9. Appendices

I.

Fmoc/Acyl protecting groups in the synthesis of polyamide (peptide) nucleic acid monomers

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The chemical synthesis of polyamide (peptide) nucleic acid (PNA) monomers **22**–**25** has been accomplished using Fmoc [*N*-(2-aminoethyl)glycine backbone], anisoyl (adenine), 4-*tert*-butylbenzoyl (cytosine) and isobutyryl/diphenylcarbamoyl (guanine) protecting-group combinations, thus allowing oligomer synthesis on *both* peptide and oligonucleotide synthesizers. An alternative method for the preparation of (N^6-anisoyladenin-9-yl)acetic acid **7** is described using partial hydrolysis of a dianisoylated derivative. Different methods were studied for guanine alkylation including (*a*) Mitsunobu reaction; (*b*) low-temperature, sodium hydride- and (*c*) *N*,*N*-diisopropylethylamine-mediated alkylation reactions to give preferentially N^9-substituted derivatives. Empirical rules are proposed for differentiating N^9/N^7-substituted guanines based on their ^{13}C NMR chemical-shift differences.

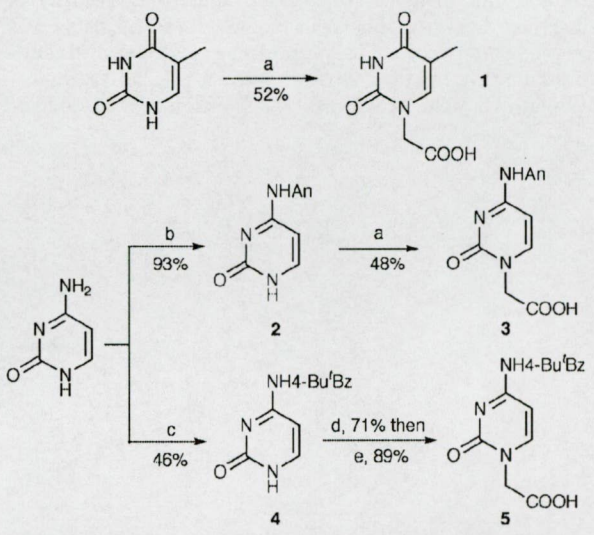
Introduction

Polyamide (or as originally referred: peptide) nucleic acids (PNA) are one of the most powerful analogues of oligonucleotides in terms of chemical and enzymic stability, double- and triple-helix formation, with potential applications in antisense diagnosis and therapeutics.[1,2] In these compounds the entire sugar–phosphate backbone is replaced with an *N*-(2-aminoethyl)glycine moiety and the nucleobases are attached through an *N*-acetyl linkage.

The chemical synthesis of PNA mostly relies on the assembly of the protected *N*-(2-aminoethyl)glycine backbone and protected nucleobase-substituted acetic acid structural units followed by standard oligomerization protocols.[3,4] The pioneering efforts of a Danish group resulted in the application of Boc (backbone) and Z (cytosine, adenine) or *O*-benzyl (guanine) protection.[5,6] Later the Uhlmann group used a monomethoxytrityl (MMTr)/acyl (anisoyl, 4-*tert*-butylbenzoyl, isobutyryl/acetyl) strategy.[7,8] All these methods require the use of (strong) acidic conditions (*e.g.*, TFA, HF) in the oligomer construction and final cleavage from the support. The need for milder methods led to the employment of the Fmoc group for backbone protection and Z[9] or MMTr groups[10] for the nucleobases. The Fmoc group is a convenient alternative to acid-sensitive backbone-protecting groups (Boc, MMTr) and allows easy monitoring of the coupling process.[11] The combination Fmoc/acyl should also be feasible since the former group can be cleaved without affecting the more stable base-protecting acyl groups.[12–14] Herein we report on our results concerning the use of Fmoc (backbone)/acyl (4-*tert*-butylbenzoyl for cytosine; anisoyl for adenine; isobutyryl/*N*,*N*-diphenylcarbamoyl for guanine) protecting groups in the synthesis of PNA monomers. The prior protecting-group combinations (with the exception of Fmoc/MMTr) were used *either* for peptide *or* oligonucleotide synthesis protocols. With biologically important PNA–DNA and PNA–peptide conjugates in mind our approach offers a substantial advantage over the existing ones since *both* oligomerization methodologies are possible with the same monomers. Beside this, use of the frequently applied urethane protecting groups (*e.g.*, Z) for nucleobases is not practical as in our experience the yields are often very low. This paper complements and details our preliminary account.[15]

Results and discussion

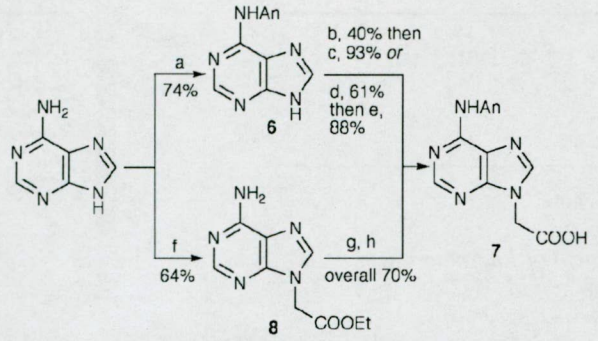
The choice of nucleobase-protecting groups was motivated by different considerations since uniform protection, though attractive, is not possible. Thymine does not require protection and our synthesis of the thymine monomer, starting from acid **1**, was based on the procedure of Thomson *et al.*[9] The anisoylated cytosine derivative **2** was alkylated to give acid **3** (Scheme 1) but the solubilities of these substances were so low



Scheme 1 *Reagents and conditions*: a, (1) $BrCH_2COOMe$, K_2CO_3, DMF; (2) NaOH, then HCl; b, AnCl, py, 80 °C; c, 4-$Bu^tC_6H_4COCl$, Et_3N, DMF; d, NaH, DMF, $BrCH_2COOEt$; e, NaOH, aq. 1,4-dioxane, then HCl. An = 4-$MeOC_6H_4CO$.

in common solvents that we had to abandon this group. The 4-*tert*-butylbenzoyl group proved to be more rewarding; acid **5**[8] was easily obtained *via* intermediate **4** and used later.

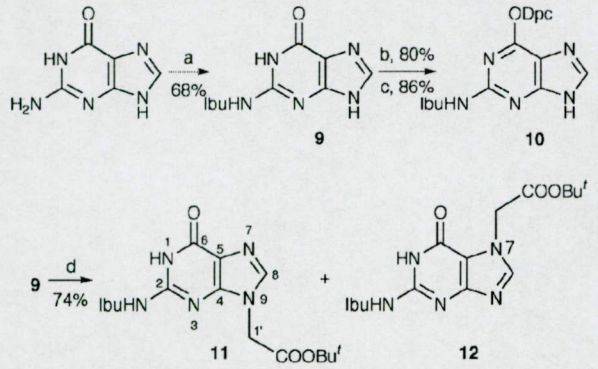
(N^6-Anisoyladenin-9-yl)acetic acid **7**[7] was prepared in 28% overall yield from adenine *via* the alkylation of compound **6** (Scheme 2). An improved overall yield (40%) was obtained when *tert*-butyl bromoacetate was used for the alkylation



Scheme 2 *Reagents and conditions*: a, 1.5 equiv. AnCl, py, 100 °C; b, NaH, DMF, $BrCH_2COOMe$; c, NaOH, then $KHSO_4$; d, NaH, DMF, $BrCH_2COOBu^t$; e, 50% (v/v) TFA–CH_2Cl_2, (±)-1,4-dithiothreitol; f, NaH, DMF, $BrCH_2COOEt$; g, 2.5 equiv. AnCl, py, 80 °C; h, NaOH, aq. MeOH, then HCl.

instead of methyl bromoacetate followed by acidolysis. In an alternative approach ethyl (adenin-9-yl)acetate **8**[5] was anisoylated with excess of anisoyl chloride and the resulting N^6,N^6-dianisoylated derivative (not isolated) was subjected to partial hydrolysis to give acid **7** in an improved yield (70%; overall 45% from adenine).

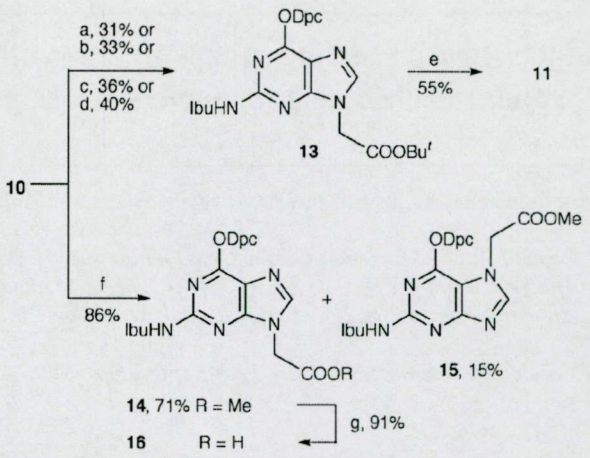
The substitution of guanine is notorious for giving N^9/N^7-regioisomers.[16] Although the 2-amino group is not really nucleophilic enough to interfere with many transformations, the poor solubility of unprotected guanine excludes its use in most reactions. The application of N^2-acyl (acetyl, propionyl, isobutyryl, *etc.*)-protected derivatives increases the solubility but N^2-acylation alone cannot solve the fundamental problem of the selectivity of alkylation.[16] Constraining guanine from its dominant 6-lactam structure to lactim (enolate) form by different groups has a beneficial effect on the ratio of N^9/N^7-regioisomers. The most successful in this respect is the *N*,*N*-diphenylcarbamoyl protecting group[17] which reportedly gives in some cases a 100:1 ratio in favour of the N^9-regioisomer.[18,19] To see how the introduction of this group alters the selectivity of alkylation, first 2-*N*-isobutyrylguanine **9**[20] was alkylated with *tert*-butyl bromoacetate in the presence of sodium hydride to afford a nearly 1:1 ratio of N^9/N^7-isomers (**11** and **12**, respectively) in 74% yield (Scheme 3). The selection of the isobutyryl



Scheme 3 *Reagents and conditions*: a, $(Pr^iCO)_2O$, DMF, 150 °C; b, Ac_2O, DMF, 100 °C; c, Ph_2NCOCl, $EtNPr^i_2$, py; d, NaH, DMF, 0 °C, $BrCH_2COOBu^t$. Ibu = Pr^iCO; Dpc = Ph_2NCO.

group was motivated by the fact that although its removal under basic conditions is more sluggish than that of other simple acyl groups (acetyl, propionyl)[17,21] it confers steric hindrance on the 2-amino group and thus prevents unwanted alkylation/glycosylation on it.[19]

Next the *N*,*N*-diphenylcarbamoyl derivative **10**[19,22] was chosen for alkylation studies under different conditions. Its transformation with *tert*-butyl glycolate[23] in the Mitsunobu

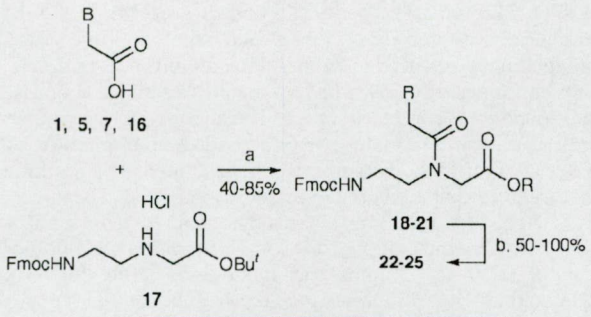


Scheme 4 *Reagents and conditions*: a, DIAD, Ph_3P, THF, $HOCH_2COOBu^t$; b, DIAD, $4\text{-}Me_2NC_6H_4PPh_2$, THF, $HOCH_2COOBu^t$; c, DIAD, Bu_3P, THF, $HOCH_2COOBu^t$; d, NaH, −20 °C, DMF, $BrCH_2COOBu^t$; e, 8% (v/v) TFA–CH_2Cl_2, $1,3\text{-}(MeO)_2C_6H_4$, 0 °C, 18 h; f, $BrCH_2COOMe$, $EtNPr^i_2$, DMF; g, NaOH, aq. 1,4-dioxane–MeOH, then HCl.

reaction[24,25] provided the product (**13**, Scheme 4) with good regioselectivity; however, its purification was very difficult and it was contaminated with significant amounts of triphenylphosphine oxide. (4-Dimethylaminophenyl)diphenylphosphine,[26,27] claimed to give a phosphine oxide which can be removed by acidic extraction,[28] proved to be unsatisfactory since the product was still contaminated with the corresponding phosphine oxide. Tributylphosphine, the oxide of which is water-soluble, gave a cleaner product but the yield was low (36%). In the next experiments sodium hydride-mediated alkylation with *tert*-butyl bromoacetate was used to obtain the desired compound. We noticed that at ambient temperature the relative proportion of N^7-regioisomer was relatively high, while lowering the temperature favoured the formation of the desired N^9-regioisomer. At −20 °C a clean reaction gave negligible amounts of the N^7-isomer but the yield of N^9-isomer was still low (40%). Acidolysis of ester **13** in dilute TFA–CH_2Cl_2 (0 °C; 18 h) removed the *N*,*N*-diphenylcarbamoyl (Dpc) group without affecting the *tert*-butyl ester functionality (⟶ **11**). Albeit there is some evidence for the lability of this group in 50% (v/v) TFA–CH_2Cl_2[8] or in the presence of Lewis acids[29] it was surprising that the Dpc group was more sensitive towards acid than was the *tert*-butyl group. The latter was expected to cleave under similar conditions.[14,30]

The application of *N*,*N*-diisopropylethylamine as a hindered base and methyl bromoacetate[8] to circumvent premature cleavage of the Dpc group in the subsequent hydrolysis gave a 71% yield of the product **14**, of which 58% was available without chromatography, along with 15% of the N^7-isomer **15**. Basic hydrolysis of ester **14** led to acid **16** in a clean transformation. The surprisingly high yield of the unwanted isomer **15** in the first reaction underlines the fact that even the sterically hindered Dpc protecting group is not sufficient to steer the reaction to complete regioselectivity. Thus the claim that the use of the Dpc group has solved the historic problem of regioselective N^9-substitution of guanine[18,19] seems to be restricted to the realm of glycosylation reactions, while alkylation transformations require further experimentation.

The coupling of nucleobase-substituted acetic acids **1**, **5**, **7**, **16** with the backbone unit **17**[9] under standard peptide-coupling conditions afforded the PNA esters **18**–**21** which were subsequently acidolysed (TFA in dichloromethane) to give the PNA monomers **22**–**25** (Scheme 5). As expected from our previous experience (**13** ⟶ **11**, Scheme 4) in the latter reaction the Dpc protecting group was removed along with the *tert*-butyl group. It is clear that in this final deprotection step the protect-

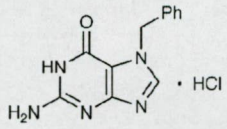


B	R = Bu^t	B	R = H
Thy	**1, 18**	Thy	**22**
$^{4\text{-Bu}^t\text{Bz}}$Cyt	**5, 19**	$^{4\text{-Bu}^t\text{Bz}}$Cyt	**23**
^{An}Ade	**7, 20**	^{An}Ade	**24**
$^{Dpc}_{Ibu}$Gua	**16, 21**	$_{Ibu}$Gua	**25**

Scheme 5 *Reagents and conditions*: a, HBTU, HOBt, DMF, $EtNPr^i_2$; b, 17–43% (v/v) TFA–CH_2Cl_2, 1,3-$(MeO)_2C_6H_4$.

ing groups of ester **21** (Fmoc, Ibu, Dpc, Bu^t) are not completely orthogonal and further research is required. Although the presence of the Dpc group is not essential in oligomer synthesis, it may confer better solubility on the guanine subunits.

UV spectroscopy is often used to locate substituents on nucleobases. However, this technique is not reliable in the case of guanines especially if only one regioisomer is available.[31] Moreover, there are no data on the influence of lactam/lactim tautomerism of guanines on UV properties. Indeed, the UV spectra of N^9/N^7-regioisomers **11/12** (lactams) and **14/15** (lactims), respectively, in buffered ethanolic solutions (pH 0, 6 and 13) revealed that there are no significant differences which would justify the assignment based solely on UV data. Therefore the site of alkylation in guanine derivatives **13–15**, **26**[32] was established in 2D NMR (HMQC[33,34] and HMBC[35,36]) experiments.



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Scrutinizing the ^{13}C NMR chemical-shift parameters of compounds **11–16**, **21**, **25**, **26** (Table 1) and a further 45 N^9/N^7-substituted guanines[19,37–39] (altogether 54 compounds) show that $\delta_{C\text{-}5}$ is the most sensitive to the N^9/N^7-substitution pattern (Fig. 1, N^9: 113.75–123.70 ppm; N^7: 104.56–115.09 ppm; for regioisomers the difference $[\Delta\delta_{C\text{-}5} = \delta_{C\text{-}5}(N^9) - \delta_{C\text{-}5}(N^7)]$ is 7.86–9.82 ppm), insensitive to the lactam/lactim tautomerism (data not shown) and this signal alone can be of diagnostic value, especially if data for both regioisomers are available. However, due to the overlapping of chemical-shift ranges for regioisomers this parameter might not be sufficient for unambiguous assignment. Kjellberg and Johansson[37] suggested that $\delta_{C\text{-}1'}$, $\delta_{C\text{-}5}$ and $\delta_{C\text{-}8}$ could be used to differentiate N^9/N^7-substituted guanines. This was also corroborated, in part, by our findings; however, some further tendencies were also observed. The utility of differential parameters $a = \delta_{C\text{-}4} - \delta_{C\text{-}5}$, $b = \delta_{C\text{-}8} - \delta_{C\text{-}5}$, $c = \delta_{C\text{-}5} - \delta_{C\text{-}1'}$, (Fig. 1) has been assessed and the following conclusions could be drawn:

1. The parameter *a* distinctly differs for the regioisomers {N^9: 28.20–35.41 ppm; N^7: 41.35–54.25 ppm; for regioisomers the difference $[\Delta a = a(N^9) - a(N^7)]$ is −9.93 to −20.53 ppm}, shows little variation for lactam/lactim tautomerism (data not shown) and presents no overlapping ranges. The diagnostic value of this observation is slightly diminished since $\delta_{C\text{-}4}$ usually cannot be simply identified without having recourse to more

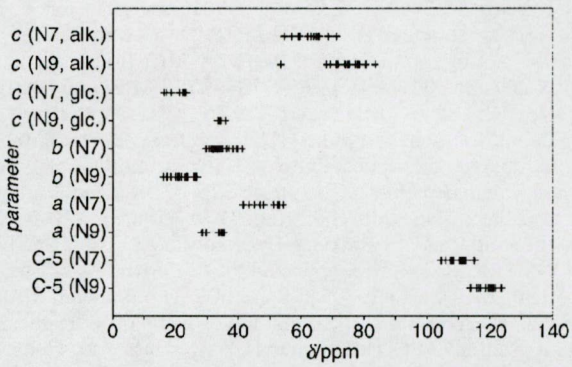

Fig. 1 ^{13}C NMR chemical-shift parameters of N^9/N^7-substituted guanines.

sophisticated assignment techniques (selective INEPT, HMQC, HMBC, *etc.*).

2. The parameter *b* shows similar characteristics {N^9: 16.10–26.91 ppm; N^7: 29.70–41.17 ppm; for regioisomers the difference $[\Delta = b(N^9) = b(N^7)]$ is −11.68 to −16.15 ppm} and its utility is further enhanced by the fact that $\delta_{C\text{-}5}$ is unmistakable among the skeletal carbons and $\delta_{C\text{-}8}$ can simply be located in a *J*-modulated spin-echo experiment.

3. The parameter *c* can be clustered according to the nature of attached substituent rather than lactam/lactim tautomerism and it gives useful values for glycosylated derivatives {N^9: 33.38–35.81 ppm; N^7: 16.30–23.38 ppm; for regioisomers the difference $[\Delta c = c(N^9) - c(N^7)]$ is 10.61–12.79 ppm} while for (cyclo)alkylated compounds it is of less use {N^9: 53.40–83.45 ppm; N^7: 54.57–71.15 ppm; for regioisomers the difference $[\Delta c = c(N^9) - c(N^7)]$ is 10.68–13.35 ppm}. The identification of $\delta_{C\text{-}1'}$, involved in this parameter, often requires more sophisticated techniques.

As a conclusion it can be seen that the values *a*, *b* [both for (cyclo)alkyl and glycosylated derivatives] and *c* (for glycosylated derivatives) are useful for characterizing the N^9/N^7-substitution pattern of guanines. From a practical point of view the parameter *b* is the most convenient one for the reasons explained above. It is noteworthy that $\delta_{C\text{-}5}$ (118.81 ppm) and the parameters *a* (35.38 ppm) and *b* (26.06 ppm) for compound **10**[19] are in good agreement with those for N^9-substituted derivatives, suggesting that its dominant tautomer is 9*H* in DMSO-d_6 solution.

Further studies relating to the application of the above monomers in the preparation of PNA oligomers and our quest for novel combinations of protecting groups are in progress and will be reported in due time.

Experimental

General

The following abbreviations are employed: diisopropyl azodicarboxylate (DIAD); *N*,*N*-diisopropylethylamine (DIPEA); 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU); 1-hydroxybenzotriazole (HOBt); trifluoroacetic acid (TFA). Chemicals were purchased from Aldrich or Fluka. Sodium hydride refers to a 55% suspension in mineral oil. Anhydrous solvents were prepared as described.[40] Light petroleum refers to the fraction with distillation range 40–60 °C. Thymine derivatives **1**, **18** and **22** were prepared using the procedure of Thomson *et al.*[9] N^7-Benzylguanine hydrochloride **26**[32] was prepared for comparison of its ^{13}C NMR parameters with those of other guanine derivatives (see Table 1). Organic solutions were dried using magnesium sulfate and evaporated in Büchi rotary evaporators. TLC: Kieselgel 60 F_{254} (Merck), visualization: UV light. Column chromatography: Kieselgel 60 (0.063–0.200 mm, Merck). Mp: Electrothermal IA 8103 apparatus. Elemental analysis: Perkin-Elmer CHN

analyzer model 2400. UV: PE Lambda 10 spectrometer, λ_{max}/nm (lg ε), sh: shoulder. IR: Bio-Rad FTS-60A (KBr pellets, ν_{max}/cm^{-1}; s, strong; m, medium; w, weak). NMR: Bruker Avance DRX 400 and 500 spectrometers (^{1}H: 400.13 MHz and 500.13 MHz; ^{13}C: 100.62 MHz and 125.76 MHz, respectively), DMSO-d_6 solutions, δ (ppm), J (Hz). Spectral patterns: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; br, broad; deut, deuterable. The superscripts *, # denote interchangeable assignments. For the 2D experiments (HMQC, HMBC) the standard Bruker software packages (INV4GSSW, INV4GSLRNDSW) were applied. For ^{13}C NMR data of guanine derivatives see Table 1. Mass spectrometry: Finnigan MAT TSQ 7000, electrospray (ESI) and atmospheric pressure chemical ionization (APCI) techniques. IUPAC names: AutoNom™ 2.1 (as implemented in ChemDraw® 5.0). Statistical analysis of ^{13}C NMR chemical-shift parameters of guanine derivatives (Fig. 1): Corel® Quattro® Pro 8.0, @ functions and SigmaPlot 4.01. Details are available upon request.

[4-(4-Methoxybenzoylamino)-2-oxo-1,2-dihydropyrimidin-1-yl]acetic acid 3

Cytosine (1.11 g, 10.0 mmol) suspended in pyridine (50 mL) was stirred at room temperature while 4-methoxybenzoyl chloride (2.56 g, 15.0 mmol) was added and the reaction mixture was stirred in an oil-bath at 80 °C. The cytosine rapidly dissolved, and then the product precipitated from the solution. After 2 h the mixture was evaporated *in vacuo* and coevaporated with methanol (2×). The residue was suspended in methanol and the mixture was filtered to afford compound **3** (2.28 g, 93%) as a white powder, mp > 260 °C. The product [4-methoxy-*N*-(2-oxo-1,2-dihydropyrimidin-4-yl)benzamide, **2**] was insoluble in practically all solvents, therefore it was used without further purification; R_f 0.58 (CH_2Cl_2–MeOH 8:2); λ_{max}[0.20% (v/v) TFA in EtOH]/nm 216 (lg ε 4.00), 276 (4.35); ν_{max}/cm^{-1} 3276w, 3150w, 3073w, 2994w, 2846w, 1714s, 1692s, 1621m, 1609m, 1578m, 1497s, 1406w, 1260s, 1189m, 801m.

The majority (2.20 g, 8.97 mmol) of this substance was suspended in anhydrous DMF (25 mL), anhydrous K_2CO_3 (1.24 g, 8.97 mmol) and methyl bromoacetate (0.86 mL, 8.97 mmol) were added and the mixture was stirred for 24 h. The reaction mixture was filtered, washed with DMF and the filtrate was evaporated. Water (9 mL) and 4 M HCl (0.4 mL) were added to the residue and stirred for 15 min. The ester was filtered off and then added to a mixture of aq. 2 M NaOH (6.5 mL, 13.0 mmol) and water (12 mL) and the reaction mixture was sonicated. The substance dissolved and after 30 min no starting material was present (TLC). The reaction mixture was acidified with 4 M HCl (3.6 mL, 14.4 mmol) and the precipitated substance was filtered off. Purification of the crude product (2.94 g) was attempted by recrystallization from methanol. 1 L of solvent was not enough to dissolve the above amount, and so 0.45 g was filtered off to give a white powder, TLC: single spot, mp 220 °C (darkens), 239 °C (decomp.). From the methanolic solution a second crop (0.86 g) was obtained as a white powder, TLC: single spot, mp 166 °C (darkens), 227 °C (decomp.). Overall yield: 1.31 g (48%); R_f 0.58 (MeCN–MeOH–AcOH 4:1:1) (Found: C, 55.5; H, 4.2; N, 13.7. Calc. for $C_{14}H_{13}N_3O_5$: C, 55.45; H, 4.3; N, 13.9%); λ_{max}(EtOH)/nm 208 (lg ε 4.58), 252 (4.55), 287 (4.27); ν_{max}/cm^{-1} 3147w, 3076w, 1711s, 1631m, 1615w, 1503m, 1419w, 1251m, 1183m, 755w; δ_H (400 MHz) 3.84 (3 H, s, OCH_3), 4.56 (2 H, s, CH_2), 7.04 and 8.03 (2 × 2 H, 2 × d, J 8.9, ArH), 7.30 (1 H, d, J 7.2, H-5*), 8.08 (1 H, d, J 7.2, H-6*), 11.03 (1 H, br s, deut, OH#), 11.97 (1 H, br s, deut, NH#); *m/z* (ESI) 304 (44%, $[M + H]^+$). This compound was mentioned by Breipohl *et al.*[8] but not described in detail.

[6-(4-Methoxybenzoylamino)purin-9-yl]acetic acid 7

A. Alkylation of [6-(4-methoxybenzoylamino)purine] and subsequent acidolysis. To [6-(4-methoxybenzoylamino)purine] **6**[7] (6.47 g, 24.0 mmol) suspended in anhydrous DMF (120 mL) was added sodium hydride (1.05 g, 24.0 mmol) in portions and the mixture was stirred at room temperature for 30 min. *tert*-Butyl bromoacetate (3.9 mL, 26.4 mmol) was added dropwise and stirring was continued for 2 h. The mixture was evaporated *in vacuo* and the residue was suspended in a mixture of water (200 mL) and dichloromethane (200 mL). The resulting precipitate was filtered off and dried (5.66 g, 61%). The majority (5.50 g, 14.3 mmol) of this substance was stirred with 50% (v/v) TFA in dichloromethane (40 mL) and (±)1,4-dithiothreitol (0.20 g, 1.3 mmol) at room temperature for 4 h. The solution was evaporated *in vacuo* and the residue was coevaporated with EtOAc (5×). The residue was dissolved in 5% (w/v) aq. $NaHCO_3$ (150 mL), filtered and acidified with 10% (w/v) aq. $NaHSO_4$ (100 mL). The precipitated solid was filtered off (4.11 g, 88%), to give 7 mp 213 °C (darkens), 250 °C (decomp.). The characteristics (^{1}H NMR and mass spectra) of this substance were in good agreement both with the published values[7] and with those of the substance prepared in procedure B.

B. Controlled hydrolysis of a dianisoylated derivative. Ethyl (adenin-9-yl)acetate **8**[5] (4.42 g, 20 mmol) was suspended in anhydrous pyridine (50 mL), heated to 80 °C for 30 min, then cooled to room temperature. 4-Methoxybenzoyl chloride (8.53 g, 50.0 mmol) was added in portions and the mixture was stirred for 18 h, then evaporated *in vacuo* and the residue was coevaporated with toluene (3×). The residue was dissolved in dichloromethane (70 mL), and the solution was washed with 10% (w/v) citric acid (2 × 30 mL), dried and evaporated *in vacuo*. The crude product (14.96 g) was dissolved in warm ethanol (100 mL), cooled to room temperature, 2 M aq. NaOH (30 mL) was added, and the solution was left at room temperature and checked from time to time by TLC. After 175 min more aq. NaOH (5 mL) was added. The reaction was stopped after 4 h by addition of 1 M HCl (35 mL), pH ≈5, and the solution was evaporated *in vacuo*. The crude product was recrystallized from methanol (1 L) to afford a white powder (4.58 g, 70%), mp 215 °C (darkens), 254 °C (decomp.) [lit.,[7] 222–223 °C (decomp.)]. The ^{1}H NMR and mass spectra of this compound were in good agreement with the published values.[7]

(2-Isobutyrylamino-6-oxo-1,6-dihydropurin-9-yl)acetic acid *tert*-butyl ester 11 and (2-isobutyrylamino-6-oxo-1,6-dihydropurin-7-yl)acetic acid *tert*-butyl ester 12

N-(6-Oxo-6,9-dihydro-1*H*-purin-2-yl)isobutyramide **9**[19,22] (1.11 g, 5.0 mmol) was suspended in anhydrous DMF and the mixture was chilled to 0 °C. Sodium hydride (0.36 g, 8.25 mmol) was added and the mixture was stirred at 0 °C for 30 min. *tert*-Butyl bromoacetate (0.81 mL, 5.5 mmol) was added and the reaction was stopped after 2 h by addition of a small amount of solid CO_2 and methanol (2 mL). The reaction mixture was evaporated *in vacuo* and the residue was chromatographed using 0–5% (v/v) methanol in dichloromethane. Eluted first was the less polar N^7-isomer **12**, (0.43 g, 26%), second a mixture (in ≈1:1 ratio as judged by TLC and ^{1}H NMR) of N^7- and N^9-isomer (0.24 g, 14%), and third the pure N^9-isomer **11** (0.56 g, 34%).

Ester **11**: white powder, mp 204 °C (decomp., from EtOH); R_f 0.13 (CH_2Cl_2–MeOH 95:5) (Found: C, 53.9; H, 6.4; N, 21.1. Calc. for $C_{15}H_{21}N_5O_4$: C, 53.7; H, 6.3; N, 20.9%); λ_{max}[50% (v/v) 1 M HCl in EtOH, pH 0]/nm 206 (lg ε 4.26), 265 (4.23); λ_{max}[50% (v/v) phosphate buffer in EtOH, pH 6]/nm 260 (lg ε 4.17), 282sh (4.02); λ_{max}[50% (v/v) 0.1 M NaOH in EtOH, pH 13]/nm 216 (lg ε 4.39), 263 (4.06); ν_{max}/cm^{-1} 3151w, 2980w, 2932w, 1753s, 1698m, 1673s, 1614m, 1562m, 1549m, 1483w, 1411m, 1233m, 1154m, 1143m, 795w; δ_H (500 MHz) 1.11 [6 H, d, J 6.8, $(CH_3)_2CH$], 1.40 (9 H, s, Bu^t), 2.78 [1 H, pseudoquintet, $(CH_3)_2CH$], 4.88

Table 1 ^{13}C NMR chemical shifts of guanine derivatives (δ, ppm)[a]

Compd.	Subst.	C-2	C-4	C-5	C-6	C-8	Other carbons
10[b,c]	—	152.34	154.19	118.81	157.41	144.87	irrelevant
11[d]	N^9	148.49	155.17	119.98	149.30	140.72	19.22 [(CH_3)$_2$CH], 28.03 [(CH_3)$_3$C], 35.01 [(CH_3)$_2$CH], 45.17 (CH_2COO), 82.69 [(CH_3)$_3$C], 167.03 (COOBu^t), 180.56 (Pr^{i}CO)
12	N^7	147.55	157.20	112.08	152.95	144.50	19.23 [(CH_3)$_2$CH], 28.00 [(CH_3)$_3$C], 35.06 [(CH_3)$_2$CH], 48.26 (CH_2COO), 82.36 [(CH_3)$_3$C], 167.20 (COOBu^t), 180.32 (Pr^{i}CO)
13[e,f]	N^9	153.24	155.88	120.47	155.95	147.01	20.08 [(CH_3)$_2$CH], 28.52 [(CH_3)$_3$C], 35.18 [(CH_3)$_2$CH], 45.82 (CH_2COO), 83.40 [(CH_3)$_3$C], 127.76, 128.15, 130.26 (arom. Cs), 142.51 (arom. quaternary C), 151.03 (OCON), 167.33 (COOBu^t), 175.97 (Pr^{i}CO)
14[e,f]	N^9	153.29	155.91	120.51	155.91	146.90	20.08 [(CH_3)$_2$CH], 35.25 [(CH_3)$_2$CH], 45.14 (CH_2COO), 53.48 (CH_3O), 127.79, 128.14, 130.26 (arom. Cs), 142.51 (arom, quaternary C), 151.03 (OCON), 168.85 (COOMe), 175.90 (Pr^{i}CO)
15[f]	N^7	149.53	164.63	112.19	141.28	150.44	19.20 [(CH_3)$_2$CH], 34.26 [(CH_3)$_2$CH], 47.50 (CH_2COO), 52.50 (CH_3O), 128.91, 129.04, 129.36 (arom. Cs), 141.28 (arom. quaternary C), 152.02 (OCON), 167.77 (COOMe), 174.94 (Pr^{i}CO)
16[e]	N^9	152.19	154.81	119.62	155.03	146.50	19.08 [(CH_3)$_2$CH], 34.22 [(CH_3)$_2$CH], 66.22 (CH_2COO), 126.90, 129.17, 129.37, 129.90 (arom. Cs), 141.49 (arom. quaternary C), 150.07 (OCON), 174.93 (Pr^{i}CO, COOH)
21[e,g]	N^9	152.01	154.72	119.40	155.05	146.31	18.98 [(CH_3)$_2$CH], 27.47/27.50 [(CH_3)$_3$C], 34.17 [(CH_3)$_2$CH], 46.56 (OCH$_2$CH), 43.73/43.99, 47.09, 48.76, 49.96 (4 × CH_2), 65.28 (OCH$_2$CH), 80.88/81.98 [(CH_3)$_3$C], 119.88, 124.79/124.88, 126.81/126.85, 126.95, 127.40, 128.71, 129.20 (arom. Cs), 140.54, 141.45, 143.61/143.65 (arom. quaternary C), 150.00 (OCON), 155.13, 155.95/156.21, 166.24/166.74, 167.69/168.32 (4 × CO), 175.00 (Pr^{i}CO)
25[e,g]	N^9	147.79, 147.82	149.16, 149.24	119.49	154.79	140.45	18.76 [(CH_3)$_2$CH], 34.58 [(CH_3)$_2$CH], 46.66 (OCH$_2$CH), 43.86/44.00, 46.85/46.97, 47.76, 49.11 (4 × CH_2), 65.43 (OCH$_2$CH), 120.01/120.04, 124.94/125.01, 126.95, 127.53 (arom. Cs), 140.64/140.68, 143.76/143.78 (arom. quaternary C), 156.07/156.30, 166.37/166.91, 170.28/170.72 (3 × CO), 180.05 (Pr^{i}CO, COOH)
26[d,f,h]	N^7	154.95	151.46	108.07	153.92	141.06	51.09 (CH_2), 128.70, 129.15, 129.62 (arom. Cs), 136.38 (arom. quaternary C)

[a] In DMSO-d_6; 125.76 MHz; *J*-modulated spin-echo experiments; for guanine numbering see ester **11**, Scheme 3. [b] Ref. 19. [c] The C-4, C-5, C-6, C-8 signals were observed only after adding trifluoroacetic acid. [d] The assignment of signals corresponding to C-2 and C-6 carbons is tentative. [e] The assignment of signals corresponding to C-4 and C-6 carbons is tentative. [f] Assignment based on HMQC and HMBC experiments. [g] Some signals were doubled due to the presence of rotamers. [h] N^7-Benzylguanine hydrochloride, prepared according to Bridson *et al.*[32]

(2 H, s, CH_2COO), 7.95 (1 H, s, H-8), 11.65 (1 H, br s, deut, NH), 12.10 (1 H, br s, deut, NH); *m/z* (ESI) 693 (20%, [2M + Na]$^+$), 671 (55, [2M + H]$^+$), 336 (100, [M + H]$^+$).

Ester **12**: white powder, mp 202.5 °C (decomp., from EtOH); R_f 0.19 (CH_2Cl_2–MeOH 95:5) (Found: C, 53.65; H, 6.15; N, 21.1%); λ_{max}[50% (v/v) 1 M HCl in EtOH, pH 0]/nm 206 (lg ε 4.24), 263 (4.20); λ_{max}[50% (v/v) phosphate buffer in EtOH, pH 6]/nm 221 (lg ε 4.24), 265 (4.11), 282sh (3.98); λ_{max}[50% (v/v) 0.1 M NaOH in EtOH, pH 13]/nm 224 (lg ε 4.31), 269 (4.01); ν_{max}/cm^{-1} 3240w, 2981w, 2937w, 1741m, 1695s, 1677s, 1604s, 1535w, 1421w, 1390m, 1370m, 1238m, 1160m, 747w; δ_H (500 MHz) 1.11 [6 H, d, *J* 6.8, (CH_3)$_2$CH], 1.39 (9 H, s, Bu^t), 2.73 [1 H, pseudo-quintet, (CH_3)$_2$CH], 5.07 (2 H, s, CH_2COO), 8.11 (1 H, s, H-8), 11.55 (1 H, br s, deut, NH), 12.14 (1 H, br s, deut, NH); *m/z* (ESI) 693 (40%, [2M + Na]$^+$), 671 (25, [2M + H]$^+$), 358 (27, [M + Na]$^+$), 336 (100, [M + H]$^+$).

[6-Diphenylcarbamoyloxy-2-(isobutyrylamino)purin-9-yl]acetic acid *tert*-butyl ester 13

A. Mitsunobu reaction, general procedure. Compound **10**[19,22] (1.00 g, 2.40 mmol) was suspended in anhydrous THF (50 mL) and the mixture was refluxed for 20 min to achieve partial dissolution of the starting material.[20] The suspension was cooled to room temperature, *tert*-butyl glycolate[23] (0.40 g, 3.0 mmol), the appropriate phosphine (3.19 mmol) and DIAD (0.62 mL, 3.19 mmol) were added dropwise, and the mixture was stirred at room temperature. The reaction mixture completely dissolved and became yellow coloured. After completion of the reaction (TLC) the solution was evaporated *in vacuo* and the residue was subjected to chromatographic purification.

A1. With triphenylphosphine.—Reaction time: 4 h at room temperature. Chromatography: 50–70% (v/v) ethyl acetate in light petroleum. Eluted first was the product **13** (0.31 g), slightly contaminated with triphenylphosphine oxide. Further fractions were also obtained containing varying proportions of the product and triphenylphosphine oxide. The different, partly crystalline fractions were triturated with methanol upon which the product crystallized. This was filtered off and washed with light petroleum. The cleanest product (0.40 g, 31%), a white powder, melted at 183.2–185.5 °C. A further crystalline crop (0.39 g) containing the product and triphenylphosphine oxide (TLC) was also obtained; R_f 0.50 (CH_2Cl_2–MeOH 95:5) (Found: C, 63.5; H, 5.5; N, 15.7. Calc. for $C_{28}H_{30}N_6O_5$: C, 63.4; H, 5.7; N, 15.8%); λ_{max}(EtOH)/nm 205 (lg ε 4.60), 229 (4.53), 258sh (4.15), 279 (4.08); λ_{max}/cm^{-1} 3462w, 3346w, 2979w, 2934w, 1738s, 1715m, 1624m, 1587m, 1524m, 1449m, 1411m, 1305m, 1240m, 1187s, 1164s, 1056m, 758w, 700m; δ_H (500 MHz) 1.09 [6 H, d, *J* 6.8, (CH_3)$_2$CH], 1.43 (9 H, s, Bu^t), 2.87 [1 H, pseudo-quintet, *J* 6.8, (CH_3)$_2$CH], 5.03 (2 H, s, CH_2), 7.29–7.53 (10 H, m, ArH), 8.45 (1 H, s, H-8), 10.69 (1 H, br s, deut, NH); *m/z* (ESI) 557 (8%, [2Ph$_3$PO + H]$^+$), 531 (100, [M + H]$^+$).

A2. With 4-(dimethylamino)phenyl(diphenyl)phosphine.[26,27]—Reaction time: 2.5 h at 0 °C. Work-up: the crude product was dissolved in dichloromethane (50 mL) and extracted successively with 4 M HCl (3 × 25 mL) and with 5% (w/v) aq. $NaHCO_3$ (50 mL). TLC revealed that most of the 4-(dimethylamino)phenyl(diphenyl)phosphine oxide remained in the organic phase. The organic phase was dried, and purified by column chromatography using 0–1% (v/v) methanol in dichloromethane. Methanolic trituration and filtration (light petroleum) afforded the product (0.42 g, 33%), mp 182.5–185.0 °C. The IR, ^1H NMR and mass spectra of this compound were in good agreement with those of the substance obtained in procedure *A1*.

A3. With tributylphosphine.—Reaction time: 1.5 h at 0 °C. Work-up: the crude product was dissolved in dichloromethane (50 mL) and extracted with water (3 × 25 mL) to remove

the tributylphosphine oxide. Chromatography: 0–1.5% (v/v) methanol in dichloromethane. Methanolic trituration and filtration (light petroleum) afforded the product (0.46 g, 36%), mp 182.2–184.8 °C. The IR, ^{1}H NMR and mass spectra of this compound were in good agreement with those of the substance obtained in procedure *A1*.

B. Low-temperature, sodium hydride-mediated alkylation. To compound **10**[19,22] (1.40 g, 3.36 mmol) suspended in anhydrous DMF (20 mL) was added sodium hydride (0.16 g, 3.70 mmol) at room temperature. After 30 min the reaction mixture was chilled to −20 °C and maintained at this temperature. *tert*-Butyl bromoacetate (0.60 mL, 4.0 mmol) was added dropwise. After 2 h the reaction was stopped by addition of a small amount of solid CO_2 and methanol, and the mixture was evaporated *in vacuo* and coevaporated with toluene (2×). The residue was dissolved in a mixture of water and dichloromethane (20 mL each). The aqueous phase was extracted with dichloromethane (3 × 15 mL), and the combined organic phases were dried, and evaporated *in vacuo*. Chromatography: 0–1% (v/v) methanol in dichloromethane. Methanolic trituration and subsequent crystallization from methanol (15 mL) afforded the product (0.70 g, 40%) as a white powder, mp 183.1–184.5 °C. The IR, ^{1}H NMR and mass spectra of this compound were in good agreement with those of the substance obtained in procedure *A1*.

Hydrolysis of [6-diphenylcarbamoyloxy-2-(isobutyrylamino)-purin-9-yl]acetic acid *tert*-butyl ester (13 ⟶ 11)

To ester **13** (0.210 g, 0.38 mmol) dissolved in anhydrous dichloromethane (6 mL) were added 1,3-dimethoxybenzene (0.070 mL, 0.53 mmol) and TFA (0.50 mL, 6.5 mmol) at 0 °C and the mixture was stirred for 18 h. The reaction mixture was diluted with dichloromethane (20 mL), and extracted with satd. aq. $NaHCO_3$ solution (3 × 10 mL) to remove the excess of acid. Chromatography: 0–10% (v/v) methanol in dichloromethane to give the lactam **11** (0.069 g, 55%) as an amorphous foam. The IR, ^{1}H NMR and mass spectra of this product were in good agreement with those of the substance obtained in a previous experiment (*vide supra*).

[6-Diphenylcarbamoyloxy-2-(isobutyrylamino)purin-9-yl]acetic acid methyl ester 14 and [6-diphenylcarbamoyloxy-2-(isobutyryl-amino)purin-7-yl]acetic acid methyl ester 15 (*cf.* ref. 8)

To compound **10**[19,22] (3.53 g, 8.47 mmol) suspended in anhydrous DMF (40 mL) was added DIPEA (2.87 mL, 16.74 mmol) and the mixture was briefly heated to 80 °C until a clear solution was obtained (10 min). The mixture was cooled to room temperature, methyl bromoacetate (0.87 mL, 9.32 mmol) was added, and the mixture was stirred for 20 h. The reaction mixture was evaporated *in vacuo* and the residue was coevaporated with methanol (3×). The partly crystalline material was suspended in methanol (40 mL) and added dropwise to water (120 mL) with vigorous stirring. The precipitate was filtered off (3.99 g, 96%) and recrystallized from EtOAc (190 mL) to afford the title products (2.01 g, 49%), mp 167.0–168.4 °C; from the mother liquor was obtained a further crop (0.37 g, 9%), mp 168.0–170.4 °C. The mother liquor was evaporated and chromatographed by using 1–4% (v/v) methanol in dichloromethane. Eluted first was ester **14** (0.54 g, 13%) then its regioisomer **15** (0.60 g, 15%). Overall yield of **14**: 2.92 g, 71%.

Ester **14**: white powder, mp 170.0–171.4 °C (from EtOAc); R_f 0.37 (CH_2Cl_2–MeOH 95:5) (Found: C, 61.35; H, 5.1; N, 17.4. Calc. for $C_{25}H_{24}N_6O_5$: C, 61.5; H, 4.95; N, 17.2%); λ_{max}[50% (v/v) 1 M HCl in EtOH, pH 0]/nm 207 (lg ε 4.50), 226 (4.53), 253sh (4.17), 279 (4.07); λ_{max}[50% (v/v) phosphate buffer in EtOH, pH 6]/nm 227 (lg ε 4.54), 259sh (4.14), 277 (4.08); λ_{max}[50% (v/v) 0.1 M NaOH in EtOH, pH 13]/nm 230 (lg ε 4.45), 276 (4.03); ν_{max}/cm^{-1} 3295w, 2996w, 2956w, 1756m, 1726s, 1681s, 1631m, 1599m, 1491m, 1384m, 1340s, 1233s, 1223s, 1192m, 1063m, 789w, 758w, 704w; δ_H (500 MHz) 1.08 [6 H, d, *J* 6.8, $(CH_3)_2CH$], 2.83 [1 H, pseudoquintet, *J* 6.8, $(CH_3)_2CH$], 3.73 (3 H, s, CH_3), 5.15 (2 H, s, CH_2), 7.31–7.49 (10 H, m, ArH), 8.43 (1 H, s, H-8), 10.64 (1 H, br s, deut, NH); *m/z* (ESI) 511 (25%, $[M + Na]^+$), 489 (100, $[M + H]^+$).

Ester **15**: amorphous foam; R_f 0.35 (CH_2Cl_2–MeOH 9:1) (Found: C, 61.3; H, 5.1; N, 17.0); λ_{max}[50% (v/v) 1 M HCl in EtOH, pH 0]/nm 207 (lg ε 4.59), 227 (4.56), 252sh (4.26), 283 (4.09); λ_{max}[50% (v/v) phosphate buffer in EtOH, pH 6]/nm 230 (lg ε 4.61), 256sh (4.17), 282 (4.02); λ_{max}[50% (v/v) 0.1 M NaOH in EtOH, pH 13]/nm 229 (lg ε 4.53), 287sh (3.90); ν_{max}/cm^{-1} 3438w, 2972w, 1753s, 1707m, 1639m, 1592w, 1493s, 1445m, 1301s, 1187s, 761m, 701m; δ_H (500 MHz) 1.11 [6 H, d, *J* 6.8, $(CH_3)_2CH$], 2.85 [1 H, pseudoquintet, *J* 6.8, $(CH_3)_2CH$], 3.56 (3 H, s, CH_3), 5.20 (2 H, s, CH_2), 7.28–7.48 (10 H, m, ArH), 8.53 (1 H, s, H-8), 10.57 (1 H, br s, deut, NH); *m/z* (ESI) 511 (40%, $[M + Na]^+$), 489 (100, $[M + H]^+$).

[6-Diphenylcarbamoyloxy-2-(isobutyrylamino)purin-9-yl]acetic acid 16 (*cf.* ref. 8)

Ester **14** (2.24 g, 4.59 mmol) was suspended under sonication in a mixture of methanol (6 mL), 1,4-dioxane (24 mL) and water (12 mL). 1 M aq. NaOH (5 mL) was added and the mixture was stirred for 30 min. The pH of the mixture was brought to ≈6 by addition of 1 M HCl and the organics were evaporated off *in vacuo*. The solution was diluted with water (120 mL) and acidified to pH ≈3 by addition of 1 M HCl. The precipitate was filtered off, and washed with ice–water to give acid **16** (1.99 g, 91%), white powder, mp 156 °C (decomp.). Attempted recrystallization from EtOAc resulted in gel formation; R_f 0.40 (MeCN–MeOH–AcOH 8:1:1) (Found: C, 60.5; H, 4.4; N, 17.4. Calc. for $C_{24}H_{22}N_6O_5$: C, 60.75; H, 4.7; N, 17.7%); λ_{max}(EtOH)/nm 229 (lg ε 4.51), 260sh (4.11), 279 (4.06); ν_{max}/cm^{-1} 3379w, 1723s, 1627m, 1591m, 1522m, 1493m, 1441m, 1411m, 1307m, 1200s, 1187s, 760w, 701m; δ_H (500 MHz) 1.09 [6 H, s, $(CH_3)_2CH$], 2.83 [1 H, m, $(CH_3)_2CH$], 5.05 (2 H, s, CH_2), 7.29–7.50 (10 H, m, ArH), 8.46 (1 H, s, H-8), 10.65 (1 H, br s, deut, NH); *m/z* [ESI ($CHCl_3$ + MeOH)], 971 (8%, $[2M + Na]^+$), 514 (8, $[M + K]^+$), 497 (20, $[M + Na]^+$), 475 (100, $[M + H]^+$).

Esters 19–21

General procedure. To acid **5**,[7] **7** or **16** (2.0 mmol) dissolved in anhydrous DMF (20 mL) were added HOBt hydrate (0.61 g, 4.0 mmol) and HBTU (1.52 g, 4.0 mmol). Meanwhile ester **17**[9] (1.95 g, 3.0 mmol for acids **5**, **7** and 1.30 g, 2.0 mmol for acid **16**) was suspended in dichloromethane (20/30 mL), extracted with satd. aq. $NaHCO_3$ (10 mL) and dried. The above dichloromethane solution of free **17** base and DIPEA (0.70 mL, 4.0 mmol) were added after 5 min and the reaction mixture was stirred at room temperature. Work-up: after evaporation of the solution *in vacuo*, the residue was dissolved in dichloromethane (30 mL), extracted with 1 M HCl (**19**: 3 × 10 mL) or with satd. aq. $NaHSO_4$ (**20**: 5 × 10 mL), and washed successively with satd. aq. $NaHCO_3$ (25 mL) and brine (25 mL). In the case of **21**, after evaporation of the reaction mixture the residue was triturated with EtOAc (5 mL) and filtered, followed by crystallization. The resulting crude products were purified chromatographically (**19**, **20**) or by crystallization (**21**).

({2-[4-(4-tert-Butylbenzoylamino)-2-oxo-1,2-dihydropyrimidin-1-yl]acetyl}-[2-(9H-fluoren-9-ylmethoxycarbonylamino)-ethyl]amino)acetic acid tert-butyl ester 19.—Reaction time: 20 h at room temperature. Chromatography: 1–3% (v/v) methanol in dichloromethane, yield 0.99 g (70%), colourless oil. In a repeated experiment the extractive work-up was omitted and the crude product was triturated with methanol (2 mL) to yield a cleaner product (0.82 g, 58%), amorphous foam; R_f 0.49 (CH_2Cl_2–MeOH 95:5) (Found: C, 67.6; H, 6.4; N, 9.7. Calc. for

$C_{40}H_{46}N_5O_7$: C, 67.8; H, 6.5; N, 9.9%); λ_{max}(EtOH)/nm 205 (lg ε 4.19), 265 (3.94), 288 (3.52), 300 (3.52); ν_{max}/cm^{-1} 3450m, 3150w, 3070w, 2966w, 2936w, 1710s, 1670s, 1629m, 1563m, 1492s, 1408w, 1367s, 1254s, 1156s, 1113s, 851w, 759w, 740w; δ_H (500 MHz, rotamers) 1.29 (9 H, s, Bu^t), 1.40 (9 H, s, Bu^t), 1.46 (2 H, s, CH_2), 3.23 (2 H, m, CH_2), 3.44 (2 H, m, CH_2), 3.97 (3 H, s, CH_3O), 4.26 (1 H, m, CH), 4.68/4.90 (2 H, 2 s, CH_2), 6.27 (1 H, s, H-5*), 7.34 (2 H, dd, *J* 7.4 and 7.2, fluorenyl CH), 7.41 (2 H, dd, *J* 7.4 and 7.2, fluorenyl CH), 7.52 (2 H, d, *J* 8.3, 4-Bu^{t}C$_6H_4$CO), 7.83 (2 H, d, *J* 7.5, fluorenyl CH), 7.87 (2 H, d, *J* 7.5, fluorenyl CH), 7.98 (2 H, d, *J* 8.3, 4-Bu^{t}C$_6H_4$CO), 8.00 (1 H, s, H-6*); *m/z* (ESI) 730 (78%, [M + Na]$^+$), 708 (100, [M + H]$^+$).

({2-(9H-Fluoren-9-ylmethoxycarbonylamino)ethyl]-{2-[6-(4-methoxybenzoylamino)purin-9-yl]acetyl}amino)acetic acid tert-butyl ester 20.—Reaction time: 1.5 h at room temperature. Chromatography: 0–20% (v/v) EtOAc in methanol, yield 1.20 g (85%), amorphous foam; R_f 0.42 (CH_2Cl_2–MeOH 95:5) (Found: C, 64.8; H, 5.4; N, 13.7. Calc. for $C_{38}H_{39}N_7O_7$: C, 64.7; H, 5.6; N, 13.9%); λ_{max}(EtOH)/nm 206 (lg ε 4.78), 266 (4.41), 278 (4.41), 289 (4.42), 300sh (4.32); ν_{max}/cm^{-1} 3065w, 2980w, 2943w, 1705m, 1670m, 1609m, 1586m, 1513w, 1458m, 1411w, 1252s, 1157m, 845s, 762m, 743m; δ_H (500 MHz, rotamers) 1.32 (9 H, s, Bu^t), 2.81 (2 H, s, CH_2), 3.25 (2 H, m, CH_2), 3.65 (2 H, m, CH_2), 3.94 (3 H, s, CH_3O), 4.50 (3 H, m, CH, CH_2), 5.25/5.44 (2 H, 2 s, CH_2), 7.16 (2 H, d, *J* 8.7, anisoyl CH), 7.39 (2 H, dd, *J* 7.4 and 7.2, fluorenyl CH), 7.49 (2 H, dd, *J* 7.4 and 7.2, fluorenyl CH), 7.76 (2 H, d, *J* 7.4, fluorenyl CH), 7.96 (2 H, d, *J* 7.4, fluorenyl CH), 8.13 (2 H, d, *J* 8.6, anisoyl CH), 8.40 (1 H, s, H-8*), 8.70 (1 H, s, H-2*), 11.10 (1 H, br s, NH); *m/z* (ESI) 706 (100%, [M + H]$^+$).

({2-[6-Diphenylcarbamoyloxy-2-(isobutyrylamino)purin-9-yl]acetyl}-[2-(9H-fluoren-9-ylmethoxycarbonylamino)ethyl]-amino)acetic acid tert-butyl ester 21.—Reaction time: 1.5 h at room temperature. The crude product was obtained as described above and was recrystallized from ethanol (750 mL) to give a white powder (1.22 g, 40%), mp 209.0–209.5 °C (decomp.); R_f 0.36 (CH_2Cl_2–MeOH 95:5) (Found: C, 66.3; H, 5.5; N, 12.9. Calc. for $C_{47}H_{48}N_8O_8$: C, 66.2; H, 5.7; N, 13.1%); λ_{max}(EtOH)/nm 221 (lg ε 4.57), 228 (4.53), 256 (4.39), 266sh (4.38), 279sh (4.27), 289sh (4.07), 300 (3.81); ν_{max}/cm^{-1} 3302m, 2979w, 1750m, 1732s, 1704s, 1656s, 1624w, 1591w, 1545m, 1493w, 1450m, 1192s, 1156m, 1055m, 762m, 697w; δ_H (500 MHz, rotamers) 1.06 [6 H, d, *J* 6.2, (C*H*$_3$)$_2$CH], 1.35/1.47 (9 H, 2 s, Bu^t), 2.83 [1.3 H, m, (CH$_3$)$_2$C*H*, NH], 3.52 (4 H, m, partly shielded by the water signal, 2 × CH_2), 3.95 (1 H, s, CH), 4.19–4.29 (2 H, m, CH_2), 4.32–4.35 (2 H, m, CH_2), 5.07/5.25 (2 H, 2 × s, guanyl CH_2), 7.22–7.48 (14 H, m, 2 × Ph, fluorenyl CH), 7.64 (2 H, d, *J* 7.3, fluorenyl CH), 7.86 (2 H, dd, *J* 7.9 and 7.9, fluorenyl CH), 8.30 (1 H, s, H-8), 10.57 (1 H, s, NH); *m/z* (ESI) 891 (1%, [M + K]$^+$); 875 (5, [M + Na]$^+$); 853 (100, [M + H]$^+$).

({2-[4-(4-*tert*-Butylbenzoylamino)-2-oxo-1,2-dihydropyrimidin-1-yl]acetyl}-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)ethyl]-amino)acetic acid 23

To ester **19** (0.40 g, 0.56 mmol) dissolved in dichloromethane (20 mL) was added 1,3-dimethoxybenzene (0.19 mL, 1.45 mmol) followed by TFA (4.0 mL, 52.3 mmol) and the mixture was stirred for 6 h at room temperature. The solution was evaporated *in vacuo*, and the residue was coevaporated with acetonitrile (5×). The residue was triturated under diethyl ether, filtered and recrystallized from methanol (20 mL) to give a white powder (0.27 g, 73%), mp 197.4–199.0 °C (decomp.); R_f 0.86 (CH_2Cl_2–MeOH 6:4) (Found: C, 66.3; H, 5.7; N, 10.6. Calc. for $C_{36}H_{38}N_5O_7$: C, 66.2; H, 5.9; N, 10.7%); λ_{max}(EtOH)/nm 205 (lg ε 4.88), 265 (4.68), 289 (4.25), 300 (4.27); ν_{max}/cm^{-1} 3147w, 3067w, 2964w, 1708s, 1664s, 1610m, 1562m, 1490s, 1409w, 1367m, 1255m, 1113w, 853w, 760w, 741w; δ_H (500 MHz, rotamers) 1.34 (9 H, s, Bu^t), 3.14/3.25 (1 H, 2 m, partly shielded by the water signal, CH_2), 3.38/3.47 (1.4 H, 2 m, partly shielded by the water signal, CH_2), 4.03/4.22 (2 H, 2 s, CH_2), 4.26 (1 H, m, CH), 4.31/4.36 (2 H, 2 d, *J* 6.7, CH_2), 4.70/4.89 (2 H, 2 s, cytosinyl CH_2), 7.27/7.41 (1 H, 2 d, *J* 7.5, H-5*), 7.33 (3 H, dd, *J* 7.5 and 6.8, fluorenyl CH, NH), 7.42 (2 H, d, *J* 6.8, fluorenyl CH), 7.53 (2 H, d, *J* 8.4, 4-Bu^{t}C$_6H_4$CO), 7.69 (2 H, dd, *J* 7.5 and 6.8, fluorenyl CH), 7.89 (2 H, d, *J* 7.5, fluorenyl CH), 7.94 (1 H, d, *J* 7.3, H-6*), 7.98 (2 H, dd, *J* 2.7, 8.4, 4-Bu^{t}C$_6H_4$CO), 11.20 (1 H, br s, NH#), 12.80 (1 H, br s, OH#); *m/z* (ESI) 652 (100%, [M + H]$^+$).

([2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)ethyl]-{2-[6-(4-methoxybenzoylamino)purin-9-yl]acetyl}amino)acetic acid 24

To ester **20** (0.92 g, 1.30 mmol) dissolved in dichloromethane (20 mL) were added 1,3-dimethoxybenzene (0.23 mL, 1.82 mmol) and TFA (15.0 mL, 196.0 mmol) and the mixture was stirred for 6 h at room temperature. The solution was evaporated *in vacuo*, and the residue was coevaporated with acetonitrile (5×). The residue was dissolved in methanol (1 mL), diethyl ether (4.5 mL) was added, and the mixture was stored at 4 °C overnight. The resulting gum was triturated with diethyl ether, filtered and recrystallized from methanol (80 mL) to afford a white powder (0.59 g, 70%), mp 160.8–163.9 °C; R_f 0.76 (CH_2Cl_2–MeOH 6:4) (Found: C, 62.8; H, 4.65; N, 14.9. Calc. for $C_{34}H_{31}N_7O_7$: C, 62.9; H, 4.8; N, 15.1%); λ_{max}(EtOH)/nm 206 (lg ε 4.74), 266 (4.43), 278 (4.43), 289 (4.44), 299sh (4.35); ν_{max}/cm^{-1} 3440m, 3222w, 3102w, 3069w, 2978w, 2946w, 1713m, 1693w, 1647m, 1603m, 1582w, 1525m, 1500m, 1411w, 1252s, 1178m, 762m; δ_H (500 MHz, rotamers) 3.15/3.59 (2 H, 2 m, CH_2), 3.18 (2 H, s, CH_2), 3.86 (3 H, s, CH_3O), 4.03/4.10 (2 H, 2 m, CH_2), 4.22 (1 H, m, CH), 4.30/4.39 (2 H, 2 m, CH_2), 5.20/5.37 (2 H, 2 s, adenyl CH_2), 7.08 (2 H, d, *J* 8.7, anisoyl CH), 7.29/7.42 (1 H, 2 br t, NH), 7.32 (2 H, dd, *J* 7.4 and 7.3, fluorenyl CH), 7.41 (2 H, dd, *J* 7.4 and 7.3, fluorenyl CH), 7.70 (2 H, d, *J* 7.4, fluorenyl CH), 7.88 (2 H, d, *J* 7.4, fluorenyl CH), 8.06 (2 H, d, *J* 8.7, anisoyl CH), 8.33 (1 H, s, H-8*), 8.62/8.67 (1 H, 2 s, H-2*); *m/z* (ESI) 650 (100%, [M + H]$^+$).

{[2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)ethyl]-[2-(2-isobutyrylamino-6-oxo-1,6-dihydropurin-9-yl)acetyl]amino}acetic acid 25

To ester **21** (0.68 g, 0.79 mmol) suspended in dichloromethane (20 mL), was added 1,3-dimethoxybenzene (0.125 mL, 0.95 mmol) followed by TFA (7.34 mL, 95.3 mmol) and the mixture was stirred for 6 h at room temperature. The solution was evaporated *in vacuo*, and the residue was coevaporated with EtOAc (4×). The solid residue was triturated with EtOAc, and filtered (0.48 g, quant.), mp 202.0–206.0 °C and then recrystallized from ethanol (40 mL) to furnish a white powder (0.21 g, 45%), mp 208.8–210.6 °C (decomp.); from the mother liquor a further crop was obtained (0.026 g, 5%), mp 206.0–208.1 °C. Overall yield of the recrystallized product: 0.236 g, 50%. This reaction was repeated on a 3.0 mmol scale and afforded a quantitative yield of the crude acid **25** (1.80 g), mp 202.0–206.0 °C; R_f 0.16 (MeCN–MeOH–AcOH 8:1:1) (Found: C, 59.7; H, 5.3; N, 16.1. Calc. for $C_{30}H_{31}N_7O_7$: C, 59.9; H, 5.2; N, 16.3%); λ_{max}(EtOH)/nm 205 (lg ε 4.79), 221sh (4.24), 256sh (4.45), 262 (4.48), 278sh (4.29), 289sh (4.13), 300 (4.03); ν_{max}/cm^{-1} 3350w, 3131w, 3067w, 2965w, 2940w, 1693s, 1674m, 1610m, 1570m, 1542m, 1485w, 1411m, 1250m, 1154w, 757w, 743w; δ_H (500 MHz, rotamers) 1.11 [6 H, d, *J* 6.6, (C*H*$_3$)$_2$CH], 2.75 [1 H, pseudoquintet, *J* 6.6, (CH$_3$)$_2$C*H*], 3.13 (1 H, m), 3.35 (1.8 H, m, partly shielded by the water signal) and 3.49 (1.2 H, m, 2 × CH_2), 4.02/4.29 (2 H, 2 s, CH_2), 4.25 (1 H, m, CH), 4.32/4.38 (2 H, 2 d, *J* 5.8, 6.5, CH_2), 4.97/5.13 (2 H, 2 s, guanyl CH_2), 7.26/7.46 (1 H, 2 br t, NH*), 7.33 (2 H, m, fluorenyl CH), 7.41 (2 H, dd, *J* 7.2 and 7.2, fluorenyl CH), 7.68 (2 H, dd, *J* 7.5, 7.2, fluorenyl CH), 7.82 (1 H, s, H-8), 7.88 (2 H, d, *J* 7.5, fluorenyl

CH), 11.59/11.65 (1 H, 2 s, NH*), 12.07 (1 H, s, NH)*, 12.50 (1 H, br s, OH*); *m/z* (APCI) 602 (100%, $[M + H]^+$).

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II.

A REGIOSELECTIVE SYNTHESIS OF ALKYL 2-(GUANIN-9-YL)ACETATES AS PNA BUILDING BLOCKS FROM 7-(4-NITROBENZYL)GUANINE DERIVATIVES

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Guanine derivatives substituted at N^7 with 4-R-benzyl groups (R = H, MeO, NO_2) have been evaluated in the regioselective N^9-alkylation of guanine. Given the capricious removal of (substituted) benzyl groups from guanine derivatives and pent-4-enoylation of guaninium hydrochloride, an improved alternative approach has been elaborated consisting in the pent-4-enoylation and per-*O*-acetylation of guanosine (**8**), 4-nitrobenzylation at N^7 followed by *N*-glycoside hydrolysis (**10**), N^9-alkylation (**12**) and deprotection with sodium dithionite to afford the peptide nucleic acid building block *tert*-butyl [N^2-(pent-4-enoyl)guanin-9-yl]-acetate (**14**) in 36% overall yield. This avoids N^7 regioisomer formation, solubility problems and any chromatographic purification. A remarkable influence of the *O*- and/or N^2-acyl groups on the stability of *N*-glycosidic bond and reactivity of 2-amino group was observed. The structure of a pyrimidine by-product **16** arising from the imidazole ring-opening of guaninium salt **4d** in alkaline medium has been elucidated by 2D NMR.

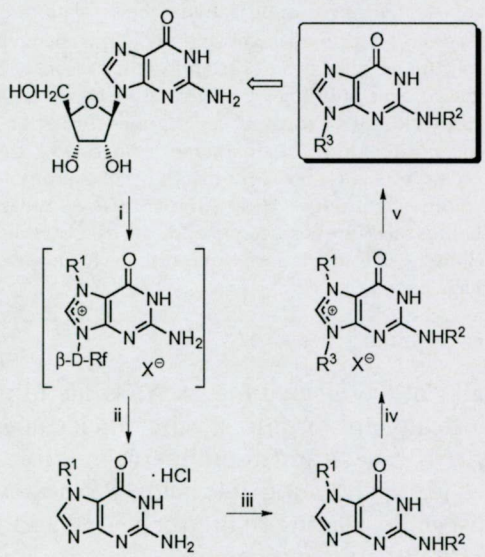
Keywords: Guanine; Regioselective alkylation; Guanosine; Nucleoside analogues; Nucleosides; Purines; Peptide nucleic acids.

The regioselective alkylation of guanine at N^9 is an important synthetic route to pharmaceutically important acyclic nucleoside analogues (e.g. acyclovir, ganciclovir, HPMPG) and monomeric building blocks of oligonucleotide analogues (e.g. peptide nucleic acids, PNA); however these reactions are rarely regiospecific, leading to mixtures of 9- and 7-alkylated products which can be very difficult to separate[1].

The regioselectivity and yield in the synthesis of 7- or 9-substituted guanosine analogues can be high in the alkylation of persilylated guanine derivatives e.g. O^6-(*N*,*N*-diphenylcarbamoyl)-N^2-isobutyrylguanine with β-*O*-activated alkylation reagents[1,2] e.g. peracylated sugars[3]. 9-Alkoxyalkyl-

ated products can be obtained in high yields even if an N^9/N^7 isomeric mixture is formed while the N^7 to N^9 rearrangement takes place upon heating[4-10] or even at room temperature in DMF [11].

The regioselectivity of the alkylation with non-β-*O*-activated, small-sized alkyl halides (e.g. alkyl haloacetates in the synthesis of PNA monomers) under basic conditions (e.g. K_2CO_3) is still inadequate. Constraining guanine into its lactim form e.g. in the case of 2-amino-6-chloropurine[12-17], 2-amino-6-(arylsulfanyl)purines[18] or O^6-(*N*,*N*-diphenylcarbamoyl) derivatives[19] improves the N^9/N^7 isomer ratio, but it is not sufficient requirement since the highest yield of N^9-isomer was around 75% and chromatographic purification could not be avoided in every case, furthermore 2-amino-6-chloropurine is mutagenic and expensive, 2-amino-6-(arylsulfanyl)purines require a strong acidic treatment, the *N*,*N*-diphenylcarbamoyl group can be labile[20,21] and this imposes limitations on its applicability. There is a third approach, however, affording exclusively 9-alkylated derivatives. Izawa et al. reported an N^9-regioselective substitution starting from guanosine[22,23]. In their approach guanosine was protected on N^7 with a (substituted) benzyl group; then, after acid hydrolysis of *N*-glycosidic bond and



SCHEME 1

The reaction sequence followed by Izawa et al.[22,23] Reagents and conditions: (i) R^1X, (ii) HCl, (iii) R^2X, (iv) R^3X, (v) removal of R^1. β-D-Rf = β-D-ribofuranosyl, R^1 = substituted benzyl, R^2 = acyl, R^3 = alkyl

acylation of the amino group, 7-substituted benzyl-N^2-acylguanines were alkylated selectively at N^9, then deprotected at N^7 in a good overall yield (ca. 80%, Scheme 1). In this case the (substituted) benzyl is not only a protective group but it activates the purine ring in alkylation[22,23].

RESULTS AND DISCUSSION

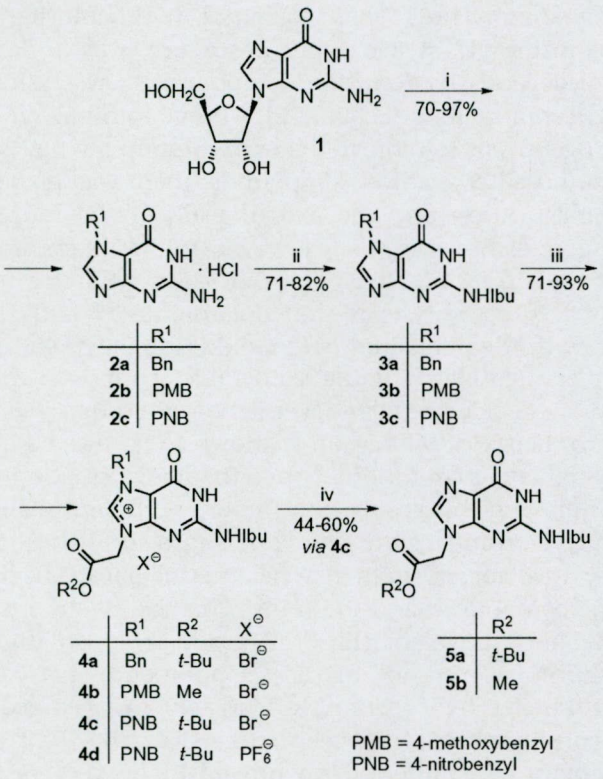
The problem of the regioselectivity of the alkylation of guanine derivatives was present in the reaction of *tert*-butyl bromoacetate with N^2-isobutyrylguanine and O^6-(*N*,*N*-diphenylcarbamoyl)-N^2-isobutyrylguanine as the first step in the synthesis of Fmoc/acyl-protected PNA monomers[24]. In our initial study the Izawa method has been adopted for the regioselective N^9-alkylation of guanine. Thus, 7-benzylguanine hydrochloride[25] (**2a**), available from guanosine (**1**) in a one-pot procedure, was acylated with isobutyric anhydride and the resulting compound **3a** was allowed to react with *tert*-butyl bromoacetate (Scheme 2). The guaninium salt **4a** was subjected to hydrogenolysis to remove the benzyl group giving rise to ester **5a**. All the steps afforded crystalline compounds, there was no need for chromatographic purification and the overall yield of the four-step process, starting from guanosine, was 37%. However, the last step often proved to be capricious and irreproducible. The notorious behavior of benzylated purines under hydrogenolysis is well documented[26] and alternative *N*-debenzylation with the potassium *tert*-butoxide–dimethyl sulfoxide–oxygen system is not compatible with the vulnerable, base-sensitive groups[27,28]. Consequently, we decided to abandon the benzyl group.

The 4-methoxybenzyl (PMB) group is known to be cleaved from ethers or amines under oxidative conditions[29] thus the above reaction was repeated (Scheme 2) employing this group with the only difference being the application of methyl bromoacetate as alkylating agent (the corresponding *tert*-butyl ester was not crystalline). The intermediates **2b**–**4b** were again nice crystalline compounds and the transformations were uneventful until the last step. The removal of the PMB group from **4b** under oxidative (cerium ammonium nitrate[30], DDQ[31], potassium peroxodisulfate[32]), catalytic[33] or transfer hydrogenolytic[34] or acid ($AlCl_3$-anisole[35,36], CBr_4-methanol[37]) conditions has not been successful. The PMB group in the guaninium moiety of compound **4b** proved to be very resistant to the above reagents.

The reductive deprotection of 4-nitrobenzyl (PNB) group is a well-established method, therefore we have embarked upon the synthesis and use of 7-(4-nitrobenzyl)guanines. The reaction sequence carried out with

benzyl and PMB groups has been repeated with 4-nitrobenzyl bromide and standard transformations led to guaninium salt **4c** (Scheme 2). The removal of PNB group has been accomplished with Zn/AcOH or In/AcOH and ester **5a** has been obtained from guanosine in 38–40% overall yield; however, the last step required chromatographic purification and removal of zinc acetate and the yellow by-product was cumbersome.

In our experience the removal of guanine isobutyryl group was not quantitative in the cleavage and deprotection of PNA oligomers[38]. Therefore a change in the protective group was needed. Pent-4-enoyl group seems to be a better choice since it can be removed, in addition to the conventional ammonia, with iodine as well[39–41].



	R^1
2a	Bn
2b	PMB
2c	PNB

	R^1
3a	Bn
3b	PMB
3c	PNB

	R^1	R^2	$X^{\ominus}$
4a	Bn	*t*-Bu	$Br^{\ominus}$
4b	PMB	Me	$Br^{\ominus}$
4c	PNB	*t*-Bu	$Br^{\ominus}$
4d	PNB	*t*-Bu	$PF_6^{\ominus}$

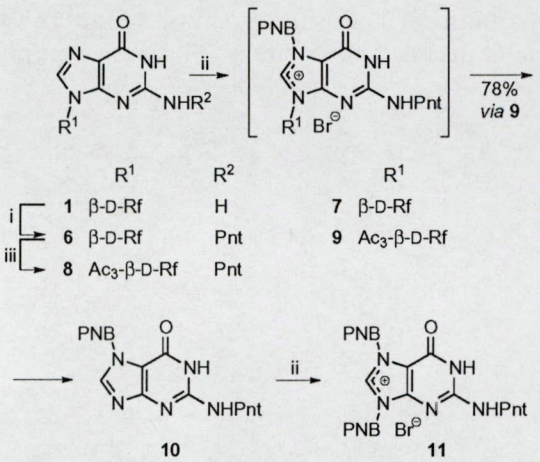
	R^2
5a	*t*-Bu
5b	Me

SCHEME 2
Reagents and conditions: (i) 1. R^1Br, DMSO; 2. HCl, then MeOH. (ii) Ibu_2O, Et_3N, DMF, 150 °C, 3 h. (iii) $BrCH_2COOR^2$, DMF, 60 °C, 24 h. (iv) Zn, aq. AcOH, r.t., 18 h

7-(4-Nitrobenzyl)guanine hydrochloride (**2c**) did not dissolve well during the pent-4-enoylation, hence another route was needed to protect the 2-amino group. Considering the high price of pent-4-enoic anhydride, the reactivity of the amino group and the solubility of compounds the sequence N^2-acylation, N^7-protection, *N*-glycoside hydrolysis, N^9-alkylation seemed to be the only good choice.

N^2-(Pent-4-enoyl)guanosine (**6**) was obtained in a high yield by temporary protection of hydroxy groups with the electron-donating trimethylsilyl group[40,42]. The 7,9-bis(4-nitrobenzyl)guaninium salt **11**, detected in the reaction mixture by TLC/MS, was the product in subsequent reaction with 4-nitrobenzyl bromide (Scheme 3), 7-(4-nitrobenzyl)-N^2-(pent-4-enoyl)guanine (**10**) could not even be detected. The formation of 7,9-bis(arylmethyl)guanines under forced conditions (elevated temperatures and prolonged reaction times) has been reported[43].

Enhancing the stability of the *N*-glycosidic bond is imperative for the monoalkylation of guanosine derivatives by introduction of electron-withdrawing, e.g. acetyl groups to the hydroxy groups of the saccharide moiety. Pent-4-enoylation of 2′,3′,5′-tri-*O*-acetylguanosine was a slow and low-yielding process even at higher temperature, therefore 2′,3′,5′-tri-*O*-



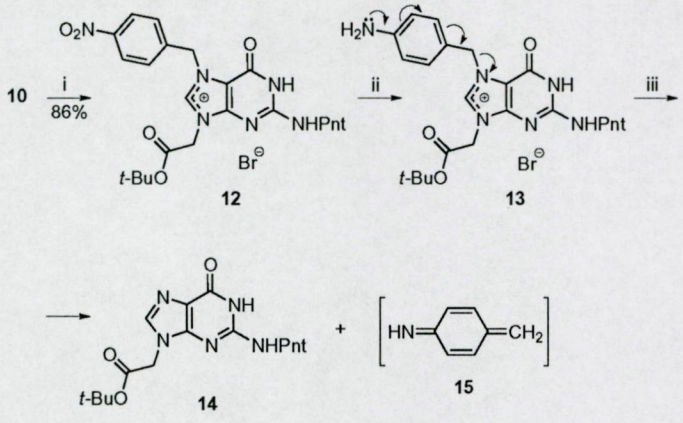
SCHEME 3

Reagents and conditions: (i) 1. TMSCl, pyridine, r.t., 1 h; 2. pent-4-enoic anhydride, pyridine, r.t., 16 h; 3. water, 0–5 °C, 5 min; 4. aq. NH_3, r.t., 30 min. (ii) 1–10 equiv. 4-nitrobenzyl bromide, DMF, r.t., up to 6 days, see the text. (iii) Ac_2O, pyridine, DMF, r.t., 16 h. (i) + (iii) 71%. β-D-Rf = β-D-ribofuranosyl, Ac_3-β-D-Rf = 2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl

acetyl-N^2-(pent-4-enoyl)guanosine (**8**) was synthesized by acetylation of N^2-(pent-4-enoyl)guanosine (**6**). The formation of guaninium salt **9** was slow but compound **11** formed only in a negligible amount. The reaction was complete after 2.5 days with an usual 4-equivalent excess of the reagent. The excess of 4-nitrobenzyl bromide was scavenged by pyridine in order to avoid the formation of the dialkylated product **11** during evaporation of the solvent (DMF). 4-Nitrobenzylpyridinium bromide and compound **10** was separated with extraction after thermolysis of the *N*-glycoside taking place at 70 °C without acid treatment.

The extent of the influence of the *O*- and/or N^2-acyl groups on the stability of *N*-glycosidic bond and the reactivity of N^2 was notable in the above reactions. 7-(4-Nitrobenzyl)guanosinium ion was stable but it decomposed spontaneously in the absence of acid after acylation on N^2. Acetylation of hydroxy groups in compound **7** stabilized the *N*-glycosidic bond at room temperature but thermolysis took place without acid at 70 °C. The acetyl groups in 2′,3′,5′-tri-*O*-acetylguanosine withdrew the electron density of the purine ring and, at the same time, from the 2-amino group to such an extent that the acylation was not complete even after a prolonged time at 100 °C. On the other hand, trimethylsilyl group activated the 2-amino group and the acylation took place smoothly.

Alkylation of compound **10** affording guaninium salt **12** (Scheme 4), with 3 equivalents of *tert*-butyl bromoacetate at 70 °C was complete overnight.



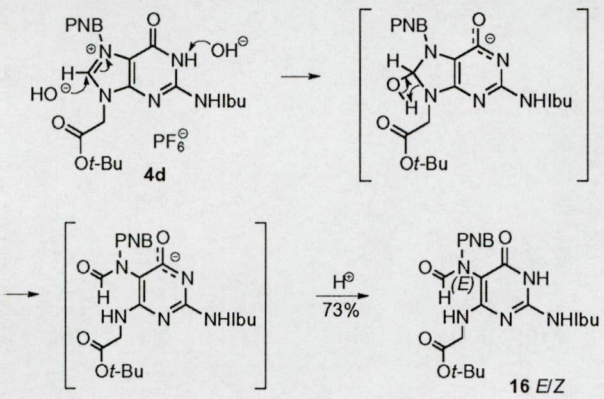
SCHEME 4

Reagents and conditions: (i) 3 equiv. *tert*-butyl bromoacetate, DMF, 70 °C, 16 h; (ii) 4 equiv. $Na_2S_2O_4$, aq. acetone, pH 7.0, r.t., 30 min; (iii) 70 °C, 16 h; (ii) + (iii) 76%

As mentioned earlier, the purification of ester **5a** after PNB removal with Zn/acetic acid was too laborious, therefore other reducing agents have been studied.

Sodium sulfide was successfully used for the deprotection of 4-nitrobenzyl esters and carbamates[44] and our initial studies were conducted with salt **4d**. In the sodium sulfide treatment, a new substance **12** (Scheme 5) emerged, the polarity of which was similar to the expected product **5a**. However, in its MS spectrum the molecular ion $[M + H]^+$ was observed at *m/z* 489 instead of at the expected value *m/z* 336. Imidazole ring opening is known to occur in alkaline solution as C-8 is electrophilic in salts[45] like **4d**. The exact position of formyl group (N^4 or N^5) has not been ascertained earlier[46] or only unconvincingly proved[47]. Our 2D NMR measurements (HSQC, HMBC) have unequivocally demonstrated (Fig. 1) that the formyl group is located on N^5 confirming that the ensuing ring scission takes place between C-8 and N-9 atoms of guaninium salt **4d**. Compound **16** exists as a 7:1 mixture of rotamers at room temperature in DMSO-d_6 solution.

Reduction of the nitro group in compound **12** was complete within 30 min in the presence of 4 equivalents of sodium dithionite even at pH 4, not only at pH 8–9 where deprotection of 4-nitrobenzyloxycarbonyl group was achieved[44]. To enhance the rearrangement of 4-aminobenzyl group leading to the deprotection, the reaction mixture was heated at 70 °C in a phosphate buffer (pH 7) and acetone was employed to obtain a homogenous solution (the intermediacy of **13** was verified by TLC/MS, Scheme 4).



SCHEME 5
Imidazole ring-opening of guaninium salt **4d** in alkaline solution

Based on the observation that electron-donating substituents on phenyl ring and/or on α-carbon atom promote the rearrangement by stabilizing the positive charge on benzyl methylene group in the removal of 4-nitrobenzyl carbamates[48], it was not suprising that heating of the reaction mixture was required for the deprotection as, in our case, a positively charged substituent was attached to the benzyl methylene group decreasing the stability of the formed cation.

Iminoquinomethane **15** was the elusive by-product of this deprotection regime. This compound has never been isolated in pure form due to its instability and tendency to polymerize[48–50]. We have been able to detect

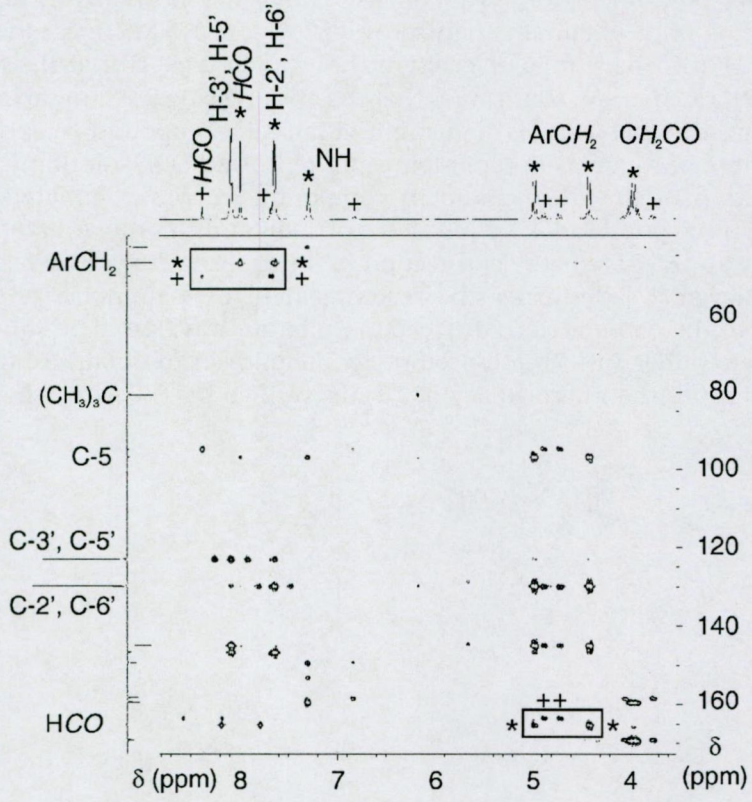


FIG. 1

HMBC spectrum (detail) of compound **16**. Crucial correlations are highlighted by boxes, primed numbers denote atoms in the benzene ring. Some signals are doubled in the major (*) and minor (+) rotamers, e.g. the formyl protons at 7.99 (*) and 8.38 ppm (+), respectively

in the ESI mass spectrum peaks of this substance at *m/z* 106 and *m/z* 138 corresponding to ions $[M + H]^+$ and $[M + MeOH + H]^+$, respectively, but our further attempts to characterize this compound have not been rewarding. The presence of this substance as a contaminant was obvious in both the yellow-colored aqueous and the organic phases. It was attempted to scavenge the substance with sulfites[49] (oxidized form of dithionite), however, it was impossible to completely remove this substance from the product. The slightly yellowish amorphous product **14** was easily obtained by washing with ethyl acetate; it was completely pure according to TLC and NMR analyses.

The site of the alkylation (N^9 vs N^7) in guanine derivatives can be unequivocally ascertained by ^{1}H [18,51,52], ^{13}C [24,51,52] and ^{15}N NMR [53] or MS/MS [54] methods. In this study our ^{13}C NMR method[24] was used. The structures of

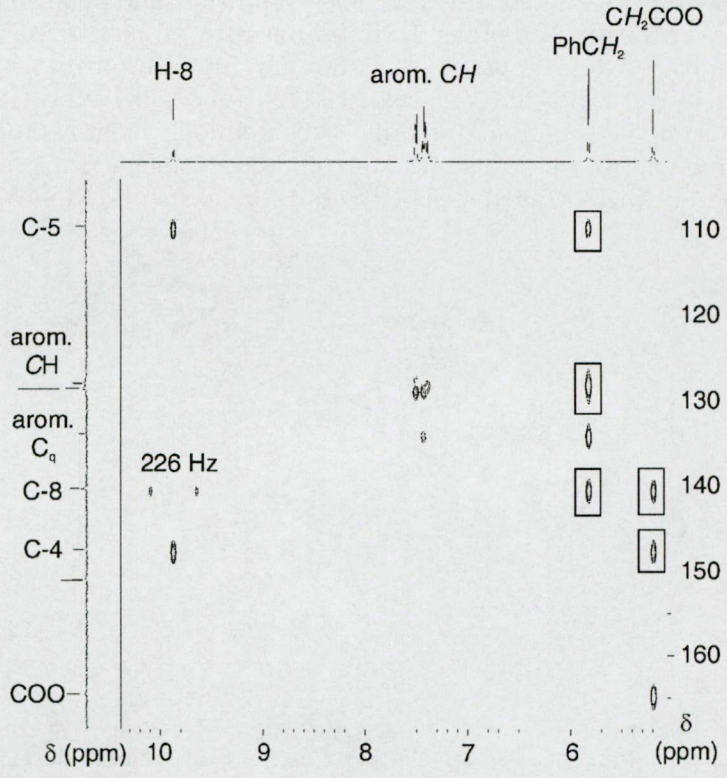
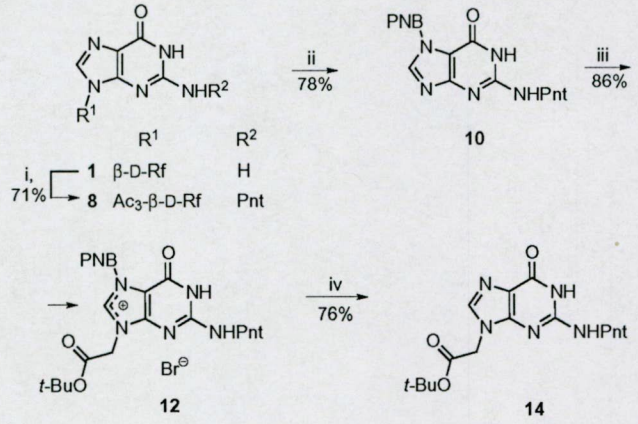


FIG. 2
HMBC spectrum (detail) of compound **4a**. Crucial correlations are highlighted by boxes

compounds **3**, **10** and **14** were corroborated by the chemical shift difference parameters $a = \delta_{C\text{-}4} - \delta_{C\text{-}5}$, $b = \delta_{C\text{-}8} - \delta_{C\text{-}5}$ and $c = \delta_{C\text{-}5} - \delta_{C\text{-}1'}$, involving C-1′, C-4, C-5 and C-8 which are the most sensitive to the site of alkylation (data not shown). It is noteworthy that the guaninium salts display an unusually large heteronuclear coupling constant between H-8 and C-8 (e.g. $^1J_{H\text{-}8,C\text{-}8}$ = 226 (**4a**) and 227 Hz (**4c**)), which indicates the imidazolium substructure[55–57]. The structure of compound **4a** has been corroborated by HSQC and HMBC investigations as well (Fig. 2).

In our study directed to 9-substituted guanines, the application of guanine derivatives substituted at N^7 with different benzyl groups (benzyl, 4-methoxybenzyl, 4-nitrobenzyl) has been evaluated. The general sequence exemplified in Schemes 1 and 2 proved to be problematic in terms of easy and reproducible removal of protecting group at N^7 and acylation of compounds **2** with pent-4-enoic anhydride. Our improved alternative approach described in this paper comprises the following steps: (i) pent-4-enoylation of guanosine at N^2 (**6**), (ii) per-*O*-acetylation (**8**), (iii) 4-nitrobenzylation at N^7 (**9**), (iv) hydrolysis of the *N*-glycoside **9** (**10**), (v) N^9-alkylation (**12**) and (vi) deprotection with sodium dithionite. This seemingly lengthy procedure can be combined into four distinct, well-reproducible steps with standard transformations affording the PNA building block *tert*-butyl [N^2-(pent-



SCHEME 6

Reagents and conditions: (i) 1. TMSCl, pyridine, r.t., 1 h; 2. 1.25 equiv. pent-4-enoic anhydride, pyridine, r.t., 16 h; 3. water, 0–5 °C, 5 min; 4. aq. NH_3, r.t., 30 min; 5. Ac_2O, pyridine, DMF, r.t., 16 h. (ii) 4 equiv. 4-nitrobenzyl bromide, DMF, r.t., 60 h. (iii) 3 equiv. *tert*-butyl bromoacetate, DMF, 70 °C, 16 h. (iv) 1. 4 equiv. $Na_2S_2O_4$, aq. acetone, pH 7.0, r.t., 30 min; 2. 70 °C, 16 h. β-D-Rf = β-D-ribofuranosyl, Ac_3-β-D-Rf = 2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl

4-enoyl)guanin-9-yl]acetate (14) with no N^7 regioisomer formation, solubility problems or chromatographic purification (Scheme 6) with the same 36% overall yield as in the case of Izawa et al. method. Main advantages of our synthesis are the acylation of N^2 amino group at room temperature due to the high solubility of the starting compound and a reproducible removal of the 4-nitrobenzyl group. Attempts at the conversion of 14 to a guanine PNA monomer are under way. The extent of the influence of the *O*- and/or N^2-acyl groups on the stability of *N*-glycosidic bond and reactivity of 2-amino group is remarkable. The site of the alkylation in 7- and 9-alkylated guanine derivatives has been corroborated by chemical shift differences of ^{13}C NMR spectra. Position of the formyl group (N^5) in the ring-opened derivative 16 formed from guaninium salt 4d under alkaline conditions (Scheme 5) has been unravelled by 2D NMR.

EXPERIMENTAL

The following abbreviations are employed: Bn (benzyl), *t*-Bu (*tert*-butyl), TMSCl (chlorotrimethylsilane), DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone), Et_2O (diethyl ether), DMF (*N*,*N*-dimethylformamide), DMSO (dimethyl sulfoxide), ESI (electrospray ionization), EtOAc (ethyl acetate), Fmoc (fluoren-9-ylmethoxycarbonyl), Ibu (isobutyryl), MeOH (methanol), PMB (4-methoxybenzyl), PNB (4-nitrobenzyl), Pnt (pent-4-enoyl), PNA (peptide nucleic acid(s)), r.t. (room temperature), TEA (triethylamine).

Chemicals were purchased from Aldrich, Fluka, Merck or Reanal (Budapest, Hungary). Pent-4-enoic anhydride was alternatively synthesized from pent-4-enoic acid as described in literature[58,59]. Compound 2a was prepared as described[25]. Compounds 2b and 2c were prepared in an analogous way[60]. 4-Methoxybenzyl bromide is prone to decomposition and it was freshly prepared prior to use from the corresponding alcohol[61]. Anhydrous solvents were prepared as described[62]. Organic solutions were dried using anhydrous $MgSO_4$ and evaporated in Büchi rotary evaporators. TLC: Kieselgel 60 F_{254} (Merck); solvent systems: CH_2Cl_2–MeOH 9:1 (S1), CH_2Cl_2–MeOH 95:5 (S2), CH_2Cl_2–iPrOH 5:0.25 (S3); visualization: UV light, H_2SO_4/ethanol. All guaninium salts gave oval spots with significant tailing. M.p.: Electrothermal IA 8103 apparatus. Elemental analysis: Perkin–Elmer CHN analyzer model 2400. UV: PE Lambda 10 spectrometer, λ_{max} in nm (log ε); sh, shoulder. Stock solutions were made in ethanol except of compounds 3b and 10, in which cases adding DMSO was necessary to achieve clear solutions. In the case of latter samples and TEAc buffer the region below 220 nm in UV spectra was uncertain, because these compounds have high absorbance in this region. NMR: Bruker Avance DRX 500 spectrometer (^1H: 500.13 MHz, ^{13}C: 125.76 MHz), DMSO-d_6 solutions; δ (ppm), *J* (Hz). Spectral patterns: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; br, broad; deut, deuterable. The superscripts *, # denote interchangeable assignments. For the 2D experiments (HMQC, HMBC), standard Bruker software packages (INV4GSSW, INV4GSLRNDSW) were used. Mass spectrometry: Finnigan MAT TSQ 7000, electrospray (ESI) technique. TLC/MS: the analyte solution has been applied onto a 5 cm wide silica gel TLC plate as a band to obtain sufficient material. After developing in a solvent system, the appropriate band was scraped off with a spatula, the silica gel was

suspended in MeOH (100 µl), sonicated, centrifuged and the supernatant was used for MS analysis.

7-Benzyl-N^2-isobutyrylguanine (3a)

Compound[25] 2a (8.393 g, 30.2 mmol) suspended in anhydrous DMF (90 ml) was treated with isobutyric anhydride (12.50 ml, 75.5 mmol) and TEA (8.43 ml, 60.4 mmol) and the mixture was heated with stirring at 150 °C for 3 h. The homogeneous solution was evaporated in vacuo, coevaporated with MeOH (2×), triturated under cold water, filtered and dried. The crude product (8.913 g) was boiled with ethanol (200 ml), the obtained solution filtered and the filtrate was evaporated to dryness. Trituration under cold ether gave the title compound (6.663 g, 70.8%) as beige crystals, m.p. 176–178 °C. R_F 0.65 (S1). For $C_{16}H_{17}N_5O_2$ (311.3) calculated: 61.7% C, 5.5% H, 22.5% N; found: 61.55% C, 5.4% H, 22.7% N. UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 202 sh (4.22), 267 (3.98); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 267 (3.97); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 272 (3.82). ^{1}H NMR: 1.12 d, 6 H, J = 6.8 ($(CH_3)_2CH$); 2.75 pseudo t, 1 H, J = 6.8 ($(CH_3)_2CH$); 5.52 s, 2 H (CH_2); 7.29–7.34 m, 5 H (C_6H_5); 8.33 s, 1 H (H-8); 11.49 s, 1 H (NH); 12.13 s, 1 H (NH). ^{13}C NMR (J-modulated spin-echo): 18.65 ($(CH_3)_2CH$); 34.52 ($(CH_3)_2CH$); 49.08 ($PhCH_2$); 110.99 (C-5); 127.32, 127.64, 128.45 (arom. CHs); 137.09 (arom. C_q); 144.08 (C-8); 147.00, 152.49, 157.07 (C-2, C-4, C-6); 179.75 (iPrCO). MS (ESI), *m/z* (rel.%): 623 (30) [2 M + H]$^+$), 312 (100) [M + H]$^+$.

N^2-Isobutyryl-7-(4-methoxybenzyl)guanine (3b)

Prepared from 7-(4-methoxybenzyl)guanine hydrochloride[60] (2b; 7.8 mmol scale) as described for compound 3a (1.989 g, 74.7%). An analytical sample was obtained by recrystallizing the product from acetonitrile (4.6 g/100 ml), m.p. 185.9–186.6 °C (acetonitrile). R_F 0.48 (S1). For $C_{17}H_{19}N_5O_3$ (341.4) calculated: 59.8% C, 5.6% H, 20.5% N; found: 59.6% C, 5.65% H, 20.3% N. UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 223 (4.13), 267 (4.08); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 267 (4.00); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 271 (3.90). ^{1}H NMR: 1.10 d, 6 H, J = 6.8 ($(CH_3)_2CH$); 2.74 pseudo t, 1 H, J = 6.8 ($(CH_3)_2CH$); 3.71 s, 3 H (CH_3O); 5.42 s, 2 H (CH_2); 6.88 d, 2 H, J = 8.5 (ArH); 7.33 d, 2 H, J = 8.5 (ArH); 8.31 s, 1 H (H-8); 11.51 s, 1 H (NH); 12.13 s, 1 H (NH). ^{13}C NMR (J-modulated spin-echo): 18.75 ($(CH_3)_2CH$); 34.60 ($(CH_3)_2CH$); 48.66 ($ArCH_2$); 54.98 (CH_3O); 110.92 (C-5); 113.92 (C-3', C-5'); 129.12, 129.17 (C-2', C-6', C-1'); 143.97 (C-8); 147.02, 152.57, 157.18, 158.86 (C-2, C-4, C-6, C-4'); 179.84 (iPrCO). MS (ESI), *m/z* (rel.%): 683 (32) [2 M + H]$^+$, 342 (100) [M + H]$^+$.

N^2-Isobutyryl-7-(4-nitrobenzyl)guanine (3c)

Prepared from 7-(4-nitrobenzyl)guanine hydrochloride[60] (2c; 8.6 mmol scale) as described for compound 3a (2.512 g, 81.8%). The substance was sufficiently pure for further transformations, however, it can be recrystallized from MeOH (2.0 g/150 ml), m.p. > 260 °C. R_F 0.68 (S1). For $C_{16}H_{16}N_6O_4$ (356.3) calculated: 53.9% C, 4.5% H, 23.6% N; found: 53.75% C, 4.6% H, 23.4% N. UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 201 (4.22), 218 (4.05), 267 (4.11); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 267 (4.15); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 272 (4.11). ^{1}H NMR: 1.10 d, 6 H, J = 6.7 ($(CH_3)_2CH$); 2.72 pseudo t, 1 H, J = 6.7 ($(CH_3)_2CH$); 5.66 s, 2 H (CH_2); 7.53 d, 2 H, J = 8.3 (ArH); 8.18 d, 2 H, J = 8.3 (ArH); 8.38 s, 1 H (H-8); 11.55 s, 1 H (NH); 12.13 s, 1 H (NH). ^{13}C NMR (J-modulated spin-echo): 18.77

($(CH_3)_2CH$); 34.64 ($(CH_3)_2CH$); 48.61 ($ArCH_2$); 111.06 (C-5); 123.72, 128.43 (arom. CHs); 144.51 (C-8); 144.64, 146.97, 147.29, 152.51, 157.36 (C-2, C-4, C-6, C-1', C-4'); 179.92 (iPrCO). MS (ESI), *m/z* (rel.%): 713 (5) [2 M + H]$^+$, 357 (100) [M + H]$^+$.

7-Benzyl-9-[(*tert*-butoxycarbonyl)methyl]-N^2-isobutyrylguaninium Bromide (4a)

Compound 3a (1.557 g, 5.0 mmol) dissolved in warm DMF (20 ml) was allowed to react with *tert*-butyl bromoacetate (0.813 ml, 5.5 mmol) at 60 °C for 24 h. The solution was evaporated in vacuo, coevaporated with EtOAc (3×) and EtOAc was added (20 ml). The resulting oil slowly crystallized in a refrigerator (1.801 g, 71%), m.p. 158 °C (dec., EtOAc). R_F ca. 0.2 (S1). For $C_{22}H_{28}BrN_5O_4$ (506.4) calculated: 52.2% C, 5.6% H, 13.8% N; found: 52.0% C, 5.7% H, 13.65% N. UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 201 (4.26), 271 (3.97); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 278 (3.70); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 243 (3.96). ^{1}H NMR: 1.12 d, 6 H, J = 6.9 ($(CH_3)_2CH$); 1.44 s, 9 H (*t*-Bu); 4.02 pseudo t, 1 H, J = 6.9 ($(CH_3)_2CH$); 5.19 s, 2 H (CH_2COO); 5.82 s, 2 H ($PhCH_2$); 7.39–7.50 m, 5 H (C_6H_5); 9.87 s, 1 H (H-8); 12.10 br s, 1 H (NH); 12.70 s, 1 H (NH). ^{13}C NMR (*J*-modulated spin-echo, HSQC, HMBC experiments): 18.56 ($(CH_3)_2CH$); 27.48 ($(CH_3)_3C$); 34.72 ($(CH_3)_2CH$); 46.40 (CH_2COO); 51.65 ($PhCH_2$); 83.57 ($(CH_3)_3C$); 109.61 (C-5); 128.16, 128.80 (arom. CHs); 134.04 (arom. C_q); 140.28 (C-8); 147.55 (C-4); 151.13 (C-2, C-6); 164.62 (COO); 180.52 (iPrCO). MS (ESI), *m/z* (rel.%): 426 (100) M$^+$, guaninium ion.

N^2-Isobutyryl-7-(4-methoxybenzyl)-9-[(methoxycarbonyl)methyl]guaninium Bromide (4b)

Prepared from compound 3b (5.33 mmol) as described for compound 4a (2.382 g, 90%), m.p. 147.3–150.6 °C (EtOAc). R_F ca. 0.2 (S1). For $C_{20}H_{24}BrN_5O_5$ (494.3) calculated: 48.6% C, 4.9% H, 14.2% N; found: 48.55% C, 5.0% H, 14.35% N. UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 200 sh (4.45), 270 (4.02); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 273 (3.83); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 273 (3.80). ^{1}H NMR: 1.11 d, 6 H, J = 6.7 ($(CH_3)_2CH$); 2.80 pseudoquintet, 1 H, J = 6.7 ($(CH_3)_2CH$); 3.74 and 3.76 each s, 6 H 2 × (CH_3O); 5.30 s, 2 H (CH_2COO); 5.72 s, 2 H ($ArCH_2$); 6.97 d, 2 H, J = 8.3 (ArH); 7.49 d, 2 H, J = 8.3 (ArH); 9.77 s, 1 H (H-8); 12.05 br s, 1 H (NH); 12.60 s, 1 H (NH). ^{13}C NMR (*J*-modulated spin-echo): 18.50 ($(CH_3)_2CH$); 34.70 ($(CH_3)_2CH$); 45.89 (CH_2COO); 51.36 ($ArCH_2$); 52.98, 55.13 (2 × CH_3O); 109.63 (C-5); 114.21, 130.19 (arom. CHs); 125.56 (C-1'*); 141.03 (C-8); 147.60 (C-4'*); 151.04, 151.10, 159.64 (C-2, C-4, C-6); 166.23 (COO); 180.49 (iPrCO). MS (ESI), *m/z* (rel.%): 414 (100) M$^+$, guaninium ion.

9-[(*tert*-Butoxycarbonyl)methyl]-N^2-isobutyryl-7-(4-nitrobenzyl)guaninium Bromide (4c) and Hexafluorophosphate (4d)

Prepared from compound 3c (10.0 mmol) as described for compound 4a. The bromide crystallized very sluggishly. It was very well soluble in EtOAc, therefore a water-insoluble hexafluorophosphate salt (4d) was prepared as follows. The crude oily product from the above reaction was dissolved in acetonitrile (20 ml) and ammonium hexafluorophosphate (1.793 g, 11.0 mmol) in water (10 ml) was added. The solution was evaporated, the residue was triturated under cold water and the precipitate was stored in vacuo over P_2O_5 (5.732 g, 93.0%). The title hexafluorophosphate dissolves well in cold MeOH, EtOAc, CH_2Cl_2 and acetonitrile but not in Et_2O or water. The salt can be precipitated from acetonitrile solution by adding water. M.p. 126.8–127.5 °C (aqueous acetonitrile). R_F (4c) ca. 0.10 (S1). For

$C_{22}H_{27}F_6N_6O_6P$ (4d; 616.4) calculated: 42.9% C, 4.4% H, 13.6% N; found: 42.65% C, 4.35% H, 13.4% N. UV (4c): λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 200 sh (4.50), 270 (4.31); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 271 (4.08); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 245 (4.11), 269 (4.06). ^{1}H NMR (4c): 1.10 d, 6 H, J = 6.7 ($(CH_3)_2CH$); 1.45 s, 9 H (*t*-Bu); 2.81 pseudoquintet, 1 H, J = 6.7 ($(CH_3)_2CH$); 5.22 s, 2 H (CH_2COO); 5.99 s, 2 H ($ArCH_2$); 7.73 d, 2 H, J = 8.3 (ArH); 8.24 d, 2 H, J = 8.3 (ArH); 9.95 s, 1 H (H-8); 12.10 br s, deut, 1 H (NH); 12.50 br s, deut, 1 H (NH). ^{13}C NMR (4c; *J*-modulated spin-echo): 18.61 ($(CH_3)_2CH$); 27.55 ($(CH_3)_3C$); 34.77 ($(CH_3)_2CH$); 46.54 (CH_2COO); 50.99 ($ArCH_2$); 83.68 ($(CH_3)_3C$); 109.72 (C-5); 123.76, 129.29 (arom. CHs); 141.42, 147.52, 147.68, 151.04, 151.21 (C-1′, C-4′, C-2, C-4, C-6, C-8); 164.64 (COO); 180.58 (iPrCO). MS (ESI) (4c), *m/z* (rel.%): 471 (100) M^+, guaninium ion, 941 (10) $[2\ M - H^+]^+$.

tert-Butyl (N^2-Isobutyrylguanin-9-yl)acetate[24] (5a)

Compound 4c (0.240 g, 0.435 mmol) was dissolved in 50% (v/v) acetic acid (5 ml), zinc powder (0.178 g, 2.72 mmol, 6.0 equivalents) was added and the mixture was stirred at room temperature for 18 h. A further portion of zinc powder (0.090 g) was added and stirring was continued for 6 h. The zinc almost completely dissolved and a yellow precipitate was formed. After filtration through a Hyflo bed and thorough washing with acetonitrile, the solution was evaporated and dissolved in a mixture of dichloromethane (25 ml) and water (25 ml). The aqueous phase was extracted with dichloromethane (25 ml) and the combined organic phases were washed with 0.05 M EDTA disodium salt (2 × 10 ml), dried and evaporated in vacuo (0.077 g). The Hyflo bed was washed again with acetonitrile and a combined crop of 0.130 g was obtained. Chromatography (CH_2Cl_2–MeOH 98:2 to 94:6) yielded 0.064 g (43.8%), m.p. 203 °C. R_F 0.13 (S2). The NMR and MS data of the product were in full agreement with those previously published[24]. In another experiment after the work-up as above, the resulting red oil was coevaporated with acetonitrile (3×) and the yellow solid obtained (0.82 g, 60%) showed a single spot on TLC. Further trituration under ether yielded a purer product (0.60 g, 44%).

2′,3′,5′-Tri-*O*-acetyl-N^2-(pent-4-enoyl)guanosine (8)

Guanosine hydrate (8.8 g, 31.1 mmol) was suspended in acetonitrile (2 × 100 ml) and evaporated to dryness. Chlorotrimethylsilane (30 ml, 234 mmol) was added dropwise (20 min) to the suspension of dried guanosine in anhydrous pyridine (150 ml) and stirred for another 40 min. Pent-4-enoic anhydride (7.10 ml, 38.9 mmol, 1.25 equivalents) was added and the reaction was stirred at room temperature for 16 h. The cooled reaction mixture was diluted with water (30 ml) and treated with ammonia solution (30 ml, 25%) for 30 min. The residue was dissolved in water (400 ml) and extracted with a mixture of Et_2O and EtOAc (1:1 v/v, 400 ml). The water phase was evaporated, then coevaporated with acetonitrile (2 × 300 ml) and used for the next step without further purification.

N^2-(Pent-4-enoyl)guanosine (6) was dissolved in a mixture of DMF (44 ml) and pyridine (22 ml). Acetic anhydride (18 ml) was added to the mixture, the pyridinium salts from the previous step were filtered off and the solution was set aside for 16 h. Ethanol (10 ml) was added to the solution, then the residue was dissolved in EtOAc (400 ml) and extracted with 1 M hydrochloric acid (2 × 300 ml) and saturated $NaHCO_3$ solution (2 × 300 ml). Evaporation in vacuo after drying gave the product as a white solid foam (10.9 g, 71%). R_F 0.30 (S2),

0.45 (S3). UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 202 (4.20), 260 (3.97), 281 (3.84); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 260 (4.00), 281 (3.87); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 266 (3.97). ^{1}H NMR: 2.03 s, 3 H (CH_3CO); 2.04 s, 3 H (CH_3CO); 2.11 s, 3 H (CH_3CO); 2.34 m, 2 H ($CH_2CH{=}CH_2$); 2.58 t, 2 H, J = 7.2 (CH_2CO); 4.29 m, 1 H (H-4'); 4.37 m, 2 H (H-5'); 4.99 d, 1 H, J = 10.2 (*cis*-$CH_2{=}CH$); 5.06 d, 1 H, J = 17.2 (*trans*-$CH_2{=}CH$); 5.48 dd, 1 H, J = 5.7, J = 3.7 (H-3'); 5.81 m, 2 H (H-1', $CH_2{=}CH$); 6.08 d, 1 H, J = 6.3 (H-2'); 8.23 s, 1 H (H-8); 11.60 br s, 1 H (NH); 12.06 br s, 1 H (NH). ^{13}C NMR (decoupled and J-modulated spin-echo spectra): 20.03, 20.25, 20.39 (3 × CH_3CO); 28.07 ($CH_2CH{=}CH_2$); 35.10 (CH_2CO); 63.00 (C-5'); 70.28 (C-3'*); 72.16 (C-2'*); 79.83 (C-4'*); 84.56 (C-1'); 115.54 ($CH_2{=}CH$); 120.37 (C-5); 136.73 ($CH_2{=}CH$); 137.74 (C-8); 148.06 (C-2#); 148.57 (C-6#); 154.65 (C-4); 169.15, 169.35, 169.99 (3 × CH_3CO); 175.46 (C_4H_7CONH). MS (ESI), *m/z* (rel.%): 492.16 (100) $[M + H]^+$.

7-(4-Nitrobenzyl)-N^2-(pent-4-enoyl)guanine (10)

Compound **8** (4.9 g, 10.0 mmol) dissolved in anhydrous DMF (60 ml) and 4-nitrobenzyl bromide (8.6 g, 40.0 mmol) were stirred at room temperature for 60 h. When the reaction was complete, pyridine (6.4 ml, 80 mmol) was added to scavenge excess of the alkylation reagent and set aside for 5 h. The reaction mixture was heated at 70 °C for 16 h to thermolyse the guaninium salt **9**. The solution was evaporated in vacuo and EtOAc (400 ml) and water (400 ml) were added to the oily residue. The product (2.3 g, 62%) was precipitated and filtered off. A further crop (0.6 g, 16%) was precipitated when the residue from the evaporated organic phase was treated with CH_2Cl_2 (10 ml). Overall yield of **10**: 2.9 g (78%), amorphous solid. R_F 0.29 (S2), 0.22 (S3). UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 267 (4.15); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 267 (4.14); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 272 (4.11). ^{1}H NMR: 2.32 m, 2 H ($CH_2CH{=}CH_2$); 2.53 t, 2 H, J = 7.2 (CH_2CO); 4.97 d, 1 H, J = 10.2 (*cis*-$CH_2{=}CH$); 5.04 d, 1 H, J = 17.1 (*trans*-$CH_2{=}CH$); 5.65 s, 2 H ($ArCH_2$); 5.77–5.85 m, 1 H ($CH_2{=}CHCH_2$); 7.52 d, 2 H, J = 8.5 (ArH); 8.17 d, 2 H, J = 8.5 (ArH); 8.37 s, 1 H (H-8); 11.57 br s, 1 H (NH); 12.08 br s, 1 H (NH). ^{13}C NMR (decoupled and J-modulated spin-echo spectra): 28.13 ($CH_2{=}CHCH_2$); 34.96 (CH_2CO); 48.60 ($ArCH_2$); 111.08 (C-5); 115.49 ($CH_2{=}CH$); 123.68, 128.44 (arom. CH); 136.73 ($CH_2{=}CH$); 144.49, 144.54, 147.00, 152.47, 157.33 (C-8, C-1', C-4', C-2, C-6, C-4); 175.26 (C_4H_7CONH). MS (ESI), *m/z* (rel.%): 368.97 (100) $[M + H]^+$.

tert-Butyl ({2-Isobutyramido)-5-[*N*-(4-nitrobenzyl)formamido]-6-oxo-1,6-dihydropyrimidin-4-yl}amino)acetate (16)

To compound **4d** (0.308 g, 0.5 mmol) dissolved in acetone (3 ml) was added sodium sulfide nonahydrate (0.48 g, 2.0 mmol) in water (1 ml) and the mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with water (20 ml) and extracted with CH_2Cl_2 (3 × 10 ml), the organic layer was dried and evaporated (0.178 g, 73%), m.p. 191 °C (dec.). R_F 0.39 (S2). For $C_{22}H_{28}N_6O_7$ (488.5) calculated: 54.1% C, 5.8% H, 17.2% N; found: 53.9% C, 5.95% H, 16.95% N. UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 201 (4.24), 232 (4.42), 276 (4.00); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 276 (3.94); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 245 (4.09), 270 (4.02). ^{1}H NMR (HSQC, HMBC, see Fig. 1, major rotamer): 1.06 d, 6 H, J = 6.5 ($(CH_3)_2CH$); 1.37 s, 9 H (*t*-Bu); 2.73 pseudoquintet, 1 H, J = 6.5 ($(CH_3)_2CH$); 3.96 m, 2 H (CH_2COO); 4.41, 4.97 2 × d, 2 × 1 H, J = 15.2 ($ArCH_2$); 7.29 t, 1 H, J = 6.0 (NH); 7.64 d, 2 H, J = 8.5 (ArH); 7.99 s, 1 H (HCO); 8.08 d, 2 H, J = 8.5 (ArH);

11.30 br s, 1 H (NH); 11.41 br s, 1 H (NH). ^{13}C NMR (HSQC, HMBC, see Fig. 1, major rotamer): 18.62 ($(CH_3)_2CH$); 27.65 ($(CH_3)_3C$); 34.58 ($(CH_3)_2CH$); 42.90 (CH_2COO); 46.82 ($ArCH_2$); 80.74 ($(CH_3)_3C$); 96.83 (C-5); 122.95 (C-3', C-5'); 129.74 (C-2', C-6'); 144.90 (C-1'); 146.60 (C-4'); 149.36 (C-2*); 158.15 (C-6*); 159.42 (C-4); 165.14 (HCO); 169.20 (COO*t*-Bu); 180.36 (iPrCO). MS (ESI), *m/z* (rel.%): 977.6 (48) [2 M + H]$^+$, 489 (100) [M + H]$^+$.

9-[(*tert*-Butoxycarbonyl)methyl-7-(4-nitrobenzyl)-N^2-(pent-4-enoyl)guaninium Bromide (12)

tert-Butyl bromoacetate (1.2 ml, 8.1 mmol) was added to a solution of compound 10 (1.0 g, 2.7 mmol) in anhydrous DMF (40 ml) and heated at 70 °C for 16 h. The residue obtained after evaporation was dissolved in a phosphate buffer (60 ml, pH 7.0) and extracted with Et_2O (60 ml). The title salt 12 (1.30 g, 86%) precipitated as a white amorphous solid. R_F 0.21 (S1). UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 201 sh (4.28), 270 (4.14); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 272 (4.02); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 245 (4.02), 269 (3.98). ^{1}H NMR: 1.41 s, 9 H ($(CH_3)_3C$); 2.30 br s, 2 H ($CH_2CH=CH_2$); 2.63 br s, 2 H (CH_2CO); 4.95 d, 1 H, J = 8.3 (*cis*-CH_2=CH); 5.02 d, 1 H, J = 16.8 (*trans*-CH_2=CH); 5.15 s, 2 H (CH_2COO); 5.81 br s, 1 H ($CH=CH_2$); 5.96 s, 2 H ($ArCH_2$); 7.72 d, 2 H, J = 7.9 (ArH); 8.19 d, 2 H, J = 7.9 (ArH); 9.81 s, 1 H (H-8); 11.32 br s, 1 H (NH); 12.20 br s, 1 H (NH). ^{13}C NMR (decoupled and *J*-modulated spin-echo spectra): 27.45 ($C(CH_3)_3$); 28.22 ($CH_2CH=CH_2$); 35.29 (CH_2CONH); 45.99 (CH_2COO); 50.36 ($ArCH_2$); 83.27 ($C(CH_3)_3$); 109.50 (C-5); 115.12 (CH_2=CH); 123.62 (ArCH); 129.26 (ArCH); 137.11 (CH_2=CH); 138.99 (C-8); 142.06 (C-1', C-4'); 147.37 (C-2*); 148.30 (C-6*); 153.83 (C-4); 164.92 (COO*t*-Bu); 173.75 (C_4H_7CONH). MS (ESI), *m/z* (rel.%): 483 (100) [M$^+$].

tert-Butyl [N^2-(Pent-4-enoyl)guanin-9-yl]acetate (14)

Sodium dithionite (0.790 g, 3.6 mmol, 80%) was added to a solution of guaninium salt 12 (0.530 g, 0.94 mmol) in acetone (15 ml) and phosphate buffer (15 ml, pH 7.0). After 30 min stirring at room temperature, the solution was heated at 70 °C for 16 h. Acetone was evaporated in vacuo and the water phase was extracted with EtOAc (50 ml). The title ester 14 (0.250 g, 76%) was obtained as a yellowish amorphous solid. R_F 0.19 (S2), 0.22 (S3). UV: λ_{max} (50% (v/v) 1 M HCl in EtOH, pH 0) 203 (4.22), 261 sh (4.03); λ_{max} (50% (v/v) 1 M TEAc in EtOH, pH 7) 260 (3.98), 280 (3.85); λ_{max} (50% (v/v) 0.1 M KOH in EtOH, pH 13) 267 (3.89). ^{1}H NMR: 1.40 s, 9 H ($C(CH_3)_3$); 2.33 q, 2 H, J = 7.0 ($CH_2CH=CH_2$); 2.56 t, 2 H, J = 7.2 (CH_2CO); 4.86 s, 2 H (CH_2COO); 4.98 d, 1 H, J = 10.1 (*cis*-CH_2=CH); 5.05 d, 1 H, J = 16.3 (*trans*-CH_2=CH); 5.82 m, 1 H ($CH=CH_2$); 7.94 s, 1 H (H-8); 11.66 br s, 1 H (NH); 12.04 br s, 1 H (NH). ^{13}C NMR (decoupled and *J*-modulated spin-echo spectra): 27.56 ($(CH_3)_3C$); 28.09 ($CH_2CH=CH_2$); 34.98 (CH_2CO); 44.76 (CH_2COO); 82.24 ($(CH_3)_3C$); 115.49 (CH_2=CH); 119.54 (C-5); 136.74 (CH_2=CH); 140.23 (C-8); 147.76 (C-2*); 148.85 (C-6*); 154.73 (C-4); 166.49 (COO*t*-Bu); 175.50 (C_4H_7CONH). MS (ESI), *m/z* (rel.%): 717.41 (38) [2 M + Na]$^+$, 695.43 (68) [2 M + H]$^+$, 370 (18) [M + Na]$^+$, 348 (100) [M + H]$^+$.

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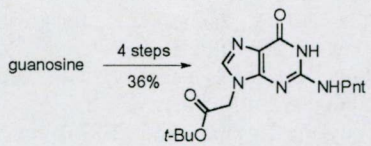
A Regioselective Synthesis of Alkyl 2-(Guanin-9-yl)acetates as PNA Building Blocks from 7-(4-Nitrobenzyl)guanine Derivatives

György Ferenc, Péter Forgó, Zoltán Kele and Lajos Kovács



III.

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Determination of N^9/N^7-isomer ratio of alkyl (guanin-9-yl)acetates by electrospray ionization tandem mass spectrometry

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N^9- and N^7-Substituted (guanyl)acetic esters were studied by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in order to determine their ratio in alkylation reactions. The loss of ammonia is significantly different for the N^9- and N^7-alkylated guanine regioisomer pairs. More importantly, the abundance of the $[MH-17]^+$ ion is in linear correlation with the N^9-isomer content. Therefore, the ratio of regioisomers can be determined in a mixture containing these compounds.

N^9-Substituted analogs of guanine nucleosides have been extensively investigated for their biological activity especially as anticancer and antiviral compounds.[1] They are important as building blocks ((guanin-9-yl)acetic acid) of antisense and antigene oligonucleotide analogs, e.g. peptide nucleic acids (PNAs).[2] Unfortunately, the glycosylation and alkylation of guanine and related compounds, e.g. 2-amino-6-chloropurine, is not regioselective, forming the unwanted N^7-regioisomers as side products.[3] The yield of the product can be improved by optimization of reaction conditions, the effectiveness of which can be improved by a fast analytical method avoiding separation of regioisomers. The sensitivity of the method is also important in order to optimize the alkylation conditions on a few mg scale accompanied by a fast thin layer chromatography (TLC) purification.

The site of the alkylation can be ascertained most conveniently by using two-dimensional nuclear magnetic resonance (2D-NMR) (^1H, ^{13}C) techniques, but the chemical shift differences of some diagnostic ^{13}C signals[4] and/or chemical shift of N-3[5] are also very informative. Unfortunately, the latter two methods, unlike ^1H NMR, give only qualitative information and at least 5 mg of pure solvent free sample is required, usually preceded by chromatographic purification (e.g. TLC). Both the purification and the NMR analysis take at least 10–20 min. Furthermore, expensive deuterated solvents are required for the latter procedure.

High-performance liquid chromatography (HPLC) analysis would be another possible approach but this should be preceded by NMR measurement to have a standard sample. Furthermore, a gradient system for the separation of regioisomers is required and a HPLC run takes 10–20 min/sample, (thus in the case of 10 parallel reactions 100–200 min) and a large volume of high purity solvent. This is not the case with a tandem mass spectrometry (MS/MS) method, in which a few μL of test reaction mixture is sufficient after dilution. The criteria of the applicability are a significant difference in the fragmentation pathways of the two regioisomers and a product ion whose abundance is proportional to the ratio of the isomers. Details of the derivatives of N^7- and N^9-alkylated guanine derivatives studied by MS/MS are collected in Table 1.

Usually, N^7- and N^9-isomer pairs are identified by their product ion spectra but in some cases there is only a slight difference between the spectra. The most unambiguous differentiation between 9- and 7-methylguanines was achieved by chemical ionization (CI)-MS/MS using NH_3 as the CI gas in which the loss of CH_3NH_2 was characteristic exclusively for the N^7-isomer.[6] In the case of β-*O*-activated alkyl groups, e.g. $CH_2OCHR^1R^2$, the abundance of ions derived from the side chains was much higher for the N^9-isomer. Therefore, the identification was based on this fact.[10] These studies have, however, afforded only qualitative information.

The fragmentation of the protonated guanine backbone was confirmed by ^{15}N and ^{16}O labelling[13] and H/D exchange,[12] proving that losses of ammonia and cyanamide are independent and principal pathways, followed by loss of CO and HCN.

(N^2-Acyl-guanin-9-yl)acetic esters **1** (9IbuMe), **3** (9IbutBu)[4] and **5** (9PnttBu)[14] have been synthesized in our laboratory as PNA monomer building blocks and studied by electrospray ionization tandem mass spectrometry (ESI-MS/MS) along with their N^7-regioisomer pairs **2** (7IbuMe) and **4** (7IbutBu), respectively[4] (Fig. 1). The side chain of these compounds is an acetic ester derivative, which is different from those previously studied. It is therefore interesting to consider how this will influence the fragmentation of the regioisomers. Our aim was to determine the N^9/N^7-isomer ratio by a simple and time- and cost-effective MS/MS method finding optimal collision-induced dissociation (CID) conditions where a

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Table 1. Structures of alkylated guanine derivatives studied by MS/MS (CI-MS/MS,[6] ESI-MS/MS,[7,12] FAB-MS/MS[8–11]) methods

Alkyl side chain[a]	The site of the alkyl group[a]
Me,[6–8] Et,[8] Pr[9]	7,[6–9] 9[6,8]
CH_2CRHOH; R = H, CH_3, CH_2OH	7[9]
$CH_2OCH_2CH_2OR$; R = H, Ac	7,[10,11] 9,[10,11] 9[12] (R = H)
$CH_2OCH(CH_2OR)_2$; R = H, Bn	7[11], 9[11]

[a] Refs. in superscripts.

significant difference can be observed in the fragmentation of regioisomers.

EXPERIMENTAL

Mass spectrometric measurements were obtained on a Finnigan TSQ-7000 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA, USA) equipped with a Finnigan ESI source. The instrument was scanned in positive ion mode over the mass range *m/z* 160–230 with a scan time of 0.1 s. Samples of 5 μL were injected into the eluent stream of a pump (Applied Biosystems 140 C (Foster City, CA, USA); flow rate: 150 μL/min, eluent: methanol/water/acetic acid 50:50:1 (v/v/v), sample concentration: 0.1 mg/mL).

The electrospray needle was adjusted to 4.5 kV and N_2 (pressure: 3.45 bar) was used as the nebulizer gas. The collision energy was varied between −10 and −70 eV to find the optimal value (−35 eV for compounds **1** (9IbuMe) and **2** (7IbuMe); −45 eV for compounds **3**–**5** (9IbutBu, 7IbutBu and 9PnttBu)). At this value the abundance of the $[M_f+H]^+$ ion was 100% and that for the $[M_f+H-17]^+$ ion was almost the same. The collision gas was argon and the pressure in the collision cell region was set at 2 ± 0.1 mTorr.

RESULTS AND DISCUSSION

Our compounds (see Fig. 1) containing different N^2-acyl protecting groups (isobutyryl in **1** (9IbuMe), **2** (7IbuMe), **3** (9IbutBu), and **4** (7IbutBu) and pent-4-enoyl in **5** (9PnttBu)) and alkyl moieties in ester groups (methyl in **1** (9IbuMe) and **2** (7IbuMe) and *tert*-butyl in **3** (9IbutBu) and **4** (7IbutBu)) could give useful information on whether these groups have an effect on fragmentation pathways. The loss of the *tert*-butyl

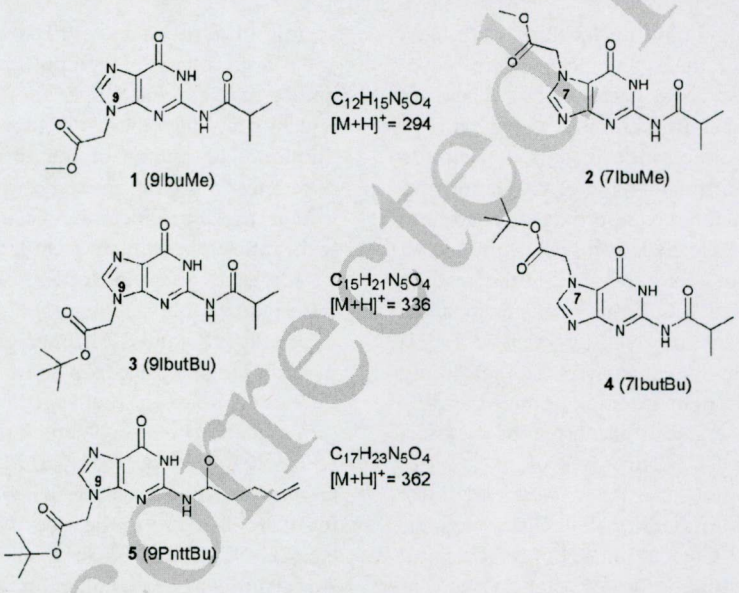

Figure 1. Structures of alkyl 2-(guanin-yl)acetates (**1**–**5**, 9IbuMe-9PnttBu) studied by ESI-MS/MS.

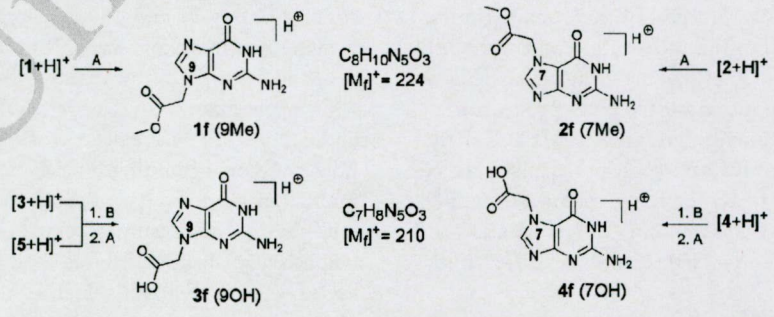

Figure 2. Structures of compounds **1f** (9Me), **2f** (7Me), **3f** (9OH) and **4f** (7OH) formed from alkyl 2-(guanin-yl)acetates (**1**–**5**) in MS/MS experiments at −30 eV. Process A: deacylation of the N^2-amino group, process B: hydrolysis of the *tert*-butyl ester. $[M_f]^+$ denotes the *m/z* value of the product ion (**1f**–**4f**).

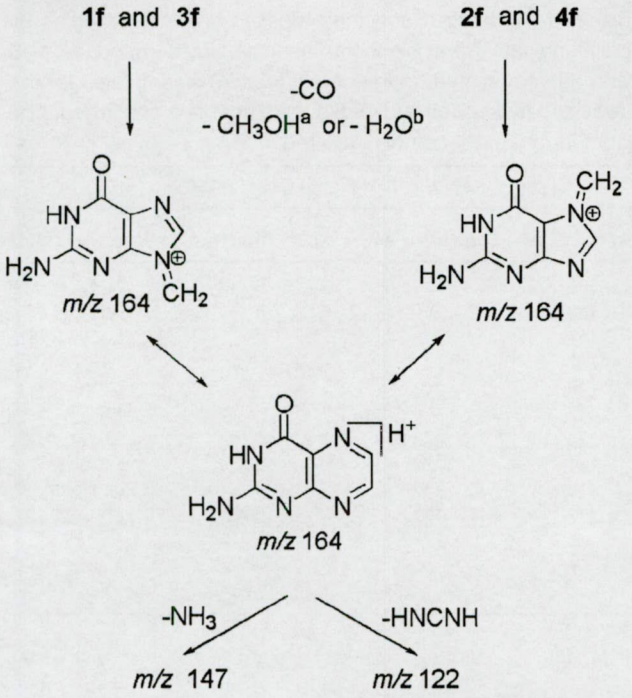

Figure 3. Formation and fragmentation of the ion at *m/z* 164 (a) from compounds **1f** (9Me) and **2f** (7Me); and (b) from compounds **3f** (9OH) and **4f** (7OH).

ester (Fig. 2, process B) was followed by dissociation of the N^2-acyl group (Fig. 2, process A). Thus the difference caused by these groups disappeared to such an extent that compounds **3** (9IbutBu) and **5** (9PnttBu) led to the same acid **3f** (9OH). Therefore, the fragmentation of differently protected compounds was similar. The free acid form (**3f** (9OH) and **4f** (7OH)) rendered the side chains unstable at higher energies in an MS/MS study.

The product ions **1f** (9Me), **2f** (7Me), **3f** (9OH) and **4f** (7OH) were the most abundant at −30 eV collision energy (for structures, see Fig. 2). The **1f**–**4f** ions are precursors of lower mass product ions; therefore, the further fragmentation steps can be more easily compared if we derive them from the f series (*m/z* values of the f series are indicated by $[M_f]^+$).

The major fragmentation pathways (losses of ammonia and cyanamide) of ions **1f** (9Me), **2f** (7Me), **3f** (9OH) and **4f** (7OH) are in good agreement with observations by Gregson and McCloskey[13] and Kamel and Munson.[12] Losses of methanol (**1f** (9Me), **2f** (7Me)) or water (**3f** (9OH) and **4f** (7OH)) and carbon monoxide also occurred, depending on the nature of side chain, leading to the ion at *m/z* 164, the rearrangement of which to pterine was suggested by Kralj *et al.*[10] (see Fig. 3). The *m/z* 164 ion loses NH_3 and cyanamide, as do the precursor ions (**1f** (9Me), **2f** (7Me), **3f** (9OH) and **4f** (7OH)), to yield ions at *m/z* 147 and 122, respectively.

When comparing the MS/MS spectra of compounds **1** (9IbuMe), **2** (7IbuMe), **3** (9IbutBu) and **4** (7IbutBu), it was found that, in both cases, the N^9-isomers (**1** (9IbuMe) and **3** (9IbutBu)) gave abundant $[M_f-NH_3]^+$ ions at *m/z* 207 and 193 that were of negligible abundance in the case of the N^7-isomers (**2** (7IbuMe) and **4** (7IbutBu)) (see boxes in Fig. 4). Therefore, the loss of ammonia from the M_f ions is a potential candidate for the quantitative determination of the regioisomer ratio.

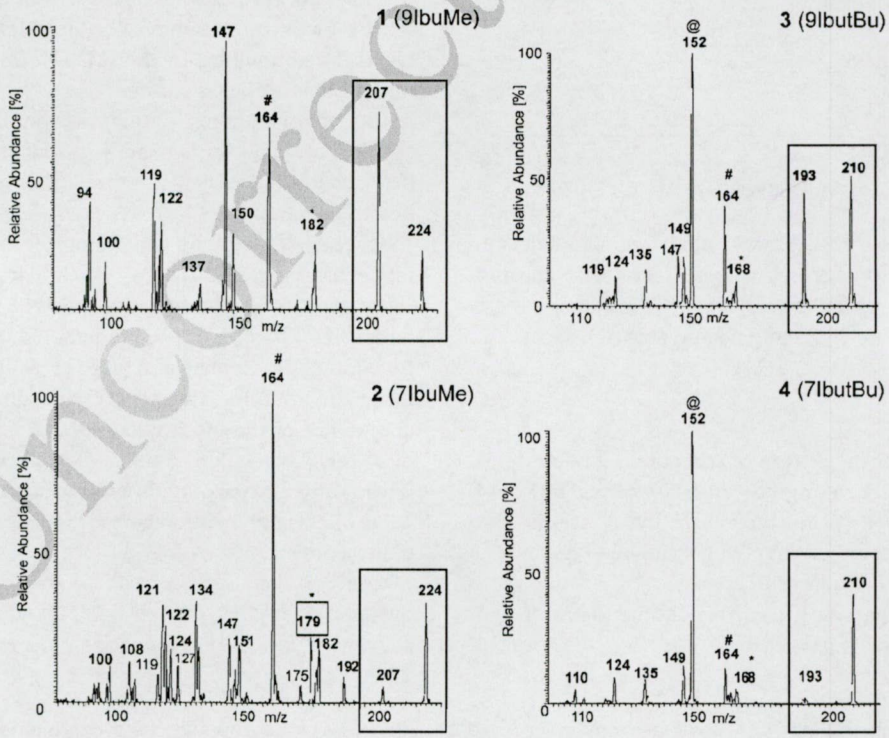

Figure 4. MS/MS spectra of regioisomer pairs **1** (9IbuMe), **2** (7IbuMe) and **3** (9IbutBu), **4** (7IbutBu) studied. The loss of ammonia, the most important fragmentation pathway, is highlighted by boxes. Other important peaks are labelled as follows: *: $[M_f\text{-}42]^+$, #: *m/z* 164 (for structure, see Fig. 3) and @: *m/z* 152. *m/z* 179 is also framed as it could be potentially interesting in future studies of N^7-isomers.

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Table 2. Ions of compounds **1** (9IbuMe), **2** (7IbuMe), **3** (9IbutBu) and **4** (7IbutBu) and their masses based on studies by McCloskey *et al.*[13] and Kamel *et al.*[12] Compounds **1f–4f** (Fig. 2) originate from initial fragmentation of compounds **1–5** [substances **3** and **5** gave an identical acid (**3f**)] The most important, deammoniated fragment (set in bold) was applied for the determination of isomer ratio. Structures of ions corresponding to italicized and underlined peaks have not been confirmed. The underlined one could be important for the characterization of N^7-isomers but further studies are required

1f (9Me), **2f** (7Me) ($[M_f]^+ = 224$)				**3f** (9OH), **4f** (7OH) ($[M_f]^+ = 210$)			
Product ion	Precursor ion	Mass difference	Fragment(s) lost	Product ion	Precursor ion	Mass difference	Fragment(s) lost
207	**224**	**17**	**NH_3**	**193**	**210**	**17**	**NH_3**
192	*224*	*32*	*MeOH*	168	210	42	NH_2CN
182	224	42	NH_2CN	*164*	*210*	*46*	*H_2O+CO*
179	207	28	CO	*152*	*210*	*58*	*$C_2H_2O_2$*
164	*224*	*60*	*MeOH+CO*	*149*	*193*	*44*	*CO_2*
150	*182*	*32*	*MeOH*	147	164	17	NH_3
147	164	17	NH_3	135	152	17	NH_3
137	164	27	HCN	124	152	28	CO
124	151	27	HCN	119	147	28	CO
122	164	42	NH_2CN	110	152	42	NH_2CN
121	164	43	NHCO				
119	147	28	CO				
94	122	28	CO				

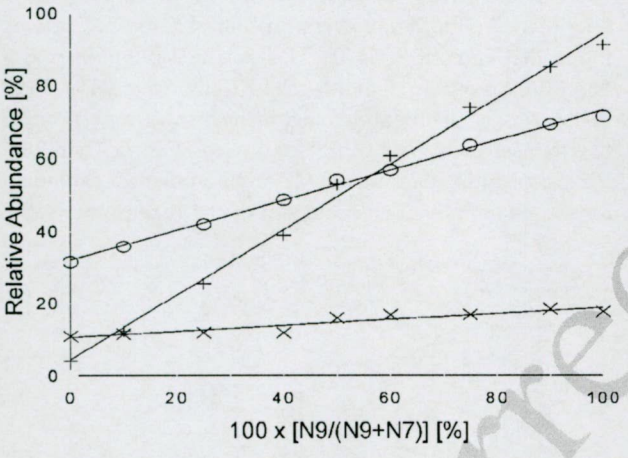

Figure 5. The linear regression study of product ion abundance as a function of isomer ratio in a mixture containing compounds **3** (9IbutBu) and **4** (7IbutBu). + series: *m/z* 193; x series: *m/z* 168 and o series: *m/z* 164.

The *m/z* 179 ion in the spectrum of compound **2** (7IbuMe) is interesting as it could be a product of *m/z* 207 formed by loss of CO. This ion could be characteristic for the N^7-isomer but further studies are required involving other alkyl (guanin-9-yl)acetates containing stable ester bonds.

The abundant ion at *m/z* 152, corresponding to protonated guanine, is the result of total side-chain loss from the free acid form of **3f** (9OH) and **4f** (7OH). This ion then loses NH_3, CO and cyanamide to yield ions at *m/z* 135, 124 and 110, respectively.

The N^9-isomer **3f** (9OH) gives a characteristic loss of ammonia as the parallel process of the side-chain loss; therefore, the ratio of regioisomers can also be determined in the case of *tert*-butyl esters.

It appears conclusive that the loss of ammonia is characteristic for N^9-regioisomers (**1** (9IbuMe) and **3** (9IbutBu)). Next, the abundance of the $[M_f\text{-}17]^+$ ion as a function of regioisomer ratio was studied.

The fragmentation pathways confirmed by Gregson McCloskey[13] and Kamel and Munson[12] have been collected in Table 2 for easier interpretation of MS/MS spectra described herein.

A series of solutions containing the isomers in different ratios has been studied by MS/MS in order to obtain a calibration curve. The abundances of the three main product ions, $[M_f\text{-}17]^+$, $[M_f\text{-}42]^+$ and *m/z* 164, were plotted as a function of the isomer ratio (see Fig. 5). The correlation was linear in all three cases but with different slopes. The abundance of the ion $[M_f\text{-}42]^+$ is almost constant; furthermore, its correlation coefficient (R^2) value shows large uncertainty for compounds **3** (9IbutBu) and **4** (7IbutBu) (see Table 3). It is not therefore appropriate for the determination of the isomer ratio.

Based on their high correlation coefficients (see Table 3), the abundances of ions $[M_f\text{-}17]^+$ and *m/z* 164 are both convenient for the determination of isomer ratios; however,

Table 3. Correlation coefficient (R^2) values from linear regression analysis of the abundances of three ions ($[M_f\text{-}17]^+$, $[M_f\text{-}42]^+$ and *m/z* 164 (for structure, see Fig. 3)) as a function of the regioisomer ratio for both pairs of regioisomers

Sample constituents	$[M_f\text{-}17]^+$	R^2	$[M_f\text{-}42]^+$	R^2	R^2 for *m/z* 164
1 (9IbuMe), **2** (7IbuMe)	*m/z* 207	0.997	*m/z* 182	0.981	0.994
3 (9IbutBu), **4** (7IbutBu)	*m/z* 193	0.995	*m/z* 168	0.875	0.996

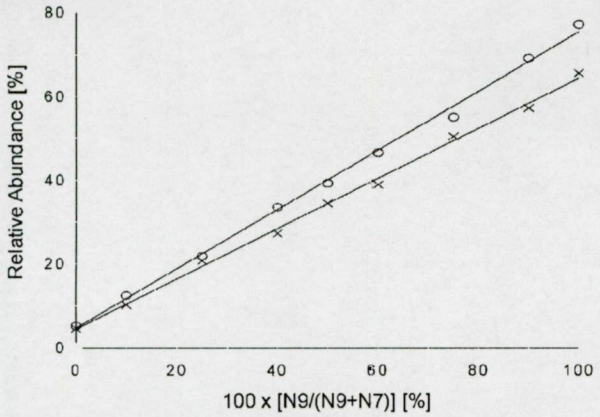

Figure 6. Calibration at two different CID gas pressures of compounds **1** (9IbuMe), **2** (7IbuMe) at 2.1 mTorr (o series, $y = 4.68 + 0.71x$, $R^2 = 0.997$) and 1.7 mTorr (x series, $y = 4.45 + 0.60x$, $R^2 = 0.997$).

as the change in abundance is twice as large for the $[M_f\text{-}17]^+$ ion than for *m/z* 164, its application would lead to more accurate results. The optimal collision energy at which the abundance of the $[M_f\text{-}17]^+$ ion is similar to that of the $[M_f]^+$ ion for the N^9-isomer and negligible for the N^7-isomer (less than 10% relative abundance) is also important for the same reason.

The slope of the calibration curves depends strongly on the collision gas pressure (see Fig. 6). Therefore, the CID pressure should be adjusted accurately to obtain comparable ion abundances. For example, a change of 0.4 mTorr led to a 0.1 difference in the slope, meaning that the relative abundance of the $[M_f\text{-}17]^+$ ion changed ca. 10% in the case of the pure N^9-isomer. This problem is increased for compounds **3** (9IbutBu) and **4** (7IbutBu), resulting in a 20% change in abundance (data not shown). To obtain accurate results every isomer determination should start with a calibration (maximum 15 min) to guarantee identical conditions in the analysis.

Alternatively, the calibration before each analysis could be avoided by use of a normalization based on an ion whose abundance is independent of the isomer ratio. Unfortunately, there was no such ion in the *m/z* 160–230 range.

The accuracy of the measurement is ca. 2.2–4.4% (**1** (9IbuMe) and **2** (7IbuMe)) and 4.8–5.0% (**3** (9IbutBu) and **4** (7IbutBu)) depending on the isomer ratio (Table 4). These values are acceptable as the aim of the optimization of reaction conditions is to increase the N^9-isomer content from ca. 50% to 90–100%. Almost the same accuracy could be achieved with ^{1}H NMR but this latter method requires cleaner and larger amounts of sample, deuterated solvents and 10–20 min/sample acquisition time. If the isomers were well resolved by HPLC then higher accuracy could be obtained, but even then there is a need for high-purity solvents and again 10-20 min/sample analysis time.

Table 4. Error of the measurement based on two points: 50 and 90% N^9-isomer content. y = the measured relative abundance of the ion $[M_f\text{-}17]^+$; x = the determined value of the isomer ratio; e.g. $x = (y - 3.28)/0.75$ for compounds **1** (9IbuMe) and **2** (7IbuMe). Error: the relative error of the determination; $\delta x = (x\text{-}x_c)/x_c$

	1 (9IbuMe) and **2** (7IbuMe) [%]		**3** (9IbutBu) and **4** (7IbutBu) [%]	
x_c	50	90	50	90
y	39.3	69.3	51.2	81.1
x	47.8	88.0	52.5	85.7
$100\delta x$	−4.4	−2.2	5.0	−4.8
Calibration curves	$y = 3.28 + 0.75x$		$y = 3.99 + 0.9x$	

CONCLUSIONS

In conclusion, we have managed to find a rapid method for the determination of the ratio of regioisomer pairs of alkyl (guanin-9-yl)acetates. Alkylated N^9- and N^7-regioisomer pairs have significantly different fragmentation pathways: deammoniation is characteristic for N^9-isomers, the abundance of which process is in linear correlation with the N^9 content in a mixture of regioisomers.

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IV.

Synthesis and analysis of peptide nucleic acid oligomers using Fmoc/acyl-protected monomers

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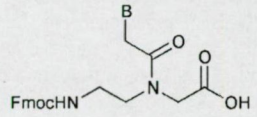
The optimization of PNA oligomer synthesis has been accomplished employing Fmoc/acyl-protected monomers on TentaGel™ and Wang resins. Among the tested activating agents (CMP, BET, HATU) the latter was of choice in solid phase syntheses. "Leakage" of TentaGel™ resin greatly hampers the solution and MS analyses. Synthesis and acyl group deprotection steps have been separately examined using Wang resin. Optimal conditions also worked well on the CPG support. HPLC and MS analyses of the PNA oligomers were carried out under various conditions.

Introduction

Peptide nucleic acids (PNA) are important tools in molecular biology and in drug design owing to their antisense and antigene effects.[1–4] They have the advantage of greater *in vivo* stability and stronger hybridization ability in comparison with native oligonucleotides. However, they enter less easily into the cells and cell nuclei and have low RNAseH activity. PNA conjugates incorporating peptides,[5] oligonucleotides,[6–9] carbohydrates[10] or lipophilic molecules,[11] respectively, might be considered as possible solutions for the above problems.

In order to synthesize PNA oligomers and their conjugates with peptides or oligonucleotides, suitably protected monomers are indispensable and they have to be compatible with both peptide and oligonucleotide synthesis protocols. To date this problem has not been solved as shown in Table 1. The monomers protected by Fmoc (backbone) and acyl (nucleobase) groups (Fig. 1)[12] could fulfil the requirements of PNA–oligonucleotide conjugate synthesis. This combination of protecting groups has not been thoroughly explored,[13–17] the advantages and pitfalls have not been characterized in detail. There are a few reports on optimization of PNA oligomer syntheses,[15,18–22] however, there are so many variables affecting the effectiveness of the synthesis (nature of solid supports and monomers including the protecting groups of the latter, coupling reagents and conditions *e.g.* bases, preactivation, reaction time, mixing sequence, monomer excess, repetition of couplings *etc.*) that it is impossible to simply establish the optimal conditions for the preparation of PNA oligomers. The present report describes our efforts directed towards the elaboration of a PNA oligomer synthesis protocol convenient for the synthesis of PNA–oligonucleotide conjugates and their HPLC and MS analysis. This paper complements and details our preliminary account on this subject.[23]



B^a	Thy	$^{4\text{-tBuBz}}$Cyt	^{An}Ade	^{Ibu}Gua
Code[b]	t	c	a	g

Fig. 1 The structure of Fmoc/acyl-protected peptide nucleic acid (PNA) monomers. a. Thy: thymin-1-yl, $^{4\text{-tBuBz}}$Cyt: N^4-(4-*tert*-butylbenzoyl)cytosin-1-yl, ^{An}Ade: N^6-anisoyladenin-9-yl, ^{Ibu}Gua: N^2-isobutyrylguanin-9-yl. b. Abbreviated notation of unprotected or protected monomer units, depending on the context.

Table 1 Compatibility of protecting group strategies with peptide and oligonucleotide protocols

Protecting groups[a]	Compatibility
Boc/Cbz[b](Benzyl)	Peptide
Fmoc/Cbz[b]	Peptide
Fmoc/Bhoc[c]	Peptide
Fmoc/Mmt[d]	Peptide
Fmoc/acyl	Oligonucleotide
Mmt[d]/acyl	Oligonucleotide

[a] The first abbreviation denotes the protecting group of the backbone while the second one that of the nucleobase. [b] Cbz: benzyloxycarbonyl. [c] Bhoc: benzhydryloxycarbonyl. [d] Mmt: monomethoxytrityl.

Results and discussion

1. Coupling experiments in the solution phase

For the efficient coupling of PNA monomers, an activating agent is required which works quickly with the lowest possible monomer excess, gives high yields and is economically viable. BET[24] and CMP[25] gave almost quantitative yield while HATU gave only 90% yield in solution phase studies in the model reaction between our t monomer and *tert*-butyl phenylalaninate.[26]

2. Initial coupling studies on the solid phase

A solid phase compatible with peptide and oligonucleotide synthesis protocol was needed for PNA oligomer synthesis. Poly(ethylene glycol) attached to polystyrene (known as TentaGel™) meets the above criteria and the removal of protecting groups and final cleavage from the support can be realized also in one step.[27]

In the following experiments coupling yields were determined by UV absorbance of dibenzofulvene-piperidine adduct.

2.1. CMP and BET. Surprisingly, these activating agents gave low yields in solid phase coupling reactions. Thus, they were no longer utilized in further studies. The reasons of this

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Table 2 Coupling yields (%) on TentaGel™ resin using 4 equiv. monomer excess[a]

	a	c	g	t
a	100	92	98, 100	99, 100, 100
c	83, 100, 86	93, 100, 89	96	100
g	100	100, 93, 100	91, 91	100, 100, 100
t	100	97, 88	92, 83	97, 97, 98

[a] Data taken from the synthesis of sequences 1–4 (*cf.* Table 4). Monomers (rows) were coupled to the monomers on the resin (columns).

discrepancy between the solution and solid phase experiments are currently still under investigation.

2.2. HATU. During the synthesis of the first sequences on FmocGly-O-TG resin 2 equiv. of monomer was not sufficient even after prolonged reaction time thus the excess was increased to 4 equiv. resulting in 95% average yield (Table 2). When the monomers were coupled to t the yield was almost quantitative in all cases. The reactions c + a and t + g were the lowest yielding (<90%) while couplings to c or g monomer were in the 91–96% range.

3. Problems with the PNA oligomer synthesis on TentaGel™

The synthesis runs smoothly according to the UV absorbances but there is a problem with the solubility of the product, consequently also with its analysis, and this problem was increased with the length of oligomer.

3.1. Ethylene glycol oligomers in the ESI-MS spectra. In the ESI mass spectra of PNA oligomers with different chain lengths synthesized on TentaGel™ a series of peaks corresponding to the cluster ions $[HO(CH_2CH_2O)_nH + 2NH_4]^{2+}$ was observed. This process can be the result of decomposition of the resin during storage, however, the above peaks were present even after thorough washing. The fracture of TentaGel™ beads resulting in the release of oligo(ethylene glycol) fragments ("leakage" of the support) has been a known phenomenon,[27] however, there are no reports on the fact that this would interfere with the synthesis of oligomers. Probably, the "leakage" causes problems only if the synthesis is performed on a low scale.

Oligo(ethylene glycol) chains make the spectra rather noisy but applying the nano-ESI technique[28] a clear spectrum is observable most likely due to separation inside the gold-coated capillary tube of the nano-ESI-MS interface as is represented by the mass spectra of H-Gly-ggtt-Gly-NH_2 (compound **3**, Fig. 2). The attempted purification of PNA samples by precipitation to separate them from the contaminating oligo(ethylene glycol) was not successful.

3.2. Incomplete removal of isobutyryl (Ibu) protecting groups (presence of $[M + 70 + H]^+$ peaks). Capped (*e.g.* Ac-gtt-Gly-NH_2) and incompletely deacylated sequences (*e.g.* peaks $[M + Ibu + H]^+$) were also present in the MS spectra beside the expected product (Fig. 2, lower panel). To determine the ratio of capped, protected sequences to that of the product an HPLC analysis was needed whereby these compounds could be separated. In order to separate the coupling and acyl deprotection steps, the polystyrene-based, acid sensitive Wang resin was employed because the protected oligomer can be cleaved with TFA and the acyl group deprotection can be realized by NH_3 treatment in solution.

4. Syntheses on Wang resin

Similar results were obtained with 4 equiv. monomer excess during the synthesis of test sequence **1**.[18] As a compromise between monomer excess and coupling efficiency, compound **5** was synthesized using 3 equiv. of monomers resulting in

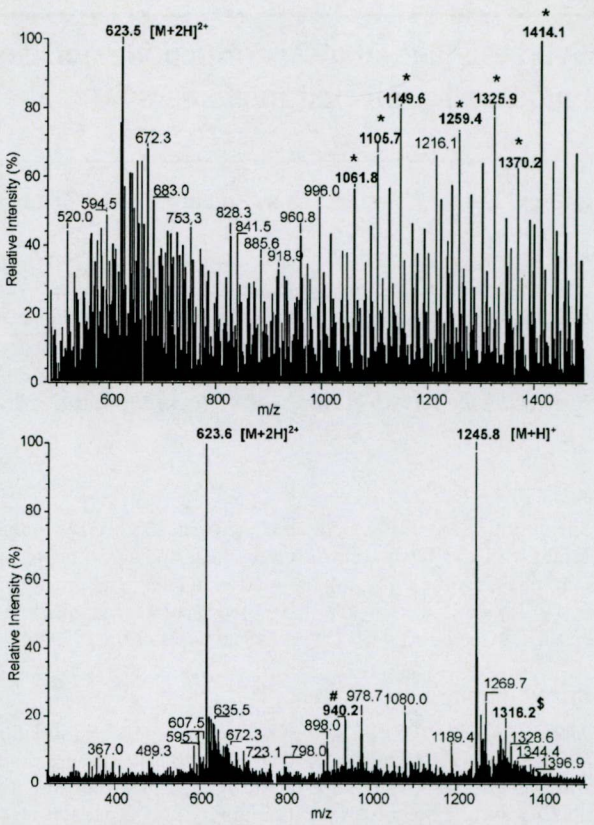

Fig. 2 Mass spectra of PNA oligomer **3** (M_{calc} 1245) synthesized on TentaGel™. Upper panel: ESI-MS spectrum. The values marked with an asterisk denote the cluster ions $[HO(CH_2CH_2O)_nH + 2NH_4]^{2+}$. Lower panel: nano-ESI-MS spectrum. Peaks denoted with # and $ signs correspond to the ions [Ac-gtt-GlyNH_2 + H]$^+$ and [M + Ibu + H]$^+$, respectively.

98% average yield per cycle and HPLC chromatogram of the products (Fig. 3) confirmed that this excess is optimal. It is noteworthy that the highest ever reported coupling yield (99.4%) was achieved at the expense of 7-fold monomer excess.[19]

To find appropriate HPLC conditions for separation of the product from capped sequences and anisoyl derivatives (An–X, X = NH_2, OH originating from the protecting group) the solution of oligomer **5** was analyzed on different HPLC column packings in order to examine the effects of pH of the eluting solution (TEAA, pH 7.0 or dilute TFA, pH ~ 2) and temperature (rt or 55 °C) on the resolution (Table 3 and Fig. 4).

TFA buffer gives better resolution than TEAA as exemplified by the separation of pentadecamer **5** on LiChrospher RP Select B column (Fig. 4, traces A and B). Apparently, this molecular size does not require 300 Å pore size for an efficient separation.

For shorter sequences better resolution was observed on Jupiter packing with very sharp peaks. However, for longer sequences the product and the anisoyl derivatives could not be separated on this packing (Fig. 4, trace C). The separation was good on the less apolar Vydac column as well but in this case the peaks were broader than on other column packings (Fig. 4, trace D). The retention times were very similar on the above-mentioned packings in TFA solution. The effect of temperature[29,30] was also studied, however, there was no significant improvement in the resolution therefore further separations were carried out at room temperature. In conclusion, the LiChrospher RP Select B column using a TFA-containing eluent provides the optimum conditions for PNA analysis and purification.

Oligomers **3**, **4**, **6**–**8** (Table 4) were synthesized to check the completeness of protecting group removal (it is well known that

Table 3 Comparison of analytical HPLC columns and conditions applied in the analysis of PNA oligomers

Column name[a]	Pore size (Å)	Reverse phase type	End-capping	Eluent[b]	t_R[c] (min)
Jupiter[d]	300	C18	Yes	TFA[e]	17.2
Vydac[f]	300	C4	—[g]	TFA[e]	16.3
LiChrospher RP Select B[h]	60	C18	Yes	TFA[e]	17.6
				TEAA[i]	20.9

[a] Size of columns: 250 × 4.6 mm. [b] Principal component of buffer. [c] Retention time of compound **5** at 25 °C. [d] Phenomenex, Torrance, CA, USA. [e] 0.1% (v/v) Trifluoroacetic acid. [f] The Separations Group, Grace Vydac, Hesperia, CA, USA. [g] Data not available. [h] Merck Ltd. Budapest. [i] 0.1 M Triethylammonium acetate.

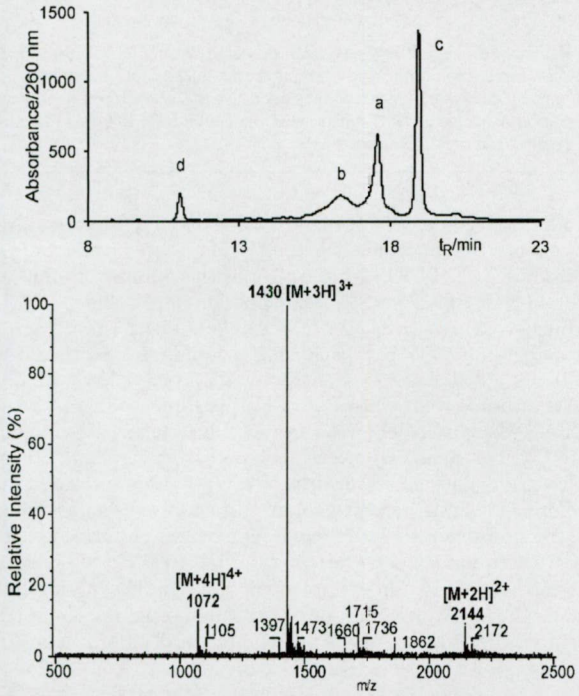

Fig. 3 Upper panel: HPLC chromatogram of the PNA sequence **5** prepared on a Wang resin (crude product, LiChrospher RP Select B column, TFA-containing eluent). Peak a: product, peak c: anisoyl derivatives, peaks b and d: unidentified. Lower panel: ESI mass spectrum (purified product, M_{calc} 4284).

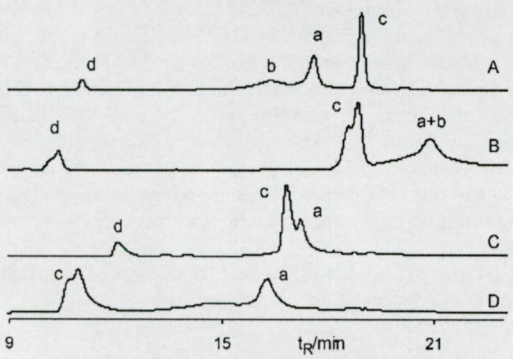

Fig. 4 HPLC chromatograms of the crude oligomer **5** under different conditions. Trace A: LiChrospher RP Select B column, TFA eluent; trace B: LiChrospher RP Select B column, TEAA eluent; trace C: Jupiter column, TFA eluent; trace D: Vydac column, TFA eluent. Peak a: product, peak c: anisoyl derivatives, peaks b and d: unidentified. (*cf.* Table 3).

in oligonucleotide synthesis the rate of deprotection decreases in the order 4-tBuBz > An > Ibu).[31]

The removal was complete in the case of anisoyl and 4-*tert*-butylbenzoyl groups but a small amount of oligomers containing residual isobutyryl groups were detected (Fig. 5).

When the deprotection time was elongated for 48 h the oligomer decomposed. The exocyclic amino group of cytosine with other bases (*e.g.* primary amines) can be converted to the corresponding amine.[15] Indeed, *n*-butylamine, hydrazine or diethylamine furnished complex product solutions even after prolonged reaction times. Thus, the routinely applied ammonia solution was used for cleavage as no better one was found.

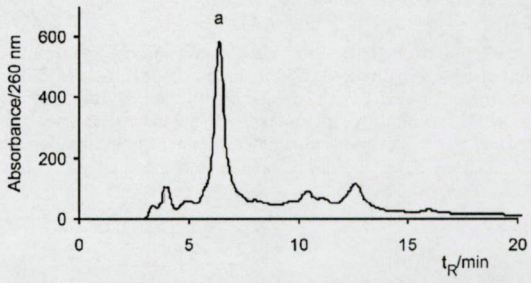

Fig. 5 HPLC chromatogram (Vydac column, TFA-containing eluent) of a crude PNA oligomer (peak a: compound **8**) after detachment from the support (Wang resin) and deprotection.

5. Synthesis on CPG

In order to prepare ON-PNA conjugates Wang resin is not an appropriate support for automated ON synthesis, thus we have turned to controlled pore glass (CPG) that is usually employed for this purpose.

Nonamers **9**, **10** and **11** were prepared on Fmoc-Gly-^{Bz}C-CPG under conditions optimized earlier, where 2′-deoxycytidine unit (^{Bz}C) is a kind of linker that was sacrificed during the cleavage. The HPLC chromatogram of the crude product **9** shows that the synthesis runs smoothly as was expected based on UV absorbances (Fig. 6). Surprisingly, both the ESI- and MALDI MS spectra of the crude product were too noisy due to the capped sequences that were present in low concentration based on HPLC analysis. The unambiguous identification of the product was achieved by MALDI-TOF analysis of the purified sample.

The *C* terminus of this substance is in both carboxy and carboxamide forms as can be seen from the isotopic pattern distribution in the MALDI-TOF-MS due to its high sensitivity. The molecular ions $[M + H]^+$ and $[M - H]^-$ were observed both in positive and negative modes which is also indicative of the presence of carboxy *C* terminus.

Conclusion

The synthesis of PNA oligomers has been elaborated using Fmoc/acyl-protected monomers. Although among the three activating agents (HATU, CMP, BET) the latter two were more effective in solution phase experiments the first one proved to be the best on the solid phase. Due to the "leakage" of TentaGel™ the product was contaminated and this rendered the mass spectrometric analysis difficult. The coupling and protecting group removal steps have been studied separately on Wang resin. Some isobutyryl group-containing PNA sequences remained in negligible amount with the classical (25% aq NH_3,50 °C, 16 h) cleavage condition.

Table 4 PNA sequences synthesized and MS data

Sequence[a]	M_{calc}	Peaks observed (ESI-MS)[b]
H-Gly-cgg act aag tcc att gc-Gly-NH_2 (**1**)[c]	4714	–[d]
H-Gly-ag tcc att gc-Gly-NH_2 (**2**)[c]	2814	–[d]
H-Gly-ggtt-Gly-NH_2 (**3**)[c]	1245	1245.8 $[M + H]^+$; 623.6 $[M + 2H]^{2+}$
H-Gly-aacc-Gly-NH_2 (**4**)[c]	1183	1184.2 $[M + H]^+$; 592.9 $[M + 2H]^{2+}$
H-Gly-gaa cat cat ggt cgt-Lys-OH (**5**)[e]	4284	2144.0 $[M + 2H]^{2+}$; 1430.0 $[M + 3H]^{3+}$; 1072.0 $[M + 4H]^{4+}$
H-Gly-a-Gly-NH_2 (**6**)[c]	406	406.9 $[M + H]^+$
H-Gly-g-Gly-NH_2 (**7**)[c]	422	423.0 $[M + H]^+$
H-Gly-g_8-Lys-OH (**8**)[e]	1658	1657.7 $[M + H]^+$; 830.3 $[M + 2H]^{2+}$
Ac-cat ggt cgt-Gly-NH_2 (**9**)[f]	2565	2565.97 $[M + H]^+$; 1283.48 $[M + 2H]^{2+}$, 856.15 $[M + 3H]^{3+}$
H-Gly-cat ggt cgt-Gly-NH_2 (**10**)[f]	2580	2581.28 $[M + H]^+$; 1291.08 $[M + 2H]^{2+}$
H-cat ggt cgt-Gly-NH_2 (**11**)[f]	2523	2524.03 $[M + H]^+$; 1262.5 $[M + 2H]^{2+}$

[a] PNA oligomer (one-letter lower-case abbreviations) and amino acid (three-letter codes) sequences have been depicted from *N* to *C* termini. *C* termini of PNA oligomers cleaved from the support with aq. NH_3 solution are both in carboxamide and carboxy forms but for simplicity only the former one is denoted. [b] Peaks obtained with the ESI technique are given for the most intensive monoisotopic peaks. For MS spectra obtained with the MALDI technique (compounds **9**–**11**) the values are given for the smallest monoisotopic peak. [c] Synthesized on TentaGel™ S–OH 130 resin. [d] Obscured by oligo(ethylene glycol) fragments. [e] Synthesized on Wang resin. [f] Synthesized on CPG support.

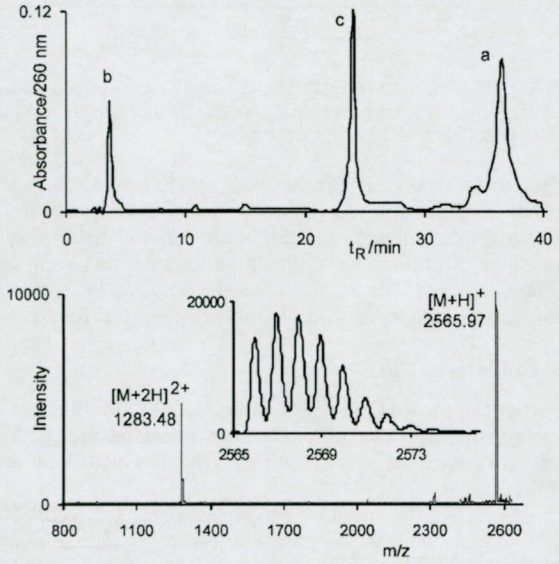

Fig. 6 Upper panel: HPLC chromatogram of the PNA sequence **9** prepared on a CPG support (crude product, LiChrospher RP Select B column, TFA-containing eluent). Peak a: product, peak c: anisoyl derivatives, peak b: 2′-deoxycytidine. Lower panel: MALDI mass spectrum (purified product, M_{calc} 2565). Inset: isotopic distribution of the molecular ion.

The PNA oligomer synthesis runs smoothly on CPG and this support is a promising candidate for the preparation of oligonucleotide–PNA conjugates with a suitable linker.

The best HPLC resolution of oligomers could be achieved with TFA solution on a LiChrospher RP Select B column.

In our experience it is not worthwhile to measure mass spectra of the crude samples because these spectra are rather noisy from the capped sequences even if the latter are present in low concentration.

Our Fmoc/acyl-protected PNA monomers, with certain limitations, can successfully be applied in the preparation of PNA oligomers and their conjugates. Alternatively, other possible methodologies should be evaluated for nucleobase deprotection and consecutive detachment from the solid support. Studies addressing these issues are in progress.

Experimental

General

Abbreviations: controlled pore glass (CPG); TentaGel™ S-OH 130 (TG-OH); triethylammonium acetate (TEAA); retention time (t_R); 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU); *N,N*-diisopropylethylamine (DIPEA); 2-bromo-3-ethylthiazolium tetrafluoroborate (BET); 2-chloro-1-methylpyridinium iodide (CMP, Mukaiyama reagent). TG-OH was obtained from Merck (catalogue no. 814786, capacity: 0.2–0.3 mmol g^{-1}, particle size: 130 μm), 500 Å CPG (~40 μmol g^{-1}) preloaded with 2′-deoxycytidine from ChemGenes Corporations (Ashland, MA, USA), whereas HATU was from PE Biosystems (Warrington, UK). Other chemicals were purchased from Aldrich, Fluka, Merck or Bachem. Anhydrous solvents were prepared as described.[32] DMF refers to an anhydrous solvent, and lutidine to the 2,6-isomer. HPLC chromatography was performed on an HP1050 (room temperature) or on an HP 1090 (55 °C) or on a Shimadzu LC 10 (in case of compounds **9**–**11**) instruments under the following conditions: (a) column packings: Rutin RP column C18, 300 Å, 250 × 4 mm (supplier: BST Ltd. Budapest, Hungary), LiChrospher RP Select B column C18, 60 Å, 250 × 4 mm (supplier: Merck Ltd. Budapest, Hungary), Vydac C4, 300 Å, 250 × 4.6 mm, Jupiter C18, 300 Å, 250 × 4.6 mm; (b) detection: 260 nm; (c) flow rate: 1.0 mL min^{-1}; (d) eluents: A: 0.1 M aq TEAA (pH 7.0), B: acetonitrile–0.1 M aq TEAA (pH 7.0) 8 : 2 (v/v); A: 0.1% aq TFA (pH 2.0), B: 0.1% in TFA acetonitrile–water 8 : 2 (v/v); (e) gradient: 5–35% (v/v) B in A during 30 min or 5–22.5% (v/v) B in A during 40 min (in case of compounds **9**–**11**). UV: PE Lambda 10 instrument. Mass spectrometry: Finnigan MAT TSQ 7000, electrospray (ESI) or nanoelectrospray[28] or Bruker Reflex III MALDI [(in case of compounds **9**, **10** and **11**) reflectron positive mode in 2–3 kDa window using sinapic or 2,5-dihydroxybenzoic acid matrices (the latter is better than sinapic acid in the 1–2 kDa region)]. Calculated molecular weights for peptide nucleic acids are given for the most intensive monoisotopic peaks.

HPLC peaks were identified by MS analysis of the fractions obtained after purification of the product.

Solution phase coupling studies

Reagents used in all coupling experiments have been utilized as stock solutions in anhydrous DMF if not stated otherwise.

To a 0.196 M solution of t monomer in DMF (10.2 μL, 2.0 μmol) one of the following solutions was added:

1. 0.77 M solution of HATU (2.6 μL, 2.0 μmol), 0.6 M solution of lutidine (3.7 μL, 2.2 μmol) and 0.4 M solution of DIPEA (5.0 μL, 2.0 μmol).

2. 0.78 M solution of BET (2.8 μL, 2.2 μmol), 0.4 M solution of DIPEA (11.0 μL, 4.4 μmol).

3. 0.51 M solution of CMP (4.3 μL, 2.2 μmol) and 0.4 M solution of DIPEA (11.0 μL, 4.4 μmol).

After 2 min preactivation, to each of the above solutions *tert*-butyl phenylalaninate (3.0 μmol) in DMF (6.0 μL) was added and the reaction mixture was made up to a total volume of 27.5 μL with DMF. After 5 min, 5 μL samples of the reaction solution were analyzed by HPLC (Rutin column, 1–80% (v/v) eluent B in A during 47 min) at 220 nm. Yields were determined by a calibration curve [peak area (5 μL solution, 220 nm) = f(concentration of t monomer)].

Solid phase synthesis (Tables 2 and 4)

Fmoc deprotection was performed with 20% (v/v) piperidine–DMF (1.5 mL) for 5 min, followed by washing with DMF (2 × 1 mL). 100 μL of this solution was added to EtOH (2 mL) and its absorbance was measured at 290 nm.[33]

The accuracy of the sample collection and absorbance determination is ±2.4% based on data from six independent measurements. [0.700; 0.717; 0.713; 0.718; 0.681; 0.730, where $\bar{x} = 0.7098$ and $\sigma = 0.0171$].

Each coupling was followed with capping with a DMF solution (1.5 mL) containing 5% (v/v) Ac_2O and 6% (v/v) lutidine for 5 min.

Coupling conditions in initial studies on TentaGel™ and Wang resins: monomer (0.1 M soln)–HATU–DIPEA–lutidine = 4 : 3.6 : 4.4 : 4.4 equiv. in DMF for 20 min after 2 min preactivation. Optimal coupling conditions: monomer–HATU–DIPEA–lutidine = 3 : 2.7 : 3.3 : 3.3 equiv. in DMF for 20 min after 2 min preactivation applied on Wang and CPG supports. PNA oligomers were synthesized manually using a home-built shaker in 3 mL Whatman syringes equipped with a 1 μm PTFE filter (catalogue no. 6984–0310) on 25–50 mg of resin on which the first unit was Gly (TG-OH, 0.25 mmol g^{-1} or H-^{Bz}C-CPG, 40 μmol g^{-1}) or Lys (Wang, 0.5 mmol g^{-1}, synthesis scale: 12.5–25 μmol). Cleavage: 25% aq NH_3, 16 h, 50 °C (TG-OH and CPG); in the case of Wang resin first a mixture of TFA and 1,3-dimethoxybenzene 9 : 1 (v/v) (1 mL) was added and it was allowed to react for 3 h. Subsequently, it was treated with aq NH_3 in the same way as above.

Using 3 equiv. monomer excess on Wang resin the following coupling yields (%) were achieved: 96 (a + a); 100 (a + c); 100, 100, 96, 100 (a + t); 94, 99, 95, 94 (c + a); 100, 100, 100 (c + g); 93, 100 (g + g); 100, 100, 96, 100 (g + t); 100, 94, 100, 100 (t + c); 100 (t + g). During synthesis on TentaGel™ there was no problem with the remaining 7 couplings which were not studied in detail on TentaGel™ resin: (a + g); (c + t); (g + a); (g + c); (t + a); (t + t) couplings, only (c + c) was a bit low (93, 100, 89%). (a + c), for example, represents coupling of monomer *a* to *c* that is connected to another monomer or amino acid on the support.

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2-Bromo-3-ethylthiazolium Tetrafluoroborate (BET)

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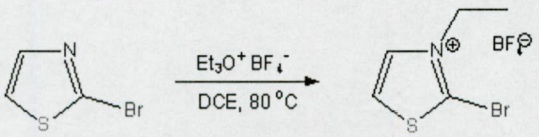

Recent progress in peptide synthesis resulted in the elaboration of novel coupling agents. One successful approach involves the application of thiazolium salts, *e.g.* 2-bromo-3-ethyl-4-methylthiazolium tetrafluoroborate (BEMT) [1-3]. This compound can be prepared from 2-bromo-3-ethyl-4-methylthiazole [1, 2]. We have found that a simpler analogue, 2-bromo-3-ethylthiazolium tetrafluoroborate (BET), can be prepared in convenient steps from the commercially available 2-aminothiazole using the procedure by Dondoni *et al.* [4] to obtain 2-bromothiazole which, in turn, was readily transformed into the title compound. In our experience BET is a highly efficient coupling reagent in the synthesis of peptide nucleic acid (PNA) oligomers in solution phase.
To a stirred solution of 2-bromothiazole [4] (1.78 mL, 20.0 mmol) in 1,2-dichloroethane (DCE, 20 mL) the solution of triethyloxonium tetrafluoroborate (11.4 g, 60.0 mmol) in DCE(60 mL) was added over 45 min. at 80 °C. When the reaction was complete (16 h) according to TLC (n-buthanol : acetic acid : water = 4:1:1) the solution was concentrated *in vacuo*. After the residue was precipitated from diethyl ether it was recrystallized from *abs.* acetonitrile/ethyl acetate to afford white plates. Yield: 4.48 g (80 %).

Mp: 137.9-139.2 °C.

^{1}H NMR (DMSO-d_6, 500 MHz, δ, ppm): 1.47 (t, J = 7.0 Hz, 3H, CH_2CH_3); 4.52 (q, J = 7.0 Hz, 2H, CH_2CH_3); 8.35 (d, J = 4.1 Hz, 1H, aryl); 8.56 (d, J = 4.1 Hz, 1H, aryl).

^{13}C NMR (DMSO-d_6, 125 MHz, δ, ppm): 14.19 (CH_2CH_3); 50.61 (CH_2CH_3); 127.45 (C-5); 137.73 (C-4); 146.50 (C-2).

ESI-MS (m/z, %): 191.8 (100, $[C_5H_7{}^{79}BrNS]^+$); 193.8 (97, $[C_5H_7{}^{81}BrNS]^+$).

Anal. cald. for $C_5H_7BBrF_4NS$ (279.891): C, 21.45; H, 2.52; Br, 28.55; F, 27.15; N, 5.00; S, 11.46; found C, 21.47; H, 2.51; Br, 28.52; F, 27.20; N, 5.01; S, 11.44.

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Sample availability: sample available from the authors and MDPI.