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Optimization of peptide nucleic acid (PNA) oligomer synthesis using Fmoc/acyl-protected monomers

Györgyi Kovács[*,1] , Zoltán Timár, Zoltán Kele and Lajos Kovács[2]

Nucleic Acids Laboratory, Department of Medicinal Chemistry, University of Szeged, Dóm tér 8, H-6720 Szeged, Hungary

E-mail: [1]gyorgyi@ovrisc.mdche.u-szeged.hu, [2]kovacs@ovrisc.mdche.u-szeged.hu; Phone: +36-62-54 51 45; Fax: +36-62-54 59 71 ; URL: http://www.mdche.u-szeged.hu/staff/nal.htm

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Abstract

The optimization of peptide nucleic acid (PNA) oligomer synthesis was carried out using Fmoc/acyl-protected monomers on NovaSyn® hydroxy-Tentagel resin and HATU/DIPEA/lutidine activation. The sequences H-Leu-ttttt-Gly-NH_2 (**1**) and H-Gly-cgg-act-aag-tcc-att-gc-Gly-NH_2 (**2**) were prepared and characterized by electrospray mass spectrometry.

Introduction

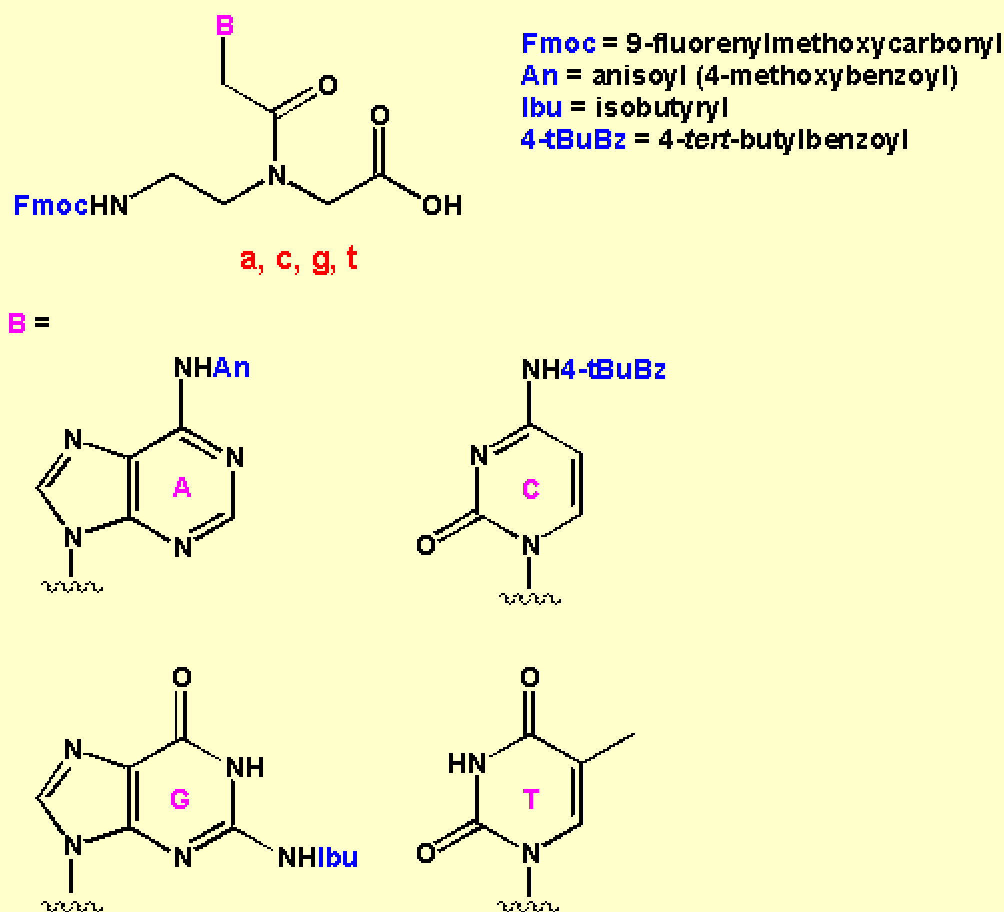
Peptide nucleic acids (PNAs) are DNA mimics with *N*-(2-aminoethyl)glycine backbone and were developed by Nielsen *et al.* [1-3]. Despite its achiral and neutral backbone, PNA was found to form very stable duplexes with Watson-Crick complementary single-stranded DNA and RNA targets and it surpassed its oligonucleotide predecessors in terms of sequence-selectivity, usually forming a PNA_2:DNA triple helix using a strand invasion mechanism. Consequently, since PNA has demonstrated both RNA and DNA binding capabilities, it has obvious potential for use in antisense and antigene strategies for controlling gene expression (treatment of a variety of human genetic disorders). Its use is restricted by poor cellular uptake, aggregation propensity and limited RNase H activity. These difficulties, in part, may be circumvented by the preparation of PNA-peptide and PNA-oligonucleotide conjugates. We aim at the elaboration of oligomerisation protocols which are compatible with the conditions of both peptide and oligonucleotide syntheses. We have recently reported the synthesis of PNA monomers with Fmoc/acyl protecting group combination [4] (Scheme 1) and now we disclose our first results on the application of these monomers in the synthesis of some PNA sequences.

The chemical synthesis of PNA oligomers was first realized using Boc (backbone) and Z (adenine, cytosine) or *O*-benzyl (guanine) protecting groups and later monomethoxytrityl (MMTr)/acyl, Fmoc/Z and Fmoc/MMTr combinations were also evaluated [5]. The combination Fmoc/acyl is well-suited for the preparation of PNA-peptide and PNA-oligonucleotide conjugates. Bergmann *et al.* [6] published their preliminary results on such a combination in some detail, but only pyrimidine oligomers have been reported. Casale *et al.* [7] applied Fmoc/benzhydryloxycarbonyl

(Bhoc) combination on a PAL-PEG-polystyrene resin using an Expedite 8909 oligonucleotide synthesizer. The Fmoc removal was carried out in the usual fashion (20 % piperidine in DMF, quantitation by UV absorbance at 301 nm) and for the coupling the combination *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), *N,N*-diisopropylethylamine (DIPEA, 0.2 M) and lutidine (0.3 M) was employed (the mixture of these bases was more effective than any of the two bases separately) and capping was performed using Ac_2O (5 %) and lutidine (6 %) in DMF. Christensen *et al.* [8] used manual and automated solid phase syntheses on a 5 mmol scale with Boc/Z combination. They applied 7-8 equiv. of the monomers, 6 equiv. HATU and 15 equiv. DIPEA.

Results and discussion

Based on these experiences, we have undertaken the manual solid phase synthesis of PNA oligomers using our Fmoc/acyl protected monomers [4] (Scheme 1) on NovaSyn® hydroxy-Tentagel resin. The first PNA sequence (H-Leu-ttttt-Gly-NH_2, **1**, the lower-case letters a, c, g and t denote the PNA monomer units) was synthesized with amino acids leucine and glycine on the *N*- and *C*-termini, respectively, (in order to prevent acyl migration and subsequent decomposition upon deprotection [9, 10]) and it was chosen to find out the best conditions for attaching the simplest t monomer to the resin (the *C*-terminal amide was the result of cleaving the oligomer from the resin with cc. ammonia).



Scheme 1. The structure of Fmoc/acyl PNA monomers a, c, g and t.

Thus, Fmoc-Gly-O-Tentagel (100 mg, 25 mmol loading capacity) was shaken with 2 x 1.25 mL of 20 % piperidine in *N*-methylpyrrolidone (NMP) for 2 x 5 min then the resin was successively washed with NMP (2 x 1 mL) and ethanol (2 x 1 mL). The t monomer (2 equiv., 0.125 M in NMP) was preactivated with HATU (1.6 equiv.), DIPEA (1.6 equiv.) and lutidine (2.2 equiv.) for 2 min. then the mixture (final volume 400 mL) with the resin was gently shaken for 30 min. Capping: Ac_2O (5 %) and lutidine (6 %) in NMP (1.110 mL). Thorough washing (3 x NMP, 3 x methanol, 3 x NMP) and suction followed each step. The coupling efficiency was monitored using UV spectroscopy by measuring

the absorbance of the Fmoc cleavage mixture at 301 nm in the consecutive steps. The first Fmoc cleavage mixture had the absorbance 0.80 which decreased to 0.38 after the fifth coupling corresponding to 85-90 % yields per coupling step. Before the final cleavage from the support (cc. ammonia, 50 °C, 16 h) the resin was washed with methanol and dichloromethane then cc. ammonia (3 mL) was added. After lyophilization HPLC assay [BST Rutin C18 300 A 250 x 4 mm, detection at 260 nm, 0-40 % eluent A (0.1 % aq. TFA) in eluent B (0.1 % TFA in 80 % aq. acetonitrile) in 40 min at eluent rate 1.0 mL/min] indicated that 51 % of the mixture was the expected product. The electrospray ionization (ESI) mass spectrometry data (Figure 1) were consistent with the correct structure.

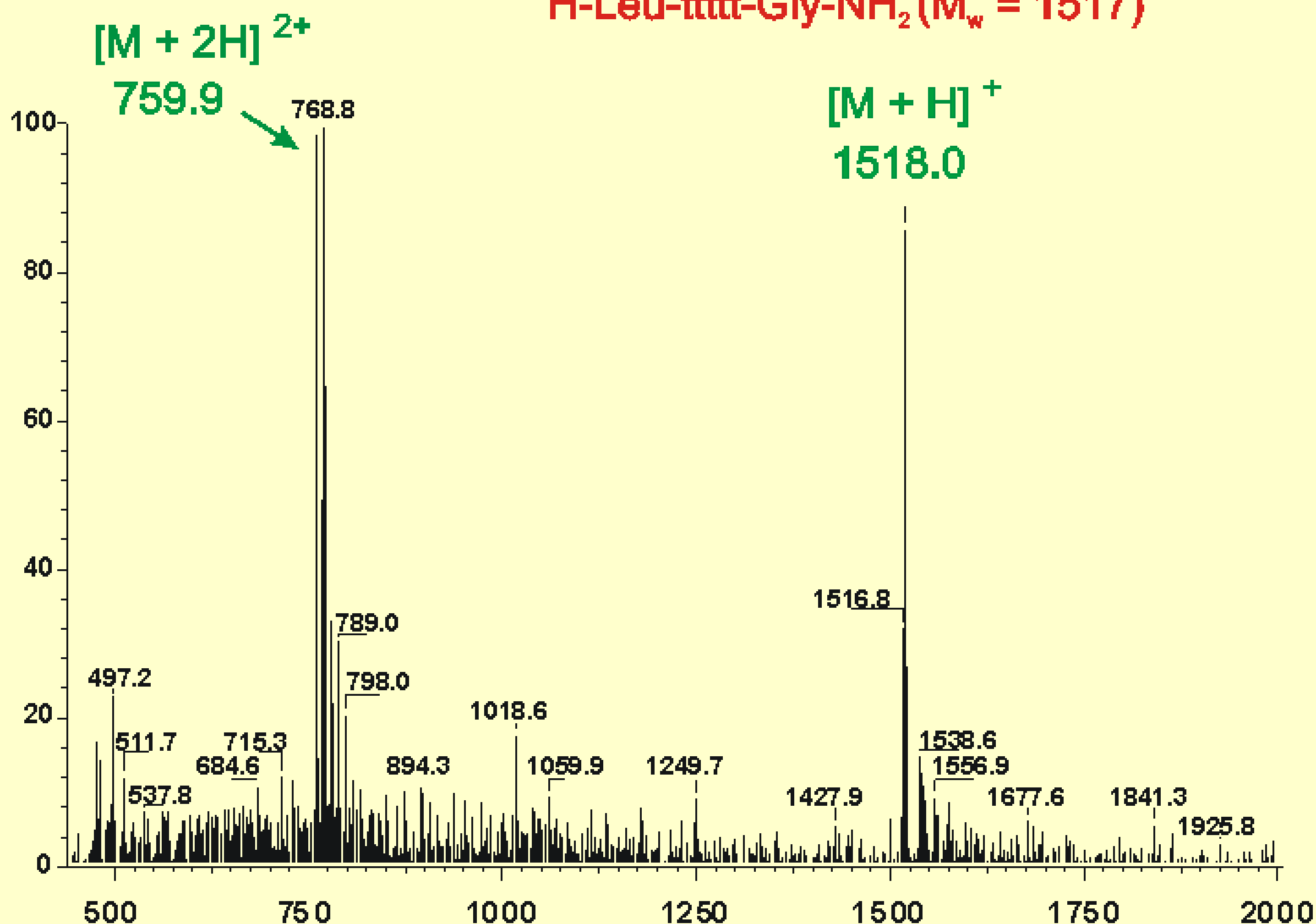


Figure 1

To optimize the coupling steps, a solution phase model reaction was performed using L-phenylalanine *tert.*-butyl ester [11] and the four monomers under the conditions given in Table 1 and the reaction course was followed using HPLC analysis. *O*-(Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*-hydroxybenzotriazole (HOBt)/*N*-methylmorpholine (NMM) (1:1:1.5) and HATU/lutidine (1:1.5) mixtures were alternatively employed and higher yield was obtained when freshly distilled anhydrous DMF was used instead of NMP. HATU performed better and after 10 min ca. 95 % coupling yield was obtained for each monomer. As it can be judged from Table 1, the nature of added bases did not influence the yields and the activating agents had only little impact.

Table 1. Solution phase optimization of PNA monomer coupling to H-L-Phe-O^{t}Bu (yields determined by integration of HPLC peaks).

Monomer	*HATU (equiv.)*	*HBTU/HOBt (equiv.)*	*Lutidine (equiv.)*	*DIPEA (equiv.)*	*Duration of coupling (min)*	*Yield (%)*
g	none	1:1	1.5	none	11	> 95

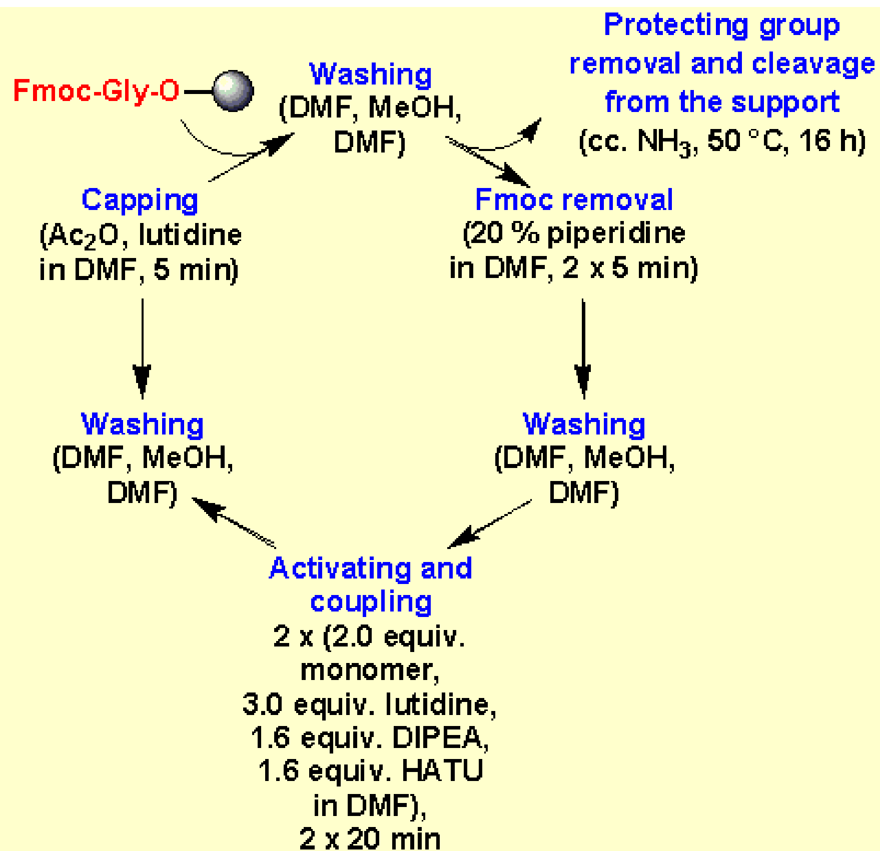
g	1	none	1.5	none	13	> 95
c	none	1:1	1.5	0.5	10	95
a	none	1:1	1.5	0.5	10	95
t	none	1:1	1.5	0.5	10	95
t	1	none	1.5	0.5	10	> 95

Next, the manual solid phase synthesis of the sequence H-Gly-cgg-act-aag-tcc-att-gc-Gly-NH_2 (**2**, suggested as a good test sequence by Christensen et al. [8]) was undertaken on Fmoc-Gly-O-Tentagel in DMF. The first five couplings were performed using HBTU/HOBt/lutidine mixture in the presence or absence of 0.5 equiv. DIPEA for 30 min. In both cases the first monomer (c^1) coupled only in 50 % and the following monomers in 80-90 % yields (UV absorbance data). The efficiency of the couplings could be improved using repeated couplings with twofold excess of the monomers (in the case of the worst-coupling c monomer the yield could be increased to 80 %, Table 2).

Table 2. Solid phase optimization of coupling the c monomer to H-Gly-O-Tentagel (yields determined by UV absorbance at 301 nm).

Coupled...	*HBTU/HOBt (equiv.)*	*Lutidine (equiv.)*	*DIPEA (equiv.)*	*Duration of coupling (min)*	*Yield (%)*
1 x	1:1	1.5	none	30	50
1 x	1:1	1.5	0.5	30	50
2 x	1:1	1.5	0.5	2 x 15	80

From the results of solid and solution phase couplings the following procedure deems to be the method of choice for the preparation of PNA oligomer **2** from Fmoc/acyl-protected monomers: The twofold excess of monomer (0.125 M) was allowed to be preactivated with HATU (1.6 equiv.), DIPEA (1.6 equiv.) and lutidine (3.0 equiv.) for 1-2 min. and this solution was added to H-Gly-O-Tentagel resin (100 mg) and the mixture was gently shaken for 20 min, then the coupling was repeated once again. The remaining steps were carried out in the same fashion as described for the t_5 sequence, the only difference being in the last coupling, where Fmoc-Gly-OH was used instead of Fmoc-Leu-OH. The synthesis cycle is outlined on Scheme 2. The UV measurements were performed at 301, 289, 265 and 256 nm and the results were practically identical for all wavelengths. The first c monomer coupled quantitatively. The absorbance after the first coupling was 0.63 which decreased to 0.27 by the end of the synthesis, which means 43 % overall yield corresponding to 95 % coupling efficiency per step for the 16 steps. The obtained sample was analyzed by ESI mass spectrometry and a series of multiply charged peaks ($[M+4H]^{4+}$ at 1179.3, $[M+5H]^{5+}$ at 943.9, $[M+7H]^{7+}$ at 674.3 etc.) were observed, which corroborate the expected structure (M_w = 4714).



Scheme 2. The synthesis cycle for preparing PNA oligomers.

In conclusion, we have described the results of our optimization experiments for preparing PNA oligomers starting from the previously synthesized Fmoc/acyl monomers [4] (Scheme 1). The influence of coupling agents, added bases (Table 1), solvents and monomer excess (Table 2) was investigated and an optimized procedure giving ca. 95 % stepwise yield was described. The prepared PNA oligomers were characterized by ESI mass spectrometry.

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