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Synthesis of Polyamide Nucleic Acids (PNAs) using a Novel Fmoc/Mmt Protecting-Group combination

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Abstract: The preparation of 9-Fluorenylmethoxycarbonyl(Fmoc) protected building blocks for the synthesis of polyamide nucleic acids (PNAs) is described. Use of 4-Methoxyphenyldiphenylmethyl (Mmt)-protecting groups for the exocyclic amino function of the nucleobases enhances the solubility of the monomers and allows final deprotection by mild acid treatment. The novel synthetic route is exemplified by the synthesis of heptameric and octameric PNAs.

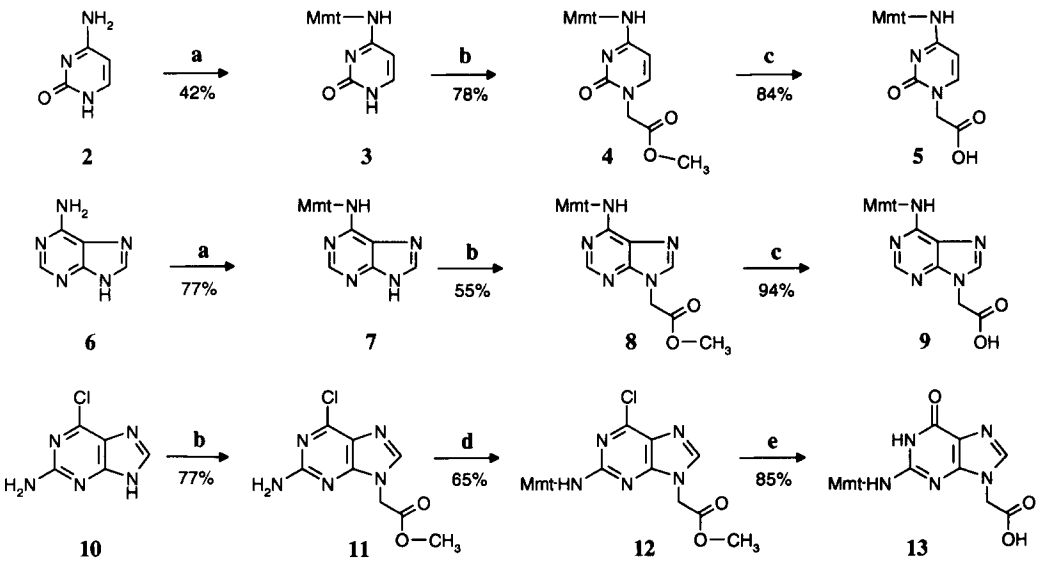
Introduction

Polyamide nucleic acids[1,2] (PNAs) are analogues of oligonucleotides where the phosphate-sugar backbone has been replaced by an uncharged achiral polyamide backbone[3] consisting of N-(2-amino-ethyl)glycine units. PNAs exhibit a remarkable behavior in several ways: they specifically recognize a complementary DNA or RNA strand and form very stable DNA/PNA duplexes and PNA/DNA/PNA triple helix structures[4]. The formed hybrids are more stable than their oligonucleotide equivalents and furthermore, PNAs are resistant to enzymatic degradation by nucleases and peptidases. They therefore have a potential to replace oligonucleotides for use in antisense therapeutics[5], molecular biology reagents and chemical diagnostics. Most syntheses of PNAs described in the literature[1,2,4] use Boc/Z-protected intermediates with repetitive TFA deprotection during chain elongation and final HF or trifluoromethane sulfonic acid cleavage to release the PNAs from the resin. One recent paper describes the Fmoc/Z protected intermediates[6]. In the final cleavage step, however, HF is needed as for the Boc-protected monomers. We have developed a milder method for the synthesis of PNAs using Fmoc protection for the N-(2-aminoethyl)glycine unit in combination with acid labile Mmt protection for the exocyclic aminofunction of the nucleobases.

Results and Discussion

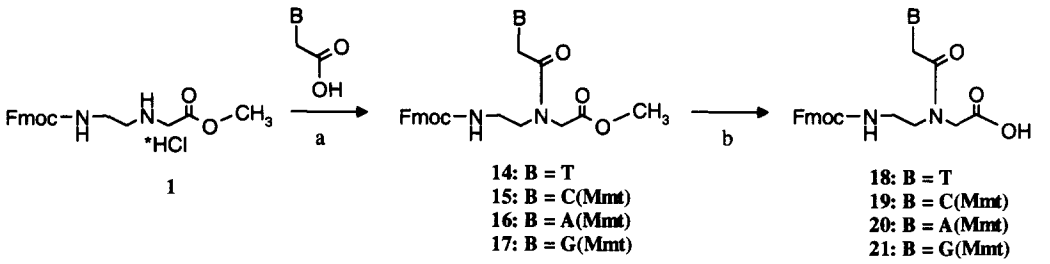
As a convenient method for synthesis of N-(2-aminoethyl)glycine (H-Aeg-OH) we used the very simple and effective reductive amination of glyoxylic acid with an excess of 1,2-diaminoethane in an alcohol/water mixture with hydrogen as reducing agent and palladium on charcoal as catalyst[7]. H-Aeg-OMe * 2HCl was

prepared according to the literature procedure[8]. Fmoc-Aeg-OMe (**1**) was prepared in dioxane/water using Fmoc-ONSu and is isolated as its hydrochloride. The Mmt-protected carboxymethyl-nucleobases were synthesized according to Scheme 1. For synthesis of the carboxymethyl cytosine (**5**) and adenine (**9**), the exocyclic amino function of the nucleobase was protected first with Mmt-chloride in a pyridine/4-N-ethylmorpholine mixture to give (**3**) and (**7**). Deprotonation with NaH in DMF and reaction with methyl bromoacetate led to (**4**) and (**8**) which were purified by chromatography. For adenine predominantly N-9 alkylation was found. Saponification of the ester with NaOH in dioxane/water gave the protected carboxymethyl-nucleobases. The guanine derivative (**13**) was prepared from 2-amino-6-chloropurine (**10**) by alkylation of the 9-nitrogen with methyl bromoacetate to give exclusively the N-9 alkylated ester (**11**). Protection of the exocyclic nitrogen function with Mmt-chloride led to (**12**). Saponification with hot 10% aequeous NaOH introduced also the 6-hydroxy function of guanine. Carboxymethyl-thymine was synthesized as described in the literature.[2]



Scheme 1: Reagents and conditions: **a**) Mmt-Cl, pyridine, 4-N-ethylmorpholine, 40°C; **b**) NaH/DMF, methylbromoacetate; **c**) NaOH, dioxane/water; **d**) Mmt-Cl, pyridine, triethylamine, flash chromatography; **e**) 10% NaOH, reflux, 3h

The carboxymethyl-nucleobases were then coupled onto the Fmoc-(2-aminoethyl)glycine methylester by standard peptide coupling procedures using uronium reagents like TOTU[9]. Saponification of the methylesters (**14-17**) proceeded smoothly under controlled conditions. Fmoc deprotection was observed as side reaction but easily reversed by addition of some Fmoc-ONSu to the reaction mixture after complete saponification. Typical procedures for some synthetic steps are given in the experimental section below.



Scheme 2: Reagents and conditions: **a**) TOTU, DMF/DIPEA; b) NaOH in dioxane/water followed by 10-30 mol% of Fmoc-ONSu

The resulting monomeric building blocks were used for the synthesis of PNAs with an Abimed 422 multiple peptide synthesizer using standard peptide synthesis conditions with 20% piperidine/DMF as deprotection and PyBOP[10] as coupling reagents. As resin we used aminomethylated polystyrene modified with our previously described amide linker[11]. Cleavage of the final product with the concomitant removal of the base protecting groups was achieved by treatment with 95% trifluoroacetic acid. As examples we synthesized H-(Aeg(T))8-Lys-NH_2, H-(Aeg(C))7-Lys-NH_2 and Ac-(Aeg(G)-Aeg(A)8-hex-OH which gave satisfactory analytical data. The latter purin-derivative was synthesized using an aminohexanol-derivatized CPG[7] and the building blocks (**20**) and (**21**). The hplc-trace of crude and the mass spectrum of purified H-(Aeg(C)7-Lys-NH_2 is shown in figure 1.

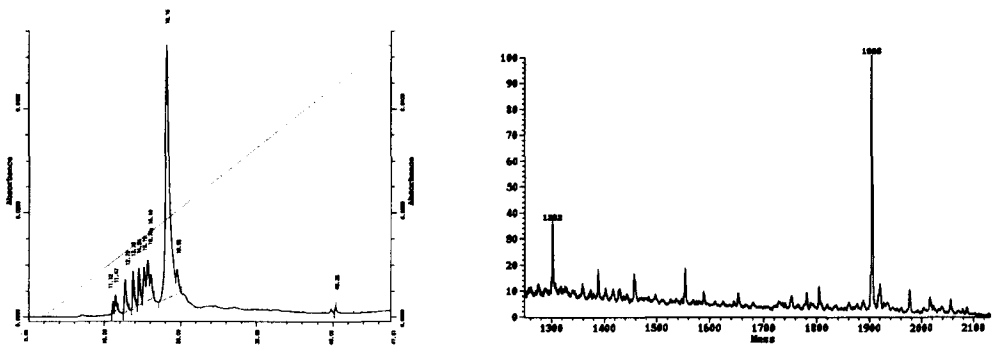


Figure 1: hplc-trace of crude and the mass spectrum of purified H-(Aeg(C))$_7$-Lys-NH_2, M (calc. for $C_{76}H_{106}N_{38}O_{22}$) 1903.92, M(exp.): 1905 $(M+H)^+$

In conclusion, we have described the synthesis of novel PNA monomers with orthogonal protecting groups in which the Fmoc group is used as a temporary protecting group, similar to Fmoc peptide synthesis, and the exocyclic amino function of the nucleobases are protected by acid-labile Mmt protecting groups. The Mmt group has the advantage that it renders monomeric building blocks of good solubility which is most important for the guanine derivative, and that it can be removed easily by TFA or 80% acetic acid. Furthermore, we have shown, that the Fmoc/Mmt protecting group combination can be successfully used for the synthesis of PNAs. In principle, this strategy may also be used for convenient synthesis of PNA-peptide conjugates.

Experimental Section

Materials and Methods: Reagents were obtained from commercial suppliers and used as received. The following abbreviations are used: 9-Fluorenylmethyloxycarbonyl (Fmoc); 4-Methoxytriphenylmethyl (Mmt); 4-N-Ethylmorpholine (NEM); N,N-Dimethylformamide (DMF); O-[(Cyano(ethoxycarbonyl)methylen)amino]-1,1,3,3-tetramethyluronium-tetrafluoroborate (TOTU); (Benzotriazol-1-yloxy)-tripyrrolidinophosphonium-hexafluorophosphate (PyBOP). HPLC analysis of PNAs was carried out on a Beckman System Gold HPLC system using a LiChro CART 100 RP-18 endcapped (5µm) (E. Merck, Darmstadt, Germany) column eluting with a linear gradient of 0-100% B in 50 min (B: 20% acetonitrile in 20 mM NH_4OAc, pH 8; A: 20 mM NH_4OAc).

Protection of the exocyclic amino function with Mmt: 20 mmol nucleobase and 30 mmol Mmt chloride were suspended in 100 mL of dry pyridine and, after the addition of 20 mmol 4-N-ethylmorpholine, this mixture was briefly heated to approximately 40 °C and left to stand overnight. Then water was added to the stirred suspension followed by dichloromethane and the resulting precipitate was filtered off.

Alkylation of the nucleobase with methyl bromoacetate: 10 mmol of Mmt-protected nucleobase were dispersed in 40 mL of dry DMF followed by addition of 10 mmol sodium hydride to the stirred suspension. After one hour 11 mmol of methyl bromoacetate were added and stirring was continued for a further 2h. Then the reaction was quenched with 2 mL of methanol and the solution was concentrated in vacuo on a rotary evaporator. The residue was triturated with water, filtered off with suction and dried to give the product which is further purified by chromatography if necessary.

Coupling of the carboxymethyl nucleobase to the backbone: 5 mmol of (**1**) were dissolved together with 5 mmol of carboxymethyl nucleobase in 10 mL of dry DMF, and 5 mmol of TOTU and 15 mmol of diisopropylethylamine were then added one after the other. The mixture was stirred at room temperature for 3h and then concentrated in vacuo on a rotary evaporator. The residue was taken up in ethyl acetate and extracted three times with solutions of sodium hydrogen carbonate and potassium hydrogen sulfate. The organic phase was concentrated and the residue was precipitated from ethyl acetate/ether. The crude product is further purified by chromatography if necessary or subjected directly to the following saponification step.

Saponification of the Fmoc-protected monomer methyl ester: 5 mmol of the Fmoc-protected monomer methyl ester were dissolved in a mixture of 15 mL of dioxane and 10 mL of water and saponified at 0 °C by the portion-wise addition of of a (1:1) mixture of 1N NaOH and dioxane. After the reaction was complete, the mixture was buffered by adding a little solid carbon dioxide. Fmoc elimination, which has taken place to some extent, was reversed by addition of of Fmoc-ONSu (10-30 mol%) and stirring for 45 min. The mixture was then adjusted to pH 6.5 with a 2M solution of potassium hydrogen sulfate. Dioxane was removed in vacuo and the solution was diluted with water, acidified to pH 5 with a solution of potassium hydrogen sulfate and extracted three times with ethyl acetate. The combined organic phases were dried over sodium sulfate and concentrated in vacuo. The residue was dissolved in a mixture of 5 mL of methanol and 15 mL of

ethyl acetate and precipitated by addition of methyl butyl ether. The product was filtered off and dried in vacuo or further purified by chromatography if necessary.

Oligomer Synthesis: H-(Aeg(T))8-Lys-NH2: The PNAs were synthesisized on an aminomethyl-polystyrene resin modified with our amide linker[11], using a multiple peptide synthesizer model AMS 422 from Abimed (Langenfeld, Germany). 14.7 mg (10 μmol) of the resin were pre-swollen in DMF and introduced into a reaction vessel of the multiple peptide synthesizer. The following reaction solutions were used in the synthesis: 1) Activator: 0.88 M PyBOP in DMF 2) Activator base: 3.95 M NEM in DMF 3): 0.65 M Fmoc-Lys(Boc)-OH in DMF 4) 0.49 M (**18**) in DMF 5) Deprotection: 20% piperidine/DMF. Following removal of Fmoc with piperidine and washing with DMF, activator solution, Fmoc derivative (5 equivalents) and base were added to the deprotected resin in the reaction vessel and coupled 40 min (average coupling yield 94%). The resin was subsequently washed 5 times with DMF and was ready for the next treatment with piperidine. After the final Fmoc-deprotection, the resin was dried and treated in portions with a total of 4 mL of 95% TFA (approximately 2.5 h). The product was precipitated by dissolving in 250 μL of TFA and addition of 3 mL of methyl tert-butyl ether. The precipitate was sedimented by centrifugation and washed several times with methyl tert-butyl ether. Yield: 221 OD_{260} ($C_{49}H_{127}N_{35}O_{33}$, 2275.26) MS(FAB, AcOH/NBA): 2276$(M+H)^+$.

H-(Aeg(C))7-Lys-NH2: The synthesis was carried out as described for H-(Aeg(T))8-Lys-NH_2 in the previous procedure but using 0.65 M (**19**) in DMF. Final deprotection was carried out as described above (crude yield: 218 OD_{260}). 70 OD_{260} of this crude product were purified on a Zorbax ODS column eluting with a linear gradient of 20-80% B in 70 min (B: 20% acetonitrile in 20 mM NH_4OAc, pH 8; A: 20 mM NH_4OAc). Yield: 44 OD_{260} ($C_{76}H_{106}N_{38}O_{22}$, 1903.92) MS(FAB, TFA/NBA): 1905 $(M+H)^+$.

Ac-(Aeg(G))-(Aeg(A))8-hex-OH: This compound was synthesized in a 1μmol scale on a modified Eppendorf Biotronik Ecosyn™ D-300 DNA synthesizer (Eppendorf Biotronik, Maintal, Germany) using an aminohexanol-derivatized CPG[7] and a 0.3 M solution of the building blocks (**20**) and (**21**) in DMF. After Fmoc cleavage, capping with Ac_2O and removal of the Mmt-protection with 3% TCA the compound was cleaved from the resin by treatment with conc. ammonia for 2.5h at room temperature. Crude yield: 30 OD_{260} ($C_{107}H_{134}N_{64}O_{21}$, 2652.66) MS($ES^+$): 2652.39 $(M)^+$.

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12. Data for the compounds are given below as follows: compound no. yield (%)/ R_f -values on Merck DC Kieselgel 60 F-254 glass plates in the solvent system mentioned (A: BuOH/AcOH/H_2O 3:1:1; B: AcOEt; C: AcOEt/MeOH 3:1 ;D: DCM/MeOH 9:1; E: DCM/MeOH 97:3; F: DCM/MeOH 7:3; G: BuOH/pyridine/ H_2O /AcOH 8:2:2:10) / mass spectrum are FAB unless otherwise stated, addition of LiCl is indicated/ ^1H NMR measured in dmso-d_6 unless otherwise stated:{**1** (63%)/ R_f(A): 0.50/ MS(+LiCl): 361.2 $(M+Li)^+$ /NMR: δ 9.23 (brs, 2H); 7.93 - 7.08 (m, 9H); 4.40 - 4.15 (m, 3H); 4.00 (s, 2H); 3.93 (s, 3H); 3.40 - 2.97 (m, 4H)} {**3** (42%)/ R_f(D): 0.45/ MS: 384.2$(M+H)^+$/ NMR: δ = 10.22 (br s, 1H); 8.22 (br s, 1H); 7.38 - 6.78 (m, 16H); 3.73 (s, 3H)} {**4** (78%)/ R_f(B): 0.39/ MS(LiCl): 462.2$(M+Li)^+$ /NMR: δ 8.38 (br s, 1H); 7.50 - 6.78 (m, 16H); 6.15 (br s, 1H); 4.34 (s, 2H); 3.73 (s, 3H); 3.63 (s, 3H)} {**5** (84%)/ R_f(B): 0.27/ MS(+LiCl): 448$(M+Li)^+$/ NMR: δ 8.30 (br s, 1H); 7.48 - 6.78 (m, 16H); 6.15 (br s, 1H); 4.20 (s, 2H); 3.73 (s, 3H)} {**7** (77%) R_f(B): 0.53/ MS: 408.2$(M+H)^+$/ NMR ($CDCl_3$): δ 8.08 (s, 1H); 7.88 (s, 1H); 7.40 - 7.20 (m, 12H); 7.00 (s, 1H); 6.80 - 6.70 (m, 2H); 3.77 (s, 3H)} {**8** (55%)/ R_f(C): 0.68/ MS(+LiCl): 480.2$(M+H)^+$; 485.2$(M+Li)^+$/ NMR: δ 8.07 (s, 1H); 7.81 (s, 1H); 7.40 - 7.10 (m, 13H); 6.92 (s, 1H); 6.80 - 6.70 (m, 2H); 4.95 (s, 2H); 3.80 (s, 3H); 3.77 (s, 3H)}{**9** (94%)/ R_f(C): 0.15/ MS(+LiCl): 465.1$(M+H)^+$; 472.1$(M+Li)^+$/ NMR: δ 8.05 (s, 1H); 7.83 (s, 1H); 7.40 - 7.10 (m, 12H); 7.03 (s, 1H); 6.80 - 6.70 (m, 2H); 4.37 (s, 2H); 3.72 (s, 3H)} {**11** (77%)/ R_f(D): 0.44/ MS(CI): 242$(M+H)^+$/ NMR: δ 8.09 (s, 1H); 6.97(s, 2H); 5.00 (s, 2H); 3.72 (s, 3H)} {**12** (65%)/ R_f(E): 0.60/ MS(ES^+): 514.2$(M+H)^+$/ NMR: δ 8.23 (br s, 1H); 8.04 (s, 1H); 7.40 - 7.10 (m, 12H); 6.80 - 6.70 (m, 2H); 4.55 (s, 2H); 3.70 (s, 3H); 3.58 (s, 3H)} {**13** (85%)/ R_f(F): 0.10/ MS(+LiCl): 488.2$(M+Li)^+$/ NMR: δ 7.80 (s, 1H); 7.40 - 7.10 (m, 12H); 6.80 - 6.70 (m, 2H); 6.52 (s, 1H); 4.26 (s, 2H); 3.74 (s, 3H)} {**14** (78%)/ R_f(A): 0.63/ MS(ES^+): 521.4$(M+H)^+$/ NMR: δ 11.28 (s, 1H);7.95 - 7.20 (m, 10H); 4.66 and 4.47 (s, 2H, two rotamers); 4.38 -4.15 (m, 4.2H); 4.06 (s, 0.8H); 3.72 and 3.83 (s, 3H, two rotamers); 3.35 - 3.05 (m, 4H; 1.73 (s, 3H)} {**15** (82%)/ R_f(G): 0.79/ MS: 778.4$(M)^+$/ NMR: δ 7.95 (s,1H); 7.92 - 6.75 (m, 24H); 5.45 (br d, 1H); 4.50(br s, 1H; 4.42 -4.15 (m, 5H); 4.02(br s, 1H); 3.72 (s, 3H); 3.62 (s, 3H); 3.45 - 3.05 (m, 4H)} {**16** (52%)/ R_f(A): 0.85/ MS: 802.4$(M+H)^+$/ NMR: δ 8.10 - 7.10 (m, 23H); 6.90 - 6.80 (m, 2H); 5.25 and 5.05 (br s, 2H, two rotamers); 4.42 -4.15 (m, 3.8H); 4.10(br s, 1.2H); 3.76 (s, 3H); 3.62 (s, 3H); 3.45 - 3.05 (m, 4H)} {**18** (89%)/ R_f(A): 0.40/ MS: 507.1873 ($C_{26}H_{26}N_4O_7$ + H requires 507.1880)/ NMR: δ 11.25 (s, 1H);7.93 - 7.20 (m, 10H); 4.64 and 4.45 (s,2H, two rotamers); 4.38 -4.15 (m, 3H); 3.95 (br s, 2H); 3.35 - 3.05 (m, 4H; 1.73 (s, 3H)} {**19** (66%)/ R_f(A): 0.80/ MS: 764.3081 ($C_{45}H_{41}N_5O_7$ + H requires 764.3084)/ NMR: δ 7.95 (s,1H); 7.92 - 6.75 (m, 24H); 5.45 (br d, 1H); 4.82 and 4.63 (s, 1H; 4.42 -4.15 (m, 3.8H); 4.06 (s, 1.2H); 3.72 (s, 3H); 3.45 - 3.05 (m, 4H)} {**20** (88%)/ R_f(A): 0.73/ MS: 788.3190 ($C_{46}H_{41}N_7O_6$ + H requires 788.3197)/ NMR: δ 8.10 - 7.10 (m, 23H); 6.90 - 6.80 (m, 2H); 5.18 and 5.02 (br s, 2H, two rotamers); 4.38 -4.18 (m, 3H); 3.97 and 3.90 (br s, 2H, two rotamers); 3.71 (s, 3H); 3.45 - 3.05 (m, 4H)} {**21** (41%)/ R_f(F): 0.35/ MS: 826.2963 ($C_{46}H_{41}N_7O_6$ + Na requires 826.2966)/ NMR: δ 10.50 (br s, 1H);7.9 - 6.8 (m, 24H); 4.50 (br s, 1.2H); 4.43 -4.15 (m, 3.8H); 3.90 and 3.78 (br s, 2H, two rotamers); 3.70 and 3.67 (s, 3H, two rotamers); 3.45 - 2.80 (m, 4H)}.

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