



The Synthesis of Polyamide Nucleic Acids using a Novel Monomethoxytrityl Protecting-Group Strategy

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Abstract: The preparation of novel monomethoxytrityl (Mmt) protected monomers for the synthesis of polyamide nucleic acids (PNAs) is described. The use of base-labile acyl-type nucleobase protecting groups and of a succinyl-linked solid-support offers a synthetic strategy similar to standard oligonucleotide synthesis conditions. This strategy has been successfully applied for the synthesis of PNAs of mixed base sequence.

In 1991, Nielsen *et al.*¹ developed a new class of oligonucleotide analogues, known as Polyamide (or Peptide) Nucleic Acids (PNAs). These are oligomers of nucleobase-derivatized N-(2-aminoethyl)glycine which recognize and bind strongly to specific DNA or RNA sequences. PNA oligomers have a number of properties which make them potentially extremely useful as antisense therapeutics and as diagnostic tools.²

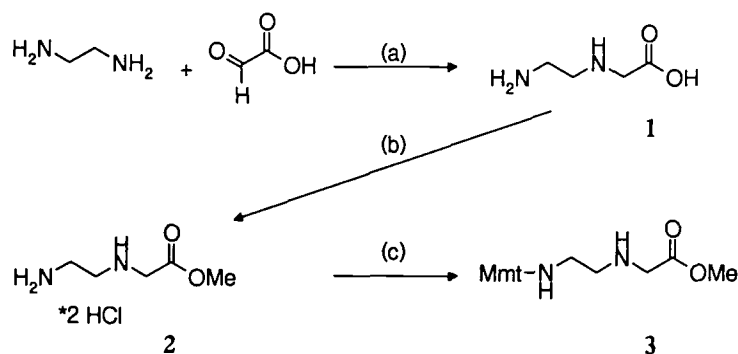
Until recently the only reported synthetic strategy for PNA synthesis was Merrifield solid-phase synthesis using a Boc/benzyloxycarbonyl protecting group strategy.^{3,4,5} The repeated treatment with TFA required for Boc deprotection, and the harsh HF or TFMSA treatment required for cleavage from the resin and deprotection render this strategy incompatible with the synthesis of many types of modified PNAs, especially the synthesis of PNA-DNA chimerae due to the sensitivity of DNA to strong acids. Very recently Thomson *et al.* reported the synthesis of Fmoc/ benzyloxycarbonyl protected PNA monomers and outlined methods for their oligomerisation.⁶ This Fmoc strategy may be combined with Fmoc peptide synthesis to allow the preparation of PNA-peptide conjugates. However, using this method, a strong acid deprotection step is also required at the end of synthesis.

In search of an alternative strategy which would open the way to a combination of PNA and oligonucleotide synthesis we have developed novel PNA monomers with orthogonal protecting groups in which the Mmt group is used as an N-terminal temporary protecting group and the exocyclic amino functions of the nucleobases are protected by base-labile acyl protecting groups. The Mmt group can be removed under mild acidic conditions (3% trichloroacetic acid), and the nucleobase protecting groups are removed at the end of synthesis using conc. aqueous ammonia. A solid support suitable for use in this synthetic strategy has also been synthesized.

RESULTS AND DISCUSSION

Monomer Synthesis

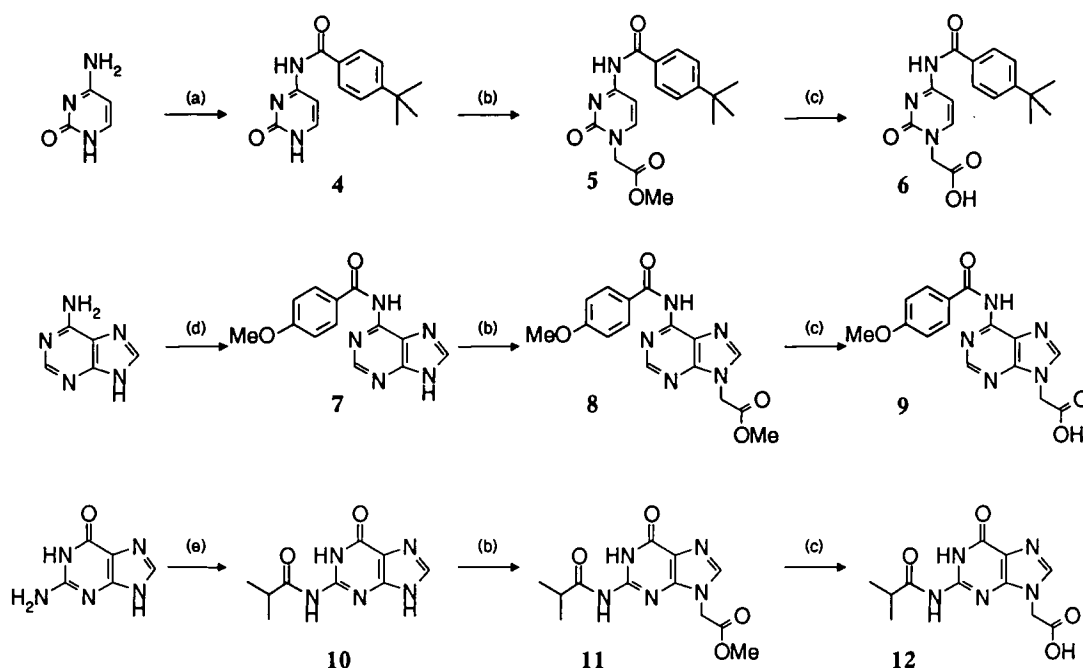
For the large-scale preparation of N-(2-aminoethyl)glycine **1** we found a very simple and effective method in the reductive amination of glyoxylic acid using an excess of 1,2-diaminoethane in alcohol/water mixtures and hydrogen with palladium on charcoal as reducing agent (Scheme 1). This procedure gives N-(2-aminoethyl)glycine in excellent purity and good yield from readily available starting materials. Methyl N-(2-aminoethyl) glycinate dihydrochloride **2** was obtained from **1** according to the literature procedure.⁹ This was then monomethoxytritylated with (4-methoxyphenyl)-diphenylmethyl chloride (Mmt-Cl) in DMF / triethylamine. The Mmt group reacted predominantly on the less hindered primary amine to give **3** as the major product. The bis-Mmt by-product was easily separated by silica gel chromatography. The position of the Mmt group in **3** was confirmed by Nuclear Overhauser Effect NMR experiments.



Reagents and Conditions: (a) H₂, Pd/C, methanol; (b) Methanol/ HCl, reflux; (c) Mmt chloride/ DMF, NEt₃.

Scheme 1: Synthesis of N-(2-aminoethyl)glycine and its Mmt-protected derivative.

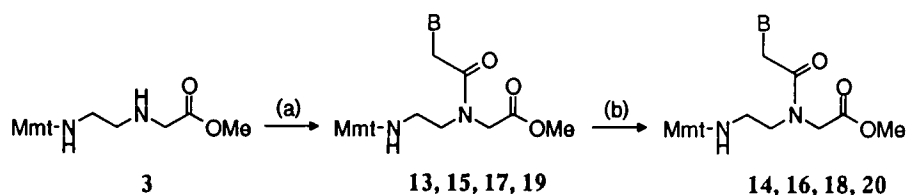
Carboxymethylated thymine was synthesized according to the procedure of Kosynkina *et al.*⁷ The acyl-protected carboxymethyl nucleobases were synthesized according to *Scheme 2*. Thus cytosine was acylated with *tert*-butylbenzoyl chloride in DMF in the presence of triethylamine to give **4**. Originally we synthesized a benzoyl protected cytosine monomer, however the tendency of this monomer to precipitate out of solution during PNA synthesis led us to chose the more lipophilic *tert*-butylbenzoyl protecting group. **4** was then converted to its sodium salt using NaH in DMF and alkylated using methyl bromoacetate. The resulting methyl ester **5** was saponified using NaOH in dioxane-water. The pH of this reaction solution was carefully controlled to prevent hydrolysis of the *N'*-protecting group. The desired product **6** was isolated by pH dependent precipitation.



Reagents and Conditions: (a) *tert*-Butylbenzoyl chloride, NEt_3 / DMF; (b) Methyl bromoacetate, NaH/ DMF; (c) NaOH, H_2O / dioxane; (d) Anisoyl chloride/ pyridine; (e) Isobutanoyl chloride, NEt_3 / DMF.

Scheme 2: Synthesis of acyl-protected carboxymethyl nucleobases.

Adenine was protected by heating with anisoyl chloride in pyridine. The resulting *N*⁶-anisoyladenine 7 was alkylated to give 8, and saponified to give 9 using procedures analogous to those used for the preparation of 6. *N*²-Isobutanoyl guanine 10 was synthesized by heating guanine with isobutanoyl chloride in DMF and triethylamine. This was then alkylated using NaH / methyl bromoacetate to give the desired *N*⁹-alkylated derivative 11 along with other regioisomers which could be separated by column chromatography. Saponification using aqueous NaOH gave the free acid 12.



13 and 14, B= thymine; 15 and 16, B= *N*⁴-*t*-butylbenzoyl cytosine
17 and 18, B= *N*⁶-anisoyl adenine; 19 and 20, B= *N*²-isobutanoyl guanine

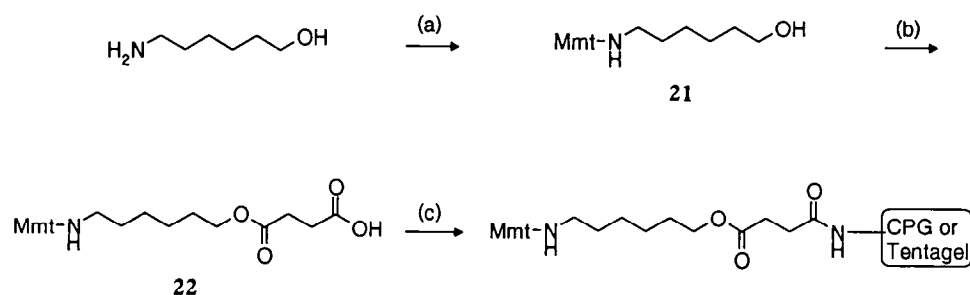
Reagents and Conditions: (a) Carboxymethyl nucleobase, DIPC, HOObt, NEM/ DMF;
(b) NaOH, H_2O / dioxane

Scheme 3: Synthesis of Mmt-protected PNA monomers.

The carboxymethylated nucleobases were coupled to the Mmt-(2-aminoethyl)glycine methyl ester backbone unit **3** using diisopropylcarbodiimide (DIPC) in DMF in the presence of HOObt and N-ethylmorpholine. Saponification of the resulting methyl esters **13**, **15**, **17** and **19** under controlled conditions gave the desired acyl-protected PNA monomers **14**, **16**, **18** and **20** (Scheme 3).

The Solid Support

Standard peptide synthesis solid supports require treatment with strong acid to cleave the peptide chain from the support. These conditions are incompatible with our orthogonal protecting group strategy and would not allow a combination of PNA and oligonucleotide synthesis. Therefore we synthesized universal solid supports which are cleavable during the ammonia treatment used for base-deprotection, and are therefore compatible with the new synthetic strategy (Scheme 4). These supports utilise an aminohexyl spacer and a base-cleavable succinyl linker. Thus 6-aminohexan-1-ol was selectively N-tritylated using Mmt chloride in pyridine to give **21**. The resulting alcohol was succinylated using succinic anhydride and DMAP in pyridine to give **22**. This was then coupled to AminoTentagel™ or Aminopropyl CPG (Controlled-Pore Glass) using TBTU / N-ethylmorpholine in DMF. PNAs are obtained after synthesis and ammonia cleavage as their C-terminal hydroxyhexylcarboxamides.



Reagents and Conditions: (a) MmtCl/ pyridine; (b) Succinic anhydride, DMAP/ pyridine; (c) Aminopropyl CPG or Amino-Tentagel, TBTU, N-ethylmorpholine/ DMF.

Scheme 4: Synthesis of Universal Solid Support for PNA Synthesis.

Solid-phase Synthesis

The monomers were used, in conjunction with the CPG or Tentagel™ solid-supports described above, for the synthesis of PNAs on a modified Eppendorf Biotronik Ecosyn™ D-300 DNA synthesizer. PNAs were synthesized on a 5-10 μmol scale. These small scale syntheses produce ample material for *in vitro* screening for biological activity. Deprotection of the Mmt group occurs by treatment with 3% trichloroacetic acid in CH₂Cl₂. Excess acid is removed by subsequent washing with acetonitrile and 0.3M N-ethylmorpholine or N,N-diisopropyl-N-ethylamine in DMF. For coupling the monomer (0.3M solutions in DMF) is activated by PyBOP or HATU (0.3M solution in DMF) in the presence of N-ethylmorpholine (0.3M solution in DMF). For this, the reagents are pre-mixed in the delivery syringe of the synthesizer and, after a short preactivation period, delivered

to the reaction vessel. After 30 minutes of coupling, unreacted amino functions are capped using a mixture of acetic anhydride/*N*-methyl imidazole in THF, which is used as capping reagent in standard DNA synthesis.

Coupling efficiencies, determined by measurement of the Mmt cation released during deprotection, are generally in the range of 95 to 99%. After synthesis is completed, the PNA is cleaved from the solid-support and deprotected with conc. aqueous ammonia solution. The cleavage and deprotection sequence: treatment with conc. aqueous ammonia, followed by 80% aqueous acetic acid for removal of the *N*-terminal Mmt group prevents the diketo piperazine cleavage reaction reported previously.⁸ The terminal Mmt protecting group can be left on if desired for 'Trityl-On' HPLC purification. *Figure 1* (A) shows the HPLC trace of crude 12mer PNA sequence H₂N-aca tca tgg tcg-(hex) on a reversed phase LiChrospher™ WP300 RP-18(e) column after detritylation. The crude, 'Trityl-Off' 12mer PNA was characterized by positive ion Electrospray mass spectrometry (*Figure 1*, C). The PNA was purified by HPLC using a RP-18 column and an acetonitrile gradient (2-7%) in 0.1% TFA in water. Following desalting by gel filtration, the PNA appears as a single peak on HPLC (*Figure 1*, B).

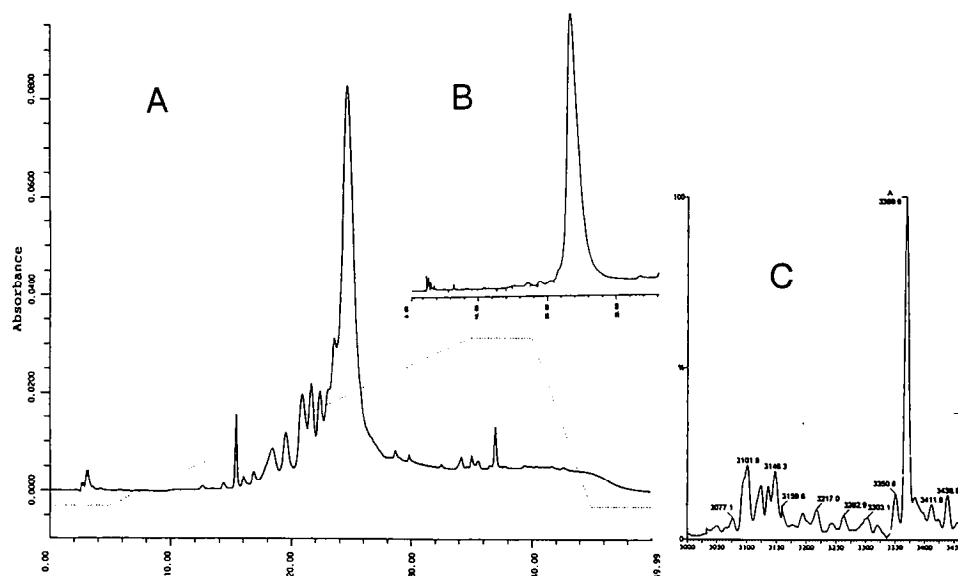


Figure 1: Reversed-phase HPLC trace and ES-MS spectrum of PNA sequence H₂N-aca tca tgg tcg-(hex).

$M(\text{calculated})$ for C₁₃₅ H₁₇₄ N₇₀ O₃₇ = 3369.31; $M(\text{experimental})=3369.6$

In conclusion, we have described the synthesis of novel Mmt protected PNA monomers, and have demonstrated that the Mmt strategy works well for the synthesis of PNAs of mixed base composition. We routinely use the Mmt strategy for the synthesis of PNAs. This synthesis strategy is milder than both the Boc- and the Fmoc-strategy. In addition the synthesis and deprotection conditions are completely compatible with oligonucleotide synthesis, and we have used this synthetic strategy for the solid-phase synthesis of PNA-DNA chimeric oligomers (manuscript in preparation).

EXPERIMENTAL

General

The following abbreviations are employed: N,N'-diisopropylcarbodiimide (DIPC); 4,4-dimethylaminopyridine (DMAP); N,N-dimethylformamide (DMF); 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOObt); O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HATU); 4-ethylmorpholine (NEM); O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium-tetrafluoroborate (TBTU); (benzotriazol-1-yloxy)-tripyrrolidinophosphonium-hexafluorophosphate (PyBOP).

The peptide coupling reagent HATU was purchased from Millipore (Eschborn, Germany); DNA/PNA synthesis reagents were purchased from MWG Biotech GmbH (Ebersberg, Germany); Tentagel™ was purchased from Rapp Polymers (Tübingen, Germany); all other reagents were obtained from Sigma-Aldrich-Fluka (Neu-Ulm, Germany). Flash chromatography was performed using Merck silica gel 60 (230-400mesh ASTM). TLC was carried out on Merck DC Kieselgel 60 F-254 glass plates. ¹H NMR was recorded in the solvents indicated at 270 Mhz. Chemical shifts (δ) are reported in parts per million down field relative to the internal TMS standard. Mass spectra were recorded using either Fast Atom Bombardment (FAB), Electrospray (ES) or Direct Chemical (DCI) ionisation. PNA synthesis was carried out on a modified Eppendorf Biotronik Ecosyn™ D-300 DNA synthesizer (Eppendorf Biotronik, Maintal, Germany). HPLC analysis of PNAs was carried out on a Beckman System Gold HPLC system using a LiChrospher™ WP300 RP-18(e) analytical column (4x250mm; E. Merck, Darmstadt; Germany), eluting with a linear gradient of eluent B in eluent A (A: 0.1% TFA in water; B: 0.1% TFA in 20% acetonitrile in water).

N-(2-Aminoethyl)glycine (1). 1,2-Diaminoethane (1.80l; 26.8mol) was dissolved in methanol (2.61l), and a solution of glyoxylic acid monohydrate (840g; 9.13mol) in water (2.61l) was then added slowly while cooling and stirring. **Caution: Exothermic reaction.** Palladium (5%) on charcoal (78g) was then added to the mixture and hydrogenation was carried out in a hydrogenation vessel at 0.3 bar and at room temperature. After 200l of hydrogen had been taken up, the reaction was complete and the catalyst was filtered off. The filtrate was concentrated *in vacuo* and the residue was subsequently distilled twice together with toluene; methanol (1.56l) was added to the residue and the mixture was stirred thoroughly at approximately 0° C. The precipitate was filtered off, then washed with methanol. A second crop was obtained from the mother liquor after concentration. The combined product was dried *in vacuo* to give **1** as a white solid in 588g (55%) yield. M.p. 149-153°C(dec.); R_f (n-butanol:acetic acid:water:ethyl acetate/1:1:1:1) 0.2; MS(DCI) 119 [M+H]⁺; ¹H NMR (D₂O) δ 3.35 (2H, s, CH₂-CO); 2.92-3.18 (4H, m, CH₂-CH₂).

Methyl N-(2-aminoethyl) glycinate dihydrochloride (2). Compound 1 (460g; 3.9mol) was suspended in methanol (14.0l), and HCl gas (500g) was passed into the suspension. **Caution: Exothermic reaction.** The mixture became hot and was then maintained under reflux for a total of 8h. The mixture was cooled to 0° C and stirred at this temperature for 3h. The product was filtered off and dried *in vacuo* to give 2 as colorless crystals in 716g (90%) yield. M.p. 190-192°C. R_f (n-butanol:acetic acid:water:ethyl acetate/1:1:1:1) 0.3; MS(DCI) 133 [M+H]⁺; ¹H NMR (D₂O) δ 4.18 (2H, s, CH₂-CO); 3.92 (3H, s, CO-OCH₃); 3.42-3.65 (4H, m, CH₂-CH₂).

Methyl N-[2-((4-methoxyphenyl)-diphenylmethyl)aminoethyl] glycinate (3). Compound 2 (14.8g; 72.2mmol) was suspended in DMF (300ml) and the suspension was treated with triethylamine (30.2ml; 220mmol), with stirring. The mixture was cooled to approximately 4°C, and a solution of Mmt-Cl (22.2g; 72.2mmol) in CH₂Cl₂ (100ml) was added dropwise, with vigorous stirring. Stirring was continued for 2.5h at room temperature. Precipitated triethylammonium hydrochloride was then filtered off, ethanol (10ml) was added to the solution, and the filtrate was concentrated *in vacuo*. The residue was then chromatographed on silica gel using a mixture of diethyl ether:petroleum ether:triethylamine/ 200:100:3. The product-containing fractions were combined and evaporated to dryness *in vacuo* to give 3 as an oil which crystallizes slowly upon standing in 18.8g (65%) yield. R_f =0.16 (diethyl ether:petroleum ether/ 2:1 + 1% triethylamine); MS(FAB/MeOH/NBA/LiCl) 411.2 [M+Li]⁺; ¹H NMR (CDCl₃) δ 7.50-6.80 (14H, m, Mmt); 4.80 (3H, s, Mmt-OMe); 4.70 (3H, s, COOMe); 3.35 (2H, s, glycine-CH₂); 2.70 (2H, t, CH₂-1); 2.25 (2H, t, CH₂-2); 1.80 (2H, br s, NH).

N^t-(4-tert-Butylbenzoyl)cytosine (4). Cytosine (11.1g; 0.10mol) was suspended in dry DMF (250 ml), and triethylamine (15.4 ml; 0.11mol) was added. 4-tert-butylbenzoyl chloride (18.6 ml; 0.10mol) was added dropwise using a syringe. Stirring was continued for 4h at room temperature and more 4-tert-butylbenzoyl chloride (3.7 ml; 0.02mol) was then added. After a further 2h, the reaction was quenched by the addition of a small amount of methanol. The solvent was removed *in vacuo*, and the residue was treated with CH₂Cl₂ and water. The resulting precipitate was filtered off and dried *in vacuo* to give 4 as a white solid in 15.7g (61%) yield. R_f = 0.55 (CH₂Cl₂:MeOH/9:1); MS(DCI) 272 [M+H]⁺; ¹H NMR (d₆-DMSO) δ 11.30 (2H, br s, NH); 8.00 (2H, d, tBuBz H-2 and 6); 7.85 (1H, d, cytosine H-6); 7.50 (2H, d, tBuBz H-3 and 5); 7.20 (1H, d, cytosine H-5); 1.30 (9H, s, tBu).

1-(Methoxycarbonylmethyl)- N^t-(4-tert-butylbenzoyl)cytosine (5). Compound 4 (15.0g; 55.3mmol) was suspended in dry DMF (250 ml), sodium hydride (1.32 g; 55.0mmol) was added in portions, and the mixture was stirred for 1h at room temperature. Methyl bromoacetate (5.20 ml; 56.0mmol) was added dropwise at room temperature using a syringe. Stirring was continued for 1h at room temperature, and methanol (10 ml) was then added. The solvent was removed *in vacuo*, and the residue was partitioned between CH₂Cl₂ and water. The organic phase was washed with water, dried (Na₂SO₄), filtered and evaporated *in vacuo*. The resulting crude product was recrystallized from isopropanol to give 5 as a white solid in 8.00g (42%) yield. M.p.: 189-193°C (dec.); R_f = 0.72 (CH₂Cl₂:MeOH/9:1); MS(DCI) 344 [M+H]⁺; ¹H NMR (CDCl₃) δ 8.70 (1H, br s, NH); 7.80 (2H, d, tBuBz H-2 and 6); 7.55 (4H, m, cytosine H-5,6; tBuBz H-3 and 5); 4.65 (2H, s, CH₂); 3.80 (3H, s, OMe); 1.30 (9H, s, tBu).

1-(Carboxymethyl)- N^t-(4-tert-butylbenzoyl)cytosine (6). Compound 5 (8.0g; 24mmol) was dissolved in a mixture of dioxane (50ml) and water (10ml), and 2N aqueous sodium hydroxide solution was added dropwise with stirring at room temperature (pH 11-12). When hydrolysis of the methyl ester was complete, the pH of the reaction solution was brought to 3 using 2M KHSO₄ solution. The precipitate which separated out was filtered off. This crude product was dissolved in aqueous NaHCO₃ solution and reprecipitated by adding 2M

KHSO₄ solution. The product was filtered off, washed with a small amount of water and dried *in vacuo* to give **6** as a white solid in 6.62g (86%) yield. M.p. 285°C (dec); R_f = 0.2 (CH₂Cl₂:MeOH/9:1); MS(DCI) 330 [M+H]⁺; ¹H NMR (d₆-DMSO) δ 8.10 (1H, d, cytosine H-6); 7.95 (2H, d, tBuBz H-2 and 6); 7.55 (2H, d, tBuBz H-3 and 5); 7.30 (1H, d, cytosine H-5); 4.55 (2H, s, CH₂); 1.30 (9H, s, tBu).

N⁶-(4-Methoxybenzoyl)adenine (7). Adenine (13.5g; 0.10mol) was suspended in dry pyridine (250 ml). 4-Methoxybenzoyl chloride (17.1g; 0.10mol) was added dropwise using a syringe. The mixture was stirred for 3h at 100°C and allowed to stand overnight at room temperature. The reaction solution was treated with methanol, and the solvent was subsequently evaporated *in vacuo*. The residue was coevaporated twice with toluene and then stirred with hot isopropanol. The mixture was allowed to cool slowly, and the product which precipitated out was filtered off and dried *in vacuo* to give **7** in 22.2g (82%) yield. M.p. 212-214°C; R_f = 0.67 (CH₂Cl₂:isopropanol/8:2); MS(+ES) 270 [M+H]⁺; ¹H NMR (d₆-DMSO) δ 11.60 (1H, br s, NH); 9.00 (1H, br s, NH); 8.75 (1H, s, adenine H-2); 8.50 (1H, s, adenine H-8); 8.15 (2H, d, Anis H-2 and 6); 7.10 (2H, d, Anis H-3 and 5); 3.90 (3H, s, Anis-OMe).

N⁶-(4-Methoxybenzoyl)-9-(methoxycarbonylmethyl)adenine (8). Compound **7** (8.01g; 29.7mmol) was suspended in dry DMF (150 ml), sodium hydride (0.75g; 31.2mmol) was added in portions, and the mixture was stirred at room temperature for 30 min. Methyl bromoacetate (2.85ml; 30.9mmol) was added dropwise at room temperature using a syringe. Stirring was continued for 2h at room temperature, and the mixture was treated with a small amount of carbon dioxide in methanol. The solvent was evaporated *in vacuo*, the residue was dissolved in CH₂Cl₂ and washed once with water. The product precipitated from the organic phase and was filtered off. The filter residue was washed with a small amount of water and then dried *in vacuo* to give **8** as a white solid in 4.03g (40%) yield. R_f = 0.76 (ethyl acetate:MeOH/8:2); MS(+ES) 342 [M+H]⁺; ¹H NMR (CDCl₃) δ 9.00 (1H, br s, NH); 8.75 (1H, s, adenine H-2); 8.10 (1H, s, adenine H-8); 8.00 (2H, d, Anis H-2 and 6); 7.00 (2H, d, Anis H-3 and 5); 5.05 (2H, s, CH₂); 3.95 (3H, s, COOMe); 3.85 (3H, s, Anis-OMe).

N⁶-(4-Methoxybenzoyl)-9-(carboxymethyl)adenine (9). Compound **8** (1.71g; 5mmol) was suspended in water (40 ml), and 2N aqueous sodium hydroxide solution was added dropwise at 0°C while maintaining pH 11, until the methyl ester was completely hydrolysed. The reaction solution was filtered, and the pH of the filtrate was brought to 3 using 2M KHSO₄ solution, causing precipitation of the product. The precipitate was filtered off, washed with a small amount of cold water and dried *in vacuo* to give **9** as a white solid in 1.52g (93%) yield. M.p. 222-223°C (dec.); R_f = 0.2 (n-butanol:acetic acid:water/ 3:1:1); MS(+ES) 328 [M+H]⁺; ¹H NMR (d₆-DMSO) δ 11.00 (1H, br s, NH); 8.70 (1H, s, adenine H-2); 8.45 (1H, s, adenine H-8); 8.05 (2H, d, Anis H-2 and 6); 7.10 (2H, d, Anis H-3 and 5); 5.10 (2H, s, CH₂); 3.90 (3H, s, Anis-OMe).

N²-(Isobutanoyl)guanine (10). Guanine (3.02g; 20mmol) was suspended in anhydrous DMF (40 ml), and triethylamine (1.45 ml; 10.4mmol) was added. Isobutanoyl chloride (2.12 g; 19.9mmol) was then added dropwise using a syringe. The mixture was stirred for 3h at 100°C. The reaction solution was then treated with methanol, and the solvent was subsequently evaporated *in vacuo*. The residue was stirred with hot isopropanol, and the precipitated product was filtered off and dried *in vacuo* to give **10** in 2.67g (60%) yield. R_f = 0.45 (n-butanol:acetic acid:water/ 3:1:1); MS (+ES) 222.2 [M+H]⁺; ¹H NMR (d₆-DMSO) δ 12.05 (1H, br s, NH); 11.50 (1H, s, NH); 8.10 (1H, s, guanine H-8); 2.75 (1H, septet, iBu-CH); 1.10 (6H, d, iBu-CH₃).

N²-(Isobutanoyl)-9-(methoxycarbonylmethyl)guanine (11). Compound **10** (4.42g; 20mmol) was suspended in dry DMF (50 ml). Sodium hydride (0.5g; 20.8mmol) was added in portions, and the mixture was stirred for 1h at room temperature. Methyl bromoacetate (1.9 ml; 20.6mmol) was subsequently added dropwise

at room temperature with a syringe. Stirring was continued for 1h at room temperature and the mixture was then treated with a small amount of carbon dioxide in methanol. The solvent was evaporated *in vacuo*, and the residue was purified by column chromatography on silica gel using CH₂Cl₂:methanol/95:5 as the eluent. The product-containing fractions were combined and concentrated *in vacuo* to give **11** as a white solid in 2.35g (40%) yield. $R_f = 0.58$ (CH₂Cl₂:MeOH/9:1); MS(DCI) 294 [M+H]⁺; ¹H NMR (CDCl₃) δ 12.25 (1H, br s, NH); 10.00 (1H, s, NH); 7.80 (1H, s, guanine H-8); 5.20 (2H, s, CH₂); 3.80 (3H, s, OCH₃); 2.80 (1H, septet, iBu-CH); 1.25 (6H, d, iBu-CH₃).

N²-(Isobutanoyl)-9-(carboxymethyl)guanine (12). Compound **11** (19.7g; 67mmol) was suspended in water (100 ml), and 2N aqueous sodium hydroxide solution was added dropwise at 0°C while maintaining pH 11, until the methyl ester was completely hydrolysed. The reaction solution was then filtered, the pH of the filtrate was brought to 3 using 2M KHSO₄ solution, and the mixture was extracted using ethyl acetate. The aqueous phase was concentrated *in vacuo* to a volume of approximately 20 ml and covered with a layer of ethyl acetate. The precipitate which separated out slowly was filtered off and dried *in vacuo* to give **12** as a white solid in 11.57g (62%) yield. $R_f = 0.34$ (n-butanol:acetic acid:water/3:1:1); MS(+ES) 342 [M+H]⁺; ¹H NMR (d₆-DMSO) δ 12.05 (1H, s, NH); 11.65 (1H, s, NH); 7.95 (1H, s, guanine H-8); 4.85 (2H, s, CH₂); 2.75 (1H, septet, iBu-CH); 1.10 (6H, d, iBu-CH₃).

Methyl N-[2-((4-methoxyphenyl)-diphenylmethylamino)-ethyl]-N-((thymine-1-yl)acetyl) glycinate (13). Compound **3** (1.00g; 2.48mmol) was dissolved in DMF (5ml). To this solution was added HOOBt (0.404g; 2.48mmol) and NEM (0.572g; 0.632ml; 4.96mmol). A solution of N-1-carboxymethylthymine (0.456g; 2.48mmol) in DMF (5ml) was then added, followed by DIPC (0.376g; 0.46ml; 3.0mmol). The reaction mixture was stirred for 20h at room temperature. The solvent was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate. This solution was washed twice with water and once with saturated aqueous KCl solution. The organic phase was dried (Na₂SO₄), filtered and evaporated *in vacuo*. The residue was applied to a silica gel chromatography column which had been equilibrated with CH₂Cl₂ containing 1% methanol and 1% triethylamine, and eluted with a gradient of 1-5% methanol in CH₂Cl₂. On concentration of the combined fractions containing the desired product a white precipitate of N,N'-diisopropylurea formed which was removed by filtration. **13** was obtained as a white foam in 1.28g (91%) yield. $R_f = 0.28$ (CH₂Cl₂:MeOH/95:5); MS (FAB/MeOH/NBA) 571.2 [M+H]⁺; ¹H NMR (d₆-DMSO) δ 11.4 (1H, s, thymine H-3); 7.45-6.85 (15H, m, Mmt and thymine H-6); 4.82 and 4.55 (2H, 2xs {rotamers}, N-CO-CH₂); 4.45 and 4.02 (2H, 2xs {rotamers}, N-CH₂-CO₂Me); 3.85 (3H, s, Mmt-OMe); 3.58 (3H, s, COOMe); 3.45 (2H, m, CH₂-N); 2.15 (2H, m, MmtNH-CH₂); 1.75 (3H, s, thymine H-5).

N-[2-((4-Methoxyphenyl)-diphenylmethylamino)ethyl]-N-((thymine-1-yl)acetyl) glycine (14). Compound **13** (1.28g; 2.25mmol) was dissolved in dioxane (10ml) and water (2ml). The solution was cooled to 0°C and 1M aqueous NaOH was added dropwise until the solution reached pH11. After 2h the solution was adjusted to pH5 by the dropwise addition of 2M aqueous KHSO₄. The product was extracted into ethyl acetate, and the aqueous phase was extracted 3 times with ethyl acetate. The combined organic phase was dried (Na₂SO₄), filtered and evaporated *in vacuo*. The residue was applied to a silica gel chromatography column and eluted with a gradient of 5-10% methanol and 1% triethylamine in CH₂Cl₂. The combined fractions containing the desired product were dried *in vacuo*, and coevaporated twice with pyridine and three times with toluene to remove excess triethylamine. **14** was obtained as a white foam in 1.07g (85%) yield. $R_f = 0.28$ (CH₂Cl₂:MeOH/8:2). MS(FAB): 557.2402 (C₃₁H₃₂N₄O₆ + H requires 557.2400); ¹H NMR (d₆-DMSO) δ 11.4

(1H, d, thymine H-3); 7.42-6.85 (15H, m, Mmt and thymine H-6); 4.82 and 4.48 (2H, 2xs {rotamers}, N-CO-CH₂); 3.95 and 3.80 (2H, 2xs {rotamers}, N-CH₂-CO₂H); 3.72 (3H, s, Mmt-OMe); 3.40 (2H, m, CH₂-N); 2.15 (2H, m, MmtNH-CH₂); 1.75 (3H, d, thymine H-5).

Methyl N-[2-((4-methoxyphenyl)-diphenylmethylamino)ethyl]-N-[(N^t-(4-*tert*-butylbenzoyl)-cytosin-1-yl) acetyl] glycinate (15). Compound 3 (1.00g; 2.48mmol) was dissolved in DMF (5ml). To this solution was added HOOBt (0.403g; 2.48mmol) and NEM (0.63ml; 4.96mmol). A suspension of 6 (0.740g; 2.48mmol) in DMF (5ml) was then added, followed by DIPC (0.47ml; 2.97mmol). The reaction mixture was stirred for 20h at room temperature. The solvent was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate. This solution was washed twice with water and once with saturated aqueous KCl solution. The organic phase was dried (Na₂SO₄), filtered and evaporated *in vacuo*. The residue was applied to a silica gel chromatography column which had been equilibrated with CH₂Cl₂:ethyl acetate/1:1, + 1% triethylamine, and eluted with CH₂Cl₂:ethyl acetate/1:1. On concentration of the combined fractions containing the desired product a white precipitate of N,N'-diisopropylurea formed which was removed by filtration. 15 was obtained as a pale yellow-white foam in 1.64g (92%) yield. R_f =0.31 (CH₂Cl₂:ethyl acetate/1:1). MS (FAB/MeOH/NBA/LiCl) 722.5 [M+Li]⁺; ¹H NMR (d₆-DMSO) δ 11.1 (1H, s, cytosine H-4); 8.00 (3H, m, 2xtBuBz H, cytosine H-6); 7.55 (2H, d, tBuBz H-3 and 5); 7.50-6.85 (15H, m, Mmt, cytosine H-5); 5.00 and 4.70 (2H, 2xs {rotamers}, N-CO-CH₂); 4.50 and 4.05 (2H, 2xs {rotamers}, N-CH₂-CO₂H); 3.75 (3H, s, Mmt-OMe); 3.58 (3H, s, COOMe); 3.40-3.50 (2H, m, CH₂-N); 2.20 (2H, m, MmtNH-CH₂); 1.35 (9H, s, tBu).

N-[2-((4-Methoxyphenyl)-diphenylmethylamino)ethyl]-N-[(N^t-(4-*tert*-butylbenzoyl)-cytosin-1-yl)acetyl] glycine (16). Compound 15 (1.63g; 2.28mmol) was dissolved in dioxane (10ml) and water (1ml). The solution was cooled to 0°C and 1M aqueous NaOH (4.56ml) was added dropwise in 2 aliquots. After 2h the solution was adjusted to pH5 by the dropwise addition of 2M aqueous KHSO₄. The precipitated salts were filtered off and washed with dioxane, and the combined filtrates were evaporated *in vacuo*. The residue was coevaporated twice with methanol and CH₂Cl₂. The residue was applied to a silica gel chromatography column and eluted with a gradient of 2-10% methanol and 1% triethylamine in CH₂Cl₂. 16 was obtained as an off-white foam in 0.83g (52%) yield. R_f =0.28 (CH₂Cl₂:MeOH/9:1). MS(FAB): 702.3282 (C₄₁H₄₃N₅O₆ + H requires 702.3292); ¹H NMR (d₆-DMSO) δ 11.1 (1H, br s, cytosine H-4); 8.00 (3H, m, 2xtBuBz-H, cytosine H-6); 7.55 (2H, d, tBuBz H-3 and 5); 7.50-6.85 (15H, m, Mmt, cytosine H-5); 5.00 and 4.65 (2H, 2xs {rotamers}, N-CO-CH₂); 4.00 and 3.80 (2H, 2xs {rotamers}, N-CH₂-CO₂H); 3.70 (3H, s, Mmt-OMe); 3.45 (2H, m, CH₂-N); 2.15 (2H, m, MmtNH-CH₂); 1.30 (9H, s, tBu).

Methyl N-[2-((4-methoxyphenyl)-diphenylmethylamino)ethyl]-N-[(N^t-(4-methoxybenzoyl)-adenin-9-yl) acetyl] glycinate (17). Compound 3 (1.24g; 3.06mmol) was dissolved in DMF (7ml). To this solution was added NEM (1.06ml; 6.12mmol), HOOBt (0.498g; 3.06mmol), 9 (1.00g; 3.06mmol) and DIPC (0.58ml; 3.67mmol). The reaction mixture was stirred for 48h at 4°C. The solvent was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate. This solution was washed twice with water and once with saturated aqueous KCl solution. The organic phase was dried (Na₂SO₄), filtered and evaporated *in vacuo*. The resulting yellow foam was dissolved in a small volume of ethyl acetate and cooled on ice to induce crystallisation of N, N'-diisopropylurea, which was then removed by filtration. The residue was applied to a silica gel chromatography column which had been equilibrated with CH₂Cl₂:ethyl acetate/1:1, + 1% triethylamine, and eluted with CH₂Cl₂:ethyl acetate/1:1. 17 was obtained as a pale yellow-white foam in 1.75g (80%) yield. R_f =0.21 (CH₂Cl₂:MeOH/95:5). MS (FAB/MeOH/NBA/LiCl) 720.3 [M+Li]⁺; ¹H NMR (CDCl₃) δ 8.95 (1H, br s, adenine

H-6); 8.75 and 8.70 (1H, 2xs {rotamers}, adenine H-2); 8.15 (1H, s, adenine H-8); 8.00 (2H, d, Anis H-2 and 6) 7.50-7.15 (12H, m, Mmt); 7.00 (2H, d, Anis H-3 and 5); 6.80 (2H, m, Mmt); 5.55 and 5.00 (2H, 2xs {rotamers}, N-CO-CH₂); 4.30 and 4.00 (2H, 2xs {rotamers}, N-CH₂-CO₂H); 3.90 (3H, s, Anis-OMe); 3.75 (3H, s, Mmt-OMe); 3.65 (5H, m, COOMe, CH₂-N); 2.35 (2H, m, MmtNH-CH₂).

N-[2-((4-Methoxyphenyl)-diphenylmethylamino)ethyl]-N-[(N⁶-(4-methoxybenzoyl)-adenin-9-yl)acetyl] glycine (18). Compound 17 (1.70g; 2.38mmol) was dissolved in dioxane (10ml). The solution was cooled to 0°C and 1M aqueous NaOH (10.3ml) was added dropwise in 5 aliquots over 2.5h. After 2h at room temperature the solution was adjusted to pH5 by the dropwise addition of 2M aqueous KHSO₄. The precipitated salts were filtered off and washed with dioxane, and the combined filtrates were evaporated *in vacuo*. The residue was coevaporated twice with ethanol and CH₂Cl₂:methanol /9:1, (v/v). The residue was applied to a silica gel chromatography column and eluted with a gradient of 10-20% methanol and 1% triethylamine in CH₂Cl₂. 18 was obtained as a white foam in 1.62g yield (85%). R_f =0.10 (CH₂Cl₂:MeOH/8:2). MS(FAB): 700.2859 (C₃₉H₃₇N₇O₆ + H requires 700.2884); ¹H NMR (CDCl₃) δ 11.00 (1H, br s, adenine H-6); 8.70 and 8.65 (1H, 2xs {rotamers}, adenine H-2); 8.35 and 8.30 (1H, 2xs {rotamers}, adenine H-8); 8.05 (2H, dd, Anis H-2 and 6); 7.50-6.85 (16H, m, Mmt and Anis H-3 and 5); 5.55 and 5.15 (2H, 2xs {rotamers}, N-CO-CH₂); 4.05 and 3.90 (2H, 2xs {rotamers}, N-CH₂-CO₂H); 3.90 (3H, s, Anis-OMe); 3.75 (3H, s, Mmt-OMe); 3.40-3.65 (2H, m, COOMe, CH₂-N); 2.20 (2H, m, MmtNH-CH₂).

Methyl N-[2-((4-methoxyphenyl)-diphenylmethylamino)ethyl]-N-[(N²-(isobutanoyl)-guanin-9-yl)acetyl] glycinate (19). Compound 3 (1.45g; 3.59mmol) was dissolved in DMF (7ml). To this solution was added NEM (1.24ml; 7.17mmol), HOOBt (0.59g; 3.59mmol), 12 (1.00g; 3.59mmol) and DIPC (0.67ml; 4.31mmol). The reaction mixture was stirred for 48h at 4°C. The solvent was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate. This solution was washed twice with water and once with saturated aqueous KCl solution. The organic phase was dried (Na₂SO₄), filtered and evaporated *in vacuo*. The resulting yellow foam was dissolved in a small volume of ethyl acetate and cooled on ice to induce crystallisation of N, N'-diisopropylurea, which was then removed by filtration. The residue was applied to a silica gel chromatography column which had been equilibrated with CH₂Cl₂:ethyl acetate/1:1, + 1% triethylamine, and eluted with CH₂Cl₂:ethyl acetate/1:1. 19 was obtained as a pale yellow-white foam in 1.18g (49%) yield. R_f =0.13 (CH₂Cl₂:MeOH/95:5). MS (FAB/MeOH/NBA/LiCl) 678.3 [M+2Li-H]⁺; 672.3 [M+Li]⁺; ¹H NMR (CDCl₃) δ 11.80 (1H, br s, guanine H-2); 7.75 and 7.70 (1H, 2xs {rotamers}, guanine H-8); 7.55-6.80 (14H, m, Mmt); 5.45 and 4.90 (2H, 2xs {rotamers}, N-CO-CH₂); 4.25 and 4.00 (2H, 2xs {rotamers}, CH₂-CO₂Me); 3.75 (8H, m, Mmt-OMe, CO₂Me and CH₂-N); 2.30 (2H, m, MmtNH-CH₂); 1.90 (1H, septet, iBu-CH); 0.85 (6H, d, iBu-CH₃).

N-[2-((4-Methoxyphenyl)-diphenylmethylamino)ethyl]-N-[(N⁶-(isobutanoyl)-guanin-9-yl)acetyl] glycine (20). Compound 19 (1.15g; 1.72mmol) was dissolved in dioxane (10ml). The solution was cooled to 0°C and 1M aqueous NaOH (10.3ml) was added dropwise in 5 aliquots over 2.5h. After 2h at room temperature the solution was adjusted to pH5 by the dropwise addition of 2M aqueous KHSO₄. The precipitated salts were filtered off and washed with dioxane, and the combined filtrates were evaporated *in vacuo*. The residue was coevaporated twice with ethanol and CH₂Cl₂:methanol/9:1, (v/v). The residue was applied to a silica gel chromatography column and eluted with a gradient of 10-20% methanol and 1% triethylamine in CH₂Cl₂. 20 was obtained as a white foam in 1.23g yield (95%). R_f =0.25 (CH₂Cl₂:MeOH/8:2). MS (-ES) 650.3 [M-H]⁻. MS(FAB): 652.2883 (C₃₅H₃₇N₇O₆ + H requires 652.2884); ¹H NMR (d₆-DMSO) δ 12.10 (1H, br s, guanine H-

2); 7.80 and 7.78 (1H, 2xs {rotamers}, guanine H-8); 7.45-6.85 (14H, m, Mmt); 5.20 and 4.95 (2H, 2xs {rotamers}, N-CO-CH₂); 3.95 and 3.80 (2H, 2xs {rotamers}, CH₂-CO₂H); 3.70 (3H, s, Mmt-OMe); 3.45 (2H, m, CH₂-N); 2.20 (3H, m, MmtNH-CH₂, iBu-CH); 0.85 (d, iBu-CH₃ and NEt₃).

1-Hydroxy-6-((4-methoxyphenyl)-diphenylmethylamino)-hexane (21). 6-Aminohexan-1-ol (1.00g; 8.55 mmol) was dissolved in anhydrous pyridine (7ml) and triethylamine(0.2ml). To this solution was added a solution of (4-methoxyphenyl)-diphenylmethyl chloride (2.50g; 8.12mmol) in anhydrous pyridine (9ml) in 3 aliquots at 45min intervals. The reaction was stirred at room temperature for a further 30min, then quenched by the addition of methanol (3ml). The solution was evaporated to a yellow syrup, and coevaporated 3 times with toluene to remove traces of pyridine. The residue was dissolved in ethyl acetate and washed with saturated aqueous NaHCO₃ solution, water and saturated aqueous KCl solution. The organic phase was dried (Na₂SO₄), filtered and evaporated *in vacuo*. The residue was applied to a silica gel chromatography column and eluted with heptane:ethyl acetate:triethylamine/49.5:49.5:1, to give 21 in 1.64g (52%) yield. R_f =0.44 (heptane:ethyl acetate/1:1), 0.73 (CH₂Cl₂:ethyl acetate /1:1), 0.11 (CH₂Cl₂:MeOH/98:2). MS (FAB/NBA/LiCl) 396.3 [M+Li]⁺, 390.3 [M+H]⁺; ¹H NMR (CDCl₃) δ 7.50-6.80 (14H, m, Mmt); 3.80 (3H, s, OMe); 3.60 (2H, t, CH₂-O); 2.15 (2H, t, CH₂-N); 1.65-1.20 (8H, m, CH₂).

6-((4-Methoxyphenyl)-diphenylmethylamino)-hex-1-yl hemisuccinate (22). Compound 21 (1.00g; 2.57mmol) was dissolved in anhydrous pyridine (10ml). To this solution was added succinic anhydride (0.26g; 2.57mmol) and DMAP (31.3mg; 0.26mmol). After stirring for 3h at room temperature further succinic anhydride (25.7mg; 0.257mmol) and DMAP (62.6mg; 0.56mmol) were added and the solution was warmed to 50°C for 6h. After a further 16h at room temperature the solution was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and washed once with an ice cold 5% aqueous solution of citric acid. The organic phase was dried (Na₂SO₄), filtered and evaporated *in vacuo*. The residue was applied to a silica gel chromatography column and eluted with 50% CH₂Cl₂/1% triethylamine in ethyl acetate, then with 5% methanol/1% triethylamine in CH₂Cl₂. 22 was obtained as a clear oil in 0.75g (60%) yield. R_f =0.30 (CH₂Cl₂:ethyl acetate/1:1). MS (-ES) 978.0 [2M-H]⁻, 488.3 [M-H]⁻. ¹H NMR (CDCl₃) δ 7.50-6.80 (14H, m, Mmt); 4.00 (2H, t, CH₂-O); 3.80 (3H, s, OMe); 2.60 (4H, m, CH₂-CH₂); 2.15 (2H, t, CH₂-N); 1.65-1.20 (8H, m, CH₂).

6-((4-Methoxyphenyl)-diphenylmethylamino)-hex-1-yl succinylamido-Tentagel. Amino-TentagelTM (0.50g) was swollen for 10 min with a solution of NEM (0.10ml) and DMF (5ml), then filtered. A solution of 22 (97.4mg; 0.165mmol), NEM (15.9mg; 0.138mmol; 17.4μl) and TBTU (52.9mg; 0.165mmol) in DMF (3ml) was prepared and immediately added to the TentagelTM NH₂. The resulting suspension was shaken for 16h at room temperature. The derivatised Tentagel was filtered off and washed with DMF (3x3ml), CH₂Cl₂ (3x1ml) and diethyl ether (3x1ml), then dried *in vacuo*. The Tentagel was capped by treatment with a solution of acetic anhydride, lutidine and 1-methyl imidazole in THF (1ml) for 1h. The derivatised and capped Tentagel was filtered off and washed with CH₂Cl₂ (3x1ml) and diethyl ether (3x1ml), then dried *in vacuo*. Qualitative Kaiser test showed the absence of free amines.

6-((4-Methoxyphenyl)-diphenylmethylamino)-hex-1-yl succinylamidopropyl-controlled-pore glass. Aminopropyl-CPG (550Å; 0.5g) was treated with a solution of NEM (0.1ml) and DMF (5ml) for 10min, then filtered. A solution of 22 (48.7mg; 0.082mmol), NEM (7.6μl) and TBTU (26.4mg; 0.082mmol) in DMF (3ml) was prepared and immediately added to the CPG. The resulting suspension was shaken for 16h at room temperature. The derivatised CPG was filtered off and washed with DMF (3x3ml), CH₂Cl₂ (3x1ml) and diethyl ether (3x1ml), then dried *in vacuo*. Capping and washing was carried out as for the Tentagel support.

Measurement of loading. The loading of the supports was determined by the spectrophotometric (478nm) determination of the concentration of Mmt⁺ cations released by detritylation of a weighed sample of support. The literature¹⁰ extinction coefficient of the Mmt cation: $\epsilon_{478}(\text{Mmt}^+) = 56 \text{ ml cm}^{-1} \mu\text{mol}^{-1}$ was used for this calculation. The loading was calculated to be $168 \mu\text{mol g}^{-1}$ and $91 \mu\text{mol g}^{-1}$ for Tentagel and CPG, respectively.

Solid-phase synthesis of 12mer PNA sequence H₂N-aca tca tgg tcg-(hex). The monomers 14, 16, 18 and 20 were used for the synthesis of H₂N-aca tca tgg tcg-(hex) on a modified Eppendorf Biotronik Ecosyn™ D-300 DNA synthesizer on a 5 μmol scale. The Mmt-protected hexyl succinylamidopropyl CPG described above was used as the solid support. The following synthesis conditions were used:

Deprotection of Mmt group: 3% Trichloroacetic acid in CH₂Cl₂ (3 min);

Neutralisation: 0.3M NEM in DMF;

Coupling of monomers: Monomer (0.3M solutions in DMF); NEM (0.3M solution in DMF); PyBOP as coupling reagent (0.3M solution in DMF), reagents pre-mixed in delivery syringe of synthesizer (15 sec); 30 min reaction time;

Capping: A: 10% Acetic anhydride/10% lutidine in THF; B: 16% N-methyl imidazole in THF (the DNA capping reagents A and B are mixed just before use); capping time 1.5 min.

After synthesis was complete, the PNA was cleaved from the solid-support (2.5 hrs ambient temperature) and deprotected (6 hrs at 50°C) with conc. aqueous ammonia solution. Finally, cleavage of the Mmt group with 80% aqueous acetic acid gave the crude 12mer PNA sequence H₂N-aca tca tgg tcg-(hex). After concentration to dryness, the solid residue (electrospray mass spectrum of crude PNA m/z 3369.6; M(calculated for C₁₃₅ H₁₇₄ N₇₀ O₃₇): 3369.31) was dissolved in 0.5ml 1M aqueous ammonia for HPLC purification. The PNA was almost insoluble in 0.5ml water. Purification on a LiChrospher™ WP300 RP-18(e) column (4x250mm; E. Merck, Darmstadt; Germany), eluting with a linear gradient of 10% to 35 % B in eluent A (A: 0.1% TFA in water; B: 0.1% TFA in 20% acetonitrile in water) resulted in 36% of homogeneous H₂N-acatcatggtcg-(hex). Positive ion electrospray mass spectrum m/z 3369.16±0.33; M(calculated for C₁₃₅ H₁₇₄ N₇₀ O₃₇): 3369.31.

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