1% TFA; solvent B = 80% Acetonitrile in 0.1% aq. TFA; flow rate = 8 mL/min; α_{max} = 260 nm). Fractions containing PNA are collected, dried by lyophilization, and stored dry at -20 °C.



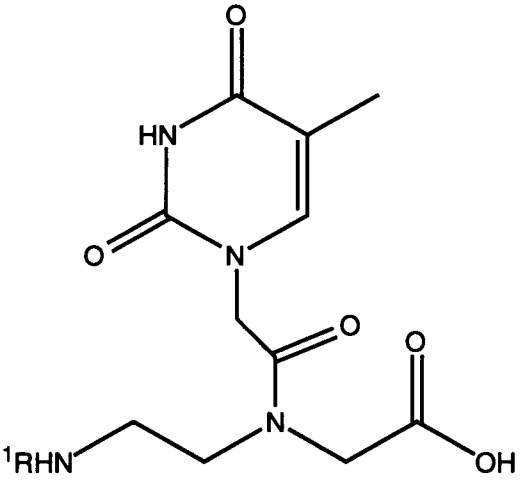
a. **Structure of Fmoc-A(Bhoc)-OH.**



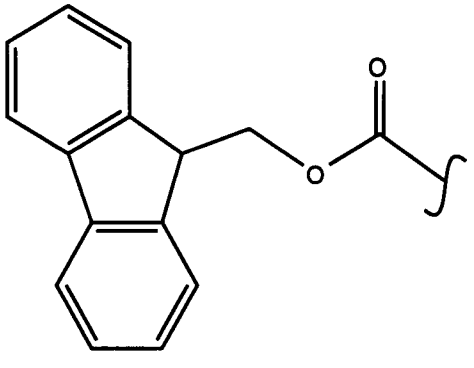
b. **Structure of Fmoc-G(Bhoc)-OH.**



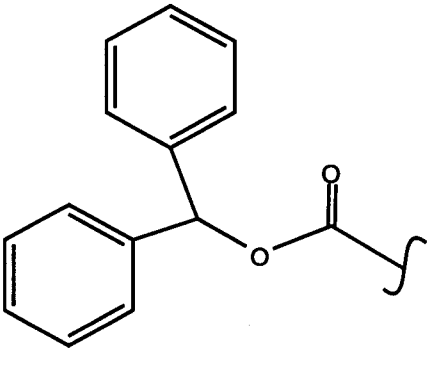
c. **Structure of Fmoc-C(Bhoc)-OH.**



d. **Structure of Fmoc-T-OH.**



e. **Structure of Fmoc Group.**



f. **Structure of Bhoc Group.**

Figure 1. The Structures of the PNA Monomers with Protecting Groups, where R^1 = Fmoc and R^2 = Bhoc.

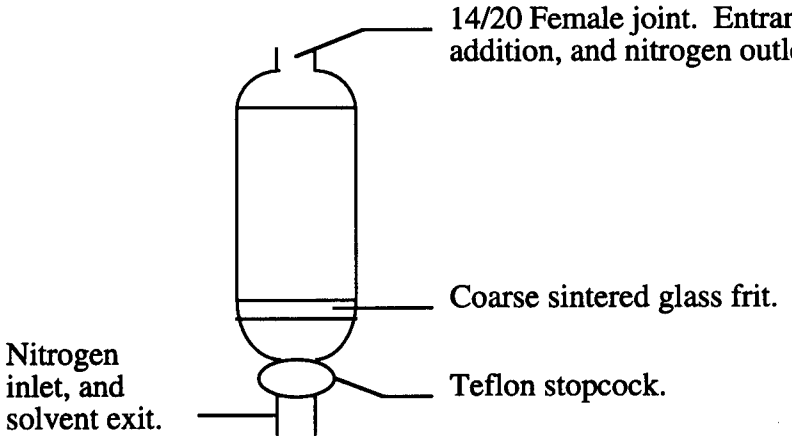


Figure 2. Diagram of Fritted Reaction Vessel.