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Solid-phase Synthesis of Peptide Nucleic Acid (PNA) Monomers and Their Oligomerization Using Disulphide Anchoring Linkers

GUDRUN ALDRIAN-HERRADA[1,2], ALAIN RABIÉ[3], REINHOLD WINTERSTEIGER[1] and JEAN BRUGIDOU[2,*]

[1] Karl-Franzens-Universität, Graz, Austria
[2] Centre National de la Recherche Scientifique, CRBM-CNRS ERS 155, Montpellier, France
[3] CNRS UPR 9055, Montpellier, France

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Abstract: A new simple solid-phase method has been developed for synthesizing Boc-protected peptide nucleic acid (PNA) monomers. An immobilized backbone **3** was built on Expansin® resin using an ester disulphide handle: 2-hydroxypropyl-dithio-2′-isobutyric acid (HPDI). The base acetic acids of thymine **5**, Z-cytosine **9**, Z-adenine **12**, and 6-*O*-benzyl guanine **17** were prepared and coupled to the immoblized backbone. The HPDI handle was cleaved under mild conditions by cyanolysis or assisted hydrolysis with tris(2-carboxyethyl)phosphine (TCEP) to give undamaged PNA monomers. These monomers were coupled to form oligomers by solid-phase method with another disulphide linkage: aminoethyldithio-2-isobutyric acid (AEDI) grafted on an amino-functionalized TentaGel® resin, using *in situ* neutralization and TBTU as activating reagent. Final cleavage of the AEDI linker gave PNA bearing a cysteamide residue that could be useful for optimizing PNA properties. Oligomers of up to 16 residues long were assembled. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Peptide nucleic acid monomers; PNA synthesis; disulphide linkers; solid-phase synthesis

INTRODUCTION

Antisense oligonucleotides have great potential as tools for modifying the function of specific genes and may eventually give rise to new ways of treating disease. They inhibit gene expression by binding to a complementary sequence in a messenger RNA, so preventing the translation of this messenger RNA into a protein [1–3]. But the actions of both DNA and RNA antisense nucleotides are limited by their poor uptake into cells and their rapid degradation by nucleases [4,5]. These limitations may be overcome by a new type of DNA analogue, the peptide nucleic acid (PNA), which was invented by Nielsen *et al.* [6]. In these molecules, the nucleic acid phosphodiester backbone is replaced by *N*-(2-aminoethyl)-glycine units bearing the nucleobases bonded via a methylene–carbonyl linker. These new compounds are not only much more stable in cells than their counterparts, but they also bind

Abbreviations: A, Ade, adenine; AEDI, aminoethyldithio-2-isobutyric acid; βAla, β-alanine; C, cytosine; Cya, cysteamide; d, decomposition; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DECA, *N*,*N*-diethylcyclohexylamine; DMAP, 4-dimethylaminopyridine; Et_3SiH, triethylsilane; ether, diethyl ether; G, guanine; Gly, glycine; HATU, *O*-(7-azabenzotriazol-1-y1)-1,1,3,3,-tetramethyluronium hexaflurophosphate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HPDI, 2-hydroxypropyl-dithio-2′-isobutyric acid; i-PrOH, iso-propanol; Lys(Z), benzyloxycarbonyl-lysine; Lys(ZCl), 2-chloro-benzyloxycarbonyl-lysine; NMM, *N*-methylmorpholine; NMP, 1-methyl-2-pyrrolidone; PNA, peptide nucleic acid; POE, polyoxyethylene; SPS, solid-phase synthesis; T, thymine; TBTU, 2-(1H-benzotriazol-1-y1)-1,1,3,3-tetramethyluronium tetrafluoroborate; TCEP, tris(2-carboxyethyl)phosphine; TFMSA, trifluoromethanesulphonic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; Z or Cbz, benzyloxycarbonyl.

* Correspondence to: Centre National de la Recherche Scientifique, Centre de Recherche de Biochimie Macromoléculaire, ERS 155, 1919, route de Mende, 34293 Montpellier Cedex 5, France.

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complementary nucleic acids with much greater affinity while retaining their specificity [7]. This paper describes a new method for synthesizing PNA monomers on a polymeric support and their oligomerization, using solid-phase strategy with disulphide linkers.

The classical way to synthesize PNA monomers starts with the Boc- or Fmoc-protected backbone *N*-(2-aminoethyl)-glycine [8]. Esterification of the carboxylic group improves work-up and purification, and methyl, ethyl or *tert*-butyl esters are generally used. The heterocyclic bases are coupled to an acetic acid moiety for subsequent attachment to the secondary amino group of the backbone. The exocylic amino groups of adenine and cytosine are usually protected with a benzyloxycarbonyl (*Z*) group. The PNA monomers are then assembled to oligomers in a stepwise manner, using the standard procedures for solid-phase synthesis [9–11].

A new strategy, submonomer solid-phase synthesis, has recently been published [12]. In this method, the backbone moiety is built on an amino-polystyrene resin, by acylation with bromoacetic anhydride followed by displacement with a monoprotected ethylenediamine. The nucleobase acetic acid is then coupled to the secondary amino group. The N-terminus amino group protection is then removed and a new cycle of acylation–displacement reactions is used to build the second residue, and so on. But this attractive approach has, as yet, only been used to prepare polythymine PNA five residues long. This is not long enough. PNA oligomers must contain around 16 residues (statistically incorporating the four nucleobases) in order to be useful and act specifically.

We have developed another way of optimizing PNA synthesis. Boc-protected PNA monomers of thymine, Z-cytosine, Z-adenine and 6-*O*-benzyl guanine were prepared by solid-phase synthesis, using an ester–disulphide linkage: 2-hydroxy-propyl-dithio-2′-isobutyric acid (HPDI). The final cleavage, with tris(2-carboxyethyl)phosphine (TCEP) or potassium cyanide, produced free carboxyl PNA monomers under mild conditions.

The PNA monomers were then linked to form oligomers by standard solid-phase synthesis, using another disulphide linker, aminoethyl-dithio-2-isobutyric acid (AEDI), which provided the mercapto amide PNA by cleavage with TCEP. A preliminary account of this study has already appeared [13].

MATERIALS AND METHODS

The solvents (acetonitrile, chloroform, DCM, DMF (stored over 4 Å molecular sieves), dioxane, ethyl acetate, hexane, iso-propanol, pentane, piperidine, THF), AcOH and TFA were obtained from S.D.S. (Peypin, France). Benzyl chloroformate, *tert*-butyl bromoacetate, cytosine, DCC, DIEA, 9-fluorenylmethyl chloroformate, lithium hydroxide, methyl bromoacetate, 4-methylmorpholine, potassium carbonate and potassium cyanide were purchased from Fluka AG (Buchs, Switzerland). 2-amino-6-chloropurine, 5-aminovaleric acid, benzyl alcohol, bromoacetic acid, cesium carbonate, *m*-cresol, DECA, DMAP, HFIP, HOBt, sodium hydride and triethylsilane were obtained from Aldrich-Chimie (St. Quentin Fallavier, France). Adenine, ethylene diamine, thymine, di-*tert*-butyl dicarbonate and pyridine were obtained from Acros (Noisy-Le-Grand, France). Other reagents were DMSO (Merck; Darmstadt, Germany), HATU (PerSeptive Biosystems; Framingham, MA), TBTU (Bachem; Budendorf, Switzerland), TFMSA (Applied Biosystems; Foster City, CA), phosphorus pentoxide, potassium hydroxide, sodium, sodium hydrogencarbonate and sodium sulphate (Prolabo; Paris, France). TCEP was prepared as described by Méry *et al.* [14]. Boc-Lys(2-Cl-Z)-OH, Boc-Lys(Z)-OH and Fmoc-βAla were obtained from Novabiochem AG (Laüfelfingen, Switzerland). Peroxides were eliminated from 1,4-dioxane and THF using an Al_2O_3 (Merck) column and DCM was neutralized over Na_2CO_3 (Prolabo) and dried over $CaCl_2$ (Prolabo). Expansin® resin was a gift from Société Expansia (Aramon, France) and TentaGel® was purchased from Rapp Polymere (Tübingen, Germany).

General Methods

Flash chromatography was carried out using Silica gel 60 (particle size 0.063–0.200 mm; Merck). Thin layer chromatography was carried out on aluminium sheets, Silica gel 60 F_{254}, layer thickness 0.2 mm (Merck); developed with ninhydrin (150°C).

Analytical HPLC was performed on Gilson equipment (Villiers-Le-Bel, France) with Brownlee aquapore RP-300 (C8, 7 μm, 220 × 4.6 mm) or Spheri-5 RP-18 (5 μm, 220 × 4.6 mm) columns. The flow rates were 1 ml/min for analytical HPLC and 3 ml/min for preparative HPLC. Effluents were monitored at 220 nm or 260 nm unless indicated. The solvent systems used was: A, H_2O/TFA 0.1% and B, CH_3CN/TFA 0.057%. Semi-preparative HPLC was

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performed similarly on Vydac C8 column, 208TP510 (Hesperia, CA, USA).

Monomer and oligomer solid-phase synthesis were carried out in a manually operated solid-phase reactor [15].

^{1}H-NMR spectra were recorded on a Brucker AM 250 spectrometer (Wissembourg, France). Chemical shifts are reported in parts per million (p.p.m., δ) down-field relative to the standard tetramethylsilane. Coupling constants are reported in Hertz (Hz). Spectral patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. Compounds **18a–25**, containing amide bonds, were obtained as mixtures of two rotamers; several of the NMR signals were doubled in the rotamer ratio as indicated. Positive ion fast atom bombardment (FAB) mass spectra were recorded on a JEOL DX 300 and SX 102 (Tokyo, Japan) spectrometer using a GT (glycerol-thioglycerol) or a NBA (*m*-nitrobenzylalcohol) matrix; electrospray mass spectra were recorded on a Fisons VG Trio 2000 spectrometer (Manchester, UK).

N-tert-butyloxycarbonyl-1,2-ethylenediamine (1)

This compound was prepared as described by Krapcho and Kuell [16]: to a solution of ethylenediamine (60 g, 1 mol) in dioxane (300 ml) was added di-*tert*-butyl dicarbonate (27 g, 0.125 mol) dissolved in dioxane (270 ml), over a period of 8 h at room temperature. The mixture was stirred at room temperature for further 8 h, the dioxane was evaporated and water (350 ml) was added to the residue. The insoluble material was removed by filtration and water was extracted with DCM. The combined extracts were dried (Na_2SO_4) and evaporated to a colourless oil (18 g; 90%). TLC (49.5% DCM/49.5% MeOH/1% AcOH); R_F 0.39, ninhydrin (150°C, violet spot). ^{1}H-NMR ($CDCl_3$): δ 4.95 (s br, 1H, N*H*-Boc), 3.15 (t, d, 2H, $J_1 = 6$ Hz, $J_2 = 6$ Hz, CH_2–NH-Boc), 2.78 (t, 2H, $J = 6$ Hz, CH_2-NH_2), 1.44 (s, 9H, tBu).

Methyl-*N*-(2-Boc-aminoethyl)glycinate (2a)

Compound **1** (8 g, 0.05 mol) was dissolved in acetonitrile (50 ml) mixed with chloroform (50 ml), and K_2CO_3 (7.2 g, 0.052 mol) was added. Methyl bromoacetate (4.6 ml, 0.05 mol), dissolved in acetonitrile (50 ml), was added very slowly over 6 h with continuous stirring under N_2. The reaction mixture was stirred at room temperature for another 30 min, and the suspension was filtered. The filtrate was evaporated to an oily residue. Purification by flash column chromatography (eluent: 80% ether/20% MeOH, with monitoring by TLC) yielded 9.6 g (83%) of transparent oil. TLC (80% ether/20% MeOH); R_F 0.47, ninhydrin (150°C, orange spot). ^{1}H-NMR ($CDCl_3$): δ 5.05 (s br, 1H, Boc-N*H*), 3.74 (s, 3H, COO–CH_3), 3.42 (s, 2H, CH_2–COO-CH_3), 3.22 (t, d, 2H, $J = 5.8$ Hz, $J' = 5.8$ Hz, Boc-NH–CH_2), 2.75 (t, 2H, $J = 5.8$ Hz, CH_2–CH_2–NH), 1.66 (s, 1H, CH_2–N*H*-CH_2), 1.44 (s, 9H, tBu). MS: m/z (FAB) = 233 (M^+ + H). The bis adduct, **2b** (10%), was also isolated: m.p. = 81–83°C; R_F 0.81, ninhydrin (150°C, yellow spot). ^{1}H-NMR ($CDCl_3$): δ 5.53 (s br, 1H, N*H*), 3.73 (s, 6H 2 × COO–CH_3), 3.56 (s, 4H, 2 × CH_2–COO-CH_3), 3.18 (t, d, 2H, $J = 5.5$ Hz, $J' = 5.5$ Hz, Boc-NH–CH_2), 2.86 (t, 2H, $J = 5.5$ Hz, CH_2–N), 1.46 (s, 9H, tBu). MS: m/z (FAB) = 305 (M^+ + H).

N-(2-Boc-aminoethyl)Gly–HPDI–Expansin® Resin (3)

Bromoacetic Acid–HPDI-Resin. Bromoacetic acid (5.670 g, 40.8 mmol) was dissolved in DCM (30 ml) and 4.704 g (22.8 mmol) of DCC were added. The mixture was stirred at 10°C for 20 min under N_2 and the precipitated dicyclohexyl urea was removed by filtration. It was washed with DCM. The filtrate was added directly to pre-swollen (in DCM) HPDI–Expansin® resin (substitution = 0.68 mmol/g) obtained from Expansin® resin (substitution = 0.79 mmol NH_2/g) as previously described [17]. DMAP (2.186 g, 17.9 mmol) dissolved in DCM (20 ml), and NMM (1.374 ml, 12.5 mmol) were added and the mixture was shaken for 3 h at room temperature. The red solution was removed by filtration and the resin was washed thoroughly with DCM (5 × 3 min), DMF (3 × 2 min), DCM (3 × 2 min), MeOH (5 × 3 min), ether (5 × 3 min) and dried under reduced pressure.

Boc-1,2-ethylenediamine Coupling. Bromoacetic acid–HPDI-resin (4 g, substitution = 0.62 mmol/g) was shaken for 4 h with 10 g (62.4 mmol) of compound **1** dissolved in DMF (18 ml). The resin was collected by filtration, washed with DMF (5 × 3 min), DCM (3 × 3 min), MeOH (4 × 3 min) and ether (5 × 3 min) and dried under reduced pressure.

Thymin-1-yl-acetic Acid Methyl Ester (4)

Methyl bromoacetate (5.5 ml, 0.06 mol) in DMF (35 ml) was slowly added to a suspension of thymine (6.3 g, 0.05 mol) in DMF (130 ml) and K_2CO_3 (8.6 g, 0.0624 mol) and the mixture was stirred overnight at room temperature under N_2. The mixture was filtered and the filtrate was evaporated to dryness, *in vacuo*. The solid residue was recrystallized from MeOH and dried over P_2O_5 under vacuum to give

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6.5 g (65.6%) of white crystalline product: m.p. = 189–191°C; ^{1}H-NMR (DMSO-d$_6$): δ 11.40 (s, 1H, N*H*), 7.49 (s, 1H, *H*$_6$–C = C), 4.48 (s, 2H, *CH*$_2$–COOCH$_3$), 3.68 (s, 3H, *CH*$_3$–O), 1.75 (s, 3H, *CH*$_3$–C = C). MS: *m*/*z* (FAB) = 199 (M$^+$ + H).

Thymin-1-yl-acetic Acid (5)

1. M NaOH (aqueous; 50 ml) was added to compound **4** (6.4 g, 32 mmol) and the reaction mixture was stirred at 0°C for 1 h and then for 4 h at room temperature. The pH was adjusted to 3.8 with 1 M HCl (aqueous) and water was evaporated. The product was dried over P_2O_5 under vacuum to give a white solid containing NaCl.
2. The direct procedure of Finn *et al.* [18] was used to obtain pure thymin-1-yl-acetic acid in good yield (86%).

m.p. > 250°C; ^{1}H-NMR (DMSO-d$_6$): δ 11.11 (s, 1H, N*H*), 7.36 (s, 1H, *H*$_6$–C═C), 4.00 (s, 2H, *CH*$_2$–COOH), 1.76 (s, 3H, *CH*$_3$–C═C). MS: *m*/*z* (FAB) = 185 (M$^+$ + H).

4-*N*-(Benzyloxycarbonyl) Cytosine (6)

4-*N*-(Benzyloxycarbonyl) cytosine was prepared as described by Dueholm *et al.* [9]. m.p. > 250°C; ^{1}H-NMR (CF$_3$COOH): δ 8.28 (d, 1H, *J* = 7 Hz, *H*$_6$), 7.40 (m, 5H, Φ), 6.68 (d, 1H, *J* = 7 Hz, *H*$_5$), 5.42 (s, 2H, *CH*$_2$). MS: *m*/*z* (FAB) = 246 (M$^+$ + H).

(4-*N*-(Benzyloxycarbonyl)cytosin-1-yl) Acetic Acid Methyl Ester (7)

Compound **6** (6.03 g, 24.6 mmol) and K_2CO_3 (3.9 g, 28.2 mmol) were suspended in DMF (90 ml) and a mixture of methyl bromoacetate (2.5 ml, 27.1 mmol) and DMF (20 ml) was added dropwise over a period of 2 h under N_2. The mixture was stirred overnight, filtered and the filtrate evaporated to dryness *in vacuo*. The residue was taken up in MeOH, the insoluble material was removed by filtration and the filtrate was evaporated. The residue was purified by flash column chromatography (eluent: 90.5% DCM/9.5% MeOH, with monitoring by TLC) to give 5.25 g (67.2%) of white solid: m.p. = 152–158°C; ^{1}H-NMR (CDCl$_3$): δ 7.76 (s br, 1H, N*H*), 7.55 (d, 1H, *J* = 7 Hz, *H*$_6$), 7.38 (m, 5H, Φ), 7.28 (d, 1H, *J* = 7 Hz, *H*$_5$), 5.21 (s, 2H, *CH*$_2$-Φ), 4.61 (s, 2H, N–*CH*$_2$), 3.78 (s, 3H, *CH*$_3$). MS: *m*/*z* (FAB) = 318 (M$^+$ + H).

(4-*N*-(Benzyloxycarbonyl)cytosin-1-yl) Acetic Acid *tert*-Butyl Ester (8)

Compound **6** (5.15 g, 21 mmol), 2.902 g (21 mmol) of K_2CO_3 and 0.684 g (2.1 mmol) of Cs_2CO_3 were stirred in suspension in DMF (85 ml) under N_2, and 3.25 ml (22 mmol) *tert*-butyl bromoacetate were added dropwise. The mixture was stirred for 29 h, filtered and the filtrate evaporated under vacuum. The residue was dissolved in $CHCl_3$ (300 ml), washed with water, dried (Na_2SO_4) and evaporated. The crude light yellow solid was crystallized from ethyl acetate to give two crops of compound **8** as a white solid (6.318 g, 83%): m.p. = 164–165°C; ^{1}H-NMR (CDCl$_3$): δ 7.69 (s br, 1H, N*H*), 7.59 (d, 1H, *J* = 7.3 Hz, *H*$_6$), 7.41 (s, 5H, Φ), 7.28 (d, 1H, *J* = 7.3 Hz, *H*$_5$), 5.24 (s, 2H, *CH*$_2$-Φ), 4.54 (s, 2H, *CH*$_2$–COO-tBu), 1.50 (s, 9H, tBu). MS: *m*/*z* (FAB) = 359 (M$^+$ + H).

(4-*N*-(Benzyloxycarbonyl)cytosin-1-yl) Acetic Acid (9)

1. 1 M NaOH (aqueous, 6.7 ml) was added to compound **7** (1.417 g, 4.46 mmol) and the mixture stirred at 0°C for 30 min and then overnight at room temperature. The pH was adjusted to 2.7 with 1 M HCl (aqueous) and the resulting white precipitate was filtered, washed with water and dried over P_2O_5 under vacuum. Yield: 1.05 g (77.5%).
2. Et$_3$SiH (2.715 ml, 17 mmol) was added to a solution of compound **8** (5.24 g, 14.58 mmol) in DCM (50 ml). The solution was cooled to 0°C, and TFA (50 ml) was added dropwise with stirring under N_2. The mixture was kept at 0°C for 30 min, then allowed to warm to room temperature for 4 h. HPLC showed the reaction to be complete. The mixture was evaporated to dryness *in vacuo* and remaining volatiles were removed by azeotroping with chloroform (4 ×), to give compound **9** as a white solid (4.42 g, 100%).

m.p. = 215°C (d); ^{1}H-NMR (DMSO-d$_6$/TFA): δ 8.05 (d, 1H, *J* = 7 Hz, *H*$_6$), 7.40 (m, 5H, Φ), 7.02 (d, 1H, *J* = 7 Hz, *H*$_5$), 5.20 (s, 2H, *CH*$_2$-Φ), 4.55 (s, 2H, N–*CH*$_2$). MS: *m*/*z* (FAB) = 304 (M$^+$ + H).

Adenin-9-yl-acetic Acid Methyl Ester (10)

NaH (2.03 g, 84.6 mmol, 60% dispersion in oil) was washed with pentane and added portion-wise to a suspension of adenine (10 g, 74 mmol) in DMF (150 ml) with stirring for about 2 h under N_2. Methyl

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bromoacetate (14 ml, 0.148 mol) in DMF (30 ml) was then added dropwise over a period of 3 h at room temperature under N_2 and the mixture stirred overnight. The reaction mixture was evaporated *in vacuo*, and water (100 ml) was added to precipitate the title compound. The solid was filtered, washed with water and recrystallized from EtOH to yield 7.36 g (48%): m.p. = 226–227°C; ^{1}H-NMR (DMSO-d_6): δ 8.15 (s, 1H, H_2), 8.13 (s, 1H, H_8), 7.32 (s, 2H, NH_2), 5.11 (s, 2H, CH_2-COO–CH_3), 3.72 (s, 3H, O–CH_3). MS: m/z (FAB) = 208 (M^+ + H).

6-*N*-(Benzyloxycarbonyl)adenin-9-yl-acetic Acid Methyl Ester (11)

A solution of compound **10** (4.97 g, 24 mmol) in warm DMF (120 ml) was added dropwise at 0°C under N_2 to 1-(benzyloxycarbonyl)-3-ethylimidazolium tetrafluoroborate (96 mmol; synthesized as described by Watkins *et al.* [19]) in DCM (130 ml). It was kept at 0°C for 10 min and then allowed to warm to room temperature, at which it was stirred overnight. The reaction was quenched by adding a saturated aqueous solution of $NaHCO_3$ (80 ml) at 0°C. The reaction mixture was evaporated to an oily residue, which was taken up in $CHCl_3$ (400 ml). Insoluble compounds were filtered off and the filtrate was washed with water (2 × 60 ml), $NaHCO_3$ (0.5 M, 60 ml), water (60 ml), $KHSO_4$ (0.5 M, 60 ml) and water (2 × 60 ml). The organic layer was dried (Na_2SO_4) and evaporated to a yellowish oily residue, which was dissolved in DCM and precipitated with pentane. The solid obtained (5.36 g) was a mixture of *Z*-adenin methyl- and ethyl-ester. Flash column chromatography of an aliquot (eluent: 86.4% ether/13.6% MeOH, with monitoring by TLC) allowed the two esters to be separated. *Z*-adenin ethyl ester: TLC (86% ether/14% MeOH); R_F 0.52 (UV); m.p. = 133–135°C, ^{1}H-NMR ($CDCl_3$): δ 8.76 (s, 1H, H_2), 7.96 (s, 1H, H_8), 7.40–7.34 (m, 5H, Φ), 5.29 (s, 2H, CH_2-Φ), 4.90 (s, 2H, CH_2–COO–CH_3), 4.24 (q, 2H, J = 7.3 Hz, CH_2–CH_3), 1.25 (t, 3H, J = 7.3 Hz, CH_2–CH_3). Z-adenin methyl ester: TLC (86% ether/14% MeOH); R_F 0.40 (UV); m.p. = 114–117°C; ^{1}H-NMR ($CDCl_3$): δ 8.75 (s, 1H, H_2), 7.96 (s, 1H, H_8), 7.42–7.34 (m, 5H, Φ), 5.28 (s, 2H, CH_2-Φ), 4.93 (s, 2H, CH_2–COO–CH_3), 3.80 (s, 3H, O–CH_3). MS: m/z (FAB) = 342 (M^+ + H).

6-*N*-(Benzyloxycarbonyl)-adenin-9-yl-acetic Acid (12)

1 M NaOH (aqueous, 19 ml) was added to compound **11** (1.55 g, 4.55 mmol) at 0°C. After 1 h the cooling was discontinued, water (18 ml) was added, and the mixture was stirred overnight at room temperature. Insoluble materials were removed by filtration and the pH was adjusted to 2 with 4 M HCl (aqueous) at 0°C. The title compound was precipitated, collected by filtration, washed with water and dried (in vacuum, over P_2O_5). The yield was 1.23 g (82.5%): m.p. = 155–160°C; ^{1}H-NMR (DMSO-d_6): δ 13.78 (s, br, 1H, COO*H*), 9.08 (s, 1H, H_2), 8.84 (s, 1H, H_8), 7.53–7.32 (m, 5H, Φ), 5.36 (s, 2H, CH_2-Φ), 5.24 (s, 2H, CH_2–COOH). MS: m/z (FAB) = 328 (M^+ + H).

6-*N*-(Benzyloxycarbonyl)adenine (13)

This compound was prepared as described by Thomson *et al.* [10] to give 5.38 g (54%): m.p. = 215°C (d); ^{1}H-NMR (DMSO-d_6): δ 13.20–10.00 (s very br, 2H, N*H*), 8.59 (s, 1H, H_2), 8.43 (s, 1H, H_8), 7.50–7.30 (m, 5H, Φ), 5.30 (s, 2H, CH_2-Φ). MS: m/z (FAB) = 270 (M^+ + H).

6-*N*-(Benzyloxycarbonyl)adenin-9-yl-acetic Acid *tert*-Butyl Ester (14)

3.610 g (13.4 mmol) of compound **13**, 1.935 g (14 mmol) of K_2CO_3 and 0.456 g (1.4 mmol) of Cs_2CO_3 were stirred together in 30 ml DMF. 2.188 ml (14.8 mmol) of *tert*-butyl bromoacetate were added dropwise under N_2 and the mixture stirred for 20 h. It was diluted with ethyl acetate and filtered. The filtrate was evaporated, *in vacuo*, to an oily residue which was dissolved in ethyl acetate (300 ml), washed with brine (2 ×), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was crystallized from ethyl acetate/hexane to give a first crop of compound **14** (3.015 g). The filtrate was evaporated, chromatographed on SiO_2 (eluent: 90% DCM/10% MeOH) and 0.580 g of pure product were recovered. Overall yield: 70%: m.p. = 135–139°C; ^{1}H-NMR ($CDCl_3$): δ 9.55 (s br, 1H, N*H*), 8.75 (s, 1H, H_2), 7.98 (s, 1H, H_8), 7.45–7.30 (m, 5H, Φ), 5.26 (s, 2H, CH_2-Φ), 4.81 (s, 2H, CH_2–COO-tBu), 1.40 (s, 9H, tBu). MS: m/z (FAB) = 384 (M^+ + H).

(2-Amino-6-chloropurin-9-yl) Acetic Acid (15)

1. Et_3SiH (0.320 ml, 2 mmol) was added to a suspension of **16** (0.5 g, 17.6 mmol) in DMF (5 ml). The mixture was cooled to −5°C, and TFA (5 ml) was added with stirring under N_2. The reaction was kept at 0°C for 1 h, allowed to warm to room temperature and stirred for further 4 h. The mixture was evaporated *in vacuo*, and remaining volatiles were removed by azeotroping with chlo-

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roform (4 ×) to give compound **15** as a white solid (0.40 g, 100%).

2. Compound **15** was also prepared as described by Nerstrom *et al.* [20].

m.p. > 300°C; ^{1}H-NMR (DMSO-d$_6$): δ 8.08 (s, 1H, H_8), 6.90 (s br, 2H, NH_2), 4.88 (s, 2H, CH_2–COOH). MS: m/z (FAB) = 228 (M$^+$ + H).

(2-Amino-6-chloropurin-9-yl) Acetic Acid *tert*-Butyl Ester (16)

1.015 g (6 mmol) of 2-amino-6-chloropurine, 0.871 g (6.3 mmol) of K_2CO_3 and 0.205 g (0.63 mmol) of Cs_2CO_3 were stirred together under N_2, in DMF (20 ml). Then *tert*-butyl bromoacetate (0.928 ml, 6.3 mmol) diluted with DMF (2 ml) was added dropwise, and the mixture was stirred for 17 h. It was filtered and the filtrate evaporated, *in vacuo*. The residue was taken up in hot $CHCl_3$ (150 ml), filtered and the filtrate evaporated to a residue which was crystallized in DCM/pentane, to give compound **16** (1.276 g, 75%): m.p. = 165°C (d); ^{1}H-NMR ($CDCl_3$): δ 7.81 (s, 1H, H_8), 5.18 (s br, 2H, NH_2), 4.74 (s, 2H, CH_2–COO–tBu), 1.47 (s, 9H, tBu). MS: m/z (FAB) = 284 (M$^+$ + H).

6-*O*-Benzylguanin-9-yl-acetic Acid (17)

1.7 g (74 mmol) of sodium were suspended in benzyl alcohol (27 ml) and tetrahydrofuran (27 ml) and refluxed under N_2 until the sodium was reacted. The mixture was cooled to 0°C and compound **15** (3.626 g, 16 mmol), in DMF (100 ml), was added. The mixture was stirred overnight at 0°C and the reaction was quenched by adding acetic acid (10 ml). The mixture was evaporated to a pasty residue which was dissolved in water (150 ml), cooled at 5°C and the pH was adjusted to 2.85 with concentrated HCl. The resulting precipitate was filtered out, washed with water, dried and recrystallized from ethanol to give 3.40 g (71%) of compound **17** as a white solid: m.p. = 192–200°C; ^{1}H-NMR (DMSO-d$_6$): δ 7.80 (s, 1H, H_8), 7.49–7.28 (m, 5H, Φ), 6.48 (s, 2H, NH_2), 5.46 (s, 2H, CH_2-Φ), 4.78 (s, 2H, CH_2–COOH). MS: m/z (FAB) = 300 (M$^+$ + H).

Methyl-*N*-(2-Boc-aminoethyl)-*N*-(thymin-1-yl-acetyl)glycinate (18b)

Compound **5** (1.84 g, 10 mmol) and TBTU (3.37 g, 10.5 mmol) were dissolved in DMF (30 ml) and NMM (1.37 ml, 12.5 mmol) was added. To this mixture was added dropwise a solution of compound **2a** (2.3 g, 10 mmol) in DMF (10 ml) and the reaction mixture was stirred for 7 h under N_2 at room temperature. The solvent was removed by evaporation and chloroform was added to the residue. The resulting precipitate was filtered out and the filtrate washed with water (6 ×), dried (Na_2SO_4) and evaporated. The resulting yellowish oil was recrystallized from ether/MeOH to yield 3.4 g (85%) of a white crystalline product: m.p. = 157°C; TLC (80% ether/20% MeOH); R_F 0.61, ninhydrin (150°C, green spot). ^{1}H-NMR ($CDCl_3$): δ 9.04 (s, 1H, NH), 7.05 (s, 0.25 H, H_6), 6.98 (s, 0.75, H_6), 5.63 (m, 0.75H, NHBoc), 5.02 (m, 0.25H, NHBoc), 4.59 (s, 1.50H, N–CH_2–CO), 4.44 (s, 0.50H, N–CH_2–CO), 4.24 (s, 0.50H, CH_2–COOCH$_3$), 4.07 (s, 1.50H, CH_2–COOCH$_3$), 3.82 (s, 0.75H, O–CH_3), 3.77 (s, 2.25H, O–CH_3), 3.57–3.53 (m, 2H, CH_2–N), 3.38–3.26 (m, 2H, Boc-NH–CH_2), 1.93 (s, 3H, CH_3–C=C), 1.45 (s, 9H, tBu). MS: m/z (FAB) = 399 (M$^+$ + H).

Methyl-*N*-((4-*N*-(Benzyloxycarbonyl)cytosin-1-yl) acetyl)-*N*-(2-Boc-aminoethyl)glycinate (19b)

Compound **9** (1.698 g, 5.6 mmol) and TBTU (1.888 g, 5.88 mmol) were suspended in DMF (42 ml) and a solution of compound **2a** (1.431 g, 6.16 mmol) in DMF (8 ml) was added, followed by NMM (850 μl, 7.7 mmol). The reaction mixture was stirred overnight under N_2 at room temperature and filtered. The filtrate evaporated to dryness, *in vacuo*, and the residue was dissolved in chloroform (180 ml), washed with water (3 × 30 ml), $NaHCO_3$ (0.1 M, 30 ml) and water (30 ml). The organic layer was dried (Na_2SO_4) and evaporated to dryness. The resulting residue was recrystallized from MeOH to give 2.03 g (70%) of a white solid: m.p. = 192–196°C; TLC (80% ether/20% MeOH); R_F 0.65, ninhydrin (150°C, green spot). ^{1}H-NMR (DMSO-d$_6$): δ 10.80 (s br, 1H, NH), 7.90 (d, 1H, J = 7.2 Hz, H_6), 7.43–7.32 (m, 5H, Φ), 7.02 (d, 1H, J = 7.2 Hz, H_5), 6.98 (m, 0.75H, NH-Boc), 6.75 (m, 0.25H, NH-Boc), 5.19 (s, 2H, CH_2-Φ), 4.82 (s, 1.50H, N–CH_2–CO), 4.64 (s, 0.50H, N–CH_2–CO), 4.36 (s, 0.50H, CH_2–COO–CH$_3$), 4.07 (s, 1.50H, CH_2–COO–CH$_3$), 3.73 (s, 0.75H, CH_3), 3.63 (s, 2.25H, CH_3), 3.44 (m, 1.50H, CH$_2$–CH_2–N), 3.33 (m, 0.50H, CH$_2$–CH_2–N), 3.20. (m, 1.50H, CH_2–NH-Boc), 3.03 (m, 0.50H, CH_2–NH-Boc), 1.38 (s, 9H, tBu). MS: m/z (FAB) = 518 (M$^+$ + H).

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Methyl-*N*-((6-*N*-(Benzyloxycarbonyl)adenin-9-yl)-acetyl)-*N*-(2-Boc-aminoethyl) glycinate (20b)

Compound **12** (1 g, 3.055 mmol) and TBTU (1.03 g, 3.2 mmol) were suspended in DMF (25 ml) and a solution of compound **2a** (780 mg, 3.36 mmol) in DMF (15 ml) was added, followed by NMM (462 µl, 4.2 mmol). The reaction mixture was stirred overnight under N_2 at room temperature. Insoluble materials were removed by filtration. The filtrate was evaporated, the residue dissolved in $CHCl_3$ (150 ml) and washed with water (3 × 20 ml), $NaHCO_3$ (0.1 M, 30 ml) and water (20 ml). The organic layer was dried (Na_2SO_4) and evaporated. The resulting yellow oil was purified by flash column chromatography (eluent: 80% ether/20% MeOH, with monitoring by TLC) to give 1.078 g (65%): m.p. = 60–63°C; TLC (80% ether/20% MeOH); R_F 0.61, ninhydrin (150°C, green spot). ^{1}H-NMR ($CDCl_3$): δ 8.72 (s, 1H, N*H*-Boc), 8.62 (s, 0.25H, H_2), 8.55 (s, 0.75H, H_2), 8.06 (s, 0.25H, H_8), 8.015 (s, 0.75H, H_8), 7.45–7.30 (m, 5H, Φ), 5.27 (s, 2H, CH_2-Φ), 5.11 (s, 1.5H, N–CH_2–CO), 4.94 (s, 0.5H, N–CH_2–CO), 4.28 (s, 0.5H, CH_2–COO–CH_3), 4.05 (s, 1.5H, CH_2–COO–CH_3), 3.81 (s, 0.75H, O–CH_3), 3.71 (s, 2.25H, O–CH_3), 3.62 (t, 1.5H, J = 6 Hz, CH_2–CH_2–N), 3.55 (t, 0.5H, J = 6 Hz, CH_2–CH_2–N), 3.39 (m, 1.5H, Boc-NH–CH_2), 3.27 (m, 0.5H, Boc-NH–CH_2), 1.39 (s, 9H, tBu). MS: m/z (FAB) = 542 (M^+ + H).

Methyl-*N*-((6-*O*-benzylguanin-9-yl)-acetyl)-*N*-(2-Boc-aminoethyl)glycinate (21b)

To a solution of compound **17** (2.144 g, 7.16 mmol) and TBTU (2.415 g, 7.52 mmol) in DMF (30 ml) was added compound **2a** (1.83 g, 7.88 mmol) in DMF (10 ml), followed by NMM (1.083 ml, 9.85 mmol). The reaction mixture was stirred overnight under N_2 at room temperature, and evaporated to a yellow oil. The oil was dissolved in $CHCl_3$ (250 ml) and washed with water (3 × 50 ml), $NaHCO_3$ (0.1 M, 60 ml) and water (50 ml). The organic layer was dried (Na_2SO_4), evaporated and the residue was recrystallized from MeOH. The title compound was collected by filtration and additional material was isolated from the mother liquor by evaporating it and recrystallizing from MeOH. The combined solids gave 2.84 g (77.2%): m.p. = 148–149°C; TLC (80% ether/20% MeOH); R_F 0.60, ninhydrin (150°C, green spot). ^{1}H-NMR (DMSO-d_6): δ 7.70 (s, 1H, H_8), 7.55–7.30 (m, 5H, Φ), 7.04 (t, 0.7H, J = 6 Hz, N*H*-Boc), 6.78 (t, 0.3H, J = 6 Hz, N*H*-Boc), 6.45 (s, 2H, NH_2), 5.50 (s, 2H, CH_2-Φ), 5.06 (s, 1.4H, N–CH_2–CO), 4.90 (s, 0.6H, N–CH_2–CO), 4.42 (s, 0.6H, CH_2–COO–CH_3), 4.08 (s, 1.4H, CH_2–COO–CH_3), 3.75 (s, 1H, O–CH_3), 3.62 (s, 2H, O–CH_3), 3.51 (t, 1.4H, J = 6 Hz, CH_2–CH_2–N), 3.33 (t, 0.6H, J = 6 Hz, CH_2–CH_2–N), 3.24 (t, d, 1.4H, J = 6 Hz, J' = 6 Hz, Boc-NH–CH_2), 3.03 (t, d, 0.6H, J = 6 Hz, J' = 6 Hz, Boc-NH–CH_2), 1.38 (s, 6.3H, tBu).1.37 (s, 2.7H, tBu). MS: m/z (FAB) = 514 (M^+ + H).

N-(2-Boc-aminoethyl)-*N*-(thymin-1-yl-acetyl) glycine (22)

Solid-phase Synthesis (SPS). To the Boc-NH–CH_2–CH_2–NH–CH_2–COO–HPDI–Expansin® resin **3** (3 g, substitution = 0.607 mmol/g), (vessel volume: 50 ml), was added a solution of compound **5** (1.370 g,7.44 mmol), TBTU (2.270 g, 7.068 mmol) and HOBt (1.082 g, 7.068 mmol) in DMSO (36 ml) and NMM (1.17 ml, 10.6 mmol) and the whole shaken for 7 h. The coupling step was repeated under the same conditions, and the resin was washed with DMSO (3 × 5 min), DMF (5 × 3 min), DCM (6 × 3 min), MeOH (2 × 3 min), water (2 × 3 min), MeOH (5 × 3 min) and ether (4 × 3 min). The resin **18a** was dried (*in vacuo*, over P_2O_5) and the product cleaved from the resin with a 4-fold excess of TCEP in 5 ml acetate buffer (0.2 M, pH 4.5, purged with nitrogen) and 1 ml i-PrOH per 100 mg resin. This reaction mixture was stirred overnight, filtered and the pH adjusted to 8–9 with 1 M NaOH (aqueous). The mixture was stirred for 5 h and the pH was adjusted to 6 with 1 M HCl (aqueous). The monomer was also released by shaking the dried resin **18a** in a 0.1 M solution of KCN in acetate buffer (0.2 M, pH 4.5, purged with nitrogen), 1 ml per 10 mg resin. The reaction was monitored by HPLC, brought to pH 4 with HCl (1N), and degassed (fume hood). Compound **22** was purified by flash column chromatography (eluent: 49.5% DCM/49.5% MeOH/1% AcOH, with monitoring by TLC). Yield: 90%.

Solution Procedure. 1 M NaOH (aqueous, 11.5 ml) was added to compound **18b** (3 g, 7.5 mmol) at 0°C and the mixture stirred for 30 min. Water (10 ml) was added and stirring was continued overnight at room temperature. The aqueous solution was washed with DCM (3 × 5 ml), cooled at 0°C and brought to pH 3.2 with 1 M $KHSO_4$. This solution was concentrated to half the original volume and ethyl acetate was added. The acid was precipitated after storage in a refrigerator for 2 days. The solid was collected by filtration, washed with water and dried *in vacuo* (over P_2O_5). The mother liquor was extracted with ethyl acetate

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(5 × 20 ml). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. The resulting solid was combined with the first filtration product to provide 2.8 g (97%) of the title compound.

m.p. = 190°C; TLC (49.5% DCM/49.5% MeOH/1% AcOH); R_F 0.47, ninhydrin (150°C, green spot). ^{1}H-NMR (DMSO-d_6): δ 11.28 (s, 0.45H, N*H*), 11.23 (s, 0.55H, NH), 7.29 (s, 0.45H, H_6), 7.24 (s, 0.55H, H_6), 7.00 (m, 0.45H, N*H*Boc), 6.98 (m, 0.55H, N*H*-Boc), 4.60 (s, 0.90H, N–CH_2–CO), 4.41 (s, 1.10H, N–CH_2–CO), 3.87 (s, 0.90H, CH_2–COOH), 3.80 (s, 1.10H, CH_2–COOH), 3.40–3.20 (m, unresolved, 2H, Boc-NH-CH_2), 3.20–3.06 (m, 0.90H, CH_2–N), 3.06–2.91 (m, 1.10H, CH_2–N), 1.73 (s, 3H, CH_3–C=C), 1.36 (s, 4.05H, tBu), 1.34 (s, 4.95H, tBu). MS: m/z (FAB) = 385 ($M^+ + H$).

N-(4-*N*-(Benzyloxycarbonyl)cytosin-1-yl)acetyl)-*N*-(2-Boc-aminoethyl) glycine (23)

SPS. To the Boc-NH–CH_2–CH_2–NH–CH_2–COO–HPDI–Expansin® resin **3** (3 g) was added a solution of compound **9** (2.256 g, 7.44 mmol), TBTU (2.270 g, 7.068 mmol) in DMF (39 ml) and DECA (1.94 ml, 10.6 mmol) and the whole shaken for 7 h. The coupling step was repeated under the same conditions. Washings, cleavage and purification of resin **19a** were carried out as described for the SPS of compound **22**, except that extra acetonitrile (1 ml per 100 mg of resin) was added to the cleavage reaction before filtration to dissolve the released product. Yield:78%.

Solution Procedure. Compound **19b** (0.9 g, 1.75 mmol) was suspended in water (36 ml), cooled to 0°C and 1 M NaOH (4 ml) and dioxane (18 ml) were added. The mixture was stirred for 5 h and the pH was adjusted to 3 with 1 M HCl (aqueous). The homogeneous solution was stored in the refrigerator for several days to precipitate the title acid. Yield: 821 mg (93%).

m.p. = 128–130°C; TLC (49.5% DCM/49.5% MeOH/1% AcOH); R_F 0.63, ninhydrin (150°C, green spot). ^{1}H-NMR (DMSO-d_6): δ 7.90 (d, 1H, $J = 7$ Hz, H_6), 7.43–7.32 (m, 5H, Φ), 7.02 (d, 1H, $J = 7$ Hz, H_5), 6.99 (m, 0.75H, N*H*-Boc), 6.75 (m, 0.25H, N*H*-Boc), 5.19 (s, 2H, CH_2-Φ), 4.81 (s, 1.50H, N–CH_2–CO), 4.63 (s, 0.50H, N–CH_2–CO), 4.23 (s, 0.50H, CH_2–COOH), 3.98 (s, 1.50H, CH_2–COOH), 3.50–2.90 (m, unresolved, 4H, NH–CH_2–CH_2–N), 1.38 (s, 9H, tBu). MS: m/z (FAB) = 504 ($M^+ + H$).

(6-*N*-(Benzyloxycarbonyl)adenin-9-yl)-acetyl-*N*-(2-Boc-aminoethyl) glycine (24)

SPS. The Boc-NH–CH_2–CH_2–NH–CH_2–COO–HPDI–Expansin® resin **3** (3 g) was shaken with a solution of compound **12** (2.435 g, 7.44 mmol), TBTU (2.270 g, 7.068 mmol) in DMF (36 ml) and NMM (1.17 ml, 10.6 mmol) for 24 h. The coupling step was repeated under the same conditions. Washings, cleavage and purification of resin **20a** were carried out as described for the SPS of compound **22**, except that extra MeOH (2 ml per 100 mg of resin) was added to the cleavage reaction before filtration to dissolve the released product. Yield: 77%.

Solution Procedure. Compound **20b** (3 g, 5.25 mmol) was suspended in water (10 ml), cooled to 0°C and 1 M LiOH (8.3 ml) was added. The reaction mixture was stirred for 2 h and allowed to warm up to room temperature for a further 1 h with stirring. The pH was adjusted to 3 with 1 M HCl (aqueous). The precipitate was collected by filtration, washed with water and dried. The compound was evaporated three times from $CHCl_3$ and reprecipitated from methanol/ether yielding 2.29 g (78.7%): m.p. = 173°C; TLC (49.5% DCM/49.5% MeOH/1% AcOH); R_F 0.60, ninhydrin (150°C, green spot). ^{1}H-NMR (DMSO-d_6): δ 13.13 (s br, 1H, COO*H*), 10.69 (s, 1H, N*H*–CO), 8.61 (s, 0.65H, H_2), 8.59 (s, 0.35H, H_2), 8.32 (s, 0.35H, H_8), 8.31 (s, 0.65H, H_8), 7.50–7.30 (m, 5H, Φ), 7.06 (t, 0.65H, $J = 5$ Hz, N*H*-Boc), 6.80 (t, 0.35H, $J = 5$ Hz, N*H*-Boc), 5.34 (s, 1.3H, N–CH_2–CO), 5.22 (s, 2H, CH_2-Φ), 5.16 (s, 0.7H, N–CH_2–CO), 4.32 (s, 0.7H, CH_2–COO–CH_3), 3.99 (s, 1.3H, CH_2–*C*OO–CH_3), 3.53 (t, 1.3H, $J = 5$ Hz, CH_2–CH_2–N), 3.31 (s, 0.7H, CH_2–CH_2–N), 3.25 (t, d, 1.3H, $J = 5$ Hz, $J' = 5$ Hz, Boc-NH–CH_2), 3.04 (t, d, 0.7H, $J = 5$ Hz, $J' = 5$ Hz, Boc-NH–CH_2), 1.39 (s, 5.85H, tBu), 1.36 (s, 3.15H, tBu). MS: m/z (FAB) = 528 ($M^+ + H$).

(6-*O*-Benzylguanin-9-yl)-acetyl-*N*-(2-Boc-aminoethyl)glycine (25)

SPS. To the Boc-NH–CH_2–CH_2–NH–CH_2–COO–HPDI-Expansin® resin **3** (3 g) was added a solution of compound **17** (2.227 g, 7.44 mmol), TBTU (2.388 g, 7.44 mmol) in DMF (39 ml) and NMM (1.022 ml, 9.3 mmol) and the whole shaken for 8 h. The coupling step was repeated under the same conditions. Washings, cleavage and purification of resin **21a** were carried out as described for the SPS of compound **22**, except that extra MeOH (2 ml per 100 mg of resin) was added to the cleavage reaction before filtration to dissolve the released product. Yield: 80%.

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Solution Procedure. Compound **21b** (850 mg, 1.68 mmol) was suspended in water (7 ml), cooled at 0°C and 1 M NaOH (aqueous, 2.55 ml) was added. The reaction mixture was stirred for 1 h and allowed to warm up to room temperature for 1 h. EtOH (10 ml) was added and stirring was continued for further 3 h. The pH was adjusted to 3 with 1 M HCl (aqueous). The resulting voluminous precipitate was collected by filtration, washed with water and dried, to give 911.2 mg (95.5%).

m.p. = 102°C (d); TLC (49.5% DCM/49.5% MeOH/ 1% AcOH); R_F 0.60, ninhydrin (150°C, green spot). ^{1}H-NMR (DMSO-d$_6$): δ 7.91 (s, 0.4H, H_8), 7.87 (s, 0.6H, H_8), 7.53–7.31 (m, 5H, Φ), 7.026 (t br, 0.6H, N*H*-Boc), 6.78 (t br, 0.4H, N*H*-Boc), 5.51 (s, 2H, CH_2-Φ), 5.08 (s, 1.2H, N–CH_2–CO), 4.92 (s, 0.8H, N–CH_2–CO), 4.31 (s, 0.8H, CH_2–COOH), 3.99 (s, 1.2H, CH_2–COOH), 3.49 (t br, 1.2H, CH_2–CH_2–N), 3.32 (t br, 0.8H, CH_2–CH_2–N), 3.24 (q br, 1.2H, Boc–NH–CH_2), 3.03 (q br, 0.8H, Boc-NH–CH_2), 1.38. (s, 5.4H, tBu), 1.37 (s, 3.6H, tBu). MS: m/z (FAB) = 500 ($M^+ + H$).

Boc-Ade(Z)-Ade(Z)-COOH (26)

The pre-swollen Boc-Ade(Z)-HPDI–Expansin® resin **20a** (100 mg, substitution = 0.0511 mmol/g) was placed in a manually operated solid-phase reaction vessel (volume: 5 ml), and the Boc-group protection was removed using TFA/*m*-cresol (95/5) (2 × 3 min). The resin was washed thoroughly with DCM/DMF (50/50) (5 × 1 min), DMF (2 × 3 min), pyridine (1 × 2 min) and DMF (2 × 3 min). A solution of compound **24** (81 mg, 0.153 mmol), TBTU (44 mg, 0.137 mmol) in DMF (2.2 ml) and DECA (56 µl, 0.3 mmol) was stirred for 5 min, added to the H_2N-Ade(Z)-HPDI-Expansin® resin and the whole shaken for 17 h. The resin was filtered, washed with DCM/DMF (50/50) (5 × 1 min), DMF (3 × 1 min), DCM (6 × 3 min), MeOH (4 × 3 min) and ether (4 × 3 min) and dried (*in vacuo*, over P_2O_5). Cleavage was carried out by shaking with a 4-fold excess of TCEP in 3 ml acetate buffer (0.2 M, pH 4.5) and 1 ml i-PrOH for 20 h. The pH was adjusted to 8 with 1 M NaOH (aqueous) and shaking was continued overnight. The pH was adjusted to 4 with 1 M HCl (aqueous), the resin was filtered off, and the filtrate was purified by semi-preparative reverse phase HPLC (monitored at 288 nm), to give the final Boc-Ade(Z)–Ade(Z)-COOH dimer, using a linear gradient from 15% to 65% B (in A) over 25 min. Yield: 45%. The dimer was analysed by FAB MS: m/z (FAB) = 937 ($M^+ + H$).

Fmoc-5-aminovaleric Acid (27)

5-Aminovaleric acid (1.757 g, 15 mmol) was dissolved in 35 ml aqueous Na_2CO_3 (3.975 g, 37.5 mmol). Dioxane (20 ml) was added, and the mixture was cooled at 0°C. 9-Fluorenylmethyl chloroformate (3.880 g, 15 mmol) was added portionwise over 1 h. The resulting mixture was stirred for 4 h at 0°C, allowed to warm to room temperature and stirred for 15 h. The reaction mixture was then poured into H_2O (500 ml). The dioxane was evaporated under vacuum and the aqueous solution was extracted twice with ether. The aqueous phase was cooled in an ice bath, and the pH adjusted to 3.8 with HCl (5 M). The precipate was collected by filtration, washed with water, and dried under vacuum, to give compound **27** (4.618 g, 90%): m.p. = 133–134°C; MS: m/z (FAB) = 340 ($M^+ + H$).

Oligomerization

PNA monomers were coupled in DMF with three equivalents of monomer, using TBTU (0.9 × quantity of the monomer) as activating reagent and DECA (six equivalents) as base, with a pre-activation time of 5 min. The coupling time was 3 h with shaking at room temperature, followed by washing with DCM/DMF (50/50) (5 × 1 min) and DMF (3 × 1 min). The Boc-group was deprotected using TFA/*m*-cresol (95/5) (2 × 3 min). The resin was washed thoroughly with DCM/DMF (50/50) (5 × 1 min), DMF (2 × 3 min), pyridine (1 × 2 min) and DMF (2 × 3 min) before the cycle was repeated. The resin was washed with DCM (3 × 2 min), MeOH (3 × 2 min) and ether (3 × 2 min) and dried under vacuum over P_2O_5 before removal of the exocyclic amino protecting groups. The resin was shaken with TFA/*m*-cresol (89/11) (180 µl per 10 mg resin), cooled at − 4°C and TFMSA (20 µl per 10 mg resin) was added under N_2 with a syringe. The resin was shaken for 1 h, washed with DMF (10 ×) and DCM (12 ×), dried and the oligomer cleaved from the solid support with a 30-fold excess of TCEP in 1 ml acetate buffer (0.2 M, pH 4.5, purged with nitrogen) and 1 ml HFIP per 10 mg resin. PNA release was monitored by HPLC. Coupling efficiency was checked by the same procedure on aliquots (3–5 mg). The PNA oligomers were purified by semi-preparative reverse-phase HPLC to give the final PNA-cysteamides. They were analysed by electrospray mass spectroscopy.

Oligomers Synthesized. $(T)_{10}$-Ala-Cya was assembled using the protocol described above on 120 mg preswollen (in DMF) Ala-AEDI-TentaGel® resin.

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Couplings were checked on aliquots after the fourth, fifth, sixth, seventh, eight, ninth and tenth cycle. The final product contained several major peaks, corresponding to 10T (29%), 9T (18%), 8T (11%), 7% (12%) and 6T (9%) oligomers.

Fmoc-NH$(CH_2)_5$-CO-A TGG AAC GAG CTG ATC-βAla-Cya was synthesized on 165 mg of βAla-AEDI-TentaGel® resin using the standard protocol. Oligomerization was checked after each coupling and finished by coupling Fmoc-5-aminovaleric acid **27**.

βAla ATG GCG TTG GTG TTC A-Lys-Cya: pre-swollen (in DMF) Lys(Z)-AEDI-TentaGel® resin (400 mg, substitution = 0.249 mmol/g) was placed in a manually operated solid-phase reaction vessel (volume: 5 ml), and oligomerization was carried out. After the last monomer, Fmoc-βAla was coupled (3-fold excess; double coupling) to prevent *N*-acyl-shift reaction. The resin was washed with DCM/DMF (50/50) (5 × 1 min) and DMF (3 × 1 min) and the Fmoc-group protection removed with 20% piperidine in DMF (1 × 2 min, 1 × 5 min and 1 × 12 min). The resin was again washed with DMF (6 × 3 min), DCM/DMF (50/50) (2 × 2 min), DCM (4 × 2 min), MeOH (6 × 2 min) and ether (5 × 2 min) and dried under vacuum over P_2O_5. The oligomer was purified by HPLC (monitored at 298 nm), using a linear gradient of 10–25% B (in A) over 17 min. Electrospray mass spectroscopy: *M* calculated for $C_{185}H_{238}N_{94}O_{54}S$ M 4670; *M* found 4670.7.

H-TG GCG TTG GTG TTC A-Lys(2-Cl-Z)-Cya: a pre-swollen (in DMF) Lys(2-Cl-Z)-AEDI-TentaGel® resin (200 mg, substitution = 0.247 mmol/g) was placed in a manually operated solid-phase reaction vessel (volume: 5 ml), and oligomerization was carried out. The oligomer was purified by HPLC (monitored at 296 nm), using a linear gradient of 3–40% B (in A) over 20 min. Electrospray mass spectroscopy: *M* calculated for $C_{179}H_{224}N_{89}O_{51}SCl$ M 4501; *M* found 4502.

RESULTS AND DISCUSSION

Solid-phase Synthesis of PNA Boc-monomers

Solid-phase synthesis makes the preparation and purification of PNA monomers much more convenient. The disulphide ester anchoring linkage, HPDI, was used as it has been thoroughly tested for peptide synthesis in our laboratory [17].This bifunctional handle can be treated by cyanolysis with KCN under mild conditions (pH 8–9), or reduced with TCEP (pH 8–9) to give C-terminal-free peptides in good yields and purities.

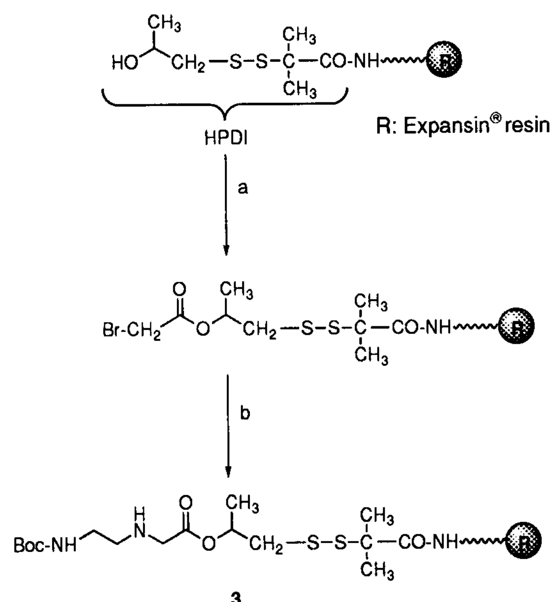

Figure 1 Reagents: (a) $(BrCH_2CO)_2$, DMAP, NMM; (b) Boc-NH$(CH_2)_2NH_2$ **1**, DMF.

Synthesis of the Backbone. The backbone unit was built by a two-step procedure similar to the first steps of the submonomer strategy [12]. The hydroxyl group of the linker was acylated with bromoacetic anhydrid using 4-dimethylaminopyridine (DMAP) as a catalyst. A subsequent displacement step with Boc-ethylenediamine **1** led to the immobilized backbone **3** (Figure 1). These reactions were optimized by synthesizing the backbone moiety **2a** in solution by a similar method. Methyl bromoacetate was slowly added to Boc-ethylenediamine **1** in excess, in the presence of K_2CO_3. However, a small fraction of the bis-derivative **2b** was produced (Figure 2). This side reaction was avoided in the solid-phase synthesis by adding to the bromoacetyl-resin

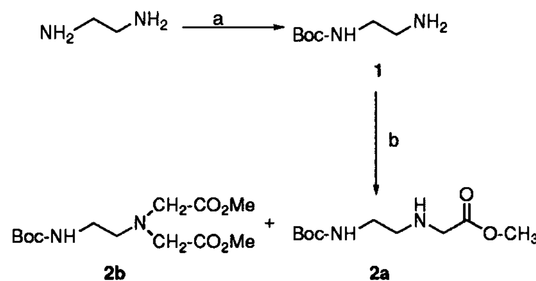

Figure 2 Reagents: (a) di-*tert*-butyl dicarbonate, dioxane; (b) $BrCH_2COOCH_3$, K_2CO_3, CH_3CN, $CHCl_3$.

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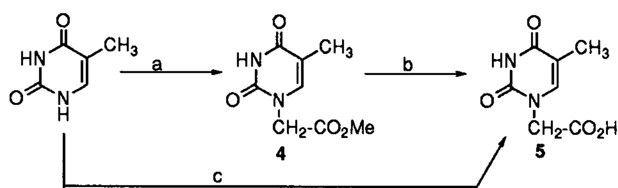

Figure 3 Reagents: (a) $BrCH_2COOCH_3$, K_2CO_3, DMF; (b) NaOH; (c) $BrCH_2COOH$, KOH, H_2O.

a large excess of Boc-ethylenediamine solution, concentrated as high as possible.

Synthesis of the Four Nucleobase-acetic Acids. *Thymin-1-yl-acetic acid* **5**. Thymin-1-yl-acetic acid was first prepared as described by Dueholm *et al.* [9] by alkylation of thymine with methyl bromoacetate. The resulting methyl ester **4** was hydrolysed and purified by chromatography. However, a bis-adduct was also formed and the final product was contamined with sodium chloride. We therefore adopted a more direct method [18] of treating thymine with bromoacetic acid and potassium hydroxide (Figure 3) to give thymin-1-yl-acetic acid **5** in good yield (86%).

4-N-Z-Cytosin-1-yl-acetic acid **9**. The exocyclic 4-amino group of cytosine was protected to prevent undesired extension at this site. This was usually done by introducing the benzyloxycarbonyl (Z) group prior to alkylation at N-1 with methyl bromoacetate in DMF [9]. Selective hydrolysis with aqueous NaOH at room temperature gave the 4-*N*-Z-cytosin-1-yl-acetic acid **9**. We initially used this method, but subsequently prepared the compound by a more reliable method [10] using *tert*-butyl bromoacetate for alkylation. The *tert*-butyl ester was removed with TFA-Et_3SiH to give 4-*N*-Z-cytosin-1-yl-acetic acid with an overall yield of 83% (Figure 4).

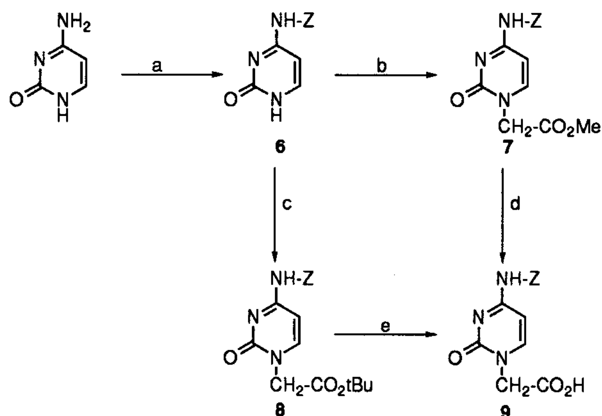

Figure 4 Reagents: (a) Cbz-Cl, pyridine; (b) $BrCH_2COOCH_3$, K_2CO_3, DMF; (c) $BrCH_2COOtBu$, K_2CO_3, Cs_2CO_3, DMF; (d) NaOH, H_2O; (e) TFA, CH_2Cl_2, Et_3SiH.

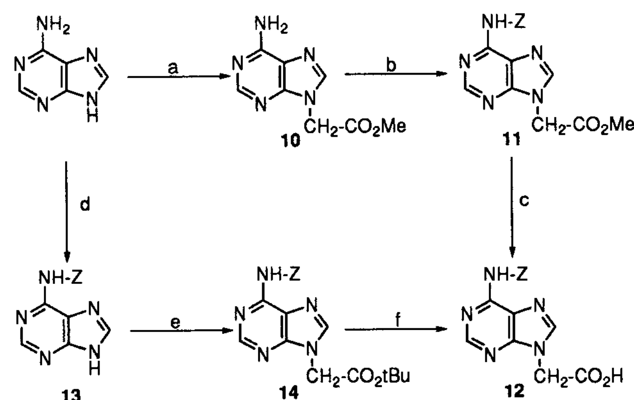

Figure 5 Reagents: (a) $BrCH_2COOCH_3$, NaH, DMF; (b) Rapoport reagent, DMF, DCM; (c) NaOH, H_2O; (d) NaH, Cbz-Cl, DMF; (e) $BrCH_2COOtBu$, K_2CO_3, Cs_2CO_3, DMF; (f) TFA, DCM, Et_3SiH.

6-N-Z-Adenin-9-yl-acetic acid **12**. We initially attempted to synthesize Z-adenine-acetic acid using the approach described by Dueholm *et al.* [9]. Adenine was alkylated with methyl bromoacetate and the benzyloxycarbonyl (Z) protective group was introduced using Rapoport's reagent [19]. The resulting mixture of Z-adenine methyl- and ethyl-ester was hydrolysed with aqueous NaOH to give Z-adenine-acetic acid. However, this method requires extensive chromatography to purify the ester. We therefore adopted the procedure described by Thomson *et al.* [10] in which the exocyclic amino group of adenine is first Z-protected by reaction with benzyl chloroformate and sodium hydride. Crystallized Z-adenine **13** was recovered with a 54% yield. Subsequent alkylation with *tert*-butyl bromoacetate gave crystallized 6-*N*-Z-Adenin-9-yl-acetic acid *tert*-butyl ester **14**, which was treated with TFA, plus Et_3SiH as scavenger, to give, quantitatively, 6-*N*-Z-adenin-9-yl-acetic acid **12**. The ^{1}H-NMR spectra for **12** and **14** were consistent with the reported data [21,22], indicating substitution at the 9-position (Figure 5).

6-O-Benzylguanin-9-yl-acetic acid **17**. The benzyl-guanin acetic acid was prepared by the approach of Dueholm *et al.* [9]. The 2-amino-6-chloropurine was used as starting material, and protection of the unreactive exocyclic 2-amino group was deliberately omitted. Alkylation with bromoacetic acid gave mainly 2-amino-6-chloropurin-9-yl-acetic acid **15**, and a small

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amount of 7-substituted product [20]. We also investigated an alternative way: alkylation of 2-amino-6-chloropurine with *tert*-butyl bromoacetate gave a satisfactory yield (75%) of crystalline 2-amino-6-chloropurin-9-yl-acetic acid *tert*-butyl ester **16**. This was hydrolysed with TFA to give quantitatively the product **15**. The solubility of this compound was improved by replacing the chloro group with a benzyloxy group, which was removed with TFA during the deprotection step of oligomerization. This second procedure was adopted because the yield of compound **17** was good, and because it was readily performed (Figure 6). The 9- and 7-isomers were assessed by ^{1}H-NMR spectroscopy. The signals from H(8) and N-CH_2 are shifted for the 9-isomer relative to the corresponding signals from the 7-isomer, whereas the NH_2 signal appeared at a lower field for the 9-isomer [21].

Monomer Synthesis. The last step in monomer synthesis was the attachment of the four nucleobase-acetic acids to the immobilized backbone unit. The main reason for using a polymer supported strategy was that the supported species are easily separated from the non-supported species by filtration. Excess reagents can therefore be used without causing separation problems, so that the reaction work-up is greatly simplified. The HPDI linker also provides an additional advantage. The disulphide bridge can be cleaved and the subsequent hydrolysis performed under mild conditions in a single one-pot procedure according to reactional mechanisms described earlier [17], and outlined in Figure 7.

Two amino-functionalized resins, TentaGel®-S-NH_2 and Expansin® resin, were tested. TentaGel® resin is a low cross-linked polystyrene backbone grafted with polyoxyethylene (POE: polyethyleneglycol). The typical POE chain length is 68 ethyleneglycol units. Expansin® is a polyacrylic resin. These polymeric supports have good swelling properties in solvents commonly used for coupling (eg. DMF, DCM or NMP), as well as in the water-isopropanol or -hexafluoropropanol mixtures required for handle cleavage. Expansin® resin was used for preparing PNA monomers because of its higher capacity (0.8 mmol/g) to obtain fair amounts of product.

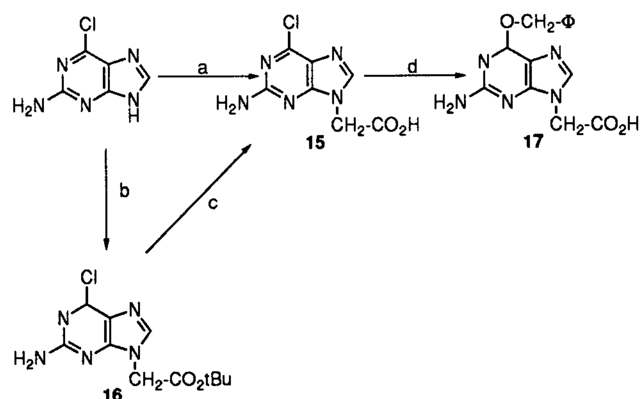

Figure 6 Reagents: (a) $BrCH_2COOH$, K_2CO_3, DMF; (b) $BrCH_2COOtBu$, K_2CO_3, Cs_2CO_3, DMF; (c) TFA, DCM, Et_3SiH; (d) Na, Φ-CH_2OH, DMF, THF.

A 4-fold excess of thymin-1-yl-, (4-*N*-Z-cytosin-1-yl)-, (6-*N*-Z-adenin-9-yl)- and (6-*O*-Bzl-guanin-9-yl)-acetic acids, **5**, **9**, **12**, **17**, were preactivated with TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3 tetramethyluronium tetrafluoroborate) and HOB*t* (1-hydroxybenzotriazole) and coupled to the immobilized N-(2-Boc-aminoethyl)glycinate **3**. Recoupling was involved in each case. Excess spent reagents and soluble by-products were thoroughly washed out, the linker cleaved by cyanolysis with KCN or by assisted hydrolysis with TCEP (Figure 7), and crude monomers, **22**, **23**, **24**, **25**, were recovered and separated from the buffer by filtration on silica gel.

Monomers were also synthesized in solution using the same coupling reagents to obtain control samples, and to compare the two methods (Table 1). The solid-phase procedure gave higher yields than the solution procedure. The mild cleavage conditions allowed by HPDI anchoring linkage are essential to preserve the structure of the monomer. An unexpected side reaction occurred during the hydrolysis of methyl-*N*-(4-*N*-Z-cytosin-1-yl)acetyl-*N*-(2-Boc-aminoethyl)glycinate, **19b**, with aqueous NaOH to which methanol was added to improve the solubility of the ester. This led to removal of the Z-protection group. The same deleterious reaction occurred during the hydrolysis of 4-*N*-Z-cytosin-1-yl-acetic acid methyl ester **7**. The milder conditions used for HPDI handle cleavage essentially eliminated this problem.

Purine monomers sometimes fail to couple efficiently during oligomerization [9,18]. A PNA dimer such as adenine–adenine can be pre-synthesized to circumvent this. The adenine-adenine dimer **26** was prepared by coupling the Boc-adenine monomer **24** with immobilized Boc-adenine monomer, first built on the polymeric support as described above.

'Submonomer' Strategy. We also attempted to assess the feasibility of the submonomer strategy [12], using immobilized N-Boc-adenine **20a** as starting material. The Boc protective group was removed

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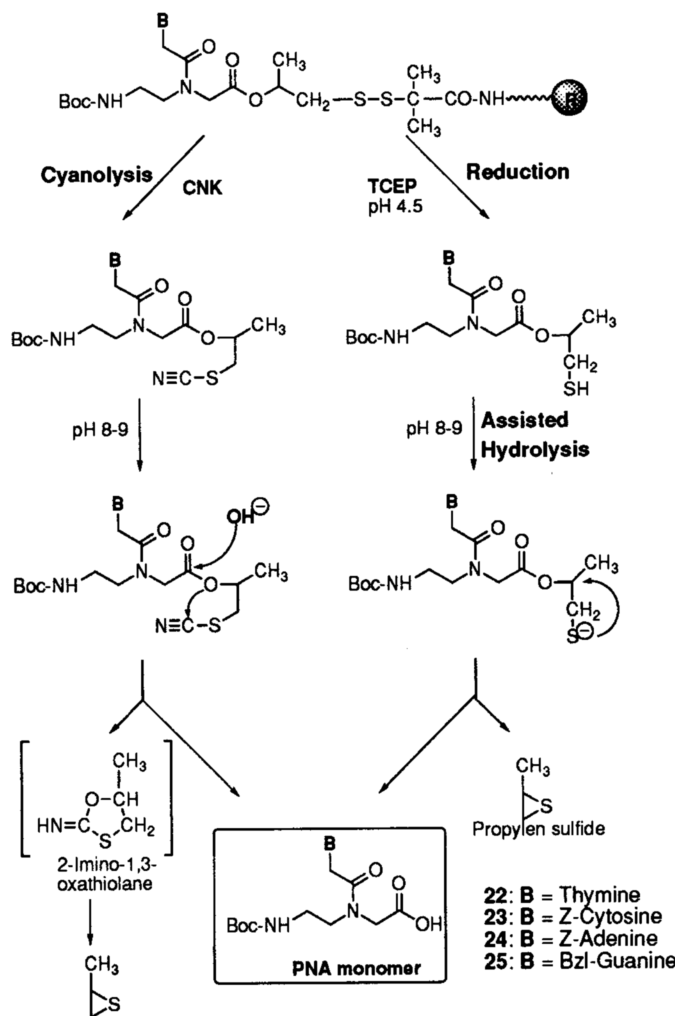

Figure 7 PNA monomer release

with TFA, bromoacetic anhydrid was reacted with the free amino group, and excess concentrated Boc-ethylenediamine in DMF was added to built the second backbone unit. Finally, 6-*N*-Z-adenin-9-yl-acetic acid **12** activated with TBTU-HOBt was coupled to obtain the PNA dimer adenine–adenine. The results were disappointing. The crude material recovered from cleavage contained several by-products according to reverse-phase chromatography, and the yield of the wanted product Ade–Ade was only 10%. This reflects the difficulties of the sub monomer approach. All reactions involved in the repeated cycle synthesis (deprotection, acylation, nucleophilic substitution and coupling) must go to near 100% completion to obtain a homogeneous product. This attractive strategy therefore requires a very careful optimization of each reaction parameter before it can be used with the HPDI linker.

Oligomerization

Oligomers were prepared on TentaGel®-S-NH_2 because the gel-like structure of this resin does not restrict growing. The anchoring linkage was AEDI, which is well suited to the solid-phase synthesis of peptides [14,23].

The recommendations of Christensen *et al.* [11] were generally followed; TBTU was used as activating reagent, and coupling additives such as HOBt and DMAP were avoided. The tertiary amine was diethylcyclohexylamine because the monomer salts dissolve in it. PNA monomers were used in a 3-fold excess over the substitution of the resin, and dissolved in neat DMF. Chain termination, which occurs when there is free TBTU in the coupling, was prevented by preactivating Boc PNA monomers with a molar amount of TBTU slightly less than that of

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Table 1 PNA monomers

Compound	Base	R	method	precursors	Yield(%)
22	Thymine	H	solid phase	18a : (5+3)	90
18b	Thymine	CH_3	solution	5+2a	85
22	Thymine	H	solution	18b	97
23	Z-Cytosine	H	solid phase	19a : (9+3)	78
19b	Z-Cytosine	CH_3	solution	9+2a	70
23	Z-Cytosine	H	solution	19b	93
24	Z-Adenine	H	solid phase	20a : (12+3)	77
20b	Z-Adenine	CH_3	solution	12+2a	65
24	Z-Adenine	H	solution	20b	78
25	Bzl-Guanine	H	solid phase	21a : (17+3)	80
21b	Bzl-Guanine	CH_3	solution	17+2a	77
25	Bzl-Guanine	H	solution	21b	95

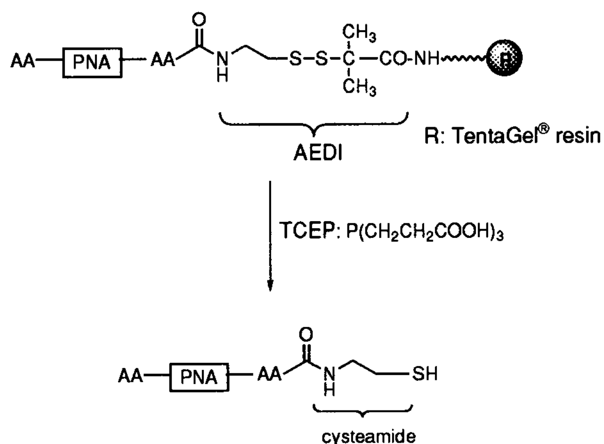

Figure 8 PNA release

monomers [24]. *N*-acyl-transfer reactions, which may occur under neutral and alkaline conditions [11], were avoided by using the *in situ* neutralization proposed by Schnölzer *et al.* [24]. Coupling was carried out in the presence of tertiary amine, without prior neutralization of the TFA salt of the deprotected terminal amino group of the growing PNA chain. Since unprotected 2-amino-6-(benzyloxy)purine was used as a precursor of guanine, capping was not performed for any coupling, according to Dueholm *et al.* [9] who observed that acetic anhydrid may react with the exocyclic amino group of 2-amino-6-(benzyloxy)purine monomer. The Z-protective groups of cytosine and adenine were removed with the low-TFMSA protocol after oligomerization [25] and the PNA was cleaved from the resin (Figure 8) by reduction of the disulphide bridge with TCEP in acetate buffer pH 4.5 [17,23].

Preliminary experiments were done with the more accessible thymine PNA monomer. We attempted to assemble ten thymine residues using alanine-AEDI-TentaGel® resin as starting material. Oligomerization was checked after the first, fourth, sixth and subsequent coupling steps by cleavage of an aliquote with TCEP. Oligomerization proceeded very well for the first four cycles, and then became sluggish. Prolonged reaction times or a double coupling protocol using HATU did not provide satisfactory results. The final product contained 10T (29%), 9T (18%), 8T (11%), 7T (12%) and 6T (9%) oligomers. The same problem was also encountered and resolved by Dueholm *et al.* [9] by including a C-terminal lysine to suppress self-association.

The heteropolymer A TGG AAC GAG CTG ATC, containing all four nucleobases, was assembled on βAla-AEDI-TentaGel® resin with no self-association problems. Oligomerization was checked after each cycle and terminated by coupling Fmoc-5-aminovaleric acid **27**. Capping the N-terminus blocked any *N*-acyl shift and cyclization side reactions, and also introduced a spacer between the PNA and other moieties that could be added later. Reverse-phase HPLC of the crude 16-mer product before removal of Z-protective groups showed a major peak (66%), which indicates an average yield of 97% for each coupling. However, the situation worsened after Z-deprotection because free nucleobases of PNA can form stable PNA–PNA duplexes [26] and consequently the work-up was complicated. Therefore, the sequence of PNAs must be carefully chosen:

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even partial hybridization must be prevented (four consecutive bases only in the example given), in the same way as the hairpins.

For the assembly of ATG GCG TTG GTG TTC A, a sequence carefully chosen to avoid the formation of duplexes, Lys(Z) was first coupled to the AEDI-TentaGel® resin to prevent self-association problem. Couplings were performed by the standard protocol, and checked after the fifth, sixth and seventh cycle, to verify that the use of unprotected 2-amino-6-(benzyloxy)purine did not cause problems. The oligomer was ended by coupling β-alanine to avoid any degradative *N*-acyl-shift reaction. The Z-protection was removed and the product cleaved from the support with TCEP. The resulting βAla-ATG GCG TTG GTG TTC A-Lys-cysteamide was better than 75% pure according to reverse phase HPLC. The identity of the product was confirmed by electrospray mass spectrometry. Synthesis of this sequence containing six guanine residues confirmed that using 2-amino unprotected (benzyloxy)purine as a precursor of guanine is trouble-free. The same sequence was synthesized again, but Lys(2Cl-Z) was first coupled to the AEDI resin. This protective group is much more resistant to removal by the low TFMSA procedure than is the Z-group. The same oligomer was obtained, but bearing a C-terminal Lys(2Cl-Z), whose lipophilic properties could be useful for vectorization.

CONCLUSION

The synthesis of PNA monomers can be simplified and improved by using a polymer support. The well-loaded polyacrylic Expansin® resin is suitable for use in organic solvents and in aqueous conditions. The HPDI linker used is cleavable under mild conditions, providing undamaged monomers. Oligomerization was carried out on TentaGel® resin because growing PNA is not restricted by steric hindrance. The AEDI anchoring linkage was used for PNA assembly because it was more robust than the HPDI linker. AEDI linkage allowed us to recover the PNA bearing a cysteamide residue, which could be very useful for optimizing the PNA properties. The very reactive thiol function could improve cell permeability and therefore bioavailability, for instance by grafting the PNA onto the *retro-inverso* peptide designed for this purpose [27]. Oligomerization can probably be improved using the recent results of Koch *et al.* [28]: HATU may be better activating reagent than TBTU, NMP-DMF could be used as coupling solvent to give better results. However, these authors used a very large seven-fold excess of monomers.

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