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A SIMPLE PROCEDURE FOR SOLID-PHASE SYNTHESIS OF PEPTIDE NUCLEIC ACIDS WITH N-TERMINAL CYSTEINE

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Abstract: Problems were encountered during attempts to prepare N-terminal cysteine-substituted peptide nucleic acids (PNAs) from commercially available, Fmoc-protected monomers. These problems have been surmounted by the use of an S-*t*-butylmercapto protecting group on the cysteine moiety. The solid-phase syntheses are carried out via a simplified procedure which should be generally useful for manual PNA synthesis.

Introduction

Peptide nucleic acids (PNAs) are N-(2-aminoethyl)glycine oligomers with the common nucleic acid bases attached to the glycine nitrogen via a methylenecarbonyl linker (Fig. 1).[1] PNAs bind tightly to complementary sequences of DNA or RNA. High melting temperatures observed for PNA–DNA and PNA–RNA duplexes may be due to decreased electrostatic repulsion from the uncharged PNA backbone. Other notable PNA attributes include resistance to nucleases and proteases, lack of chirality, and amenity to preparation using well-established peptide synthesis protocols.[2] A major limitation to the use of PNAs has been their inability to cross cell membranes, although recent progress in this area with PNA–peptide conjugates has been reported.[3] An interest in probing the mechanism of enzymes which interact with nucleic acids (various helicases, telomerase, etc.) led us to consider the preparation of PNAs suitably substituted for attachment to peptides or nucleic acids.[4] The Corey group has reported a manual, solid-phase synthesis of N-terminal cysteine-substituted PNA oligomers using *t*-Boc-protected monomers, with subsequent conjugation to a protein through a disulfide linkage.[5] We now report a convenient solution to a problem involving incorporation of a cysteine moiety on the N-terminus of PNAs prepared from commercial Fmoc-protected monomers.

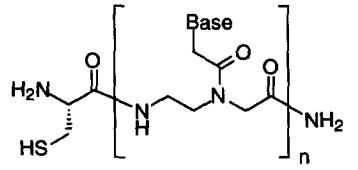

Figure 1. PNA structure with N-terminal cysteine.

Results and Discussion

General Strategy. Our initial objective was the preparation of PNAs with an N-terminal cysteine for the purpose of preparing PNA–DNA conjugates via disulfide bond formation. For maximum flexibility in scale and variety of the PNA preparations, a manual synthesis was deemed desirable, and an Fmoc-based strategy appeared to be most convenient. At present, the only commercially-available Fmoc-protected PNA monomers (Perseptive Biosystems) have a benzhydryloxycarbonyl (Bhoc) protecting group on the primary amino group

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of the A, C and G nucleobases. Using these PNA monomers and a properly protected cysteine monomer, the application of standard coupling protocols[6] has led to a successful synthesis of the requisite PNAs as detailed below.

Synthesis of a PNA 5-Mer. The target of our first PNA synthesis was cysGGGTT, and an Fmoc-protected, side chain-tritylated cysteine monomer was employed. The synthesis was carried out in an inexpensive polypropylene column at the ambient temperature using commercial Fmoc-protected monomers, HATU-activated couplings, and a polyethylene glycol-polystyrene resin (see Experimental section). The cocktail for resin cleavage and cation scavenging was a 4:1 mix of trifluoroacetic acid (TFA) and *m*-cresol. Reverse-phase HPLC analysis of the product revealed primarily two components, the minor of which was shown by MALDI-TOF MS[7] to be the desired product, while the major component had a mass which was 166 Daltons higher. We surmised that a benzhydryl cation liberated from the Bhoc protecting group on the nucleobases had been recaptured by the cysteine sulfhydryl group. Little improvement was noted in several subsequent cleavage trials with addition of various scavengers (e.g., ethanedithiol and/or thioanisole), changes in concentration, and lengthened reaction times. The solution to this vexing problem was found by using a cysteine monomer with a protecting group other than trityl on the sulfhydryl.

Successful Incorporation of N-Terminal Cysteine. A plethora of protecting groups has been devised for the cysteine sulfhydryl group in peptide synthesis, often as a prelude to conjugation through a disulfide bond.[8] Among these, the *t*-butylmercapto (*t*-butylsulfenyl; S-*t*-Bu) group was reported to be resistant to TFA and amine bases, but easily removed under mild and selective conditions using thiols or phosphines.[9] We prepared PNA pentamer GGGTT, then coupled Boc-S-*t*-butylmercapto-L-cysteine (Fluka) to the N-terminus. Boc deprotection was concomitant with cleavage from the resin. HPLC analysis revealed a single major peak, which MALDI-TOF MS confirmed as the desired product (see Fig. 2). In stark contrast to our previous synthesis, none of the benzhydrylated (M+166) by-product was detected. Cysteine deprotection was carried out by reduction of the disulfide in aqueous dithiothreitol. PNA (~0.5 mg) was dissolved in 200 μL of TrisCl buffer (5 mM, pH 7.5) containing dithiothreitol (20 mM) and left standing overnight at the ambient temperature.

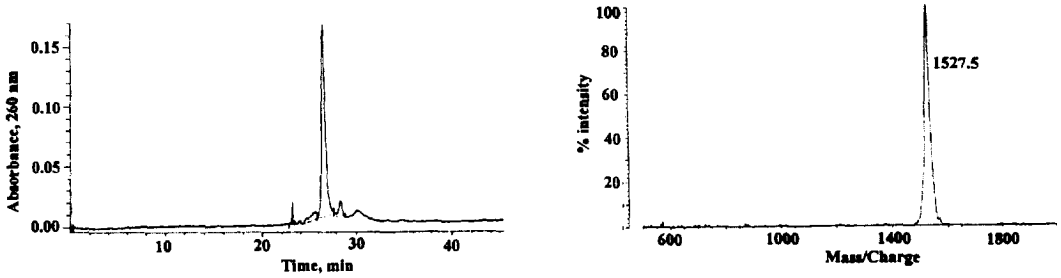

Figure 2. HPLC chromatogram and MALDI-TOF mass spectrum of unpurified cysGGGTT.

Synthesis of a PNA 15-Mer. As a more challenging test of the generality of this synthesis procedure, we attempted the preparation of a PNA 15-mer, cysCATCATGCAGGACAG. As judged by HPLC and mass spectrometry, the synthesis was successful, although unacceptable amounts of C-deletion 14-mers were present. The synthesis was repeated with double coupling followed by capping (acetic anhydride) for each C monomer addition. The MALDI-TOF mass spectrum of the unpurified product exhibited only one peak, namely that corresponding to the desired product.

Conclusion

A potentially general route for the successful incorporation of cysteine at a PNA N-terminus has been developed. The simplified apparatus and protocol should prove useful for manual solid-phase synthesis of any PNA. (We have also recently used this manual procedure for the submonomer solid-phase synthesis of peptoids.[10]) Further developments in PNA synthesis using this approach will reported in due course, as will the use of resultant PNAs in mechanistic studies of the action of various enzymes.

Experimental

Manual Synthesis Apparatus. This synthesis should be carried out in a good fume hood. For manual PNA synthesis, we used empty, disposable, polypropylene columns with porous, polyethylene bed supports (available from Bio-Rad Laboratories as "Econo-Pac" columns). One opening of a three-way polypropylene stopcock with Luer fittings was attached to the column, a second opening was plugged, and the third opening was fitted with a six-inch, 18G stainless steel syringe needle. The column was supported with a tri-grip microclamp, and the needle was inserted through a rubber septum attached to a 250-mL filter flask. The side arm of the flask was attached with vacuum tubing via a three-way stopcock to the house vacuum. For retention of liquid in the column both stopcocks were closed, while both were opened for filtration (stopcock redundancy provided more control and flexibility in operation). Agitation was provided by intermittent manual stirring with either a Teflon or a polyethylene rod. PNA monomers were purchased from Perseptive Biosystems.

Synthesis of PNA Oligomers. For PNA synthesis, the polyethylene glycol-polystyrene resin (Fmoc-PAL-PEG-PS; Perseptive Biosystems) was weighed and added to the column. (This resin has a linker which yields a C-terminal amide upon TFA cleavage of the PNA.) Syntheses were routinely carried out on a 10–20 μmol scale based upon the stated loading capacity of the resin. The resin was rinsed with N-methylpyrrolidinone (NMP), reacted with two successive portions of 20% piperidine in NMP for 10 min (Fmoc removal), then rinsed three times with 1:1 NMP:dichloromethane (DCM). Coupling was achieved at room temperature with occasional stirring by treating the resin for 20 min with a fourfold excess of monomer in NMP (0.1 M) containing four equivalents each of O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; Perseptive), diisopropylethylamine (DIPEA), and 2,4,6-collidine. (For synthesis of the 15-mer, coupling was repeated for addition of each C monomer, followed by capping with 5% acetic anhydride and 6% collidine in NMP for 5 min.) The resin was washed thrice with 1:1 DCM:NMP, then the appropriate number of iterations of the deprotection/coupling cycle was performed as described above. The resin was then washed thrice with 1:1 DCM:NMP.

If the resin-bound PNA was to be stored, the N-terminal protecting group was left intact, the resin was washed thrice with DCM, and after lyophilizing was stored conveniently in the capped polypropylene synthesis column at –20 °C. Resin-bound PNA oligomers may be safely stored in the freezer indefinitely when dry, if the N-terminal protecting group is left intact. This is also a convenient point to split the resin for divergent synthetic purposes. For resin cleavage when the N-terminal monomer is an Fmoc-protected PNA, Fmoc removal is accomplished by two cycles of piperidine/NMP treatment as described above, followed by two 1:1 DCM:NMP washings and three washings with DCM. Alternatively if a *t*-Boc-protected amino acid has been added at the N-terminus, deprotection will be concomitant with TFA cleavage from the resin.

Resin Cleavage and PNA Isolation. For cleavage, the dry resin was weighed and transferred to a small plastic scintillation vial containing a Teflon-coated spin bar. A mixture of 4:1 TFA:*m*-cresol (CAUTION) was added (approx. 200 μL/10 mg of resin), an unlined plastic cap was attached, and the mixture was stirred at the ambient temperature for 2 h. PNA was isolated by transferring the cleavage solution to a Pasteur pipet

containing a small glass wool plug for resin filtration, and allowing the filtrate to drip into ice-cold ether (approximately 15 mL per 1 mL of cleavage cocktail) contained in a tared 50 mL plastic centrifuge tube. The scintillation vial and glass wool were rinsed with a few drops of TFA. The tube was capped and the mixture was vortexed, followed by centrifugation at 2500 rpm for 3 min at the ambient temperature. The supernatant was carefully removed with a Pasteur pipet. The white precipitate was diluted with fresh, cold ether, vortexed, and centrifuged. This process was repeated twice more and the resulting white solid residue was dried first by house vacuum, then lyophilized. The PNA was stored at –20 °C in the tightly-sealed centrifuge tube.

RP-HPLC and MALDI-TOF MS. Reverse-phase HPLC was carried out on a Beckman System Gold instrument using an Alltech Econosil C_{18} 10 × 250 mm, 10 micron column at the ambient temperature with a flow rate of 1 mL/min and detection at 260 nm. Water was purified using a Super Q System (Millipore). The HPLC solvent buffer system was 0.1% TFA in H_20 (buffer A) and 0.1% TFA in acetonitrile (buffer B). The program consisted of 100% buffer A initially. Upon injection of the sample, buffer B was increased in a linear fashion to 100% over 40 min and held for 10 min. Mass spectra were obtained on a Kratos Kompact MALDI 2 V5.2.1 instrument using sinapinic acid as the matrix. The calculated and observed molecular weights (Mw + 1; monocations) were 1615.7 and 1614.6 for (S-*t*-Bu)cysGGGTT, respectively, and were 4288.3 and 4288.2 for (S-*t*-Bu)cysCATCATGCAGGACAG.

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