Journal of Peptide Science
J. Peptide Sci. **7**: 402–412 (2001)
DOI: 10.1002/psc.333

PNA Synthesis Using a Novel Boc/Acyl Protecting Group Strategy

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Received 26 April 2000
Accepted 26 March 2001

Abstract: The synthesis of novel Boc/acyl protected monomers for the synthesis of peptide nucleic acid (PNA) is described. The oligomerization protocol using these new monomers has been optimized with regard to coupling reagents. The use of base-labile acyl protecting groups at the exocyclic amines of the heterocyclic bases (isobutyryl for guanine and benzoyl for adenine and cytosine) and a PAM-linked solid support offers an attractive alternative to the present procedures used in PNA synthesis. This strategy has been applied for the synthesis of a test 17mer PNA on both control pore glass (CPG) and a polystyrene MBHA support and was used in the preparation of PNA–DNA chimeras. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: nucleic acid analogues; peptide nucleic acid monomers; PNA synthesis; protecting groups; solid-phase synthesis

INTRODUCTION

The original synthesis of peptide nucleic acid (PNA) was based on a modified Merrifield peptide synthesis strategy using Boc/Z protected PNA monomers [1]. This strategy is very robust and has been used successfully in the synthesis of both PNA and PNA-peptide chimeras. The combination of PNA and DNA in one molecule results in PNA–DNA chimeras with interesting new properties. Unfortunately, the harsh acidic conditions required during deprotection using the Boc/Z strategy are incompatible with the synthesis of PNA–DNA chimeras. The orthogonal Fmoc/Z strategy reported by Thomson *et al.* [2] is also restricted in this issue. Therefore, a series of different synthesis strategies have been developed, in which the PNA monomers were prepared with the main focus on PNA–DNA chimera preparation. Breipohl *et al.* [3–5] developed a Fmoc/Mmt and a Mmt/acyl strategy, and Fmoc has also been used in combination with acyl protecting groups on the exocyclic amines [6].

One of the main requirements for an oligomerization process is the demand for nearly quantitative coupling efficiency in each coupling steep. The average coupling yields for the above-mentioned strategies are reported to be between 94 and 99%. In the Fmoc/Mmt strategy reported by Breipohl *et al.* [5] an average coupling yield of 94% was reported. A somewhat higher yield (97%) was observed for the Fmoc/Z strategy [2]. For the Mmt strategy [3] the average yield is in the order of 95–99%, but the highest average coupling yields have been reported for the Boc/Z strategy. Christensen *et al.* [7] reported an average coupling yield of 97% and a recently improved protocol has been published [8] which repeatedly provides coupling yields above

Abbreviations: Ac, acetyl; Boc, *tert*-butyloxycarbonyl; CPG, control pore glass; DCC, *N*,*N*-dicyclohexylcarbodiimide; DIC, *N*,*N*-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; Dmt, di-(*p*-methoxyphenyl)phenylmethyl; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 3-hydroxy-1,2,3-benzotriazole; Mmt, mono-*p*-methoxyphenyldiphenylmethyl; MBHA, methylbenzhydrylamine; NHS, *N*-hydroxysuccinicamide; NMM, *N*-methylmorpholine; PAM, phenylacetamidomethyl; PyAOP, 7-azabenzotriazol-1-yloxy-tris(pyrrolidino)-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; Z, benzyloxycarbonyl.

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99%. The reason for the high coupling yields for the Boc/Z strategy is presumably twofold: (i) the Boc group is removed with TFA, which provides a fast and nearly quantitative deprotection; and (ii) TFA disrupts aggregations in the growing oligomer chain, which solvates the oligomers completely thereby providing optimal conditions in the subsequent coupling step.

Fmoc strategies seem to give the lowest coupling yields for which there are several explanations. In PNA synthesis where aggregations are frequently encountered [9,10] difficult deprotection of the Fmoc group becomes an issue. Furthermore, the conditions needed during Fmoc deprotection, 15% piperidine in polar aprotic solvents, induces side reactions in PNA. The side reactions are related to the nucleophilic character of the liberated *N*-terminal amine [2,7,8] and result in detachment of the terminal monomer or in acyl migration. For a recent review on PNA see Uhlmann *et al.* [11].

Based on the current knowledge on PNA synthesis we have designed a new strategy, which is suitable for both normal PNA synthesis and for most chimera syntheses. Boc was chosen for backbone protection for the reasons mentioned above and acyl was chosen for base protection. In this paper we describe the synthesis of these new Boc/acyl monomers and an optimized synthesis protocol used in the oligomerization process. To demonstrate the utility of this new strategy we synthesize a test 17mer PNA on both a CPG and MBHA solid support, and finally show how the strategy can be used in the synthesis of PNA–DNA chimeras. We also estimate the overall recovered yield of PNA and compare this with the yield of the standard Boc/Z strategy.

MATERIALS AND METHODS

HOBt and HBTU were purchased from Nova-Biochem, PyAOP was from PerSeptive Biosystems, CPG was from Solid Phase Sciences and 6-(Mmt-amino)hexyl phosphoramidite was from Clonetech. All other reagents were purchased from Sigma-Aldrich-Fluka.

^{1}H-NMR spectra were recorded in d_6-DMSO at 250 MHz. MALDI-TOF MS data were recorded on a HP G2025A LD-TOF spectrometer. A mixture of diammonium hydrogen citrate and 2,6-dihydroxyacetophenone was used as matrix solution for chimeras. For all other samples *trans*-3,5-dimetoxy-4-hydroxycinnamic acid was used as matrix. RP HPLC was carried out on a Hewlett-Packard 1050 system using a Deltapack C_{18} column for analytical HPLC (100 Å, 5 μm, 3.9 × 150, 1 ml/min) and a Vydac 218TP510 column for semipreparative HPLC (100 Å, 5 μm, 10 × 310, 3 ml/min). For pure PNA-oligomers two eluents were used: (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. A linear gradient from 0 to 40% of B in 30 min was used. For chimeras the eluents used were (A) 0.1 M triethylammonium acetate (pH 7.0) and (B) acetonitrile. A linear gradient from 0 to 40% of B in 30 min was used.

Synthesis of Boc/acyl Monomers and Linkers

***N*-Boc-ethylene diamine (1)**. Ethylene diamine (13.5 kg, 225 mol) was dissolved in DCM (64 l). A solution of di-*tert*-butyldicarbonate (6.6 kg, 30 mol) in DCM (6 l) was added under vigorous stirring over a period of 1 h. The reaction mixture was stirred for 1 h while cooling with water to 20°C. Water (38 l) was slowly added to the reaction mixture over a period of 45 min. The mixture was stirred for another 15 min before phase separation and removal of the water phase. Water (20 l) was added and the pH was adjusted to 1.5 with conc. HCl. The mixture was stirred for 15 min followed by phase separation and removal of the organic phase. DCM (32 l) was added and the pH adjusted to 12 with 28% NaOH solution (4.9 kg, 34 mol). The mixture was stirred for 15 min before removal of the organic phase. The water phase was extracted with DCM (32 l) and the combined organic phases were evaporated to dryness *in vacuo*. This yielded 3.6 kg (75%) of crude *N*-Boc-ethylene diamine (**1**) that had a purity of > 99% (as measured by GC). This crude product was used without further purification.

Methyl N-(2-Boc-aminoethyl)glycinate (2). *N*-Boc-ethylene diamine (3.6 kg, 22.5 mol) was dissolved in a mixture of acetonitrile (50 l) and triethylamine (3.0 kg, 29.7 mol). Methyl bromoacetate (3.8 kg, 24.8 mol) was added under vigorous stirring over 30 min. The reaction mixture was stirred for another 2 h. The reaction mixture was concentrated *in vacuo* to 15 l and water (16 l) was added to the remanense. The solution was cooled to 5°C and the pH was adjusted to 10.5 with 28% NaOH solution (1.45 kg, 10 mol). The solution was extracted with DCM (35 l). The pH of the water phase was adjusted to 11.2 with 28% NaOH (1.45 kg, 10 mol), and then extracted with DCM (35 l). The combined organic phases were concentrated *in vacuo*. This gave 4.4 kg of crude product that was purified by dissolving in ethyl

acetate (45 l) and filtration through silica gel 60 (4 kg). After concentration *in vacuo* 3.6 kg (67%) of the crude product was obtained. The purity of the crude material was 88% (measured by GC). The methyl *N*-(2-Boc-aminoethyl)glycinate can be used without any further purification or if required purified by vacuum distillation: 120°C/0.03 mmHg. ^{1}H-NMR (d_6-DMSO): δ 5.46 (1H bs, NH); 3.72 (3H, s, CH_3); 3.43 (2H, s, CH_2CO); 3.21 (2H, q, CH_2); 2.74 (2H, t, CH_2); 1.88 (1H, s, NH); 1.44 (9H, s, *t*-Bu).

Ethyl 2-adenin-9-ylacetate (3). Adenine (50 g, 0.37 mol) was suspended in DMF (750 ml). Sodium hydride (16 g, 0.40 mol, 60% suspension) was added in portions over 1 h at 10°C with stirring. The mixture was stirred at room temperature for 1 h. Ethyl bromoacetate (123 g, 82 ml, 0.74 mol) was added dropwise over 3 h. The mixture was left at room temperature for 20 h. The yellow solution was evaporated to dryness *in vacuo* and stirred for 2 h with water (500 ml). The crude product was collected by filtration, washed with water and absolute ethanol. Yield 36 g (44%). ^{1}H-NMR (d_6-DMSO): δ 8.14 (1H, s, H-8); 8.12 (1H, s, H-2); 7.27 (2H, s, NH_2); 5.07 (2H, s, CH_2CO); 4.17 (2H, q, CH_2); 1.21 (3H, t, CH_3).

Ethyl (bis-N^6-(benzoyl)adenin-9-yl)acetate (4). Benzoylchloride (13 g, 11.5 ml, 9.9 mmol) was added dropwise at 0°C to a solution of ethyl adenin-9-ylacetate (**3**) (10 g, 4.5 mmol) in pyridine (150 ml). The reaction mixture was stirred over night at room temperature. Methanol (10 ml) was added and the mixture was evaporated to dryness *in vacuo*. The crude product was purified by crystallization from absolute ethanol (250 ml). After filtration and washing with ethanol and diethyl ether, the product **4** was isolated. Yield 9.0 g (62%). ^{1}H-NMR (d_6-DMSO): δ 8.69 (1H, s, H-8); 8.59 (1H, s, H-2); 7.89–7.44 (10H, m, 2 × Bz); 5.24 (2H, s, CH_2CO); 4.18 (2H, q, CH_2); 1.20 (3H, t, CH_3): MALDI-TOF MS $[M + H]^+$ calcd. 430.4, found 430.0.

(N^6-Benzoyladenin-9-yl)acetic acid (5). Compound **4** (9.0 g, 21 mmol) was dissolved in water (200 ml), followed by dropwise addition of 2 M NaOH solution (60 ml) at 0°C. After 30 min the temperature was allowed to reach room temperature and the reaction mixture was left for 1 h. The pH was adjusted to 2.5 with 2 M $NaHSO_4$ whereupon the product precipitates. After filtration and washing with cold water (*N*^{6}-benzoyladenin-9-yl)acetic acid (**5**) was isolated. Yield 3.5 g (56%). ^{1}H-NMR (d_6-DMSO): δ 11.15 (1H, bs, NH); 8.73 (1H, s, H-8); 8.45 (1H, s, H-2); 8.08–7.50 (5H, m, Bz); 5.11 (2H, s, CH_2CO): MALDI-TOF MS $[M + H]^+$ calcd. 298.3, found 298.1.

Benzyl (2-amino-6-chloropurin-9-yl)acetate (6). A solution of 2-amino-6-chloro-purine (50 g, 295 mmol) and potassium carbonate (81.6 g, 590 mmol) in DMF (1000 ml) was placed in a 2-l round-bottom flask. After 1 h at room temperature, benzyl bromoacetate (74.4 g, 51.5 ml, 325 mmol) was added in one portion and the mixture was left at room temperature for 2 h. Celite (25 g) was added and the mixture was filtered through celite. The Celite was washed with DMF (50 ml). The solution was concentrated *in vacuo* to a volume of 200 ml and after cooling at 15°C the mixture was filtrated and the filtrate was evaporated to dryness *in vacuo*. The residue was added to a mixture of ethyl acetate (500 ml) and water (100 ml) and stirred for 10 min. The crude product was collected upon filtration, washed with cold ethanol (2 × 200 ml) and dried over night at 70°C. Yield 70 g (76%). ^{1}H-NMR (d_6-DMSO) δ 8.13 (1H, s H-8); 7.41–7.35 (5H, m, Ph); 7.00 (2H, s, NH_2); 5.21 (2H, s, CH_2CO); 5.08 (2H, s, CH_2Ph): MALDI-TOF MS $[M + H]^+$ calcd. 318.7, found 318.0.

(2-Amino-6-O-benzylpurin-9-yl)acetic acid (7). Fresh cut pieces of sodium (10.1 g, 440 mmol) were added to benzylalcohol (1000 ml) placed in a 2 l round-bottom flask equipped with mechanical stirring. The exothermic reaction was left for 1 h at room temperature until the sodium was completely reacted. Benzyl (2-amino-6-chloropurin-9-yl)acetate (50 g, 157 mmol) was added in one portion and the mixture was left over night. After addition of water (1 l) and washing with diethyl ether (2 × 500 ml), the pH was adjusted to 2 using 2 M $NaHSO_4$. The white precipitate was collected and dissolved in 4 l of hot ethanol:water (3:1). The pure product was obtained after cooling and filtration. Yield 39 g (83%). ^{1}H-NMR (d_6-DMSO): δ 7.82 (1H, s, H-8); 7.52–7.34 (5H, m, Ph); 6.49 (2H, s, NH_2); 5.50 (2H, s, CH_2Ph); 4.80 (2H, s, CH_2CO): MALDI-TOF MS $[M + H]^+$ calcd. 300.3, found 300.1.

Ethyl (2-amino-6-O-benzylpurin-9-yl)acetate (8). (2-Amino-6-*O*-benzylpurin-9-yl)acetic acid (76.7 g, 256 mmol) was suspended in ethanol (1000 ml) and stirred during addition of HOBt (46 g, 340 mmol) and DIC (46 ml, 37.1 g, 294 mmol). After 4 h at room temperature, the mixture was cooled to 0°C and the white precipitate was collected by filtration. After washing with cold ethanol, the product was dried at 60°C over night. Yield 68 g (81%). ^{1}H-NMR (d_6-DMSO): δ 8.21 (1H, s, H-8); 7.55–7.36 (5H, m, Ph); 5.52 (2H, s, CH_2Ph); 4.99 (2H s, CH_2CO); 4.17 (2H, q, CH_2); 1.21 (3H, t, CH_3): MALDI-TOF MS $[M + H]^+$ calcd. 328.3, found 328.5.

Ethyl (N²-(isobutyryl)-2-amino-6-O-benzylpurin-9-yl)acetate (9). A solution of ethyl (2-amino-6-*O*-benzylpurin-9-yl)acetate (68 g, 208 mmol) in pyridine (6000 ml) was cooled to 0°C. Isobutyrylchloride (26.6 g, 26 ml, 250 mmol) was added dropwise and the mixture was stirred at 0°C for 1 h giving a yellow mixture. Methanol (20 ml) was added and the solution was evaporated to dryness. The crude residue was co-evaporated with toluene (3 × 100 ml) and dissolved in DCM. The organic phase was washed with 0.1 M HCl and with saturated aqueous NaCl. After drying over $MgSO_4$, filtration and concentration, the crude product was crystallized from ethyl acetate. Yield 66.2 g (80%). ^{1}H-NMR (d_6-DMSO): δ 10.4 (1H, s, NH); 8.20 (1H, s, H-8); 7.60–7.32 (5H, m, Ph); 5.63 (2H, s, CH_2Ph); 5.07 (2H, s, CH_2CO); 4.17 (2H, q, CH_2); 2.92 (1H, m, *i*-Bu-CH); 1.22 (3H, t, CH_3); 1.09 (6H, d, *i*-Bu-CH_3): MALDI-TOF MS $[M + H]^+$ calcd. 398.4, found 399.2.

(N²-(isobutyryl)-2-amino-6-O-benzylpurin-9-yl)-acetic acid (10). Ethyl (N^2-(isobutyryl)-2-amino-6-*O*-benzylpurin-9-yl)acetate (57.7 g, 145 mmol) was suspended in water (500 ml) and 1 M LiOH (350 ml) was added dropwise during stirring. After stirring for 90 min the pH was adjusted to 3 using 2 M $NaHSO_4$ (approximately 150 ml). The product was isolated by filtration and washed with diethyl ether. The product was dried over night at 70°C. Yield 50 g (93%). ^{1}H-NMR (d_6-DMSO): δ 10.40 (1H, s, NH); 8.17 (1H, s, H-8); 7.59–7.35 (5H, m, Ph); 5.63 (2H, s, CH_2Ph) 4.92 (2H, s, CH_2CO); 2.92 (1H, m, *i*-Bu-CH); 1.09 (6H, d, *i*-Bu-CH_3): MALDI-TOF MS $[M + H]^+$ calcd. 370.4, found 370.5.

Methyl N-((N⁶-(benzoyl)adenin-9-yl)acetyl)-N-(2-Boc-aminoethyl)glycinate (11a). Boc-backbone **2** (1.0 g, 4.3 mmol) was dissolved in DMF (5 ml) and HOBt (670 mg, 5 mmol), (N^6-benzoyladenin-9-yl)acetic acid (1.21 g, 4.1 mmol) and DCC (1.0 g, 4.8 mmol) were added. The mixture was stirred for 3 h. After addition of DCM (20 ml) and cooling at 0°C, the mixture was filtered to removed *N*,*N*-dicyclohexyl urea. The filtrate was washed with aqueous $NaHCO_3$ (2 × 20 ml) and with aqueous $NaHSO_4$ (2 × 20 ml). After drying over $MgSO_4$, the crude product was isolated by evaporation *in vacuo* and **11a** was recovered as white foam. Yield 1.41 g (67%). ^{1}H-NMR (d_6-DMSO): δ 11.10 (1H, bs, NH); 8.71 (1H, s, H-8); 8.33 (1H, s, H-2); 8.07–7.51 (5H m, Ph); 7.05–6.69 (1H, m, NH); 5.38/5.20 (2H, 2 × s (rotamers), CH_2CO); 4.47/4.10 (2H, 2 × s (rotamers), CH_2CO); 3.62 (3H, s, CH_3O); 3.60–3.06 (4H, m, 2 × CH_2); 1.40 (9H, s, *t*-Bu): MALDI-TOF MS $[M + H]^+$ calcd. 512.5, found 512.4.

N-((N⁶-(Benzoyl)adenin-9-yl)acetyl)-N-(2-Boc-aminoethyl)glycine (12a). Compound **11a** (900 mg, 1.76 mmol) was dissolved in a mixture of THF (2.5 ml) and water (5 ml), followed by addition of 1 M LiOH (5 ml). After stirring at room temperature for 1 h THF was removed *in vacuo*. The product precipitated after adjusting the pH to 2.8 with 2 M $NaHSO_4$. The crude product was dissolved in DCM and precipitated during addition to *n*-hexane (200 ml). This gave **12a** as a white powder. Yield: 550 mg (62.8%). ^{1}H-NMR (d_6-DMSO): δ 11.16 (1H, bs, NH); 8.71 (1H, s, H-8); 8.33 (1H, s, H-2); 8.07–7.50 (5H, m, Ph); 7.06–6.72 (1H, m, NH); 5.37/5.19 (2H, 2 × s (rotamers), CH_2CO); 4.33/4.00 (2H, 2 × s (rotamers), CH_2CO); 3.54–3.06 (4H, m, 2 × CH_2); 1.39 (9H, s, *t*-Bu): MALDI-TOF MS $[M + H]^+$ calcd. 498.5, found 497.6.

Methyl N-((6-O-benzyl-N²-(isobutyryl)-2-aminopurin-9-yl)acetyl)-N-(2-Boc-aminoethyl)glycinate (11b). Boc-backbone **2** (37 g, 159.5 mmol) and DMF (400 ml) was placed in a round-bottom flask. After addition of (N^2-(isobutyryl)-2-amino-6-*O*-benzylpurin-9-yl)acetic acid (**10**) (49 g, 132.6 mmol), HOBt (21.50 g, 159.1 mmol) and DCC (33 g, 159.9 mmol), the mixture was stirred at room temperature for 2 h. The reaction was quenched by addition of DCM (500 ml) and after cooling at 0°C, the mixture was filtered to removed *N*,*N*-dicyclohexyl urea. The organic phase was washed with 0.5 M $NaHCO_3$ (3 × 500 ml), 2 M $NaHSO_4$ (2 × 500 ml) and with saturated aqueous NaCl. The organic phase was dried over $MgSO_4$ and after filtration the organic phase was concentration *in vacuo* to provide 60 g of crude product **11b**. The product was purified by column chromatography using DCM/MeOH 19:1 as eluent. Yield 50 g (65%). ^{1}H-NMR (d_6-DMSO): δ 10.35 (1H, bs, NH); 8.19 (1H, s, H-8); 7.58–7.33 (5H, m, Ph); 7.05–6.72 (1H, m, NH); 5.63 (2H, s, CH_2Ph); 5.20/5.03 (2H, 2 × s (rotamers), CH_2CO); 4.46/4.09 (2H, 2 × s (rotamers), CH_2CO); 3.62 (3H, s, CH_3O); 3.60–3.25 (4H, m, 2 × CH_2); 2.96 (1H, m, *i*-Bu-CH); 1.33 (9H, s, *t*-Bu); 1.10 (6H, d, *i*-Bu-CH_3): MALDI-TOF MS $[M + H]^+$ calcd. 584.6, found 584.3.

N-((6-O-Benzyl-N²-(isobutyryl)-2-aminopurin-9-yl)acetyl)-N-(2-Boc-aminoethyl)glycine (12b). Compound **11b** (7.5 g, 12.85 mmol) was dissolved in a mixture of THF (20 ml) and water (30 ml). A 1 M aqueous LiOH solution (32 ml, 32 mmol) was added dropwise over 30 min. After 1 h at room temperature, water (100 ml) was added and the THF was removed by evaporation *in vacuo*. The pH of the solution was

adjusted to 2.5 by dropwise addition of 2 M $NaHSO_4$. After standing at 5°C for 1 h the precipitate was filtered off and dried *in vacuo*. The crude product **12b** was further purified by crystallization from ethanol/*n*-hexane. Yield 5.0 g (68%). ^{1}H-NMR (d_6-DMSO): δ 10.39 (1H, bs, NH); 8.08 (1H, s, H-8); 7.59–7.32 (5H, m, Ph); 7.05–6.78 (1H, m, NH); 5.63 (2H, s, CH_2Ph); 5.19/5.01 (2H, 2 × s (rotamers), CH_2CO); 4.27/3.99 (2H, 2 × s (rotamers), CH_2CO); 3.53–2.89 (5H, m, *i*-Bu-CH, 2 × CH_2); 1.34 (9H, s, *t*-Bu); 1.09 (6H, d, *i*-Bu-CH_3): MALDI-TOF MS $[M + H]^+$ calcd. 570.6, found 570.5.

Boc-aminoacetyl-4-(oxymethyl)phenylacetic acid (Boc-Gly-PAM) (13). To a solution of Boc-glycine (1.12 g, 6.4 mmol) in DMF (5 ml) was added 4-bromomethylphenyl-acetic acid phenacyl ester (1.5 g, 4.3 mmol) dissolved in acetonitrile (30 ml), followed by addition of potassium fluoride dihydrate (900 mg, 9.56 mmol). The mixture was stirred at 50°C for 20 h. The precipitated inorganic material was removed by filtration and ethyl acetate (60 ml) and water (60 ml) were added. The aqueous layer was extracted with ethyl acetate (3 × 50 ml) and the combined organic phases were washed with water (3 × 50 ml). Excess Boc-glycine was removed by washing with a buffer solution (3 × 100 ml) (6.9 g K_2CO_3 and 8.4 g $NaHCO_3$ in 300 ml of water). After drying over $MgSO_4$ the solvent was removed by evaporation *in vacuo* to give 1.7 g (90%) of Boc-aminoacetyl-4-(oxymethyl)-phenylacetic acid phenacyl ester as a yellow solid. The product (1.6 g, 3.6 mmol) was dissolved in 40 ml of acetic acid and water (85:15) followed by addition of zinc dust (5 g). The mixture was stirred at room temperature for 4 h. After filtration, ethyl acetate (150 ml) and water (150 ml) were added, and the water phase was made acidic (pH ~ 1.5) by addition of aq. HCl (0.5 M). The water phase was extracted with ethyl acetate (3 × 100 ml) and the combined organic phases were washed with water (10 × 50 ml). The crude product was obtained after drying over $MgSO_4$ and concentration *in vacuo*. The crude product **13** was dissolved in a minimum of ethyl acetate and precipitated by dropwise addition to *n*-hexane. Yield 850 mg (73%). ^{1}H-NMR (d_6-DMSO): δ 7.32–7.22 (4H, m, Ph); 5.10 (2H, s, CH_2O); 3.72 (2H, d, CH_2NH); 3.56 (2H, s, CH_2CO); 1.38 (9H, s, *t*-Bu).

6-O-Dmt-hexanoic acid NHS-ester (14). ε-Caprolactone (5.7 g, 0.05 mol) in NaOH (100 ml, 2 M) was stirred for 1 h at room temperature. The pH of the solution was adjusted to 2.8 by adding $NaHSO_4$ (2 M). The solution was extracted with DCM (2 × 50 ml). The combined organic phases were extracted with brine (50 ml) and dried over $MgSO_4$. The organic phase was evaporated to dryness giving ε-capronic acid (5.65 g, 42.5 mmol, 85%). The crude product was dissolved in pyridine (100 ml) and, after addition of Dmt-chloride (14.4 g, 42.5 mmol) the mixture was stirred over night at room temperature. *N*-hydroxy-succinimide (4.76 g, 42.5 mmol) and DCC (8.75 g, 42.5 mmol) were added and stirred for a further 2 days. The reaction mixture was filtrated, the solution concentrated and purified by silica gel chromatography (200 g) with EtOAc/hexane (1:1) as eluent. Yield 18.4 g (71%). ^{1}H-NMR: δ 6.85–7.40 (13H, m); 4.19 (2H, m, CH_2O); 3.73 (6H, s, CH_3O); 2.57–2.70 (6H, m, CH_2-NHS, CH_2CO); 1.54–1.77 (6H, m, CH_2).

Synthesis of PNA and PNA–DNA Chimeras Using Boc/acyl Monomers

Solid phase synthesis of 17mer PNA on MBHA resin. The test 17mer PNA (Ac-CGGACTAAGTCCATTGC-Gly-NH_2) was synthesized using Boc-T monomer, Boc-benzoyl-C monomer, Boc-benzoyl-A monomer and Boc-isobutyryl-*O*-benzyl-G monomer (all 0.26 M) on an ABI 433A peptide synthesizer (5 μmol scale). A standard synthesis cycle as previously described [8] was used except for changing the activator reagent and neutralization reagent from HATU and DIEA to 0.2 M PyAOP in NMP and 0.5 M NMM in pyridine, respectively. The final piperidine wash in each cycle following capping was omitted due to the lability of the PAM linker during basic conditions. The PAM linker was introduced by a double coupling in the first condensation using Boc-Gly-PAM monomer synthesized as described above. All other monomers were only coupled ones. The PNA oligomer was acetylated in the final step, to prevent rearrangement [7,8] of the final monomer during the basic deprotection treatment. After synthesis the acyl protecting groups of the bases were removed and the entire PNA was cleaved from the solid support by treatment with either ammonia (32%) or methylamine (40%). Ammonia deprotection proceeded for 20 h at 60°C, whereas methylamine deprotection went at room temperature in 4 h. After filtration and washing of the resin with water, the filtrate was concentrated *in vacuo* to give the crude product. The total yield of PNA was estimated by dissolving the crude product in 5% acetic acid and measuring the UV absorption at 260 nm. The total yield was approximately 68% (3.4 μmol): MALDI-TOF MS $[M + H]^+$ calcd. 4704.5, found 4705.3.

Solid phase synthesis of 17mer PNA on CPG resin. The aminopropyl-CPG support was downloaded with Boc-Gly-PAM monomer by treating the CPG (500 mg, 500 Å) with a solution of 0.26 M Boc-Gly-PAM (1 ml) and DIC (100 μl) for 20 h. The CPG was filtered off and washed with DCM (3 × 2 ml), DMF (3 × 2 ml) and dried *in vacuo*. The reaction vessel was loaded with 100 mg of the resin. The test 17mer PNA was synthesized on the peptide synthesizer as described for the MBHA support, except that all vortex steps were omitted to avoid destruction of the CPG resin. The vortexing was replaced by extended 'waiting times', to ensure adequate reaction times. The final PNA was cleaved from the CPG support and deprotected during ammonia treatment as described above: MALDI-TOF MS $[M + H]^+$ calcd. 4704.5, found 4712.2.

Synthesis of ^{H}PNA^{NH}–$^{5'}$DNA$^{3'}$ chimeras. The DNA part (only pyrimidines) was synthesized on an ABI 394 DNA/RNA synthesizer using standard phosphoramidite synthesis on CPG support. The 5′-amino functionality was introduced using a special 6-(Mmt-amino)hexyl phosphoramidite. The Mmt-group was removed and the resin was transferred to the peptide synthesis reaction vessel and installed on the peptide synthesizer. The PNA monomers were added using the same cycles as used in the synthesis of PNA on CPG (again without vortex). The Boc-group of the final PNA-monomer was removed and labelled with biotin for detection reasons and to prevent rearrangement during removal of the chimera from the solid support. All chimeras were purified by HPLC, the purity was checked by analytical HPLC and the mass confirmed by MALDI-TOF mass spectroscopy.

The following chimeras were prepared (PNA in *italic*):

Biotin-*TGTTAAATCAGCTCA*-NH-$(CH_2)_6$-TTTTT

MALDI-TOF MS $[M-H]^-$ calcd. 5907.4, found 5902.8.

Biotin-*TGTTAAATCAGCTCA*-NH-$(CH_2)_6$-TTT

MALDI-TOF MS $[M-H]^-$ calcd. 5299.0, found 5298.9.

Synthesis of $^{5'}$DNA$^{3'}$–^{H}PNA^{NH_2} chimeras. The PNA part was synthesized as described above on a CPG support. The support was transferred to a manual reaction container and after deprotection reacted with 6-*O*-Dmt-hexanoic acid NHS-ester (**14**) for 20 h. After filtration and washing with DCM and DMF, the resin was transferred to a DNA-reaction vessel and the DNA part was synthesized on the DNA/RNA synthesizer. The final chimera was obtained after deprotection and removal from the resin using ammonia:

5′-ATGCAGCT-O-$(CH_2)_5$CO-*TGCACTATGT*-Gly-NH_2

MALDI-TOF MS $[M-H]^-$ calcd. 5359.4, found 5357.1.

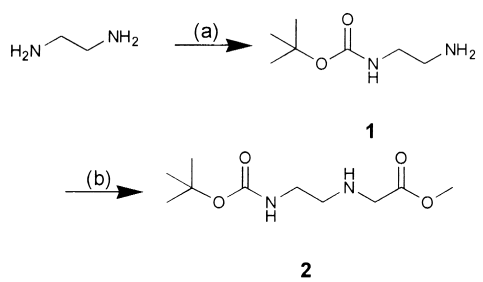
RESULTS AND DISCUSSION

Monomer and Linker Synthesis

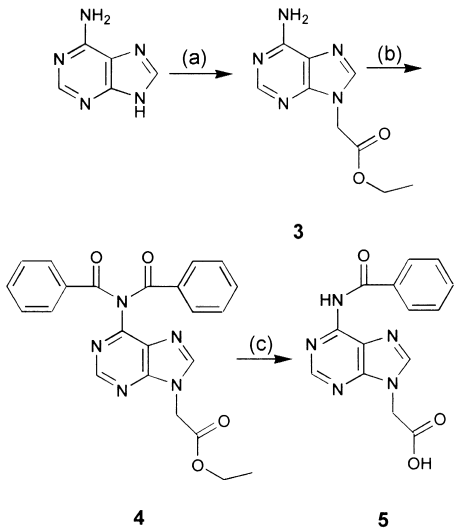
Boc-backbone synthesis. Breipohl *et al.* [4] reported a large-scale backbone synthesis using ethylene diamine as starting material and in which the protecting groups on the amine and the carboxylic acid both were removable with strong acid (TFA). The large-scale backbone synthesis reported here also makes use of ethylene diamine as starting material but provides a protocol for orthogonal backbone protection (Scheme 1).

Ethylene diamine was mono-Boc protected by using di-*tert*-butyl dicarbonate [12]. Excess ethylene diamine was removed by aqueous extraction. The product was isolated and evaporated *in vacuo* to give a 99% pure *N*-Boc-ethylene diamine (**1**), which was used directly in the subsequent alkylation with methyl bromoacetate. This gave the target molecule **2** in an overall yield of 51%. The crude backbone contained bis-alkylated mono-Boc diamine as a minor by-product. However, the crude product was used without further purification in the subsequent Boc-monomer syntheses. If required **2** can be purified by vacuum distillation at 120°C.

Adenine and guanine monomer synthesis. Ethyl adenin-9-ylacetate (**3**) (Scheme 2) was synthesized according to the procedure described by Dueholm *et al.* [13].



Scheme 1 Synthesis of methyl *N*-(2-Boc-aminoethyl)-glycinate. Reagents: (a) di-*tert*-butyldicarbonate, DCM; (b) methyl bromoacetate, CH_3CN, NEt_3.

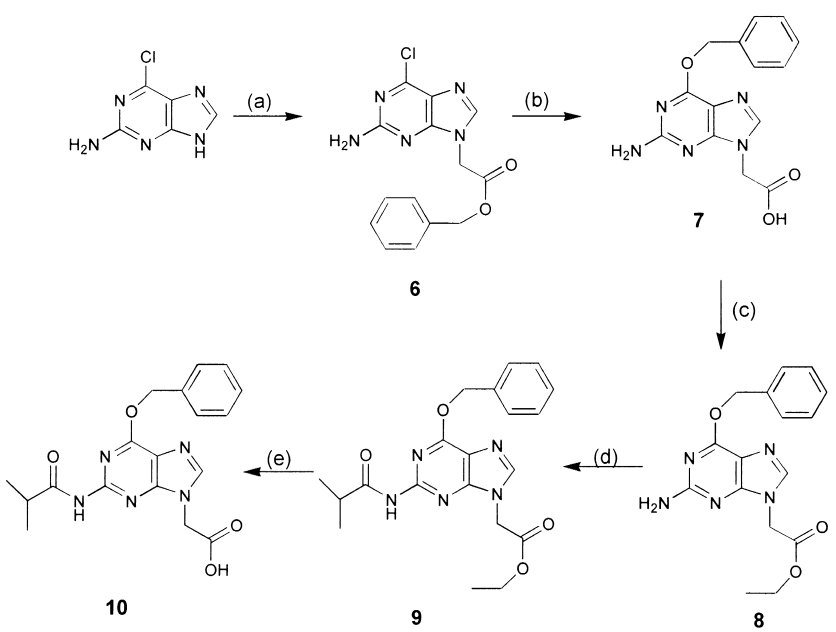


Scheme 2 Synthesis of (N^6-benzoyladenin-9-yl)acetic acid. Reagents: (a) ethyl bromoacetate, NaH, DMF; (b) benzoylchloride, pyridine; (c) NaOH, H_2O.

Benzoyl protection was performed in pyridine by adding benzoylchloride at 0°C and the bis-benzoylated derivative **4** was isolated in 62% yield. The ethyl ester group and one benzoyl group was removed during saponification with sodium hydroxide and (N^6-denzoyladenin-9-yl)acetic acid (**5**) was isolated by filtration after adjusting the pH to 2.5. The product was used without further purification.

The commercial available 6-chloro-2-aminopurine gives good regio-selectivity in alkylation reactions and is frequently used as starting material in the synthesis of guanine derivatives. The selective alkylation at the N^9-position was achieved when treating 6-chloro-2-aminopurine with benzyl bromoacetate in the presence of potassium carbonate to yield **6**. To increase the solubility of the final guanine monomer a lipophilic group was introduced at the 6-position. This was obtained by exchanging the chloro with the benzyloxy group. Reacting **6** with sodium benzylate, which simultaneously removed the benzyl ester group, performed this aromatic nucleophilic alkylation. The following protection of the 2-amino group by direct treatment of compound **7** with isobutyrylchloride gave (N^2-(isobutyryl)-2-amino-6-*O*-benzylpurin-9-yl) acetic acid (**10**) but in a low yield. After column chromatography only 30% of **10** could be recovered.

Therefore, another strategy was chosen in which the ethyl ester was formed prior to the isobutyrylchloride treatment (Scheme 3). After protecting the amino functionality and saponification in lithium hydroxide, the final (N^2-(isobutyryl)-2-amino-6-*O*-benzylpurin-9-yl)acetic acid (**10**) was obtained. The overall yield for the three steps (c)–(e) was 60% and purification of all products was achieved by crystallization.



Scheme 3 Synthesis of (N^2-(isobutyryl)-2-amino-6-*O*-benzylpurin-9-yl)acetic acid. Reagents: (a) K_2CO_3, benzyl bromoacetate, DMF; (b) Na, benzyl alcohol; (c) EtOH, DIC, HOBt; (d) isobutyrylchloride, pyridine; (e) LiOH, H_2O.

The protected carboxymethyl purines were coupled to the backbone using HOBt and DCC (65–67% yield) (Scheme 4). Finally, the monomer methyl ester **11** was hydrolysed with lithium hydroxide and the final adenine **12a** and guanine **12b** monomers were isolated by precipitation. Both monomers are easily dissolved in the coupling solvents at the concentrations used for PNA synthesis and are stable in solution for months.

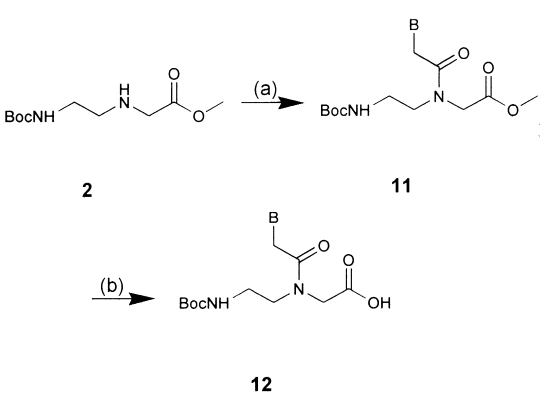
Cytosine monomer synthesis. Boc-N^4-benzoyl-cytosine monomer was synthesized according to the procedure previously described by Christensen *et al.* [14].

PAM linker and DNA–PNA linker synthesis. Preparation of Boc-aminoacetyl-4-(oxymethyl)-phenylacetic acid (**13**) (Figure 1) (Boc-Gly-PAM) was analogous to the method described by Tam *et al.* [15]. The overall yield for the two-step synthesis was 65%. The 6-*O*-Dmt-hexanoic acid NHS-ester (**14**) (Figure 1) used as linker in 5′-DNA–PNA-3′ chimera synthesis was prepared in the following way.

Afier treatment of ε-caprolactone with sodium hydroxide, the ring-open salt was treated with Dmt-chloride in pyridine to form the *O*-Dmt-protected compound. This was transformed into the NHS-ester after treatment with *N*-hydroxysuccinimide and DCC. The final product was isolated after column chromatography in 71% yield.

Oligomerization

PNA synthesis. The Gly-PAM unit (Boc-Gly-PAM monomer) was used as base labile linker on both an aminopropyl-CPG and on an MBHA solid support. The performance of the new monomers in PNA synthesis was examined in the synthesis of a test 17mer PNA (Ac-CGG-ACT-AAG-TCC-ATT-GC-gly-NH_2) during standard Boc/Z conditions [7,8]. C, T, and G monomers coupled in high yields (> 95%) but the A monomer showed a much lower coupling yield. The mass spectrum of the raw product showed that all major deletion fragments were found right before A couplings and the residues were capped with either acetyl or benzoyl. This unexpected coupling pattern was examined in a model system. In the model system Boc/acyl A monomer was coupled on a T monomer linked to the Gly-PAM-MBHA resin. Coupling was allowed to proceed for 15 min followed by acetic anhydride capping. After capping, the dimer was removed from the resin by methylamine treatment and the ratio between Ac-T-Gly-NHMe/Benzoyl-T-Gly-NHMe (uncoupled equivalents) and Boc-A-T-Gly-NHMe (coupled equivalent) was quantified by HPLC. The coupling efficiency was tested with several combinations of activator reagents and bases (Table 1).



Scheme 4 General flow scheme of Boc-acyl monomer synthesis. Reagents: (a) acyl-purine, HOBt, DCC, DMF; (b) LiOH, THF, H_2O.

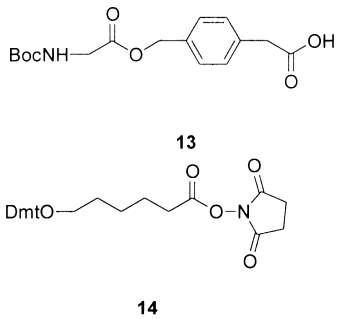


Figure 1 Structure of Boc-Gly-PAM and 6-*O*-Dmt-hexanoic acid NHS-ester.

As seen from Table 1 the lowest coupling yields (81.2 and 77.7%) were actually found under the conditions which normally provided the highest coupling yields in standard Boc/Z PNA synthesis (HATU/DIEA). An explanation for this observation may be that the A monomer, when activated in the presence of a strong amine like DIEA, is deprotonated at the benzoyl-amide which makes the nitrogen much more nucleophilic. It is well known that HATU reacts readily with nitrogen nucleophiles [16] and therefore, it is likely that HATU reacts with the deprotonated N^6-amine during adenine monomer activation (Scheme 5). This reactivity was also reflected in the protection step during the monomer synthesis where the N^9-alkylated adenine was turned directly into the bis-benzoylated product (see Scheme 2).

To test this hypothesis Boc/Benzoyl-A-monomer methyl ester (**11a**) was exposed to HATU/DIEA and

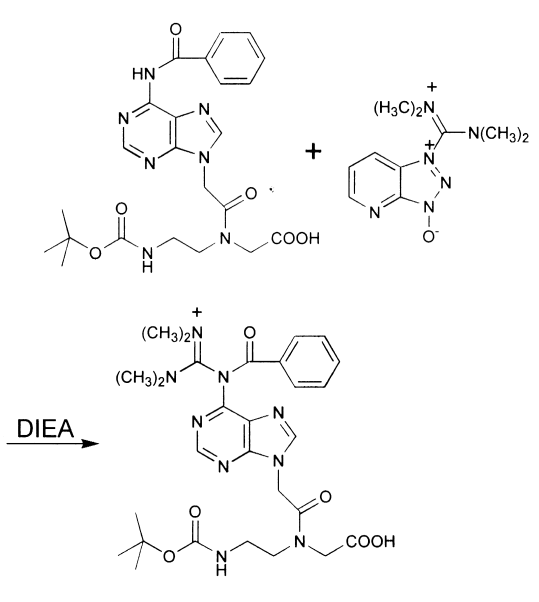
Table 1 Coupling Experiments on a T-Gly-PAM-MBHA Resin[a]

Coupling conditions	Ac-T-Gly (%)	Benzoyl-T-Gly (%)	Boc-A-T-Gly (%)
HATU and DIEA	12.1	6.7	81.2
HATU and 2× DIEA	13.5	8.8	77.7
DIC	2.7	0	97.3
HBTU and DIEA	9.7	5.9	84.4
HATU and NMM	5.1	1.9	93.0
HBTU and NMM	5.7	2.3	92.0
PyAOP and DIEA	3.5	0	96.5
PyAOP and NMM	1.4	0	98.6

[a] Unless otherwise stated all experiments were performed with Boc-bebzoyl-A monomer (0.26 M, 75 μl), base (0.5 M, 0.75 μl) and coupling reagent (0.202 M, 75 μl).

the mixture was followed by electrospray MS. The results was very clear; after 2 min the molecular ion (**11a**; $[M+H]^+ = 512.2$) was starting to be transformed into the guanidinium alkylated Boc/Bz-A monomer methyl ester ($[M+H]^+ = 610.3$) and the guanidinium alkylated Boc-A-monomer methyl ester ($[M+H]^+ = 506.3$) (results not shown). After 30 min approximately half the starting material was converted into these two molecules.

This reaction between the guanidinium salt and the A monomer will have two effects; it reduces the concentration of the activated monomer and produce a guanidinium alkylated A monomer **15** (Scheme 5). The reduced concentration of activated monomer is reflected in the low coupling yield of the A monomer giving rise to high amounts of acetyl capped deletion fragments. The guanidinium alkylation places a strong electron-attracting group on the N^6-position that weakens the amide bond. The increased lability of the bond is reflected in the mass spectrum where a significant amount of benzoyl capping is detected. It was interesting to see that both guanidinium salts (HATU and HBTU) gave the same reaction pattern.



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Scheme 5 Proposed reaction between HATU with Boc/Bz-A monomer in the presence of DIEA.

To improve the coupling yield DIEA ($pK_a \approx 11.5$) was replaced with the much weaker base *N*-methylmorpholine (NMM) ($pK_a \approx 7.4$). This replacement increased the coupling yield in the model system from 81.2 to 93.0% and clearly demonstrated that the strength of the base is important during activation. The coupling with NMM in combination with HATU/HBTU gave approximately 2% of benzoyl capped T-Gly species. This indicates that even during the activation with a weak base some alkylation occurs. Therefore, further improvement of the coupling yield was found by using a more suited activation reagent. Phosphonium salts like PyAOP are widely used in peptide chemistry and do not alkylate amines. Thus, PyAOP was tested in combination with NMM and gave the highest recorded coupling yield (98.6%).

To test the results from the model system the test 17mer was synthesized under three sets of conditions. As seen from Figure 2 the overall yield is significantly improved when changing the base from DIEA (A) to NMM (B) and a further improvement is obtained when the coupling reagent HATU is exchanged with PyAOP (C). Finally, we also tested the synthesis of the 17mer using Boc/acyl monomers and NMM/PyAOP conditions on the CPG support using an ABI peptide synthesizer with vortex free cycles. The overall yield is not as good as with MBHA resin but is useful in the synthesis of PNA–DNA chimeras.

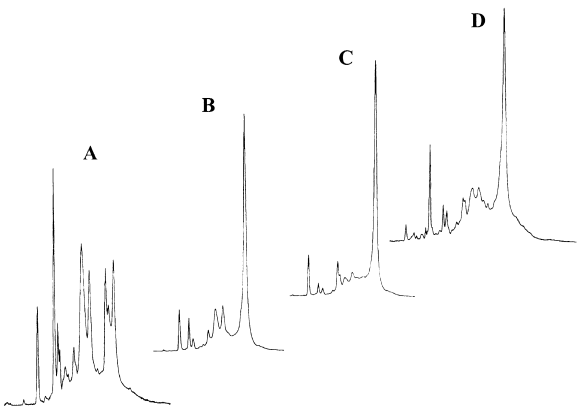

Figure 2 HPLC traces of crude Ac-CGG-ACT-AAG-TCC-ATT-GC-Gly-NH_2 synthesized on MBHA and on CPG. Coupling conditions: (A) HATU/DIEA; (B) HATU/NMM; (C) PyAOP/NMM; (D) PyAOP/NMM on CPG.

Cleavage from the resin. In the Boc/Z strategy the target PNA is normally cleaved from the resin by very strong acid (HF or TFMSA). The yield of crude PNA is often low, which is believed to be due to acid induced cross-linking reactions of the PNA to the solid support. In the Boc/acyl strategy the oligomer is removed from the resin by either ammonia or alkylamine treatment. In our test typically 65–70% of the 17mer was recovered after work-up. In a parallel Boc/Z synthesis only 21% of the test 17mer was recovered.

Chimera synthesis. All chimeras were synthesized on CPG support as described above. The chimeras were synthesized in two orientations: $^{5'}DNA^{3'}$-linker-$^{H}PNA^{NH_2}$ (5′-DNA–PNA-3′) and $^{H}PNA^{NH}$-linker-$^{5'}DNA^{3'}$ (5′-PNA–DNA-3′). In the latter case only pyrimidines in the DNA strand can be used since purines are degraded during repeated TFA treatment. When 5′-PNA–DNA-3′ chimeras were synthesized, commercially available supports were used loaded with the 3′-end residue. The DNA part was synthesized using standard phosphoramidite chemistry on a DNA/RNA synthesizer (ABI 394). The 6-(Mmt-amino)hexyl phosphoramidite was introduced at the 5′-end using extended coupling time (4 min). The solid support bound DNA was removed from the plastic column and transferred to the reaction vessel on the ABI 433A peptide synthesizer. The PNA part was made using the Boc/acyl monomers and following the procedure described above using a vortex free program and PyAOP/NMM as activation mixture.

The synthesis of 5′-DNA-PNA-3′ chimeras was performed on CPG downloaded with Boc/Gly-PAM. The loading was estimated to be approximately 50 mmol/g. After synthesis of the PNA part on the peptide synthesizer, the 6-*O*-Dmt-hexanoic acid was introduced as linker to the DNA part. Due to the slow reaction of the NHS ester **14** the reaction mixture was left for 20 h at room temperature. The resin was transferred to a DNA/RNA plastic column and the DNA part was synthesized on a DNA synthesizer according to the phosphoramidite protocol. For both types of chimeras the final products were obtained after treatment with ammonia (33%) for 20 h at 60°C. The chimeras were purified by HPLC using a gradient of triethylammonium acetate/acetonitrile.

CONCLUSION

We have shown that the Boc/acyl strategy is well suited for routine PNA synthesis. The orthogonal protecting group strategy combines the advantages of Boc chemistries during PNA synthesis with a facile preparation of the protected monomers. The ammonia/methylamine deprotection procedure is significantly more convenient than, for example, the HF or the TFMSA procedure that is required for the Boc/Z strategy and the amount of the recovered crude PNA is significantly higher. This last issue is of importance when large amounts of PNA are prepared. The strategy offers additionally the option of making 5′-DNA–PNA-3′ chimeras of all base combinations and 5′-PNA–DNA-3′ chimeras containing DNA pyrimidines.

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