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Development of an efficient and low-cost protocol for the manual PNA synthesis by Fmoc chemistry

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ABSTRACT

An efficient and low-cost protocol for the manual synthesis of Peptide Nucleic Acids is reported here. The protocol relies on coupling reactions carried out with 2.5 equiv of PNA monomers activated with HOBT/HBTU, in the presence of pyridine/NMM. The protocol has been tested on four PNA oligomers with a length ranging from 9 to 12 bases and a purine content up to 67%.

1. Introduction

Peptide Nucleic Acids (PNAs) are DNA analogues with a peptide-like backbone, having the nucleobases attached to the backbone through a methylene carbonyl linker.[1] The ability to bind complementary DNA and RNA with high affinity, and the resistance to degradation by nucleases and proteases stimulated the research on the therapeutic applications of PNAs as antisense, antigene and decoy.[2,3] Furthermore the specificity of binding towards DNA and RNA opened the way to biotechnological applications of PNAs, including the identification of single nucleotide polymorphisms.[4]

Synthesis of PNA oligomers has been carried out using a variety of monomers and coupling conditions. Examples of protecting groups employed for the PNA monomers are represented by Boc/*Z*, Fmoc/*Z*, Fmoc/Boc, Mmt/acyl, Fmoc/acyl, Dde/Mmt, NVOC/acyl, azide/Bhoc and Fmoc/Bhoc.[2,5–10] So far, only two kinds of PNA monomers are commercially available, protected with the Boc/*Z* and the Fmoc/Bhoc. Oligomerization conditions are usually set up considering the stability of the exocyclic amine-protecting groups, the specific base sequences, the nature of the resin, other than the protecting group on the backbone amine. The Fmoc/Bhoc combination of protecting groups is largely the most employed, due to the mild treatments for the cleavage of the oligomer from the resin and removal of Bhoc groups. Yields of oligomers depend on several factors, including the type of activator, the reaction time, the monomer excess, the pre-activation time, the times the coupling is repeated, the length of the oligomers and the temperature.[11] Syntheses of sequences with a high content of purines often give poor yields due to the difficulty of coupling very hindered monomers; furthermore especially when using Fmoc-protected monomers low yields may originate from aggregation of the peptide nucleic acid and stacking of the fluorenylmethoxycarbonyl with the nucleobases. Nowadays PNA syntheses are usually carried out on automated synthesizers, under controlled and standardized conditions.

Protocols for the manual synthesis are often an adaptation of protocols employed in automated synthesis. The synthesis by Fmoc chemistry of PNA oligomers relies on the protocol developed by Egholm and Casale for the automated synthesis of PNA oligomers using Fmoc/Bhoc protected PNA monomers.

In this protocol 5 equiv of PNA monomer is initially pre-activated with HATU in the presence of the bases DIPEA and 2,6 lutidine and coupled for 20 min.[12] Published protocols report manual synthesis with a number of PNA monomer equivalents for coupling ranging from 3 to 8.[13–15] When PNA monomers are coupled manually adapting the Egholm procedure, the coupling times are extended at least to 1 h to increase the reaction yield. The coupling time is extended to 6 h when 3 equiv of PNA monomers is used, with HOBT/HBTU as activators and DIPEA as a base to get quantitative coupling. When polypurine stretches have to be synthesized couplings need to be repeated to obtain the desired oligomer. Overall, the combination of large excesses of PNA monomers, the use of HATU as an activator, the increase in reaction times result in a very expensive and time-consuming synthesis.

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Table 1
Sequences and yields

Sequence (name)	% Purine	% Yields			
		Standard protocol	Protocol 1	Protocol 2	Protocol 3
CACACTGTC (PNA 1)	33	19	75	18	45
ACGCACACTGTC (PNA 2)	42	0	45	<10	56
AGACGACCCA (PNA 3)	60	63	68	41	56
GGCCGGGACACA (PNA 4)	67	24	65	50	75

SP, standard protocol: 5 equiv of PNA, 4 equiv HATU, 5 equiv of DIPEA, 7.5 equiv 2,6 lutidine. P1, protocol 1: 5 equiv of PNA, 5 equiv of HOBT/HBTU, 5 equiv NMM, pyridine (25% of the total coupling volume); P2, protocol 2: 2.5 equiv of PNA, 2.5 equiv of HOBT/HBTU 2.5 equiv NMM, pyridine (25% of the total coupling volume); P3, protocol 3: 2.5 equiv of PNA, 2.5 equiv of HOBT/HBTU, 5 equiv NMM, pyridine (40% of the total coupling volume).

2. Results and discussion

With the aim of developing an efficient and low-cost protocol for the manual synthesis of PNA oligomers by Fmoc chemistry, we have explored a new combination of activators and bases. We synthesized for reference PNA oligomers by standard conditions, using 5 equiv of PNA monomers activated with HATU (4 equiv), in the presence of DIPEA (5 equiv) and 2,6 lutidine (7.5 equiv) for 1 min and coupled for 20 min. The deprotection was carried out by treatment of the resin-bound PNA with 20% piperidine in DMF, and capping steps were always carried out with a single treatment with a solution composed of 5/6/89 acetic anhydride/2,6 Lutidine/DMF v/v/v. Cleavage and deprotection were carried out with TFA/*m*-cresol 80:20 v/v, 90 min. Four PNA sequences with a length ranging from 9 to 12 bases were obtained, following the standard and the new protocols (see Table 1 for sequences). As sequences containing long stretches of purines are hard to synthesize, due to the toughness of coupling sterically hindered monomers such as adenine and guanine to each other, we tested the protocols on PNA oligomers having a purine content ranging from 30% to 67%. In all the cases only guanine monomers were double coupled. We started our studies checking protocols for Fmoc peptide and PNA synthesis, looking for conditions in which very little amounts of aminoacid/PNA and different combinations of bases and activators were employed. In this regard a rich font of information is represented by protocols for the coupling of modified PNAs, as those bearing side chains on the backbone. A protocol described by Le Chevalier Isaad et al., reports the efficient synthesis of a peptide by manual coupling using only 2.5 equiv of aminoacids activated with an equal amount of HOBT/TBTU, in the presence of *N*-methyl morpholine (NMM) as a base.[16] For the synthesis of PNAs, we initially referred to the protocol reported by Gogoi (3 equiv of Fmoc/Bhoc monomers activated with HOBT/HBTU in the presence of DIPEA—coupling time 6 h)[13] We reasoned that we could start our investigations changing the activator HATU for the combination HOBT/HBTU and improve the coupling conditions with the use of different bases. The first trials were carried out on two PNA sequences: one 9-mer and one 12-mer with content of purines, respectively, of 33% and 42% (Table 1, PNA 1 and PNA 2). The synthesis scale was 2 μmol; PNAs were elongated on the PAL-PEG PS resin (0.19 mmol/g). Our efforts were initially devoted to replace HATU using 5 equiv of PNA monomers, activated with HOBT/HBTU. We dissolved the PNA monomers (5 equiv) in a solution of HOBT/HBTU (5 equiv) in DMF adding as a base *N*-methyl morpholine (NMM) (5 equiv) dissolved in DMF (pre-activation time 1 min). Oligomers were obtained using repetitive cycles of deprotection, coupling and capping. Coupling time was set to 20 min. Yields were judged at the end of the synthesis after analysis of the LC–MS profiles of the crudes. The results obtained with these protocols were unsatisfactory. We did not obtain the desired oligomers, while we could see many deletes. NMM is a weak base, with a Kb around 10^{-7}, much lower as compared to the Kb for DIPEA. We thought we could combine it with a larger amount of another very weak base, pyridine (Kb 10^{-9}), which is also a good co-solvent for PNA monomers. So we investigated the combination of NMM with pyridine. Syntheses were carried out as described earlier, but coupling was carried out using a solution of NMM (5 equiv) in pyridine.

The amount of pyridine in the coupling mixture is 25% (protocol 1); this quantity does not cause cleavage of the N-terminal Fmoc. Interestingly we obtained the desired oligomers. A comparison of

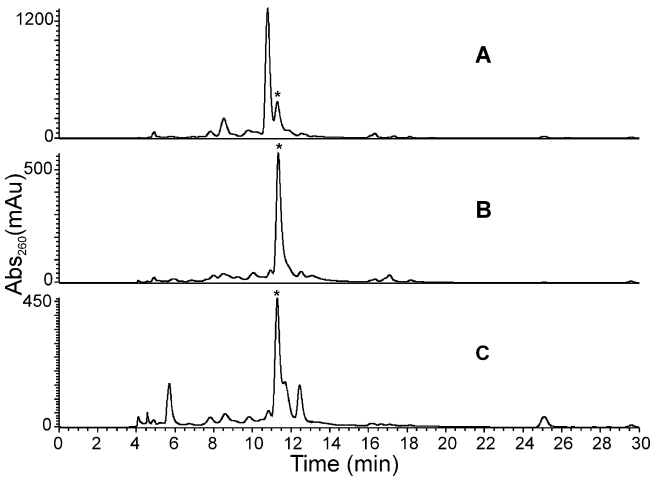

Figure 1. LC profile for the crude obtained for PNA1 with standard protocol (A), protocol 1 (B) and protocol 3 (C). The peak labelled with the asterisk corresponds to the desired product.

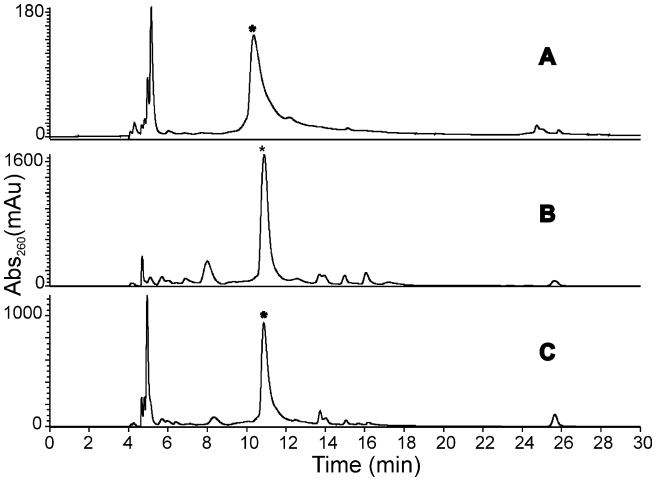

Figure 2. LC profile for the crude obtained for PNA3 with standard protocol (A), protocol 1 (B) and protocol 3 (C). The peak labelled with the asterisk corresponds to the desired product.

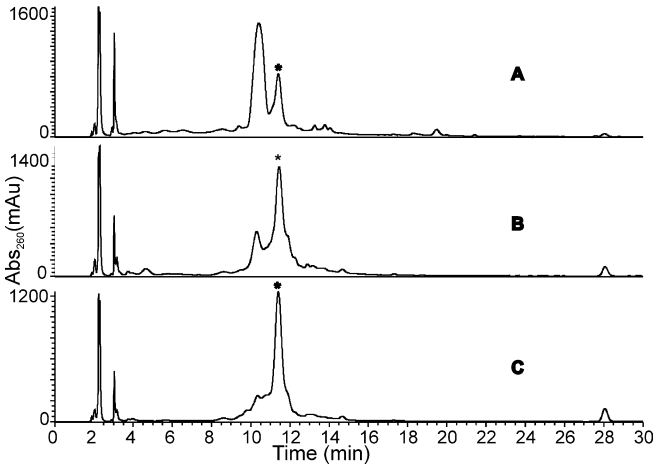

Figure 3. LC profile for the crude obtained for PNA4 with standard protocol (A), protocol 1 (B) and protocol 3 (C). The peak labelled with the asterisk corresponds to the desired product.

the LC–MS profiles of the crudes obtained with the new protocol (HOBT/HBTU/NMM/pyridine) and the standard protocol (HATU/DIPEA, 2,6 lutidine) revealed that yields using the new coupling conditions were significantly improved, as demonstrated by the LC profile in which the desired oligomer now corresponds to the major peak (for PNA 1 see Figure 1, compare panels A and B). Encouraged by these results we tested the same protocol on two more sequences, a 10-mer and a 12-mer with content of purines, respectively, of 60% and 67% (PNA 3 and 4). The PNA oligomers were successfully obtained. The LC–MS profiles in fact show a major peak, containing a single product with the mass corresponding to the desired oligomer. The LC profile for PNAs 3 and 4 is shown in Figures 2 and 3, (compare panel A with B). Next we explored the synthesis of our PNA oligomers reducing the amount of PNA equivalents from 5 to 2.5. All oligomers were synthesized using 2.5 equiv of PNA monomers, activated with an equal amount of HOBT/HBTU and 2.5 equiv of NMM in pyridine as a base (protocol 2). Syntheses yielded the desired products, although in reduced yields, as compared to those obtained using protocol 1 (Table 1). Finally we investigated the effect of increasing the amount of bases. All PNAs were synthesized using 2.5 equiv of PNA monomers, activated with an equal amount of HOBT/HBTU and 5 equiv of NMM in pyridine (protocol 3).

The percentage of pyridine in the coupling mixture is now 40. We found that yields of PNAs doubling the amount of bases with 2.5 equiv of PNA monomers (protocol 3) were in average comparable to those obtained with 5 equiv of PNA and lower content of bases (protocol 1) (Table 1). It is likely that doubling the NMM speeds up the formation of the intermediate active ester (as demonstrated for peptide synthesis) and this effect counterbalances the decrease of equivalents of PNA monomers.[17] The comparison of the LC profiles obtained with protocol 3 is shown for three sequences, respectively, in Figures 1C, 2C and 3C. Furthermore in all cases yields obtained using protocol 3 were comparable or higher than to those obtained with the standard protocol, even for sequences with higher content of purines (Figure 3C and Table 1).

These results suggest that HATU is not necessary for the PNA coupling; the mixture HOBT/HBTU gives very good yields of coupling especially when combined with pyridine/NMM.

3. Conclusions

In conclusion we demonstrated that the manual coupling of Fmoc/Bhoc PNA monomers is very efficient using HOBT/HBTU as activators in the presence of NMM/pyridine as bases. The protocol developed is robust and low cost and can be executed by an automated PNA synthesizer. Interestingly the addition of large amounts of pyridine contributes to increase the yields of oligomers with high content of purines, as demonstrated for PNA 4 (content of purine 67%), in which very high yields are obtained even when the equivalents of monomer per coupling are halved. We believe that the protocol developed will allow the obtainment with good yields of difficult sequences, avoiding expensive activators.

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Supplementary data

Supplementary data (experimental protocols) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.05.026.

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