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Optimization of a Solid-Phase Synthesis of a PNA Monomer

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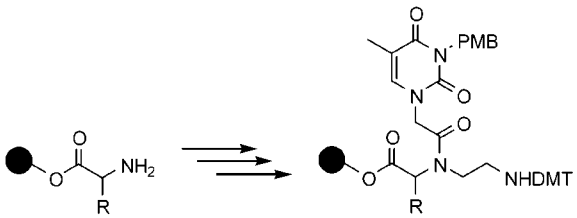
ABSTRACT



A new scheme for the synthesis of peptide nucleic acid (PNA) is described. First, a resin-bound amino acid is alkylated under Fukuyama–Mitsunobu conditions. A nucleobase is then incorporated via an acid fluoride. Subsequently, oligomerization may be achieved by deprotection, coupling of another amino acid, and repetition of the cycle. Each step of the submonomer synthesis has been optimized to provide essentially quantitative yield.

Peptide nucleic acid or PNA is perhaps one of the most successful synthetic analogues of DNA or RNA. Since its introduction in 1991,[1] there have been over 600 publications about PNA, from such diverse fields as chemistry, biochemistry, biotechnology, and medicine.[2]

The success of PNA as a nucleic acid mimic lies in its structure. The sugar−phosphate backbone of natural nucleic acids is replaced by a polyamide backbone consisting of *N*-(2-aminoethyl)glycine units. A nucleobase is connected to the internal nitrogen via a methylene−carbonyl linker. This structure maintains the bond spacing of DNA/RNA (six covalent bonds along the backbone, with the nucleobase three bonds away from the backbone). As well, the amide bonds confer some degree of conformational rigidity to a PNA strand, aiding in complex formation. As a result of this design, PNA forms exceptionally stable and sequence-specific duplexes and triplexes with complementary DNA, RNA, or PNA. Also, PNA is stable to degradation by nucleases and proteases.

There are however a few drawbacks which complicate the direct use of PNA in many applications, the most severe being low water solubility and poor cellular uptake. These problems are most frequently addressed by the conjugation of various moieties to the end of a PNA strand. An alternative to this would be to construct a PNA with an *N*-(2-aminoethyl)X backbone, where X, rather than glycine, could be any readily available α-amino acid. Such modifications can impart chirality, charge, or functionality, all without disturbing the bond configuration required for the desired binding properties.

While some PNA oligomers of this sort have been studied,[3] we feel there is room for further investigation in this area. The construction of such oligomers first requires the synthesis of the appropriate PNA monomers. This can either be done by reductive alkylation[4] or Fukuyama−Mitsunobu alkylation[5] of the desired α-amino acid, followed by acylation with the desired nucleobase−acetic acid derivative. These processes can be tedious and time-consuming. In addition, when one

(1) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497.

(2) For more about the applications, properties, and standard syntheses of PNA, see the following review and references therein: Nielsen, P. E. *Acc. Chem. Res.* **1999**, *32*, 624.

(3) Sforza, S.; Haaima, G.; Marchelli, R.; Nielsen, P. E. *Eur. J. Org. Chem.* **1999**, 194.

(4) Dueholm, K. L.; Egholm, M.; Buchardt, O. *Org. Prep. Proc. Int.* **1993**, *30*, 1927.

(5) Fukuyama, T.; Jow, C.-K.; Cheung, M. *Tetrahedron Lett.* **1995**, *36*, 6373.

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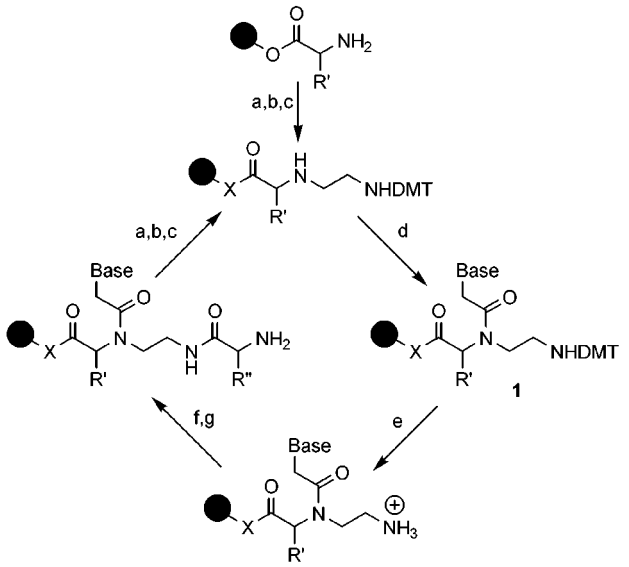
considers the multitude of readily available α-amino acids, natural nucleobases, and nucleobase analogues, the number of possible monomers to synthesize becomes unmanageable.

As a solution to this problem, we envision a submonomer synthesis of PNA oligomers (Scheme 1). On the basis of the Fukuyama−Mitsunobu alkylation, a resin-bound amino acid is first sulfonylated and then alkylated under Mitsunobu conditions with an N-protected 2-aminoethanol and then desulfonylated. The secondary amino group is then acylated with the desired protected nucleobase−acetic acid derivative, yielding the resin-bound PNA monomer **1**.

Scheme 1. Submonomer Synthesis Cycle Based on Fukuyama−Mitsunobu Amine Synthesis[6] [a]



[a] (a) *o*-NsCl, DIEA, CH_2Cl_2; (b) $DMTNHCH_2CH_2OH$, TMAD, PBu_3, DIEA, THF; (c) KSPh, NMP, H_2O; (d) $BaseCH_2COF$, DIEA, DMAP, NMP; (e) TFA, MeOH, CH_2Cl_2; (f) Fmoc-amino acid, peptide coupling; (g) piperidine, DMF. X = O or NH.

At this point, rather than cleaving and purifying the monomer for use in an oligomer synthesis, the next monomer is constructed on the first one by deprotection of the amino group, coupling of another amino acid, which is deprotected, and repetition of the cycle. A PNA oligomer synthesis is thus carried out, with each step incorporating a moiety smaller than a single monomer or a submonomer.[7] While PNA monomers have been constructed via similar scheme,[8] certain challenges are associated with the synthesis of oligomers by this method. First, all protecting groups must be removable under orthogonal conditions, all of which must not affect the resin linkage itself. Moreover, even the nucleobase derivatives must be protected from Mitsunobu alkylation. In addition, since the cycle is to be repeated several times, each step must proceed in very good yield with no resin-bound byproducts and, preferably, as quickly as possible. The purpose of this Letter is to communicate the results of our efforts to optimize each step in the submonomer synthesis scheme.

Our resin of choice contains the *p*-benzyloxybenzyl alcohol (Wang) linker, which offers enhanced stability toward nucleophiles (such as thiophenolate ion, required in the desulfonylation step) over the standard Merrifield resin, while also remaining stable to the dilute acid required for the detritylation step in our synthetic scheme. In addition, the Wang resin is extremely well-suited for solid-phase optimization studies. Perhaps one of the more challenging aspects to solid-phase chemistry is the monitoring of reactions. Reactions on the Wang resin were conveniently and quantitatively monitored by periodically removing a small amount (5−10 mg) of resin from the reaction, thoroughly rinsing and drying, and then cleaving directly into a deuterated solvent, collecting the ^{1}H NMR spectrum and comparing integrations of the compounds of interest.

The first reaction to be studied was sulfonylation. While many solvents, bases, and concentrations were tried, it was found that 2 equiv each of 2-nitrobenzenesulfonyl chloride (nosyl chloride) and *N*,*N*-diisopropylethylamine (DIEA) at a concentration of 0.2 M in dichloromethane gave the best results. Beginning with the commercially available Fmoc-glycine derivatized Wang resin, the Fmoc group was removed under standard conditions, and the nosyl chloride and DIEA solution was added. Every 2 min for 10 min, a small amount of resin was removed from the reaction and thoroughly rinsed. After 10 min, the resin was rinsed and fresh reagents were added for a further 10 min. The 10 samples were dried and cleaved using $CF_3COOD/CDCl_3$, and the ^{1}H NMR spectra were recorded. The results are shown in Figure 1a. Interestingly, the reaction was very rapid, rising to nearly 90% completion within the first 2 min. However, the starting material, glycine, was not completely consumed until 4 min after the second addition of reagents. Perhaps even more interesting though was that the complete disappearance of glycine did not correspond to complete reaction, as dinosylated glycine was formed, amounting to nearly 20% of the material by the end of 20 min. To our knowledge, there has been no previous report of a side reaction of this kind. This could either be due to the use of milder sulfonylation conditions or the fact that the dinosylated product could be hydrolyzed to the desired mononosyl compound on extractive workup or HPLC analysis or purification. Nevertheless, under these conditions dinosylation does occur, and if left untreated, the disulfonylated material would not be alkylated in the subsequent Mitsunobu reaction, significantly decreasing the yield of the cycle. Fortunately, a very brief treatment with piperidine solution completely converts all dinosylated product to the desired mononosylated product (see Figure 1b). Considering all of

(6) The following abbreviations are used: *o*-Ns or nosyl (2-nitrobenzenesulfonyl); DEAD (diethyl azodicarboxylate); DIAD diisopropyl azodicarboxylate), TMAD [*N*,*N*,*N*′,*N*′-tetramethyl azodicarboxamide or 1,1′-azobis(*N*,*N*-dimethylformamide)]; PyBroP (bromo-tris-pyrrolidino-phosphonium hefaxfluorophosphate); PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate); HBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; HATU [2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate].

(7) For a different submonomer synthesis of PNA, see: Richter, L. S.; Zuckermann, R. N. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1159.

(8) Davis, P. W.; Swayze, E. E. *Biotechnol. Bioeng.* **2000**, *71*, 19.

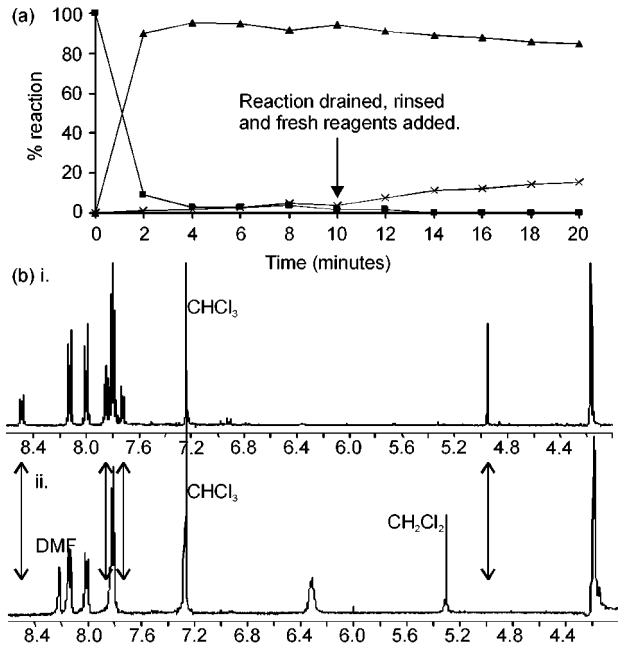


Figure 1. (a) Proportions of glycine derivatives at 2 min intervals in the nosylation of glycine-derivatized Wang resin, as determined by integration of the methylene peaks in the ^{1}H NMR spectrum of the crude cleavage material: ■, unreacted glycine, δ 4.10 ppm; ▲, mononosyl glycine, δ 4.16 ppm; ×, dinosyl glycine, δ 4.94 ppm. (b) i. ^{1}H NMR spectrum of the product after 2 × 10 min under nosylation conditions (recorded in 20% $CF_3COOD/CDCl_3$). The arrows indicate the signals due to the undesired dinosylated product. ii. ^{1}H NMR spectrum of material cleaved from the same resin after a 1 min treatment with 30% piperidine in DMF (recorded in 7.5% $CF_3COOH/CDCl_3$). Unlabeled peaks are due to the desired mononosylated glycine.

the above, nosylations are now routinely carried out with two 3 min treatments of nosyl chloride/DIEA, followed by a 1 min treatment with 30% piperidine in DMF, affording 95−100% conversion.

The next reaction studied was the Mitsunobu alkylation.[9] The protecting group of choice for the nitrogen of the aminoethyl group is the dimethoxytrityl or DMT group. This was chosen for its stability under basic/nucleophilic conditions, its ease of removal under brief acid treatment (through which the resin linkage is stable), and for the ease of synthesis of DMT−ethanolamine. Mitsunobu alkylation of nosyl glycine by DMT−ethanolamine was attempted under a number of conditions. The standard conditions (DEAD/PPh_3) were found to be completely ineffective. The results were improved with the use of TMAD/PBu_3,[10] but even this reagent system did not afford complete alkylation within a reasonable time period. Only upon addition of excess base (DIEA) was the reaction seen to proceed to completion within a few hours. To determine how quickly the reaction proceeds, resin-bound nosyl glycine was treated with 3 equiv each of DMT−ethanolamine, TMAD, and PBu_3 and 10 equiv of DIEA for an hour, removing a small amount of resin every 10 min, followed by a second treatment with fresh reagents for a second hour. Again, the extent of reaction was determined by cleaving from the resin directly into deuterated solvent and comparing ^{1}H NMR signal integrations of alkylated and nonalkylated products. For comparison, the experiment was repeated with DEAD and with DIAD. The results are shown in Figure 2. As can be seen, the TMAD

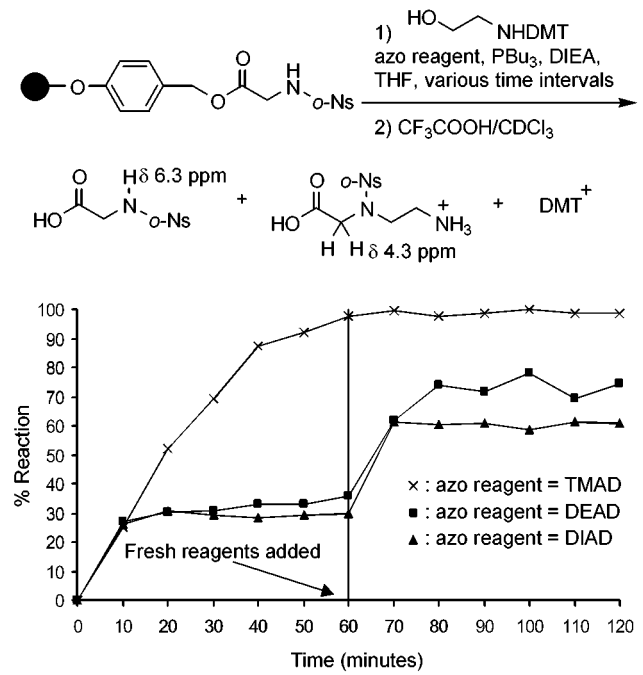


Figure 2. Conditions for optimization of the Mitsunobu reaction, and results, as determined by integration of the ^{1}H NMR signals of the indicated protons.

alkylation proceeds nearly linearly for the first 40 min, after which the effectiveness of the reagent tapers off. The azodiester reagents, on the other hand, seem to lose their effectiveness after only 10 min under these conditions. Given the above results, our protocol is to use two 40 min treatments with TMAD, which routinely affords essentially quantitative conversion.

The last stage of the Fukuyama−Mitsunobu synthesis to be optimized was the desulfonylation reaction. To examine this reaction by ^{1}H NMR, the DMT protecting group first had to be removed, to clarify the aromatic region of the NMR spectrum. This was accomplished by successive 30 s treatments with TFA/MeOH/CH_2Cl_2 (1:2:97).[11] By the third treatment, no orange color, characteristic of the DMT cation, was generated, which was taken as an indication of complete detritylation. The primary amino group was then acetylated. While many reagents were tested for the denosylation reaction, we found that 0.5 M KSPh in NMP/H_2O (24:1)

(9) Mitsunobu, O. *Synthesis* **1981**, 1.
(10) Tsunoda, T.; Otsuka, J.; Yamamiya, Y.; Ito, S. *Chem. Lett.* **1994**, 539.
(11) Alsina, J.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1996**, *37*, 4195.

gave the best results. The *N*-(acetamidoethyl)-*N*-nosylglycine resin was treated with 7 equiv of the KSPh solution for 2 × 30 min, sampling every 10 min, for analysis by ^{1}H NMR. This time, the very first sample (t = 10 min) showed no sign of the DMT or nosyl groups, confirming both that the visual test for detritylation was accurate and that denosylation occurs rapidly and is complete within 10 min. Subsequent experiments have shown that denosylation is also complete within 10 min when the DMT protecting group is in place.

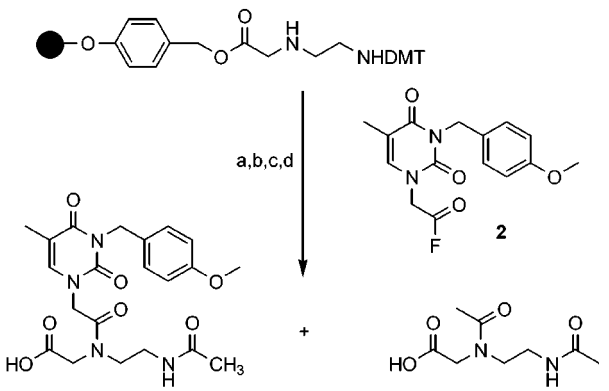
The last step of the submonomer synthesis scheme to require attention was the incorporation of the nucleobase submonomer. It should be noted that even thymine which remains unprotected for standard PNA syntheses requires a protecting group for our synthesis scheme, as the N3 position is efficiently alkylated under our Mitsunobu conditions (see Supporting Information).

Investigation of a few different protecting groups led us to the *p*-methoxybenzyl (PMB) group. This can be easily prepared and can be quantitatively deprotected by treatment with 0.6 M $AlCl_3$ in anisole followed by alkaline aqueous workup.[12]

Incorporation of the PMB-protected thymine submonomer proved to be a difficult step to optimize. Although it appears to be a straightforward acylation, the common acylating agents PyBroP, PyBOP, HBTU, and HATU were all ineffective, perhaps due to the steric effect of the DMT protecting group. The acid fluoride[13] was prepared, isolated, and used in combination with DIEA and catalytic DMAP to afford the desired product. This step was also optimized according to Scheme 2. Resin-bound *N*-(DMT-aminoethyl)glycine was treated with a 0.2 M solution of the acid fluoride and DIEA with a catalytic amount of DMAP for 1 h. Every 15 min, a small amount of resin was removed from the reaction, detritylated under the conditions described above, and then acetylated under standard conditions, cleaved, and examined by ^{1}H NMR. A sample of the expected byproduct, the peracetylated PNA backbone, was prepared by standard solution-phase methods in order to determine its precise chemical shifts. The ^{1}H NMR spectrum showed no sign of this compound, consisting mainly of the desired PMB-protected, acetylated thymine PNA monomer. Acylation by the acid fluoride then is concluded to be complete within 15 min.

In summary, we have demonstrated that solution-phase ^{1}H NMR can be conveniently used to monitor solid-phase reactions on Wang resin. We have devised a submonomer synthesis strategy for PNA which can in principle incorporate any α-amino acid and any nucleobase at any position in the oligomer. This scheme includes a protection strategy in which all deprotection conditions are completely compatible with other protecting groups which must remain in place, including the nucleobase protecting group and the resin linkage. We have optimized each step in the scheme so that it proceeds as quickly and cleanly as possible. In particular, with respect to the Fukuyama−Mitsunobu process, we have discovered the following: (1) Nosylation can be made to occur extremely rapidly, but undesired dinosylated product is formed, which can be reversed by a brief treatment with piperidine; (2) sluggish Mitsunobu reactions can be accelerated by the addition of excess base; (3) desulfonylation can also be made to occur quite rapidly. Work is currently underway to demonstrate the cyclic yield and overall efficiency of the scheme by the preparation and quantitation of a deprotected PNA oligomer. Also, our results toward the incorporation of other nucleobases and α-amino acids will be reported in due course.

Scheme 2. Nucleobase Submonomer Incorporation Experiment[a]



[a] **2**, DIEA, catalytic DMAP, NMP, various time intervals; (b) TFA, MeOH, CH_2Cl_2, 3 × 30 s; (c) Ac_2O, DIEA, CH_2Cl_2, 1 hl (d) 95% TFA/H_2O, 16 h, evaporate, then 5% CF_3COOD/DMSO-d_6.

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Supporting Information Available: Experimental procedures and spectral data for key compounds, as well as experimental procedures for optimization experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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