REGIOSELECTIVE SYNTHESIS AND MS/MS STUDY OF ALKYL (GUANIN-9-YL)ACETATES. SYNTHESIS OF PEPTIDE NUCLEIC ACID OLIGOMERS

Ph. D. thesis

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Abbreviations

Ade	adenine
aeg	*N*-(2-aminoethyl)glycyl
An	anisoyl
APICID	atmospheric pressure ionisation collision-induced dissociation
BET	2-bromo-3-ethylthiazolium tetrafluoroborate
Bn	benzyl
Bu^t	*tert*-butyl
CI	chemical ionisation
CID	collision-induced dissociation
CMP	2-chloro-1-methylpyridinium iodide (Mukaiyama reagent)
CPG	controlled pore glass
Cyt	cytosine
DIAD	diisopropyl azodicarboxylate
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimetylformamide
DMSO	dimethylsulfoxide
Dpc	*N,N*-diphenylcarbamoyl
ESI	electrospray ionization
Et_2O	diethyl ether
EtOAc	ethyl acetate
FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
Gua	guanine
HATU	2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetra-methyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
Ibu	isobutyryl
MALDI	matrix-assisted laser desorption/ ionisation
Me	methyl
MeOH	methanol
MS/MS	tandem mass spectrometry
Ph	phenyl
PMB	4-methoxybenzyl
PNA	peptide nucleic acid(s)
PNB	4-nitrobenzyl
Pnt	pent-4-enoyl
Pr^i	isopropyl
rt	room temperature
4-tBuBz	4-*tert*-butylbenzoyl
TEA	triethylamine
TEAA	triethylammonium acetate
TFA	trifluoroacetic acid
TG-OH	TentaGel™ S-OH 130
THF	tetrahydrofuran
Thy	thymine
TLC	thin layer chromatography
TMSCl	chlorotrimethylsilane
TOF	time-of-flight
t_R	retention time

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I. Z. Timár, L. Kovács, **Gy. Kovács** and Z. Schmél: Fmoc/Acyl protecting groups in the synthesis of polyamide (peptide) nucleic acid monomers, *J. Chem. Soc., Perkin Trans. 1*, 19-26 (2000)

II. **Gy. Ferenc**, P. Forgó, Z. Kele and L. Kovács: A regioselective synthesis of alkyl 2-(guanin-9-yl)acetates as PNA building blocks from 7-(4-nitrobenzyl)guanine derivatives, *Collect. Czech. Chem. Commun.*, in print (2004)

III. **Gy. Ferenc**, Z. Kele and L. Kovács: Determination of the N^9/N^7-isomer ratio of alkyl (guaninyl)acetates by electrospray ionization tandem mass spectrometry, *Rapid Commun. Mass Spectrom.*, in print (2005)

IV. **Gy. Kovács**, Z. Timár, Z. Kupihár, Z. Kele and L. Kovács: Synthesis and analysis of peptide nucleic acid oligomers using Fmoc/acyl-protected monomers, *J. Chem. Soc., Perkin Trans. 1*, 1266-1270 (2002)

V. **Gy. Kovács**, Z. Kele, P. Forgó and L. Kovács, 2-Bromo-3-ethylthiazolium tetrafluoroborate, *Molecules*, **6**, M219-M219 (2001), http: //www.mdpi.org/molbank/m0219.htm

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1.1. L. Kovács, Z. Timár, **Gy. Kovács**, Z. Schmél, G. Kiss, P. Benke, G. Pócsfalvi, R. Rónyai (1999): Nukleobázisok alkilezési reakciói. MTA Bioorganikus Kémiai Munkabizottság. (Szeptember 9-10, Visegrád)

1.2. L. Kovács, Z. Timár, **Gy. Kovács**, Z. Schmél, G. Kiss, P. Benke, G. Pócsfalvi, R. Rónyai (1999): Nukleobázisok alkilezési reakciói. VI. Szent-Györgyi Napok. (Szeptember 30 - Október 2, Szeged)

1.3. L. Kovács, **Gy. Kovács**, P. Forgó, Z. Kele (2002): Regioselective substitution of guanine. Czech Chemical Society. (3 October, Brno)

1.4. **Gy. Kovács**, P. Forgó, Z. Kele, L. Kovács (2003): A guanin szelektív N-9-alkilezése N-7-(4-nitrobenzil)guanin alkalmazásával. MTA Nukleotidkémiai Munkabizottsági Ülés, (Május 27, Balatonszemes)

1.5. **Gy. Ferenc**, P. Forgó, Z. Kele, L. Kovács (2003): Selective N-9 alkylation of guanine using N7-(4-nitrobenzyl)guanine. Nucleic Acid Chemical Biology (NACB) PhD Summer School/ Symposium. (17-20 June, Odense, Denmark)

1.6. **Gy. Ferenc**, P. Forgó, Z. Kele, L. Kovács (2003): Selective N-9 alkylation of guanine using N^7-(4-nitrobenzyl)guanine. 19th International Congress of Heterocyclic Chemistry, 11-PO-51. (10-15 August 2003, Fort Collins, Colorado, USA)

2.1. **Gy. Ferenc,** Z. Kele, L. Kovács (2004): Az alkilezett guanin származékok ESI-MS/MS vizsgálata. MTA Nukleotidkémiai Munkabizottsági Ülés, (Május 26, Balatonszemes)

3.1. **Gy. Kovács,** Z. Timár, Z. Kele, L. Kovács (2000): Optimization of peptide nucleic acid (PNA) oligomer synthesis using Fmoc/acyl-protected monomers. 4th Electronic Conference on Synthetic Organic Chemistry, B0003-B0003. (1-30 September), http://www.unibas.ch/mdpi/ecsoc-4/b0003/b0003.htm

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3.5. **Gy. Kovács,** Z. Timár, Z. Kupihár, Z. Kele, P. Forgó, L. Kovács (2001): Synthesis of peptide nucleic acid (PNA) oligomers and their conjugates. Hungarian-German-Italian-Polish Joint Meeting on Medicinal Chemistry, P76. (2-6 September, Budapest)

3.6. **Gy. Kovács,** P. Pádár, Z. Timár, Z. Kupihár, Z. Kele, L. Kovács (2002): Synthesis and MS analysis of peptide nucleic acid (PNA) oligomers and conjugates. National Center for Biomolecular Research. (2 October, Brno)

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4. P. Pádár, **G. Kovács** and L. Kovács, *Molbank,* 2004, M381-M381.

5. **G. Ferenc**, Z. Kupihár, Z. Kele and L. Kovács, *Nucleosides Nucleotides Nucleic Acids,* 2005, accepted for publication.

6. A. Horváth, Z. Kupihár, **G. Ferenc**, P. Pádár, M. Hornyák, L. Kovács and J. Aradi, *J. Med. Chem.*, 2005, in preparation.

1. Introduction and Aims

Peptide nucleic acids (PNA), oligonucleotide analogues developed by Nielsen *et al.* in 1991, are important tools in molecular biology and in drug design owing to their antisense and antigene effects by physically interfering with the function of the mRNA and forming triplex invasion loops.[1-6] Their advantages are greater *in vivo* stability and stronger hybridization ability in comparison with native oligonucleotides. The latter property is essential in the field of genetic diagnoses as well *e.g.* in PNA-fluorescence *in situ* hybridization (PNA-FISH) probes,[7] in MALDI-TOF-MS study[8] and in PCR (polymerase chain reaction).[9]

Unfortunately, PNAs enter less easily into the cells and cell nuclei and have low RNAseH activity. Conjugating them to peptides,[10-12] oligonucleotides,[13-17] carbohydrates[18] or lipophilic molecules,[19,20] respectively, might be considered as possible solutions for the above problems.

In order to synthesize PNA conjugates with peptides or oligonucleotides, suitably protected monomers and the conditions of the oligomer synthesis, which should be compatible with both peptide and oligonucleotide synthesis protocols, are indispensable. To date this problem has not been solved (Table 1).

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

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

Our aim was to synthesize PNA oligomers by Fmoc (backbone) and acyl [anisoyl (adenine), 4-tert-butyl-benzoyl (cytosine), isobutyryl (guanine)] protecting group strategy form our own monomers that could fulfil the requirements of the synthesis of conjugates. [I, IV]

The regioselectivity of guanine alkylation was one of the main problem in the synthesis of PNA monomers, leading to mixtures of N^9- and N^7-regiosiomers of alkyl [N^2-(acyl)guaninyl]acetates which can be very difficult to separate.[22,23]

The simplest synthesis of alkyl [N^2-(acyl)guanin-9-yl]acetates consists of alkylation of N^2-

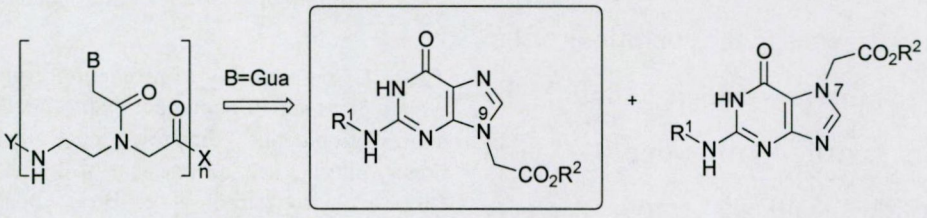
isobutyrylguanine but the yield of N^9-regioisomer is usually low, *ca.* 40%.[24] The alkylation of the lactim form of guanine in N^2-acyl-O^6-(*N,N*-diphenylcarbamoyl)guanine[25] or that of 2-amino-6-chloropurine[26,27] significantly improved the yield of N^9-regioisomers to 70-75%.

Our aim was to elaborate a synthesis of alkyl [N^2-(acyl)guanin-9-yl]acetates with higher regioselectivity [I, II].

In addition, a fast, simple and quantitative analytical method was required for the determination of regioisomer ratio to speed up the optimization of regioselective alkylation conditions.

The disadvantages of NMR and HPLC analysis are the long analysis time, 10-20 min/sample (thus in the case of 10 parallel reactions 100-200 min) and a large volume of deuterated or high purity solvent, in addition, the former requires a pure and solvent free sample, the latter the separation of regioisomers. MS is a faster and cheaper technique but the basic condition is to find a product ion, the abundance of which is in linear correlation with the ratio of regioisomers.

Our aim was to investigate the N^9/N^7-regioisomer pairs with the time- and cost-effective tandem mass spectrometry to find the optimal CID conditions under which significant difference can be observed in the fragmentation of the regioisomers in order to determine their ratio [III].



Scheme 1 Summary of this PhD thesis: Synthesis of PNA oligomers, including the synthesis of N^9-alkylated guanine derivatives and MS/MS study of N^9 and N^7 regioisomers. R^1: isobutyryl (Ibu) or pent-4-enoyl (Pnt). R^2: methyl (Me), *tert*-butyl (Bu^t) or H. Monomer: n=1; Y=Fmoc; X= OH and B= ^{Ibu}Gua. Oligomer: n>1; Y=H; X= NH_2/ OH and B= Gua; Ade; Cyt; Thy.

2. A regioselective synthesis of alkyl [N^2-(acyl)guanin-9-yl]acetates

2.1. Introduction

Guanine has problematic chemistry including poor solubility and low regioselectivity in reactions *e.g.* alkylations and glycosylations due to its polyfunctionality.

Constraining guanine into its lactim form *e.g.* in the case of 2-amino-6-chloropurine,[26-31] 2-amino-6-(arylsulfanyl)purines,[32] 2-amino-6-(1,2,4-triazol-4-yl)purines[33] or O^6-(*N,N*-diphenylcarbamoyl) derivatives[25] improves the N^9/N^7-isomer ratio, but this is not sufficient requirement since the highest yield of N^9-isomer was around 75%[25] and a chromatographic purification could not be avoided in every case. In addition, 2-amino-6-chloropurine is mutagenic, expensive and poorly regioselective in some cases,[34,35] conversion of 2-amino-6-(arylsulfanyl)purines and 2-amino-6-(1,2,4-triazol-4-yl)purines to guanine derivatives requires a strong acidic or alkalic treatment, and *N,N*-diphenylcarbamoyl group is acid labile[36,37] and these facts impose limitations on their applicability.

The most successful protecting group, in respect of regioselectivity, is the *N,N*-diphenylcarbamoyl[38] which reportedly gives in some cases 100:1 ratio in favour of the N^9-regioisomer.[39,40] The yield of N^9-regioisomer strongly depends on the properties of alkylating reagent *e.g.* presence of β-*O*-activation and size.[41]

2.1.1. Alkylation with β-*O*-activated reagents

High regioselectivity in alkylation of guanine derivatives (*e.g.* N^2, N^9-diacetylguanine[42] or per (trimethylsilyl)-N^2-isobutyryl-O^6-(*N,N*-diphenylcarbamoyl)guanine[40]) can be obtained with β-*O*-activated reagents (*e.g.* 2-oxa-1,4-butanediol diacetate[42] or peracylated sugars[40]). The N^9-isomer is the main product under termodynamic conditions, in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) at an elevated temperature. The N^7-isomer can be isolated in a large amount from the kinetic reaction mixture, below room temperature in the presence of a $SnCl_4$ or $TiCl_4$ catalyst.[40,42] 9-Alkoxyalkylated products can be obtained in good yields even if an N^9/N^7- isomeric mixture is formed while an N^7 to N^9 rearrangement takes place upon heating[43-46] or even at room temperature in DMF.[47]

2.1.2. Alkylation with non-β-*O*-activated reagents

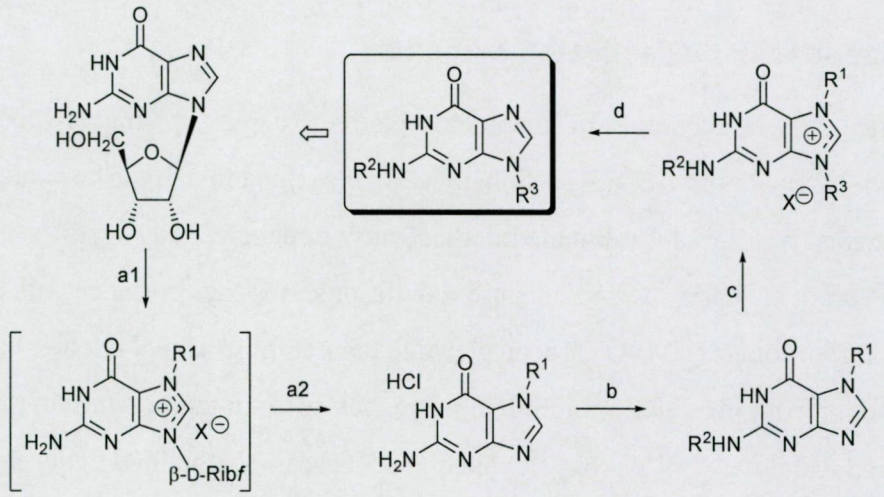
Under alkaline conditions. The regioselectivity is still inadequate even in the alkylation of the lactim form of guanine [*e.g.* N^2-acetyl-O^6-(*N,N*-diphenylcarbamoyl)-guanine] with non-β-*O*-activated, small-sized alkyl halides (*e.g.* methyl bromoacetate) under alkaline conditions (*e.g.* DIPEA) since the best yield of N^9-isomer was around 75%.[25]

In Mitsunobu reaction. Mitsunobu reaction, an important alternative to alkylation in alkaline solution, proved to be highly regioselective leading only to the N^9-alkylated product of N^2-acetyl-O^6-(2-{4-nitro-phenyl})ethylguanine.[48]

The effect of the size of alkylating reagent on regioselectivity. The regioselectivity was 94% in alkylation of 2-amino-6-chloropurine with 5-(2-bromoethyl)-2-phenyl-1,3-dioxane. This result can be attributed to the bulky phenyl group in the dioxan ring since the N^7-isomer was formed in 10% with the sterically less hindered *tert*-butyl group.

2.1.3. The method by Izawa *et al.*

Izawa *et al.* reported an N^9-regioselective substitution starting from guanosine.[49,50] In their approach guanosine was protected on N^7 with a (substituted) benzyl group; then, after acid hydrolysis of *N*-glycosidic bond and acylation of the amino group, 7-(substituted benzyl)-N^2-acyl-guanines were alkylated selectively at N^9, then deprotected at N^7 in a good overall yield (ca. 80%,



Scheme 2 The reaction sequence followed by Izawa *et al. General reagents and conditions:* a. (1) R^1X, (2) HCl. b. R^2X. c. R^3X. d. removal of R^1. β-D-Rib*f* =β-D-ribofuranosyl; R^1 = (substituted) benzyl; R^2 = various acyl; R^3 = various alkyl.

Scheme 2). In this case the (substituted) benzyl is not only a protective group but it activates the purine ring towards alkylation.[49,50]

Kobe *et al.*[51,52] have developed an almost identical approach as Izawa *et. al.* did. The main difference between them is that the method by Kobe *et al.* provides N^7-regioisomers as well through N^9-benzylguanine derivatives.

2.1.4. Synthesis of alkyl [*N* ^{2}-(acyl)guanin-9-yl]acetates

The simplest synthesis of alkyl [*N* ^{2}-(acyl)guanin-9-yl]acetates is alkylation of N^2-isobutyrylguanine but the yield of N^9-regioisomer was low, *ca.* 40%.[24] The alkylation of the lactim form of guanine in N^2-acetyl-O^6-(*N,N*-diphenylcarbamoyl)-guanine[25] or that of 2-amino-6-chloropurine[26,27] significantly improved the yield of N^9-regioisomers to 70-75%.

2.1.5. Aims

The synthesis of alkyl [*N* ^{2}-(acyl)guanin-9-yl]acetate:

- by a highly regioselective Mitsunobu reaction from N^2-acyl-O^6-(*N,N*-diphenyl-carbamoyl)-guanine, in 3 steps from guanine
- by the method by Izawa *et al.*, leading exclusively to the N^9-isomer through N^2-acyl-N^7-(substituted benzyl)-guanine, in 4 steps form guanosine.

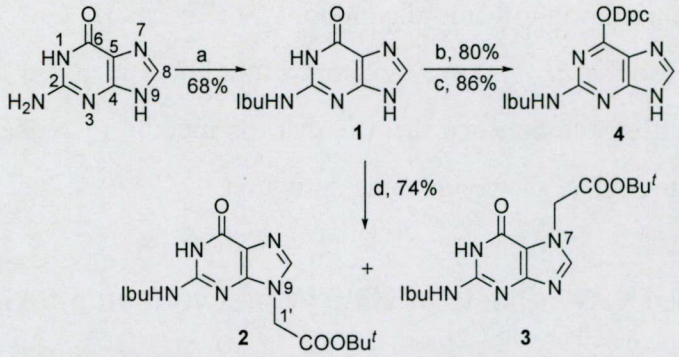
2.2. Results and discussion

The isobutyryl group was selected as a protective group for the *N* ^{2}-amino group of alkyl [N^2-(acyl)guanin-9-yl]acetates because it fits into the Fmoc/acyl strategy of PNA oligomer synthesis and, although its removal under basic conditions is more sluggish than that of other simple acyl groups (acetyl, propionyl)[38,53] it confers steric hindrance on the 2-amino group and thus prevents unwanted alkylation/glycosylation on it.[40]

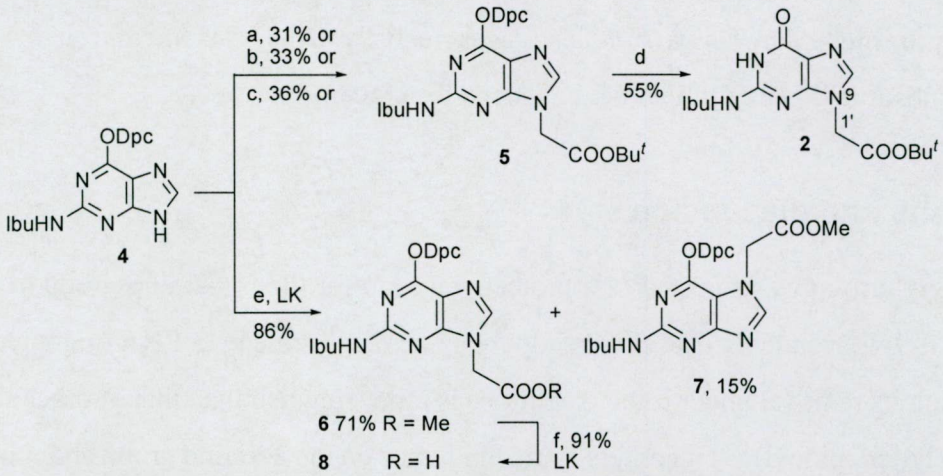
In order to see how the introduction of *N,N*-diphenylcarbamoyl group alters the selectivity of alkylation, first N^2-isobutyrylguanine **1**[54] was alkylated with *tert*-butyl bromoacetate in the presence of sodium hydride to afford nearly 1:1 ratio of N^9/N^7-isomers (**2** and **3**, respectively) in

74 % yield (Scheme 3).

Next, the *N,N*-diphenylcarbamoyl derivative **4**[29,40] was alkylated with *tert*-butyl glycolate[55] in a highly regioselective Mitsunobu reaction[56,57] and this provided the product (**5**, Scheme 4) with good regioselectivity, however, the purification was very difficult and ester **5** was contaminated with significant amounts of triphenylphosphine oxide. (4-Dimethylaminophenyl)diphenylphosphine,[58,59] claimed to give a phosphine oxide which can be removed by acidic extraction,[60] 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%, 17% from guanine). In conclusion, Mitsunobu reaction was not convenient in spite of its high regioselectivity because of the problematic removal of contaminating phosphine oxides.



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.



Scheme 4 *Reagents and conditions:* a. DIAD, Ph_3P, THF, $HOCH_2COOBu^t$. b. DIAD, 4-$Me_2NPhPPh_2$, THF, $HOCH_2COOBu^t$. c. DIAD, Bu_3P, THF, $HOCH_2COOBu^t$. d. 8% (v/v) TFA/DCM, 1,3-$(MeO)_2C_6H_4$, 0 °C, 18 h. e. $BrCH_2COOMe$, $EtNPr^i_2$, DMF. f. NaOH, aq. dioxane/MeOH then HCl. LK= results by L. Kovács

In the meantime my supervisor alkylated compound **4** with methyl bromoacetate[25] to

circumvent premature cleavage of Dpc group in the subsequent hydrolysis in the presence of *N,N*-diisopropylethylamine as a hindered base. The N^9-isomer (33% overall yield from guanine) was obtained in 71% yield along with 15% of the N^7-isomer (Scheme 4, route e). The surprisingly high yield of the unwanted isomer 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 Dpc group has solved the historic problem of regioselective N^9-substitution of guanine[39,40] seems to be restricted to the realm of glycosylation reactions while alkylation transformations require further experimentation.

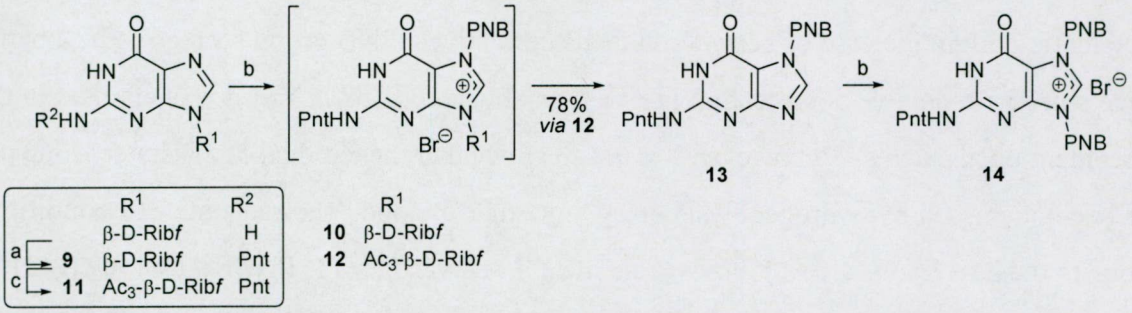
In our further study the Izawa method has been adopted for the regioselective N^9-alkylation of guanine. The reaction steps were high-yielding until the deprotection on N^7, which was problematic both in the case of benzyl and PMB derivatives. PNB group seemed to be a better choice since its reductive deprotection is a well-established method, and it was claimed in the earlier mentioned patents,[51,61] however, it is not clear whether the removal of this latter group has ever been attempted by hydrogenolysis or by any other method. The reagents and conditions leading to the best results are as follows (Scheme 2, L. Kovács): a. (1) PNBBr, DMSO; (2) HCl, then MeOH (97%). b. Ibu_2O, Et_3N, DMF, 150 °C, 3 h (82%). c. $BrCH_2COOBu^t$, DMF, 60 °C, 24 h (93%). d. Zn, aq. AcOH, rt, 18 h (44-60%). The overall yield of N^9-isomer from guanosine was 33-44%. Note that the guaninium salt can be obtained in 74% from guanosine therefore an improvement of the deprotection of N^7 is an important task for a better overall yield of N^9-isomer. [II]

Meanwhile, we have turned to the monomethoxytrityl/pent-4-enoyl (MMTr/Pnt) protective group strategy in the PNA synthesis. The N^2-protecting group of guanine had to be changed from isobutyryl because the deprotection of 2-amino group was not quantitative during the cleavage and deprotection of PNA oligomers (*cf.* the results in section 4. Synthesis and analysis of PNA oligomers). [IV] The advantage of the pent-4-enoyl group is that it can be removed with iodine as well[62-64] beside the conventional ammonia and this could be useful in the future synthesis of PNA-peptide conjugates.

7-(4-Nitrobenzyl)guanine hydrochloride did not dissolve well during the acylation reaction with pent-4-enoic anhydride, hence another route was needed to protect the 2-amino group.

Considering the high price of the 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-enoylguanosine (**9**) was obtained in a good yield by temporary protection of hydroxy groups with the electron-donating trimethylsilyl group.[63,65] The 7,9-bis(4-nitrobenzyl)guaninium salt **14**, detected in the reaction mixture by TLC/MS, was the product in subsequent reaction with 4-nitrobenzyl bromide (Scheme 5), 7-(4-nitrobenzyl)-N^2-pent-4-enoyl-guanine (**13**) 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.[66]



Scheme 5 *Reagents and conditions:* a. (1) TMSCl, pyridine, rt, 1 h; (2) pent-4-enoic anhydride, pyridine, rt, 16 h; (3) water, 0-5 °C, 5 min; (4) aq. NH_3, rt, 30 min. b. 4 equiv. 4-nitrobenzyl bromide, DMF, rt, 2.5 days. c. Ac_2O, pyridine, DMF, rt, 16 h, a+c: 71%. β-D-Rib*f* = β-D-ribofuranosyl; Ac_3-β-D-Rib*f* = 2',3',5'-tri-*O*-acetyl-β-D-ribo-furanosyl.

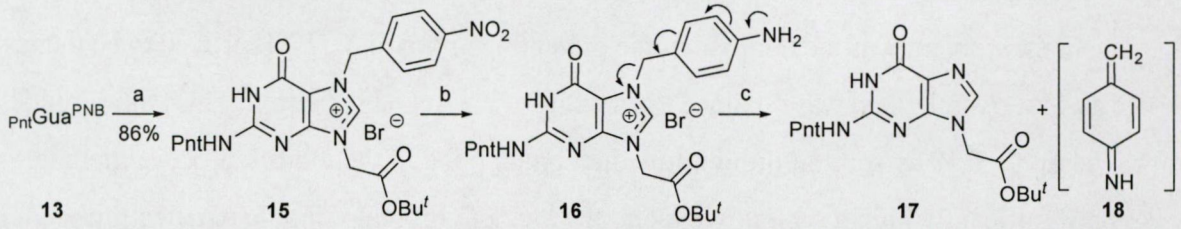
Enhancing the stability of the *N*-glycosidic bond is imperative for the monoalkylation of guanosine derivatives by introduction of electron-withdrawing, *e.g.* acetyl groups onto the hydroxy groups of the carbohydrate moiety. Pent-4-enoylation of 2',3',5'-tri-*O*-acetylguanosine was a slow and low-yielding process even at higher temperature therefore 2',3',5'-tri-*O*-acetyl-N^2-(pent-4-enoyl)guanosine (**11**) was synthesized by acetylation of N^2-(pent-4-enoyl)guanosine (**9**). The formation of guaninium salt **12** was slow but compound **14** formed only in a negligible amount. The reaction was complete after 2.5 days with an usual 4 equiv. excess of the reagent. The excess of 4-nitrobenzyl bromide was scavenged by pyridine in order to avoid the formation of the dialkylated product **14** during evaporation of the solvent (DMF). 4-Nitrobenzylpyridinium bromide and compound **13** were 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 groups activated the 2-amino group and the acylation took place smoothly.

Alkylation of compound **13** affording guaninium salt **15** (Scheme 6), with 3 equiv. of *tert*-butyl bromoacetate at 70 °C, was complete overnight.

Purification of the product was too labourious after PNB removal with Zn/acetic acid (*cf.* paper II) therefore other reducing agents have been studied.

Reduction of the nitro group in compound **15** was complete within 30 min in the presence of 4 equiv. of sodium dithionite even at pH 4, not only at pH 8-9 where deprotection of 4-nitrobenzyloxycarbonyl group was observed.[67] To enhance the rearrangement of 4-aminobenzyl group leading to 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 **16** was verified by TLC/MS, Scheme 6). Based on the observation that electron-donating substituents on phenyl ring and/or on α-carbon atom promote the rearrangement by stabilising the positive charge on benzyl methylene group in the removal of 4-nitrobenzyl carbamates,[68] 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



Scheme 6 *Reagents and conditions:* a. 3 equiv. *tert*-butyl bromoacetate, DMF, 70 °C, 16 h. b. 4 equiv. $Na_2S_2O_4$, aq. acetone, pH 7.0, rt, 30 min. c. 70 °C, 16 h, b+c: 76%. $_{Pnt}Gua^{PNB}$ (**13**): *N*-[7-(4-Nitrobenzyl)-6-oxo-6,7-dihydro-1H-purin-2-yl]pent-4-enamide

formed cation.

Iminoquinomethane **18** 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.[68-70] We have been able to detect in the ESI mass spectrum the 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-coloured aqueous and the organic phases. It was attempted to scavenge the substance with sulfites[69] (oxidized form of dithionite), however, it was impossible to completely remove this substance from the product. The slightly yellowish amorphous product **17** was easily obtained by washing with ethyl acetate; it was completely pure according to TLC and NMR analyses.

2.3. Conclusion I

tert-Butyl [N^2-(acyl)guanin-9-yl]acetate was synthesized:

1. from guanine through the promising N^2-isobutyryl-O^6-(*N,N*-diphenylcarbamoyl)-guanine (**4**)

a) in a highly regioselective Mitsunobu reaction but the yield of N^9-isomer was below 40% because of the problematic removal of phosphine oxides even in the case of water-soluble tributylphosphine oxide.

b) in alkylation with methyl bromoacetate in the presence of *N,N*-diisopropylethylamine as a hindered base. The N^9-isomer was obtained in 71% yield, in 33% overall yield from guanine (results by L. Kovács).

2. from guanosine employing the method by Izawa *et al.* leading exclusively to the N^9-isomer without column chromatography.

The main problem was the removal of the protecting group of N^7. PNB proved to be the best choice as 44% overall yield was obtained even with the Zn/AcOH reduction. This value could be improved up to 56% by sodium dithionite reduction, applied in the modified Izawa method.

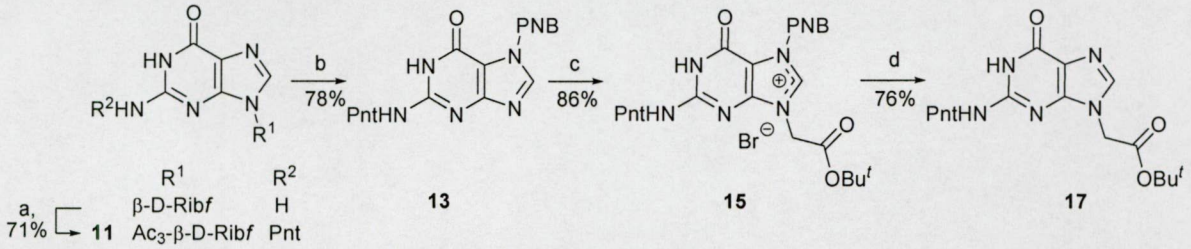
The significantly higher overall yield and the lack of chromatographic purification renders the Izawa method the best regioselective alkylation procedure despite the extra reaction step.

These methods are not compatible with sensitive acylating reagents and to overcome the low solubility caused by the free or protonated 2-amino groups of guanine and N^7-benzylguaninium hydrochloride high temperatures are required. Guaninium hydrochloride did not dissolve at all in pent-4-enoylation in contrast to isobutyrylation.

The alkylation step			The overall method		
Starting material	Condition	Ratio of N^9/N^7-isomers	No of reaction steps [a]	Overall yield of N^9-isomer [%]	Scheme No.
$_{Ibu}$Gua (**1**)	alkaline, NaH	1:1	2	25	3
$^{Dpc}_{Ibu}$Gua (**4**), LK	alkaline, DIPEA	1:0.2	3	33	4
$^{Dpc}_{Ibu}$Gua (**4**)	Mitsunobu	*ca.* 1:0	3	17	4
$_{Ibu}$Gua^{PNB}, LK	*Izawa et al.*	*1:0*	*4*	*33-44 (56 [b])*	*2*
$_{Pnt}$Gua^{PNB} (13)	***modified Izawa method***	***1:0***	***4 [c]***	***36***	***7***

Table 2 Comparison of the alkylation methods of guanine.
[a] starting from guanine or guanosine; [b] theoretical yield, calculating with the yield obtained in the PNB removal with sodium dithionite in the case of compound **15**; [c] 6 reactions can be combined into 4 distinct, well-reproducible steps The row in italic is the method by Izawa *et al.* with 100% regioselectivity requiring no chromatographic purification. The bold one is the only suitable method for heat-sensitive acylation transformations. $^{Dpc}_{Ibu}$Gua (**4**): (2-isobutyramido-9H-purin-6-yl)diphenylcarbamate; $_{Ibu}$Gua^{PNB}: *N*-[7-(4-Nitro-benzyl)-6-oxo-6,7-dihydro-1H-purin-2-yl]isobutyramide; $_{Pnt}$Gua^{PNB} (**13**): *N*-[7-(4-Nitrobenzyl)-6-oxo-6,7-dihydro-1H-purin-2-yl]pent-4-enamide

In order to avoid the formation of hydrochloride salts the general sequence of the Izawa method was changed in the following ways: (1) pent-4-enoylation of guanosine at N^2 (**9**), (2) per-*O*-acetylation (**11**), (3) 4-nitrobenzylation at N^7 (**12**), (4) *N*-glycoside hydrolysis (**13**), (5) N^9-alkylation (**15**) and (6) deprotection with sodium dithionite (Scheme 7).



	R^1	R^2
11	β-D-Rib*f*	H
13	Ac_3-β-D-Rib*f*	Pnt

Scheme 7 *Reagents and conditions:* a. (1) TMSCl, pyridine, rt, 1 h; (2) 1.25 equiv. pent-4-enoic anhydride, pyridine, rt, 16 h; (3) water, 0-5 °C, 5 min; (4) aq. NH_3, rt, 30 min; (5) Ac_2O, pyridine, DMF, rt, 16 h. b. (1) 4 equiv. 4-nitrobenzyl bromide, DMF, rt, 60 h; (2) 8 equiv. pyridine, 5h; (3) 70 °C, 16 h. c. 3 equiv. *tert*-butyl bromoacetate, DMF, 70 °C, 16 h. d. (1) 4 equiv. $Na_2S_2O_4$, aq. acetone, pH 7.0, rt, 30 min; (2) 70 °C, 16 h. β-D-Rib*f* = β-D-ribofuranosyl; Ac_3-*O*-β-D-Rib*f* = 2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl.

This is the optimal sequence of reactions since the influence of *O*- and/or N^2-acyl groups on the stability of *N*-glycosidic bond and reactivity of 2-amino group is remarkable. The electron-withdrawing property of the acetyl group is responsible for the stability of guaninium salt **12** at room temperature and the decreased reactivity of 2',3',5'-tri-*O*-acetylguanosine in N^2-acylation.

This seemingly lengthy procedure can be combined into 4 distinct, well-reproducible steps with standard transformations and 36% overall yield. This yield is a modest value but the solubility of each guanine derivative involved was good and this procedure can be the method of choice for heat-sensitive substrates.

3. An ESI-MS/MS study of N^9- and N^7-alkylated guanine derivatives

3.1. Introduction

As it has been mentioned earlier, 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 by-products.[22] The yield of the product can be enhanced by optimization of reaction conditions, the effectiveness of which can be improved by a rapid analytical method avoiding the 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) (^{1}H, ^{13}C) techniques but chemical shift difference of some diagnostic ^{13}C signals[21] and/or chemical shift of N-3[71] are very informative as well. Unfortunately, the latter two methods, unlike ^{1}H 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 minutes. 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 an NMR identification of isomer peaks to have a standard sample, in addition, a gradient system for the separation of regioisomers is required and an 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 an 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 isomers.

Details of the derivatives of N^7 - and N^9 -alkylated guanine derivatives studied by MS/MS are collected in Table 3.

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.[72] 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.[76] These studies have, however, afforded only qualitative information.

Alkyl side chain[a]	The site of the alkyl group[a]
Me[72-74], Et[74], Pr[75]	7[72-75], 9[72,74]
CH_2CRHOH, R=H, CH_3, CH_2OH	775
$CH_2OCH_2CH_2OR$, R=H, Ac	7[76,77], 9[76,77], 9[78] (R=H)
$CH_2OCH(CH_2OR)_2$, R=H, Bn	7[77], 9[77]

Table 3 The structure of alkylated guanine derivatives studied by MS/MS (CI-MS/MS,[72] ESI-MS/MS,[73,78] FAB-MS/MS[74-77]) methods. [a] ref. in superscripts

The fragmentation of the protonated guanine backbone was confirmed by ^{15}N and ^{16}O labelling[79] and H/D exchange,[78] 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 **19** (9IbuMe), **2** (9IbutBu)[21] and **17** (9PnttBu)[80] 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 **20** (7IbuMe) and **3** (7IbutBu), respectively[21] (Figure 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, time- and cost-effective MS/MS method finding optimal collision-induced dissociation (CID) conditions where a significant difference can be observed in the fragmentation of regioisomers.

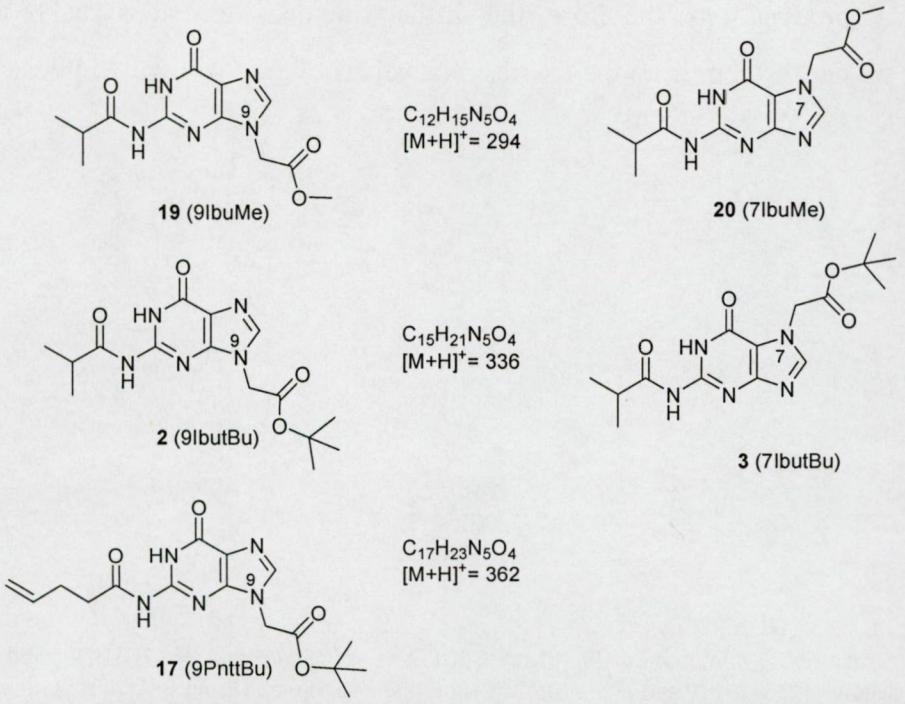


Figure 1 Structures of alkyl (guaninyl)acetates (**2**, **3**, **17**, **19** and **20**) studied by ESI-MS/MS.

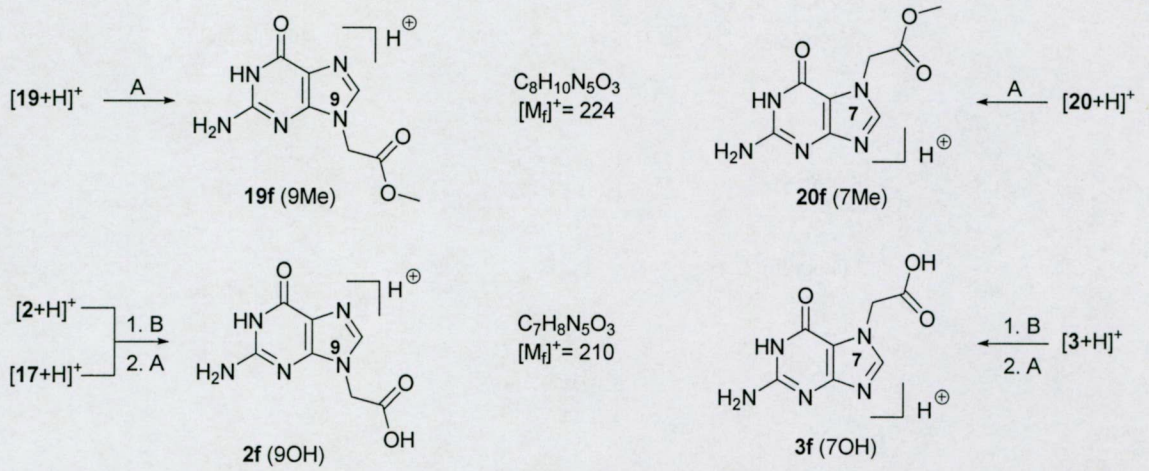
3.2. Results and discussion

3.2.1. Qualitative studies of regioisomer pairs

Our compounds (Figure 1) containing different N^2-acyl protecting groups [isobutyryl in **19** (9IbuMe), **20** (7IbuMe), **2** (9IbutBu), **3** (7IbutBu) and pent-4-enoyl in **17** (9PnttBu)] and alkyl moieties in ester groups [methyl in **19** (9IbuMe) and **20** (7IbuMe) and *tert*-butyl in **2** (9IbutBu) and **3** (7IbutBu)] could give useful information on whether these groups have an effect on fragmentation pathways. The loss of the *tert*-butyl ester (Scheme 8, process B) was followed by dissociation of the N^2-acyl group (Scheme 8, process A). Thus the difference caused by these groups disappeared to such an extent that compounds **2** (9IbutBu) and **17** (9PnttBu) led to the same acid **2f** (9OH). Therefore, the fragmentation of differently protected compounds was similar. The free acid form [**2f** (9OH) and **3f** (7OH)] rendered the side chains unstable at higher energies in an MS/MS study.

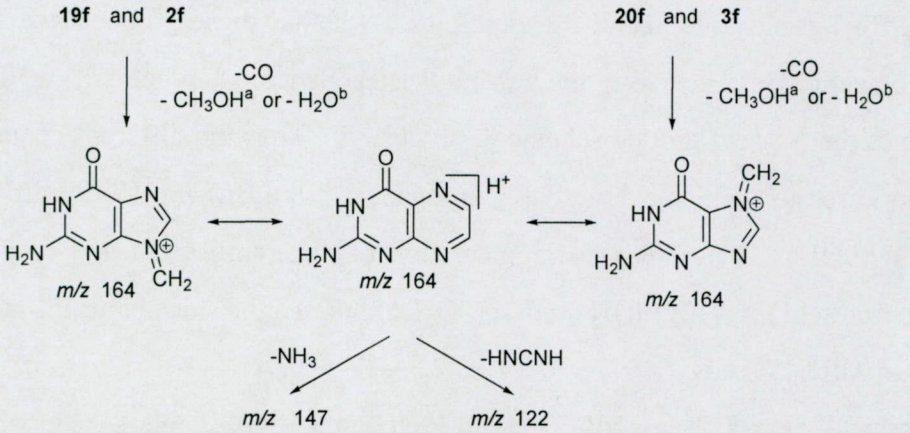
The product ions **19f** (9Me), **20f** (7Me), **2f** (9OH) and **3f** (7OH) were the most abundant at -30 eV collision energy (for structures see Scheme 8). The **19f**, **20f**, **2f** and **3f** ions are precursors

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 $[M_f]^+$).



Scheme 8 Structures of compounds **19f** (9Me), **20f** (7Me), **2f** (9OH) and **3f** (7OH) formed from alkyl (guanin-yl)acetates (**2**, **3**, **17**, **19** and **20**) in MS/MS experiments at -30 eV. Process A: deacylation of 2-amino group, process B: hydrolysis of *tert*-butyl ester. $[M_f]^+$ denotes the *m/z* value of the product ion (**2f**, **3f**, **19f** and **20f**)

The major fragmentation pathways (losses of ammonia and cyanamide) of ions **19f** (9Me), **20f** (7Me), **2f** (9OH) and **3f** (7OH) are in good agreement with observations by McCloskey *et al.*[79] and Kamel *et al.*[78] Losses of methanol [**19f** (9Me) and **20f** (7Me)] or water [**2f** (9OH) and **3f** (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.*[76] (see Scheme 9). The *m/z* 164 ion loses NH_3 and cyanamide, as do the precursor ions [**19f** (9Me), **20f** (7Me),



Scheme 9 The formation and fragmentation of ion at *m/z* 164. (a) from compounds **19f** (9Me) and **20f** (7Me); and (b) from compounds **2f** (9OH) and **3f** (7OH).

2**f** (9OH) and **3f** (7OH)], to yield ions at *m/z* 147 and 122, respectively.

When comparing the MS/MS spectra of compounds **19** (9IbuMe), **20** (7IbuMe), **2** (9IbutBu) and **3** (7IbutBu), it was found that, in both cases, the N^9-isomers [**19** (9IbuMe) and **2** (9IbutBu)] gave abundant $[M_f\text{-}NH_3]^+$ ions at *m/z* 207 and 193 with negligible abundance in the case of N^7-isomers [**20** (7IbuMe) and **3** (7IbutBu)] (see boxes in Figure 2). Therefore, the loss of ammonia is a potential candidate for the quantitative determination of the regioisomer ratio.

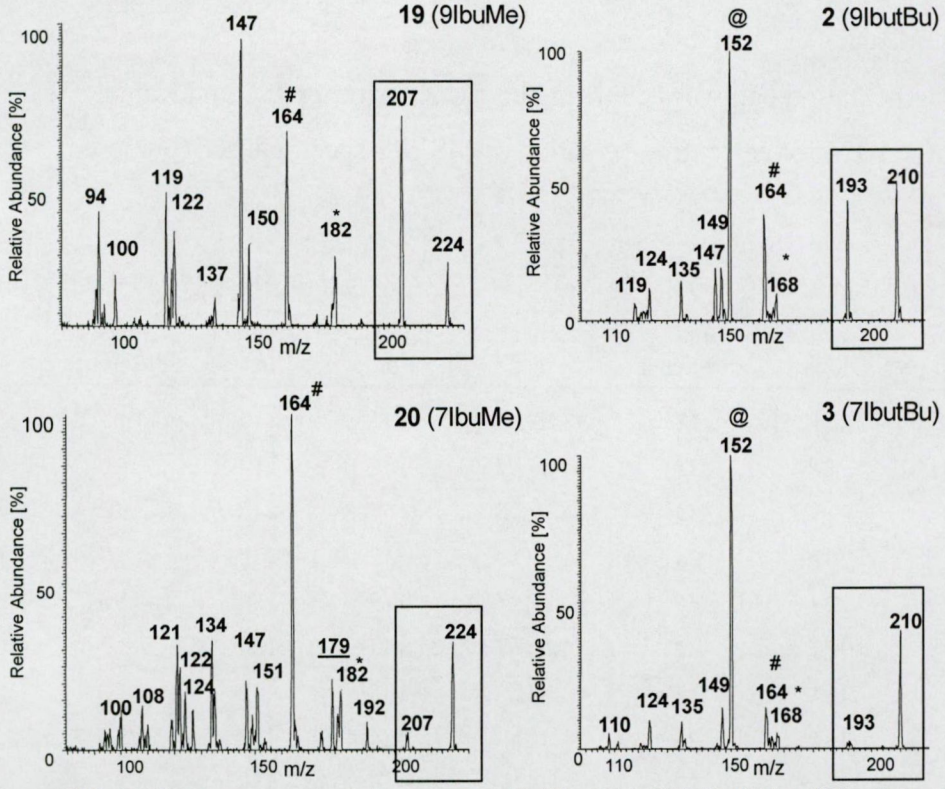


Figure 2 The MS/MS spectra of regioisomer pairs **19** (9IbuMe), **20** (7IbuMe), **2** (9IbutBu) and **3** (7IbutBu) studied. The loss of ammonia, the most important fragmentation pathway, is highlighted by boxes. Other important peaks are labelled as follows: *: $[M_f\text{-}42]^+$, #: *m/z* 164 (for structure see Scheme 9) and @: *m/z* 152. *m/z* 179 is underlined as it can be useful in future studies of N^7-regioisomers.

The *m/z* 179 ion in the spectrum of compound **20** (7IbuMe) is interesting as it could be a product ion of *m/z* 207 formed by loss of CO molecule. This ion could be characteristic for the N^7-isomer but further studies are required involving other alkyl (guaninyl)acetates containing stable ester bond.

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 **2f** (9OH) and **3f** (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 **2f** (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 [**19** (9IbuMe) and **2** (9IbutBu)]. Next, the abundance of the $[M_f\text{-}17]^+$ ion as a function of regioisomer ratio was studied.

The fragmentation pathways confirmed by McCloskey *et al.*[79] and Kamel *et al.*[78] have been collected in Table 4 for easier interpretation of MS/MS spectra described herein.

19f (9Me), **20f** (7Me) ($[M_f]^+$=224)				**2f** (9OH), **3f** (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				

Table 4 The ions of compounds **19** (9IbuMe), **20** (7IbuMe), **2** (9IbutBu) and **3** (7IbutBu) and their masses based on studies by McCloskey *et al.*[79] and Kamel *et al.*[78] Compounds **19f**, **20f**, **2f** and **3f** (Scheme 8) originate from the initial fragmentation of compounds **2**, **3**, **17**, **19** and **20** [substances **2** and **17** gave an identical acid (**2f**)] The most important, deammoniated fragment (set in bold) was applied for determination of isomer ratio. The 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.

3.2.2. Quantitative studies of regioisomer pairs

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 ions, $[M_f\text{-}17]^+$, $[M_f\text{-}42]^+$ and *m/z* 164 were plotted as a function of the isomer ratio (see Figure 3). The correlation was linear in all three cases but with different slopes. The abundance of the ion $[M_f\text{-}42]^+$ was almost constant; furthermore, its correlation coefficient (R^2) value shows large uncertainty for compounds **2** (9IbutBu) and **3** (7IbutBu) (see Table 5). Therefore, it is not appropriate for the determination of isomer ratio.

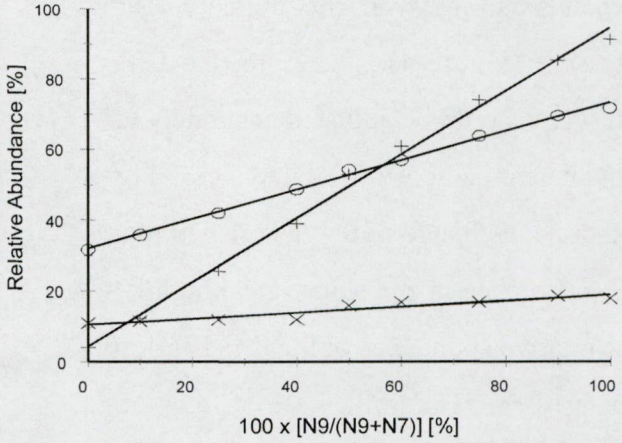


Figure 3 The linear regression study of product ion abundance as a function of isomer ratio in a mixture containing compounds **2** (9IbutBu) and **3** (7IbutBu). + series: *m/z* 193; **x** series: *m/z* 168 and **o** series: *m/z* 164.

Based on their high correlation coefficients (see Table 5) the abundances of ions $[M_f\text{-}17]^+$ and *m/z* 164 are both convenient for the determination of isomer ratios; however, as the change in abundance is twice as large for the $[M_f\text{-}17]^+$ ion than for the *m/z* 164, its application would lead to more accurate results. The optimal collision energy at which the abundance of $[M_f\text{-}17]^+$ 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.

sample constituents	$[M_f\text{-}17]^+$	R^2	$[M_f\text{-}42]^+$	R^2	R^2 for *m/z* 164
19 (9IbuMe), **20** (7IbuMe)	*m/z* 207	0.997	*m/z* 182	0.981	0.994
2 (9IbutBu), **3** (7IbutBu)	*m/z* 193	0.995	*m/z* 168	0.875	0.996

Table 5 Correlation coefficient (R^2) values from linear regression analysis of the abundance of three ions ($[M_f\text{-}17]^+$, $[M_f\text{-}42]^+$ and *m/z* 164 (for structure see Scheme 9)) as a function of the regioisomer ratio for both pairs of regioisomers.

The slope of the calibration curves depends strongly on the collision gas pressure (Figure 4). 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 **2** (9IbutBu) and **3** (7IbutBu), resulting in a 20% change in abundance (data not shown). To obtain accurate results every isomer determination should start with calibration (maximum 15 minutes) to guarantee identical conditions in the analysis.

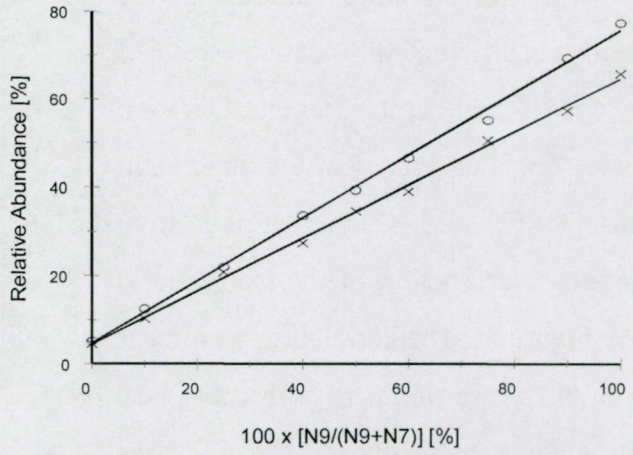


Figure 4 Calibration of compounds **19** (9IbuMe), **20** (7IbuMe) at two different CID gas pressures at 2.1 mTorr (**o** series, y=4.68+0.71x, R^2: 0.997) and 1.7 mTorr (**x** series, y=4.45+0.60x, R^2: 0.997)

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

The accuracy of the measurement is *ca.* 2.2-4.4% [**19** (9IbuMe) and **20** (7IbuMe)] and 4.8-5.0% [**2** (9IbutBu) and **3** (7IbutBu)] depending on the isomer ratio (Table 6). 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

	19 (9IbuMe) and **20** (7IbuMe) [%]		**2** (9IbutBu) and **3** (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
100δx	-4,4	- 2.2	5.0	- 4.8
calibration curves	y=3.28+0.75x		y=3.99+0.9x	

Table 6 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 **19** (9IbuMe) and **20** (7IbuMe). δx: the relative error of the determination; $\delta x = (x\text{-}x_c)/x_c$

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.

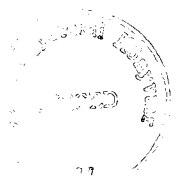
3.3. Conclusion II

We have managed to find a rapid method for the determination of regioisomer ratio of alkyl (guaninyl)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 is in linear correlation with the N^9 content in a mixture of regioisomers.

tert-Butyl esters fragmented easily to the free acid, which proved to be unstable leading to the loss of the side chain. Fortunately, the N^9 - regioisomer even so gave the important $[MH-17]^+$ ion, in addition, its abundance changed linearly with the isomer content.

There is an interesting $[MH-45]^+$ peak in the MS/MS spectrum of N^7 -regioisomers which could be a characteristic for them, but further studies are needed including other compounds as well containing stable ester bonds in their side chain.

This method can be used for other types of alkylated guanine compounds as well, except benzyl and glycosyl derivatives, in which the loss of side-chain is rather easy due to the formation of stable cations. The optimal collision energy has to be found for the actual regioisomer pair, and also a mass spectrometer and CID pressure at which the fragmentation is different for the regioisomers and hopefully there is a fragment, *e.g.* the $[MH-17]^+$ ion with an abundance in linear correlation with the N^9 -isomer content of the mixture.

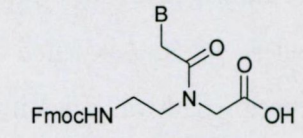


4. Synthesis and analysis of PNA oligomers

4.1. Introduction

In order to synthesize PNA oligomers and their conjugates with peptides or oligonucleotides, suitably protected monomers are indispensable which should be compatible with both peptide and oligonucleotide synthesis protocols. To date, this problem has not been solved (Table 1).

The monomers protected by Fmoc (backbone) and acyl (nucleobase) groups (Figure 5) [I] could fulfil the requirements of PNA-oligonucleotide conjugate synthesis. This combination of protecting groups has not been thoroughly explored,[81-85] the advantages and pitfalls have not been characterized in detail.



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

Figure 5 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.

PNA having a pseudopeptide backbone could be synthesized by standard peptide synthesis protocols but note that the β-amino group takes place in the formation of the amide bond and nucleobases are in the side chains which can hybridize leading to low solubility especially if it contains a high proportion of guanine.

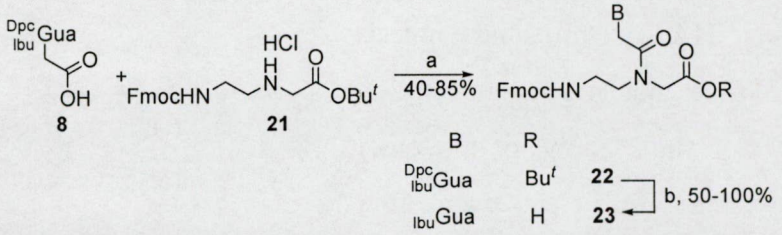
There are a few reports on optimization of PNA oligomer syntheses,[83,86-90] however, there are so many variables affecting the effectiveness of the synthesis (nature of solid supports and monomers including the protecting groups of the latter, coupling reagents and conditions *e.g.* bases, preactivation, reaction time, mixing sequence, monomer excess, repetition of couplings *etc.*) that it is impossible to simply conclude as to what the optimal conditions for the preparation of PNA oligomers are. The answer for this question greatly depends on the specific set of the above variables.

Most PNA oligomer sequences published so far did not contain guanine or just a few of them. This proves that the synthesis of these monomers is more laborious, and the presence of guanine in the oligomer decreases its solubility. 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.

4.2. Results and discussion

4.2.1. The synthesis of monomer

The last two steps of the monomer synthesis (shown in Scheme 10 taking guanine derivatives as an example) are the coupling of nucleobase-substituted acetic acid (**8**) with the backbone unit **21**[30] under standard peptide coupling conditions affording the PNA ester (**22**) which was subsequently acidolysed (TFA in dichloro-methane) to give the PNA monomer (**23**) (Scheme 10). As expected from our previous experience (**5** → **2**, Scheme 4) in the latter reaction the Dpc protecting group was removed along with the *tert*-butyl group.



Scheme 10 *Reagents and conditions:* a. HBTU, HOBt, DMF, $EtNPr^i_2$. b. 17-43% (v/v) TFA/DCM, 1,3-$(MeO)_2C_6H_4$.

4.2.2. Coupling experiments in solution phase

For the efficient coupling of PNA monomers an activating agent was required which is working fast with the lowest possible monomer excess, giving high yields and being economically viable. BET[91] and CMP[92] gave almost quantitative yield while HATU only 90% in solution phase studies in the model reaction between our t monomer and *tert*-butyl phenylalaninate.[93]

4.2.3. Initial coupling studies on 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^{TM}) meets the above criteria and the removal of protecting groups and final cleavage from the support can be realized also in one step.[94]

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

CMP and BET. Surprisingly, these activating agents gave low yields in solid phase coupling reactions. The reason of this discrepancy is a capping reaction taking place along with coupling. The intensive peaks of capped sequences were easily determined in the mass spectra of these oligomers. The higher reactivity of the β-amino group is the reason for this side reaction. Even the preactivation of monomer did not help in avoiding this side reaction in the case of CMP and BET that worked well in the case of HATU. This is proving again the higher reactivity of the former coupling reagents.

HATU. During the synthesis of first sequences on FmocGly-O-TG resin 2 equiv. of monomer was not sufficient even after prolonged time thus the excess was elevated to 4 equiv. resulting in 95% average yield (Table 7). 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 91-96% range.

	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

Table 7 Coupling yields (in %) on TentaGel™ resin using 4 equiv. monomer excess. Data taken from the synthesis of sequences **24-27** (*Cf.* Table 9). Monomers (rows) were coupled to the monomers on the resin (columns).

4.2.4. Problems with the PNA oligomer synthesis on TentaGel™

The synthesis run smoothly based on UV absorbances but there was a problem with the solubility of the product (consequently, also with its analysis) and this problem was increased with the length of oligomer.

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 $[HO(CH_2CH_2O)_nH + Na^+]$ was observed. This process can be the result of decomposition of the resin during storage, however, the above peaks were present even after thorough wash. The fracture of TentaGel™ beads resulting in the release of oligo(ethylene glycol)

fragments ("leakage" of the support) has been a known phenomenon,[94] 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[95] a clear spectrum is observable most likely due to separation inside the gold-coated capillary tube of the nano-ESI-MS interface as it is represented by the mass spectra of H-Gly-ggtt-Gly-NH_2 (compd **26**, Figure 6). The attempted purification of PNA samples by precipitation to separate them from the contaminating oligo(ethylene glycol) was not successful.

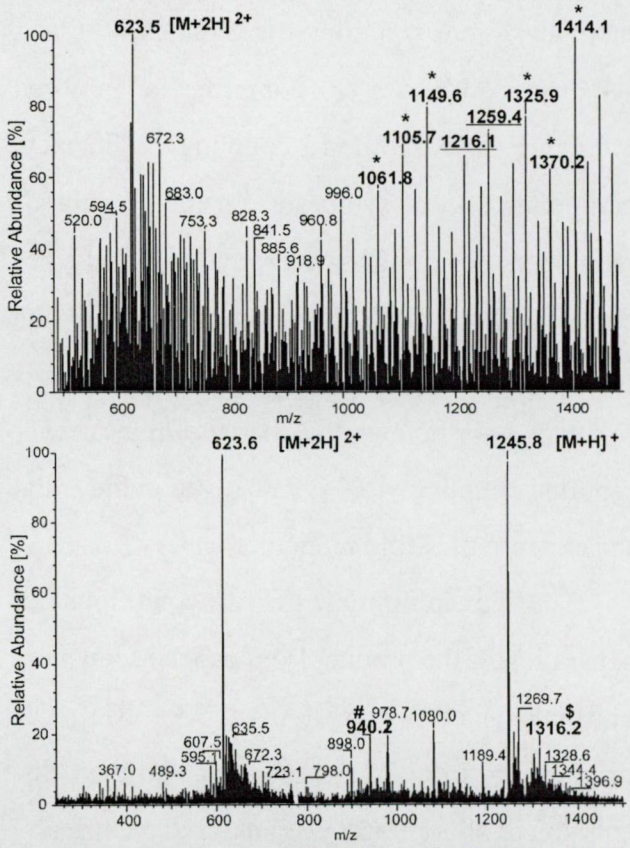


Figure 6 Mass spectra of PNA oligomer **26** (M_{calc} 1245) synthesized on TentaGel™. Upper panel: ESI-MS spectrum. The values marked with an asterisk denote the cluster ions $[HO(CH_2CH_2O)_nH + 2NH_4]^{2+}$, the underlined values the ions $[HO(CH_2CH_2O)_nH + Na + NH_4]^{2+}$. Lower panel: nano-ESI-MS spectrum. Peaks denoted with # and $ signs correspond to the ions [Ac-gtt-GlyNH_2 + H]$^+$ and [M + Ibu + H]$^+$, respectively.

Incomplete removal of isobutyryl (Ibu) protecting groups (presence of [M + 70 + H]$^+$ peaks). Capped (*e.g.* Ac-gtt-GlyNH_2) and incompletely deacylated sequences (*e.g.* peaks [M + Ibu + H]$^+$) were also present in MS spectra beside the expected product (Figure 6, lower panel). This was noticed by Rosenbohm *et al.*[96] as well therefore they have turned to the more base sensitive dimethylformamidine protective group. To determine the ratio of capped and unprotected sequences and the product, an HPLC analysis condition was required to separate these compounds. In order to separate the study of coupling and acyl deprotection steps the polystyrene-based, acid sensitive Wang resin was employed as the protected oligomer that can be cleaved with TFA and acyl group deprotection can be realized by NH_3 treatment in solution.

4.2.5. Syntheses on Wang resin

Similar results were obtained with 4 equiv. monomer excess during the synthesis of test sequence **24**.[86] As a compromise between monomer excess and coupling efficiency, compound **28** was synthesized using 3 equiv. of monomers resulting in 98% average yield per cycle and the HPLC chromatogram of the products (Figure 7) 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.[87]

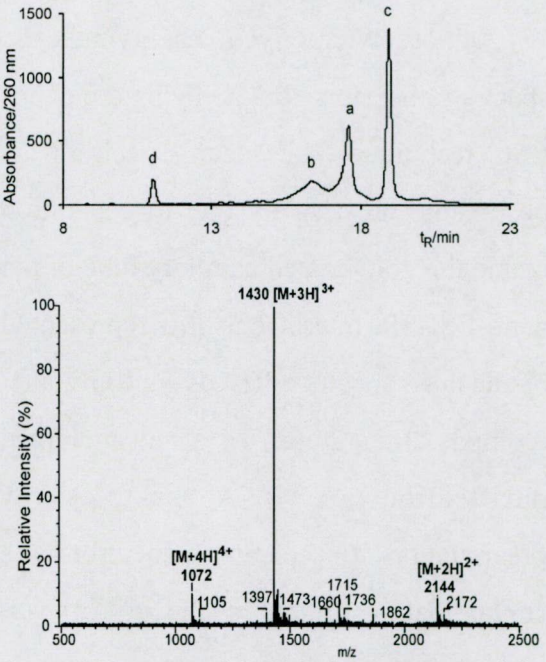


Figure 7 Upper panel: HPLC chromatogram of the PNA sequence **28** prepared on a Wang resin (crude product, LiChrospher RP Select B column, TFA-containing eluent). Peak a: product, peak c: anisoyl derivatives, peaks b and d: unidentified. Lower panel: ESI mass spectrum (purified product, M_{calc} 4284).

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 groups) the solution of oligomer **28** was analyzed on different HPLC column packings in order to examine the effects of pH of the eluting solution (TEAA, pH 7.0 or dil. TFA, pH ~ 2) and temperature (rt or 55 °C) on the resolution (Table 8 and Figure 8).

Column name[a]	Pore size (Å)	RP 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

Table 8 Comparison of analytical HPLC columns and conditions applied in the analysis of PNA oligomers. a. Size of columns: 250×4.6 mm. b. Principal component of buffer. c. Retention time of compound **28** at 25 °C. d. Phenomenex. e. 0.1% (v/v) trifluoroacetic acid. f. The Separations Group. g. Data not available. h. Merck Ltd. Budapest. i. 0.1 M triethylammonium acetate.

TFA buffer gives better resolution than TEAA as exemplified by the separation of pentadecamer **28** on LiChrospher RP Select B column (Figure 8, 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 (Figure 8, trace C). The separation was good on the less unpolar Vydac column as well but in this case the peaks were wider than on other column packings (Figure 8, trace D). The retention times were very similar on the above-mentioned packings in TFA solution. The effect of elevated temperature[97,98] 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 using TFA-containing eluent is the best-suited for PNA analysis and purification.

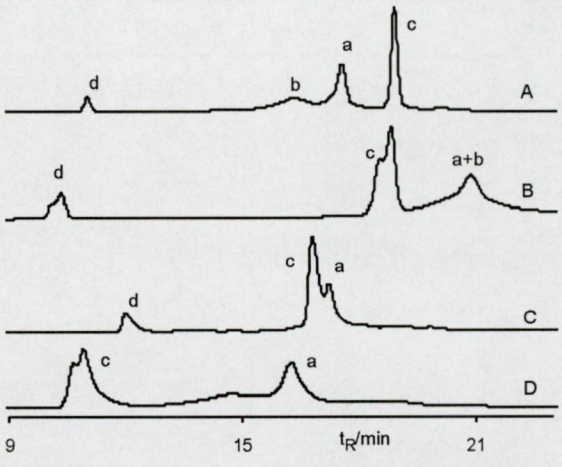


Figure 8 HPLC chromatograms of the crude oligomer **28** under different conditions. Trace A: LiChrospher RP Select B column, TFA eluent; trace B: LiChrospher RP Select B column, TEAA eluent; trace C: Jupiter column, TFA eluent; trace D: Vydac column, TFA eluent. Peak a: product, peak c: anisoyl derivatives, peaks b and d: unidentified. (*cf.* Table 8).

Oligomers **26**, **27**, **29-31** (Table 9) were synthesized to check the completeness of protecting group removal (it is well known that in oligonucleotide synthesis the rate of deprotection decreases in the order 4-tBuBz > An > Ibu).[99]

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 (Figure 9).

When the reaction time was elongated for 48

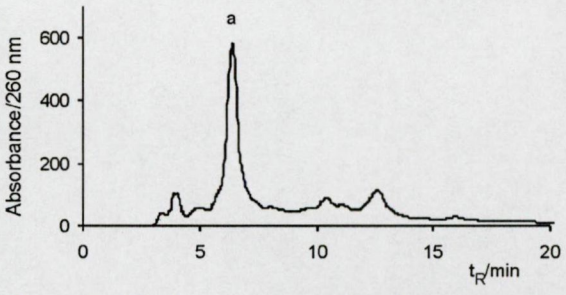


Figure 9 HPLC chromatogram (Vydac column, TFA-containing eluent) of a crude PNA oligomer (peak a: compound **31**) after detachment from the support (Wang resin) and deprotection.

h the oligomer decomposed. The exocyclic amino group of cytosine with other bases (*e.g.* primary amines) can be converted to the corresponding amine.[83] 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.

Sequence[a]	M_{calc}	Peaks observed (ESI-MS)[b]
H-Gly-cgg act aag tcc att gc-Gly-NH_2 (**24**)[c]	4714	-[d]
H-Gly-ag tcc att gc-Gly-NH_2 (**25**)	2814	-[d]
H-Gly-ggtt-Gly-NH_2 (**26**)[c]	1245	1245.8 $[M+H]^+$; 623.6 $[M+2H]^{2+}$
H-Gly-aacc-Gly-NH_2 (**27**)[c]	1183	1184.2 $[M+H]^+$; 592.9 $[M+2H]^{2+}$
H-Gly-gaa cat cat ggt cgt-Lys-OH (**28**)[e]	4284	2144.0 $[M+2H]^{2+}$; 1430.0 $[M+3H]^{3+}$; 1072.0 $[M+4H]^{4+}$
H-Gly-a-Gly-NH_2 (**29**)[c]	406	406.9 $[M+H]^+$
H-Gly-g-Gly-NH_2 (**30**)[c]	422	423.0 $[M+H]^+$
H-Gly-g_5-Lys-OH (**31**)[e]	1658	1657.7 $[M+H]^+$; 830.3 $[M+2H]^{2+}$
Ac-cat ggt cgt-Gly-NH_2 (**32**)[f]	2565	2565.97 $[M+H]^+$; 1283.48 $[M+2H]^{2+}$, 856.15 $[M+3H]^{3+}$
H-Gly-cat ggt cgt-Gly-NH_2 (**33**)[f]	2580	2581.28 $[M+H]^+$; 1291.08 $[M+2H]^{2+}$
H-cat ggt cgt-Gly-NH_2 (**34**)[f]	2523	2524.03 $[M+H]^+$; 1262.5 $[M+2H]^{2+}$

Table 9 PNA sequences synthesized and MS data thereof.
[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 carboxyl forms but for simplicity only the former one is denoted. [b] Except for compound **32** (MS spectra were obtained with MALDI technique and values are given for the smallest monoisotopic peak). Peaks obtained with ESI technique are given for the most intensive monoisotopic peaks. [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.

4.2.6. 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.

Nonamer **33** was 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 shows that the synthesis run smoothly as it was expected based on UV absorbances (Figure 10). Surprisingly, both the ESI- and MALDI MS spectra of the crude product were too noisy from 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.

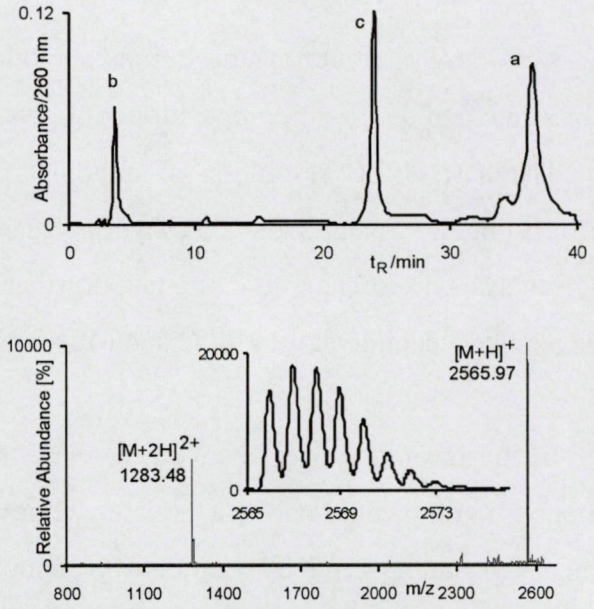


Figure 10 Upper panel: HPLC chromatogram of the PNA sequence **32** prepared on a CPG support (crude product, LiChrospher RP Select B column, TFA-containing eluent). Peak a: product, peak c: anisoyl derivatives, peak b: deoxycytidine. Lower panel: MALDI mass spectrum (purified product, M_{calc} 2565). Inset: isotopic distribution of the molecular ion.

The *C* terminus of this substance is in both carboxyl and carboxamide forms as it 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 in positive and negative modes that is also indicative of the presence of carboxyl *C* terminus.

4.3. Conclusion III

The synthesis of PNA oligomers has been elaborated using Fmoc/acyl-protected monomers. Among the three activating agents (HATU, CMP, BET), the latter two were too reactive leading to capping the *N*-terminal amino group, therefore the first one proved to be the best for the

synthesis of PNA oligomer. 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 a Wang resin. Some isobutyryl group-containing PNA sequences remained in negligible amount with the classical (25% aq NH_3/50 °C/16h) cleavage condition.

The PNA oligomer synthesis runs smoothly on CPG as well rendering this support a promising candidate for the preparation of oligonucleotide-PNA conjugates with a suitable linker.

The best HPLC resolution of oligomer could be achieved with TFA solution on a LiChrospher RP Select B and 2,5-dihydroxybenzoic acid was an optimal matrix in the MALDI-TOF analysis. These supports the assumption that PNA oligomers with a pseudopeptide backbone behave like a peptide in the HPLC and MS analysis.

In the meantime we have turned to the MMTr/Pnt protecting group strategy because isobutyryl group could not be completely removed from the guanine residues, in addition, the Fmoc/acyl strategy is not compatible with peptide synthesis as the final deprotection requires an ammonia treatment at 50 °C which would lead to the racemization of the peptide moiety. The experiences gathered during this work will be useful in the elaboration of a PNA oligomer synthesis by the MMTr/Pnt strategy.

5. Summary

The synthesis of PNA oligomers has been elaborated using our own Fmoc/acyl-protected monomers.

To have an appropriately protected guanine monomer, first the regioselective synthesis of the *tert*-butyl [N^2-(acyl)guanin-9-yl]acetate had to be developed.

Starting from N^2-isobutyryl-O^6-(*N,N*-diphenylcarbamoyl)-guanine (**4**) in the Mitsunobu alkylation the regioselectivity was good but the yield of the N^9-isomer was below 40% because of the problematic removal of phosphine oxides even in the case of the water-soluble tributyl-phosphine oxide.

The N^9-isomer was synthesized exclusively from guanosine by method by the Izawa *et al.* The main problem was the removal of the protecting group of N^7. PNB proved to be the best choice as 44% overall yield was obtained even with the Zn/AcOH reduction. This value could be improved up to 56% by sodium dithionite reduction, applied in the modified Izawa method. The significantly higher overall yield and the avoidance of chromatographic purification renders the Izawa method the best alternative for the regioselective alkylation of guanine.

These methods were not compatible with sensitive acylating reagents and to overcome the low solubility caused by the free or protonated 2-amino groups of guanine and N^7-benzylguaninium hydrochloride high temperatures were required.

In order to avoid the formation of hydrochloride salts the general sequence of the Izawa method was changed in the following way: (1) pent-4-enoylation of guanosine at N^2 (**9**), (2) per-*O*-acetylation (**11**), (3) 4-nitrobenzylation at N^7 (**12**), (4) *N*-glycoside hydrolysis (**13**), (5) N^9-alkylation (**15**) and (6) deprotection with sodium dithionite.

This is the optimal sequence of reactions since the influence of *O*- and/or N^2-acyl groups on the stability of *N*-glycosidic bond and reactivity of 2-amino group is remarkable. The electron-withdrawing property of the acetyl group is responsible for the stability of guaninium salt **12** at room temperature and the decreased reactivity of 2',3',5'-tri-*O*-acetylguanosine in N^2-acylation.

This seemingly lengthy procedure can be combined into 4 distinct, well-reproducible steps with standard transformations and 36% overall yield. This yield is a modest value but the

solubility of each guanine derivative involved was good and this procedure can be the method of choice for heat-sensitive substrates.

We have managed to find a rapid method for the determination of regioisomer ratio of alkyl (guaninyl)acetates. This can be useful in the optimization of alkylation conditions of guanine *e.g.* under alkaline or Mitsunobu conditions from a small aliquot of the reaction mixture.

Alkylated N^9 -and N^7- regioisomer pairs have significantly different fragmentation pathways: deammoniation is characteristic for the N^9- isomers, the abundance of which is in linear correlation with the N^9 content in a mixture of regioisomers.

tert-Butyl esters fragmented easily to the free acid which proved to be unstable leading to the loss of the side chain. Fortunately, the N^9 -regioisomer even so gave the important $[MH-17]^+$ ion, in addition, ita abundance changed linearly with the isomer content.

There is an interesting $[MH-45]^+$ peak in the MS/MS spectrum of N^7 -regioisomer which could be diagnostic for it but further studies are needed including other compounds as well containing stable ester bonds in their side chain.

This method can be used for other types of alkylated guanine compounds as well, except benzyl and glycosyl derivatives, in which the loss of side chain is rather easy due to the formation of stable cations. The optimal collision energy has to be found for the actual regioisomer pairs, and also a mass spectrometer and CID pressure at which the fragmentation is different for the regioisomers and hopefully there is a fragment, *e.g.* the $[MH-17]^+$ ion with an abundance in linear correlation with the N^9 -isomer content of the mixture.

The regioselective alkylation of guanine was followed by monomer and oligomer synthesis. In the latter, first a cheap and reactive coupling reagent was searched for. Among the three activating agents (HATU, CMP, BET), the latter two were too reactive resulting in capping of the *N*-terminal amino group therefore the first one proved to be the best for the synthesis of PNA oligomers. Due to the "leakage" of TentaGel™ the product was contaminated and this rendered the routine mass spectrometric analysis difficult. A clear spectrum is observable by nano-ESI-MS interface most likely due to separation inside the gold-coated capillary tube. The coupling and

protecting group removal steps have been studied separately on a Wang resin. Some isobutyryl group-containing PNA sequences remained in negligible amount with the classical (25% aq NH_3/50 °C/16h) cleavage condition.

The PNA oligomer synthesis runs smoothly on CPG as well rendering this support a promising candidate for the preparation of oligonucleotide-PNA conjugates with a suitable linker.

The best HPLC resolution of oligomer could be achieved with TFA solution on a LiChrospher RP Select B and 2,5-dihydroxybenzoic acid was an optimal matrix in the MALDI-TOF analysis. These support the assumption that PNA oligomers with a pseudopeptide backbone behave like a peptide in the HPLC and MS analysis.

In the meantime we have turned to the MMTr/Pnt protecting group strategy because isobutyryl group could not be completely removed from the guanine residues, in addition, the Fmoc/acyl strategy is not compatible with peptide synthesis as the final deprotection requires an ammonia treatment at 50 °C which would lead to the racemization of the peptide moiety. The experiences gathered during this work will be useful in the elaboration of a PNA oligomer synthesis by the MMTr/Pnt strategy.

6. Experimental

6.1. General

Chemicals were purchased from Aldrich, Fluka, Merck, Bachem or Reanal (Budapest, Hungary). TG-OH was obtained from Merck (catalogue no. 814786, capacity: 0.2-0.3 mmol/g, particle size: 130 mm), 500 Å CPG (≈40 μmol/g) preloaded with 2'-deoxycytidine from ChemGenes Corporations (Ashland, MA, USA), HATU from PE Biosystems (Warrington, UK). Pent-4-enoic anhydride was alternatively synthesized from pent-4-enoic acid as described by Cabre-Castellvi *et al.*[100,101] Sodium hydride refers to a 55 % suspension in mineral oil. DMF refers to an anhydrous solvent in the case of the oligomer synthesis, lutidine to the 2,6-isomer. Anhydrous solvents were prepared as described.[102] Organic solutions were dried using magnesium sulfate 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-Pr^iOH 5:0.25 (S3), 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 (lgε), 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 (^1H: 500.13 MHz; ^{13}C: 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.

Mass spectrometry: Finnigan MAT TSQ 7000, electrospray (ESI) or nanoelectrospray techniques[95] or Bruker Reflex III MALDI [(in case of compounds **32, 33** and **34**) 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.

TLC/MS: the analyte solution has been applied to 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.

IUPAC names: as implemented in ChemDraw® 9.0.

HPLC chromatography was performed on a HP1050 (room temperature) or on a HP 1090 instrument (55 °C) or on Shimadzu LC 10 (in case of compound **32**) 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; (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 compound **32**). HPLC peaks were identified by MS analysis of the fractions obtained after the purification of the product.

6.2. Materials and methods

6.2.1. Synthesis of N^9-alkylated guanine derivatives

***tert*-Butyl 2-(2-Isobutyramido-6-oxo-1,6-dihydropurin-9-yl)acetate (2) and *tert*-Butyl 2-(2-Isobutyramido-6-oxo-1,6-dihydropurin-7-yl)acetate (3)**

N-(6-Oxo-6,9-dihydro-1H-purin-2-yl)isobutyramide[29,40] (1.11 g, 5.0 mmol) was suspended in anhydrous DMF and chilled to 0 °C. Sodium hydride (0.36 g, 8.25 mmol) was added and 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 adding a small amount of dry ice 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 more polar N^7-isomer (**3**, 0.43 g, 26%) second the mixture (in ca. 1:1 ratio as judged by TLC and ^{1}H NMR) of N^7- and N^9-isomers (0.24 g, 14%) and third the pure N^9-isomer (**2**, 0.56 g, 34%).

Ester **2**: white powder, mp 204- °C (decomp., from EtOH); R_F 0.13 (S2); (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 (lgε 4.26), 265 (4.23); λ_{max}(50 v/v% phosphate buffer in EtOH, pH 6)/nm

260 (lgε 4.17), 282sh (4.02); λ_{max}(50 v/v% 0.1 M NaOH in EtOH, pH 13)/nm 216 (lgε 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; (C*H*$_3$)$_2$CH); 1.40 (9 H, s, Bu^t); 2.78 (6 H, pseudoquintet, (C*H*$_3$)$_2$CH); 4.88 (2 H, s, CH$_2$COO); 7.95 (1 H, s, H-8); 11.65 (1 H, br s, deut, NH); 12.10 (1 H, br s, deut, NH); δ_C (125.76 MHz) 19.22 ((*C*H$_3$)$_2$CH); 28.03 ((*C*H$_3$)$_3$C); 35.01 ((CH$_3$)$_2$*C*H); 45.17 (*C*H$_2$COO); 82.69 ((CH$_3$)$_3$*C*); 167.03 (*C*OOBu^t); 180.56 (Pr^i*C*O); *m/z* (ESI) 693 (20%, [2M+Na]$^\oplus$); 671 (55, [2M+H]$^\oplus$); 336 (100, [M+H]$^\oplus$).

Ester **3**: white powder, mp 202.5- °C (decomp., from EtOH); R_F 0.19 (S2); (Found: C, 53.65; H, 6.15; 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 (lgε 4.24), 263 (4.20); λ_{max}(50 v/v% phosphate buffer in EtOH, pH 6)/nm 221 (lgε 4.24), 265 (4.11), 282sh (3.98); λ_{max}(50 v/v% 0.1 M NaOH in EtOH, pH 13)/nm 224 (lgε 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; (6 H, pseudoquintet, C*H*$_3$)$_2$CH); 1.39 (9 H, s, Bu^t); 2.73 (1 H, pseudoquintet, (CH$_3$)$_2$C*H*); 5.07 (2 H, s, CH$_2$COO); 8.11 (1 H, s, H-8); 11.55 (1 H, br s, deut, NH); 12.14 (1 H, br s, deut, NH); δ_C (125.76 MHz) 19.23 ((*C*H$_3$)$_2$CH); 28.00 ((*C*H$_3$)$_3$C); 35.06 ((CH$_3$)$_2$*C*H); 48.26 (*C*H$_2$COO); 82.36 ((CH$_3$)$_3$*C*); 167.20 (*C*OOBu^t); 180.32 (Pr^i*C*O); *m/z* (ESI) 693 (40%, [2M+Na]$^\oplus$); 671 (25, [2M+H]$^\oplus$); 358 (27, [M+Na]$^\oplus$); 336 (100, [M+H]$^\oplus$).

tert-Butyl 2-[6-(diphenylcarbamoyloxy)-2-isobutyramido-9H-purin-9-yl]acetate (5)

Mitsunobu reaction, general procedure. Compound **4**[29,40] (1.00 g, 2.40 mmol) was suspended in anhydrous THF (50 mL) and refluxed for 20 min to achieve partial dissolution of the starting material.[54] The suspension was cooled to room temperature, *tert*-butyl glycolate[55] (0.40 g, 3.0 mmol), the appropriate phosphine (3.19 mmol) and diisopropyl azodicarboxylate (0.62 mL, 3.19 mmol) were added dropwise and 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 subjected to chromatographic purification.

1. With triphenylphosphine. Reaction time: 4 h at room temperature. Chromatography: 50 → 70 v/v% ethyl acetate in light petroleum. Eluted first was the product (**5**; 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 and washed with light petroleum. The cleanest product (0.40 g, 31%), 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 (S2); (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 (lgϵ 4.60), 229 (4.53), 258sh (4.15), 279 (4.08); ν_{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, $(CH_3)_2CH$); 1.43 (9 H, s, Bu^t); 2.87 (1 H, pseudoquintet, *J* 6.8, $(CH_3)_2CH$); 5.03 (2 H, s, CH_2); 7.29-7.53 (10 H, m, arom.); 8.45 (1 H, s, H-8); 10.69 (1 H, br s, deut, NH); δ_C (125.76 MHz) 20.08 ($(CH_3)_2CH$); 28.52 ($(CH_3)_3C$); 35.18 ($(CH_3)_2CH$); 45.82 (CH_2COO); 83.40 ($(CH_3)_3C$); 127.76; 128.15; 130.26 (arom. C's); 142.51 (arom. quaternary C); 151.03 (OCON); 167.33 (*C*OOBu^t); 175.97 (Pr^i*C*O); *m/z* (ESI) 557 (8%, $[2Ph_3PO+H]^{\oplus}$), 531 (100, $[M+H]^{\oplus}$).

2. With (4-dimethylaminophenyl)diphenylphosphine.[58,59] Reaction time: 2.5 h at 0 °C. Work-up: the crude product was dissolved in dichloromethane (50 mL) and extracted with 4 M HCl (3×25 mL) then with 5 m/v% $NaHCO_3$ solution (50 mL). TLC revealed that most of the (4--dimethylaminophenyl)diphenylphosphine 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, ^{1}H NMR and mass spectra of this compound were in good agreement with those of the substance obtained in procedure *1*.

3. 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 *1*.

Hydrolysis of *tert*-Butyl 2-(6-(diphenylcarbamoyloxy)-2-isobutyramido-9H-purin-9-yl)-acetate (5→2)

To ester **5** (0.210 g, 0.38 mmol) dissolved in anhydrous dichloromethane (6 mL), 1,3--dimethoxybenzene (0.070 mL, 0.53 mmol) and TFA (0.50 mL, 6.5 mmol) were added at 0 °C and stirred for 18 h. The reaction mixture was diluted with dichloromethane (20 mL), extracted with satd. $NaHCO_3$ solution (3×10 mL) to remove the excess acid. Chromatography: 0 → 10 v/v% methanol in dichloromethane (0.069 g, 55%), amorphous foam. The IR, ^1H NMR and mass spectra of this acid were in good agreement with those of the substance obtained in a previous experiment (*vide supra*).

2',3',5'-tri-*O*-Acetyl-N^2-(pent-4-enoyl)guanosine (11)

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 equiv.) was added and the reaction was stirred for 16 h at rt. 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.

2-(Pent-4-enoyl)guanosine (**9**) 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 remaining from the previous step were filtered off and the solution was set aside for 16 h. Ethanol (10 mL) was added to the solution before evaporation, then the residue was dissolved in EtOAc (400 mL) and successively extracted with 1 M hydrochloric acid (2 × 300 mL) and satd. $NaHCO_3$ 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); δ_H (500 MHz, DMSO-d_6) 2.03 (3 H, s, CH_3CO), 2.04 (3 H, s, CH_3CO), 2.11 (3 H, s, CH_3CO), 2.34 (2 H, m, $CH_2CH=CH_2$), 2.58 (2 H, t, *J* 7.2, CH_2CO), 4.29 (1 H, m, H-4'), 4.37 (2 H, m, H-5'), 4.99 (1 H, d, *J* 10.2, *cis*-CH_2=CH), 5.06 (1 H, d, *J* 17.2, *trans*-

CH_2=CH), 5.48 (1 H, dd, *J* 5.7, 3.7, H-3'), 5.81 (2 H, m, H-1', CH_2=C*H*), 6.08 (1 H, d, *J* 6.3, H-2'), 8.23 (1 H, s, H-8), 11.60 (1 H, br s, NH), 12.06 (1 H, br s, NH); δ_C (125 MHz, DMSO-d_6, decoupled and *J*-mod. spin-echo spectra) 20.03, 20.25, 20.39 (3 × *C*H_3CO), 28.07 (*C*H_2CH=CH_2), 35.10 (*C*H_2CO), 63.00 (C-5'), 70.28 (C-3'*), 72.16 (C-2'*), 79.83 (C-4'*), 84.56 (C-1'), 115.54 (*C*H_2=CH), 120.37 (C-5), 136.73 (CH_2=*C*H), 137.74 (C-8), 148.06 (C-2#), 148.57 (C-6#), 154.65 (C-4), 169.15, 169.35, 169.99 (3 × CH_3*C*O), 175.46 (C_4H_7*C*ONH); *m/z* (ESI) 492.16 (100%, $[M+H]^+$).

N-[7-(4-Nitrobenzyl)-6-oxo-6,7-dihydro-1H-purin-2-yl]pent-4-enamide (13)

Compound **11** (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 rt 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 **12**. The solution was evaporated *in vacuo*, EtOAc (400 mL) and water (400 mL) were added to the oily residue. The product (2.3 g, 62%) was precipitated and filtered out. 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 **13**: 2.9 g (78%), amorphous solid, R_f 0.29 (S2), 0.22 (S3); δ_H (500 MHz, DMSO-d_6) 2.32 (2 H, m, C*H*$_2$CH=CH_2), 2.53 (2 H, t, *J* 7.2, C*H*$_2$CO), 4.97 (1 H, d, *J* 10.2, *cis*-C*H*$_2$=CH), 5.04 (1 H, d, *J* 17.1, *trans*-C*H*$_2$=CH), 5.65 (2 H, s, ArC*H*$_2$), 5.77-5.85 (1 H, m, CH_2=C*H*CH_2), 7.52 (2 H, d, *J* 8.5, ArH), 8.17 (2 H, d, *J* 8.5, ArH), 8.37 (1 H, s, H-8), 11.57 (1 H, br s, NH), 12.08 (1 H, br s, NH); δ_C (125 MHz, DMSO-d_6, decoupled and *J*-mod. spin-echo spectra) 28.13 (*C*H_2=CHCH_2), 34.96 (*C*H_2CO), 48.60 (Ar*C*H_2), 111.08 (C-5), 115.49 (*C*H_2CH), 123.68, 128.44 (arom. *C*H), 136.73 (CH_2*C*H), 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_7*C*ONH); *m/z* (ESI) 368.97 (100 %, $[M+H]^+$).

9-[(*tert*-Butoxycarbonyl)methyl-7-(4-nitrobenzyl)-N^2-(pent-4-enoyl)guaninium bromide (15)

tert-Butyl bromoacetate (1.2 mL, 8.1 mmol) was added to the solution of compound **13** (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 **15** (1.30 g, 86%) precipitated as a white amorphous solid, R_f 0.21 (S1); δ_H (500 MHz, DMSO-d_6) 1.41 (9 H, s, $(CH_3)_3C$), 2.30 (2 H, br s, $CH_2CH=CH_2$), 2.63 (2 H, br s, CH_2CO), 4.95 (1 H, d, *J* 8.3, *cis*-CH_2=CH), 5.02 (1 H, d, *J* 16.8, *trans*-CH_2=CH), 5.15 (2 H, s, CH_2COO), 5.81 (1 H, br s, $CH=CH_2$), 5.96 (2 H, s, $ArCH_2$), 7.72 (2 H, d, *J* 7.9, ArH), 8.19 (2 H, d, *J* 7.9, ArH), 9.81 (1 H, s, H-8); 11.32 (1 H, br s, NH), 12.20 (1 H, br s, NH) δ_C (125 MHz, DMSO-d_6, decoupled and *J*-mod. 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 (*C*OOBu^t), 173.75 (C_4H_7CONH); *m/z* (ESI): 483 (100%, M^+).

***tert*-Butyl 2-(6-oxo-2-pent-4-enamido-1,6-dihydropurin-9-yl)acetate (17)**

Sodium dithionite (80%, 0.790 g, 3.6 mmol) was added to the solution of guaninium salt **15** (0.530 g, 0.94 mmol) in acetone (15 mL) and phosphate buffer (15 mL, pH=7.0). After 30 min stirring at rt 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 **17** (0.250 g, 76%) was obtained as a yellowish amorphous solid, R_f 0.19 (S2), 0.22 (S3); δ_H (500 MHz, DMSO-d_6) 1.40 (9 H, s, $C(CH_3)_3$), 2.33 (2 H, q, *J* 7.0, $CH_2CH=CH_2$), 2.56 (2 H, t, *J* 7.2, CH_2CO), 4.86 (2 H, s, CH_2COO), 4.98 (1 H, d, *J* 10.1, *cis*-CH_2=CH), 5.05 (1 H, d, *J* 16.3, *trans*-CH_2=CH), 5.82 (1 H, m, $CH=CH_2$), 7.94 (1 H, s, H-8), 11.66 (1 H, br s, NH), 12.04 (1 H, br s, NH); δ_C (125 MHz, DMSO-d_6, decoupled and *J*-mod. 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 (*C*OOBu^t), 175.50 (C_4H_7CONH); *m/z* (ESI) 717.41 (38%, $[2M+Na]^+$), 695.43 (68, $[2M+H]^+$), 370 (18, $[M+Na]^+$), 348 (100, $[M+H]^+$).

6.2.2. An ESI-MS/MS study of N^9 and N^7-alkylated guanine derivatives

The mass spectrometer 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 a nebulizer gas. The collision energy was varied between -10 and -70 eV to find the optimal value [-35 eV for compounds **19** (9IbuMe), **20** (7IbuMe); -45 eV for compounds **2, 3** and **17** (9IbutBu, 7IbutBu and 9PnttBu)]. At this value the abundance of the $[M_f]^+$ was 100 % and that for the $[M_f-17]^+$ 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.

6.2.3. PNA monomer synthesis

***tert*-Butyl 2-[*N*-(2-{[(9H-fluoren-9-yl)methoxy]carbonylamino}ethyl)-2-(2-isobutyr-amido-6-oxo-1,6-dihydropurin-9-yl)acetamido]acetate (22)**

To acid **8** (synthesized by L. Kovács[21]) (2.0 mmol) dissolved in anhydrous DMF (20 mL), HOBt hydrate (0.61 g, 4.0 mmol) and HBTU (1.52 g, 4.0 mmol) were added. Meanwhile ester **21**[30] (1.30 g, 2.0 mmol for acid **8**) was suspended in dichloromethane (20/30 mL), extracted with satd. $NaHCO_3$ solution (10 mL) and dried. The above dichloromethane solution of **21** and DIPEA (0.70 mL, 4.0 mmol) were added after 5 min and the reaction mixture was stirred for 1.5 h at room temperature. Work-up: after evaporating the reaction mixture the residue was triturated with EtOAc (5 mL) and filtered following the ensuing crystallization. The resulting crude product was purified by crystallization 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 (lgϵ 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, $(CH_3)_2CH$); 1.35/1.47 (9 H, 2×s, Bu^t); 2.83 (1.3 H, m, $(CH_3)_2CH$, 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);

δ_C (125.76 MHz, rotamers) 8.98 ($(CH_3)_2CH$); 27.47/27.50 ($(CH_3)_3C$); 34.17 ($(CH_3)_2CH$); 46.56 (OCH_2CH); 43.73/43.99; 47.09; 48.76; 49.96 (4×CH_2); 65.28 (OCH_2CH); 80.88/81.98 ($(CH_3)_3C$); 119.88; 124.79/124.88; 126.81/126.85; 126.95; 127.40; 128.71; 129.20 (arom. C's); 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^iCO); *m/z* (ESI): 891 (1%, $[M+K]^{\oplus}$); 875 (5, $[M+Na]^{\oplus}$); 853 (100, $[M+H]^{\oplus}$).

2-[*N*-(2-{[(9H-fluoren-9-yl)methoxy]carbonylamino}ethyl)-2-(2-isobutyramido-6-oxo-1,6-dihydropurin-9-yl)acetamido]acetic acid (23)

To ester **22** (0.68 g, 0.79 mmol) suspended in dichlorometane (20 mL), 1,3-dimethoxybenzene (0.125 mL, 0.95 mmol) was added 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*, coevaporated with EtOAc (4×). The solid residue was triturated with EtOAc, filtered (0.48 g, quant.), mp 202.0-206.0 °C and 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 affording a quantitative yield of the crude acid **23** (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 (lgε 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, $(CH_3)_2CH$); 2.75 (1 H, pseudoquintet, *J* 6.6, $(CH_3)_2CH$); 3.13 (1 H, m, CH); 3.35 (2.8 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*); δ_C (125.76 MHz, rotamers) 18.76 ($(CH_3)_2CH$); 34.58 ($(CH_3)_2CH$); 46.66 (OCH_2CH); 43.86/44.00; 46.85/46.97; 47.76; 49.11 ($4\times CH_2$); 65.43 (OCH_2CH); 120.01/ 120.04; 124.94/125.01; 126.95; 127.53 (arom. C's); 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^iCO, COOH); *m/z* (APCI) 602 (100%, $[M+H]^+$).

6.2.4. PNA oligomer synthesis

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 soln of t monomer (10.2 mL, 2.0 mmol) one of the following solutions was added:

1. 0.77 M soln of HATU (2.6 mL, 2.0 mmol), 0.6 M soln of lutidine (3.7 mL, 2.2 mmol) and 0.4 M soln of DIPEA (5.0 mL, 2.0 mmol);

2. 0.78 M soln of BET (2.8 mL, 2.2 mmol), 0.4 M soln of DIPEA (11.0 mL, 4.4 mmol);

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

After 2 min preactivation to each of the above solutions *tert*-butyl phenylalaninate (3.0 mmol) in DMF (6.0 mL) was added and the reaction mixture was completed to 27.5 mL of total volume with DMF. After 5 min 5 mL 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 mL solution, 220 nm) = f(concentration of t monomer)].

Solid phase synthesis (Tables 7 and 9)

Fmoc deprotection 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.[103]

The accuracy of the sample collection and absorbance determination was ±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 σ=0.0171 { (σ/$\bar{X}$)*100=2.41}].

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

Coupling condition 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 condition: monomer/HATU/DIPEA/lutidine = 3/2.7/3.3/3.3 equiv. in DMF for 20 min after 2 min reactivation 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 or H-C^{Bz}-CPG, 40 μmol/g) or Lys (Wang, 0.5 mmol/g, synthesis scale: 12.5/25 mmol). 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 the synthesis on TentaGel™ there was no problem with the missing values for (a+g); (c+t); (g+a); (g+c); (t+a); (t+t) couplings, only (c+c) was a bit lower (93, 100, 89%). Meaning of *e.g.* (a+c): coupling of monomer *a* to *c* that is connected to another monomer or amino acid on the support.

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9. Appendices

I.