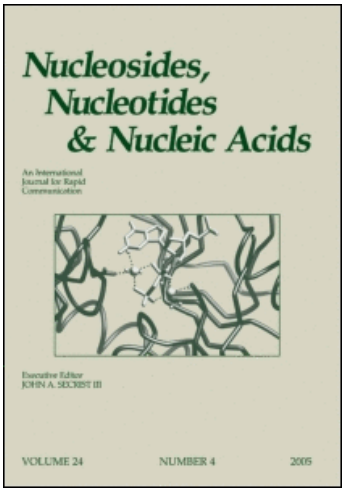

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A. Van Aerschot[a]; P. Herdewijn[a]; G. Janssen[a]; H. Vanderhaeghe[a]
[a] Rega Institute, Katholieke Universiteit Leuven, Leuven, Belgium

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PROTECTION OF THE LACTAM FUNCTION OF 2'-DEOXYINOSINE WITH A 2-(4-NITROPHENYL)-ETHYL MOIETY

A. Van Aerschot*, P. Herdewijn, G. Janssen and H. Vanderhaeghe

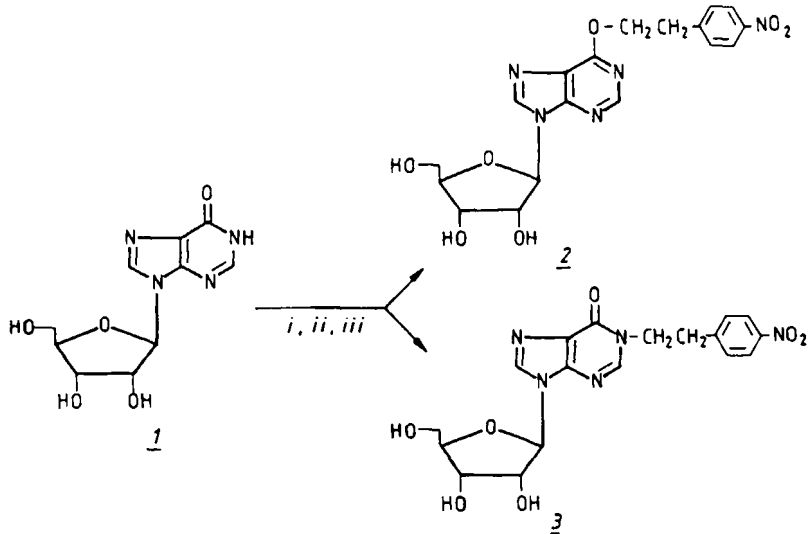
Rega Institute, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Abstract - The reaction of O-protected inosine with p-nitrophenyl ethanol under Mitsunobu conditions yields a mixture of the 1- and O^6-alkylated derivatives. 2'-Deoxyinosine protected on O^6-, can be synthesized fairly easy from deoxyguanosine with a Mitsunobu reaction followed by reductive deamination.

INTRODUCTION

DNA probes are very useful for isolation of clones coding for a certain protein. Since amino acid codons are apt to degeneration, DNA probes for a specific amino acid sequence should contain all possible codon sets. Of obvious importance is an ideal base analogue which would substitute for all four natural nucleosides without much destabilisation, thus obviating the need for mixed-sequence probes. Probes with the phenyl ring as the base substituent have failed[1], but during our work deoxyinosine has been reported to be a suitable alternative[2].

Deoxyinosine was a base we had in mind as a substitute for the natural nucleosides. In contrast with Ohtsuka *et al.*[2], we looked for a practical protecting group for the lactam function of the base moiety, since side reactions on deoxyguanosine and thymidine during the phosphotriester synthesis were well known[3]. The work of Adamiak *et al.*[4] who reported side reactions on the hypoxanthine lactam system during the phosphotriester synthesis, demonstrated this to be a good decision. Our preliminary results have been communicated already[5], and this paper describes the detailed procedures for preparing the necessary building blocks.



i: Benzoylchloride-pyridine ; ii: Triphenylphosphine , DEAD , NPE - OH ; iii : NH_3 - MeOH

Fig. 1. Protection of the lactam function.

RESULTS AND DISCUSSION

Protection of the lactam function. The 2-(4-nitrophenyl)-ethyl group was chosen for protection of 2'-deoxyinosine, as it was already successfully used for 2'-deoxyguanosine[6]. The usefulness of this group with respect to its ease of introduction and quantitative elimination were tested first on the riboside of hypoxanthine. Mitsunobu reaction[7] on perbenzoylated inosine, followed by ester hydrolysis, gave two nucleoside products, which were separated on silica gel and isolated in 55 % and 24 % yield. The products were identified respectively as the O^6- (2) and 1-alkylated (3) inosine derivative. Guanosine on the other hand gives selectively the O^6-protected nucleoside,[6] because of steric hindrance. The use of different solvents (dioxane, benzene, pyridine, methylene chloride), and lower temperature (0° and -70°C), didn't change the ratio of both products (about 5 on 2).

Identification of 2 and 3 was straightforward, using different techniques. Infrared analysis gave no C:O stretching vibration anymore around 1680 cm^{-1} for 2, present in 3 and in inosine. ^1H NMR of 3 showed the α-methylene protons of the 2-(4-nitrophenyl)-ethyl group attached to N-1 at higher field in comparison with those attached to an oxygen as in

2. Likewise the ^{13}C NMR signal for the α-carbon of the protecting group was shown upfield for 3 as compared to 2. Also remark the more downfield position of C-6 in 2, as compared to C-6 in inosine and in 3.

As final identification proof a comparison was made with the methylated inosine analogues. Therefore, inosine was alkylated under Mitsunobu conditions with methanol. This gave 69 % of the known 1-methylinosine,[8] and only 5 % of the O^6-methylinosine[9], which is in contrast with the isolated yield for 2 and 3. ^1H and ^{13}C NMR and IR analysis gave analogous signals as for 2 and 3, confirming the right identification of the latter. UV analysis doesn't give information on the site of alkylation, as the maxima for both products are coinciding (λ_{max}=253 nm for 2 and 3). In contrast with alkylation with 2-(4-nitrophenyl)-ethyl alcohol, there is though a strong preference for N-1-methylation.

Removal of the protecting group. Elimination of the O^6-2-(4-nitrophenyl)-ethyl group was possible with 0.5 M DBU in pyridine. After 2 h the starting product had reacted completely but a small percentage of the isomer 3 was detected on TLC, which gradually increased to about 20 % after 24 hours. Treating the 1-alkylated derivative 3 under the same conditions, also yielded a 1:4 ratio of 1-alkylated and dealkylated inosine. t-Butylamine-pyridine (1:9), and MeOH saturated with ammonia hardly caused any deprotection. A 1 M tetramethylguanidine-o-nitrobenzaldoxime solution (TMG - oximate) in dioxane-water (1:1) at 70°C though, gave complete deprotection of 2 and 3 in 3 h and 5 h respectively, without side reactions.

Quantitative studies were done after 5'-monomethoxytritylation of 2 and 3. This facilitates isolation of the deprotected compounds. 0.5 M DBU in pyridine gave dealkylation of 4 to 6, and concomitant formation of 5. A ratio of about 4 to 1 was reached after 4 h, staying unchanged for 48 h. Neutralisation and chromatographic purification yielded 62 % of 6 and 19 % of 5. Likewise treatment of 5 gave the same result after 24 h. Reaction at 70°C with a 1 M oximate-TMG solution gave after 4 h complete dealkylation of 4 without side reactions, and 77 % of cristalline 6 was isolated. 5 Likewise gave only 6 after 18 h under the same conditions (analytical, TLC controlled).

The ease of deprotection of the 1-alkyl derivative and the formation of 5 starting from 4, were somewhat surprising. The seemingly uncomplete deprotection of 4 and 5 with DBU, can be explained by a Michael

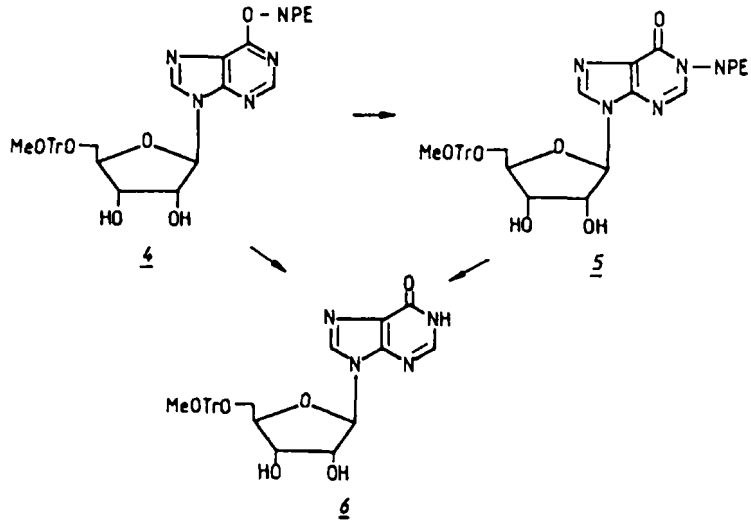


Fig. 2. Elimination of the 2-(4-nitrophenyl)-ethyl protecting group.

type addition on the formed p-nitrostyrene, regenerating 5. In the presence of p-thiocresol as a nucleophile, dealkylation with DBU only gives 6, without formation of 5. It was demonstrated that the p-nitrostyrene formed in the β-elimination reaction with DBU, could be captured with p-thiocresol to form the [2-(4-nitrophenyl)-ethyl]-(p-tolyl)-thioether[10] (β-elimination of 2-(4-nitrophenyl)-ethyl as a phosphate protecting group). When we repeated this procedure for our problem, the reaction slows down considerably, and is complete only after 5 days with 0.5 M DBU and 0.5 M p-thiocresol in dry pyridine. After workup the thioether was isolated and identified with UV and ^{1}H NMR, identical with the thioether in ref. 10. In these circumstances the deprotection went to completion.

Deoxyinosine protection. Deoxygenation of 2 to 10 according to the method of Robins et al.[11] was unsuccessful. As shown with inosine, Mitsunobu reaction on dI would lead to a mixture of the O^6- and N-1 protected derivatives. Therefore an alternative strategy was chosen by synthesizing the O^6-protected deoxyinosine 10 from deoxyguanosine. After selective acylation[12] of the sugar moiety to 7, Mitsunobu reaction yielded 8 in 68 % yield, although chromatographic purification was cumbersome because of the presence of triphenylphosphine oxide and the di-(N,N'-ethoxycarbonyl)-hydrazine. Compound 8 was the starting material for synthe-

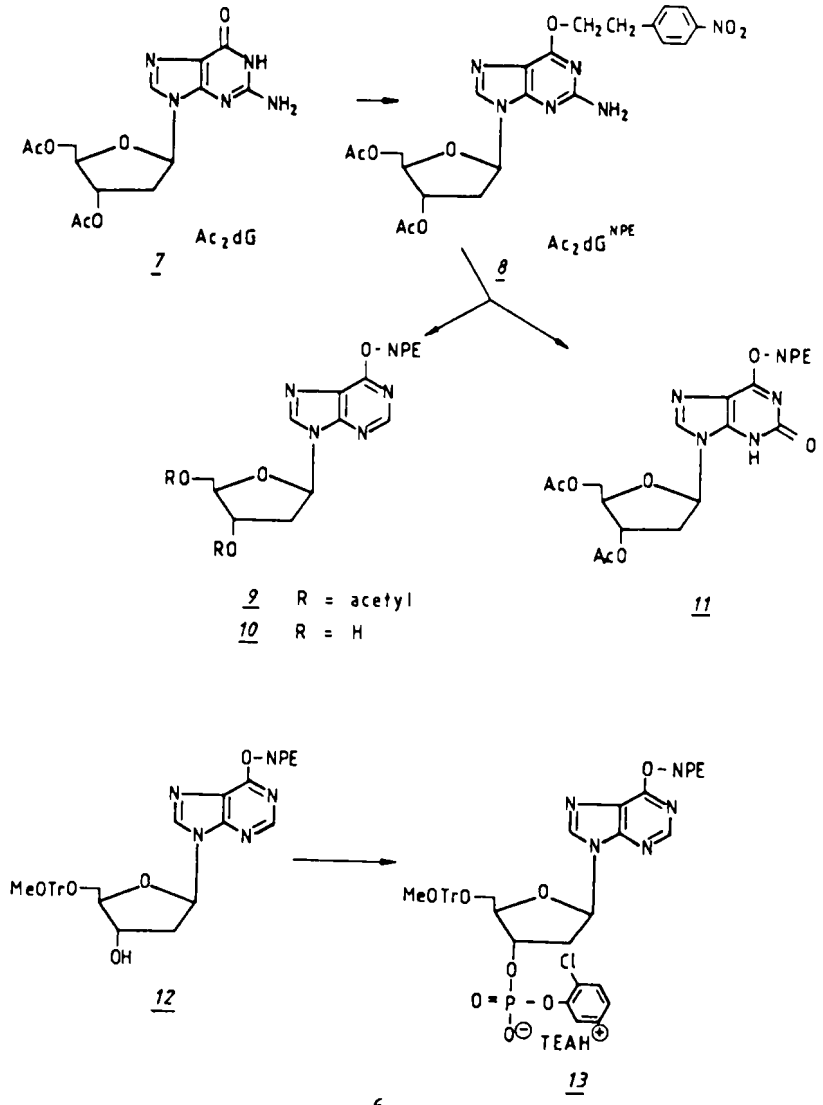


Fig. 3. Synthesis of the O^6-protected deoxyinosine monomer.

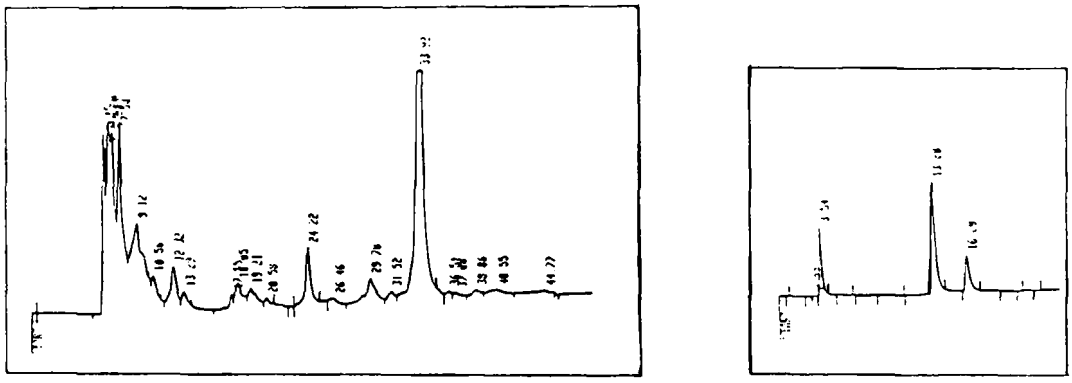


Fig. 4. A) HPLC purification on a Partisil 10 SAX column of the deprotected heptamer (TITIITT). Buffer A, 0.01 M KH_2PO_4, pH 6.3. Buffer B, 0.3 M KH_2PO_4, pH 6.3, both in formamide-water (6:4). Gradient, 0-50 % B in 45 min, 0.5 ml/min, ambient temperature. The peak which eluted after 33 min was collected, desalted and lyophilized.

B) Analysis of the isolated heptamer on a Partisil 10 SAX column after nuclease P1 digestion. Buffer A, 0.01 M KH_2PO_4, pH 4.0. Buffer B, 0.2 M KH_2PO_4, pH 4.0. Gradient, 0 % B for 5 min, 0-50 % B in 20 min. Order of elution: thymidine, thymidine 5'-phosphate, 2'-deoxyinosine 5'-phosphate.

sis of the protected deoxyinosine and deoxyxanthosine monomers (see accompanying paper). The same product was synthesized recently by Eritja et al.[13] They first protected the 2-amino function with a dimethoxytrityl group. Mitsunobu reaction and subsequent detritylation afforded the same compound in 3 steps.

Deamination of 8 according to Nair and Chamberlain[14] with isoamyl nitrite in THF at 50°C, gave after 48 h 55 % of the deoxyinosine derivative 9 and 21 % of the deoxyxanthosine 11. The main product 9 and its deesterified derivative 10 were identified with UV, MS and NMR analysis, which were comparable with the analysis for 2. The O^6-protected deoxyxanthosine 11 was identified mainly by a weak molecular ion at m/e = 501, a strong downfield migration of the C-2 signal in ^{13}C NMR compared to 9, and the presence of a deuteriumoxide exchangeable proton.

Monomethoxytritylation of 10 and phosphorylation with o-chlorophenyl dichlorophosphate[15] gave the protected monomer 12, which could be used as such for incorporation into oligonucleotides.

Incorporation into oligonucleotides. To evaluate the incorporation into oligonucleotides, a heptamer with 3 internal deoxyinosine residues

TABLE 1. Synthesis cycle

CH_2Cl_2 wash	2 min
CH_2Cl_2-iPrOH 85:15	2 min
saturated $ZnBr_2$-0.02 M triazole in CH_2Cl_2-iPrOH 85:15[17]	5 min
CH_2Cl_2-iPrOH 85:15	2 min
MeOH wash	2 min
Pyridine wash	5 min
Condensation	20 min
Pyridine wash	2 min
Capping	5 min
Pyridine wash	2 min

The condensation mixture consists of 30 µmol monomethoxytrityl nucleoside phosphodiester and 200 µmol MSNT in 250 µl pyridine - N-methylimidazole (10:1). Capping is done with a 1 ml solution of pyridine - Ac_2O - N-methylimidazole (8:1:1).

(TITIITT) was synthesized and enzymatically degraded with nuclease P1 to its mononucleotide components. The occurrence of side reactions, or the incomplete removal of the new protecting group, would clearly show on analysis of the degraded heptamer. Two deoxyinosine residues were also put next to each other, to evaluate the coupling and deprotection yield, which are known to be rather low in the core of two 2'-deoxyguanosines. Synthesis was done with a manual bench top synthesizer (Omnifit) on a silica gel functionalised with a double glycine spacer[16]. The support was put in the apparatus and the cycle shown in Table 1 was carried out. The coupling yields were determined spectrophotometrically after each detritylation, and were as usual (85-95 % per step).

After synthesis the solid support was divided in two. One part was treated with a 1 M TMG-oximate solution at 70°C for 18 h, giving deprotection of the base labile groups. The second part was treated with a two step procedure involving 0.5 M TMG-oximate treatment at 37°C for 15 h, followed by 0.5 M DBU in pyridine for 15 h at RT. Detritylation of both parts was done with 80 % acetic acid.

HPLC purification on a Partisil 10 SAX column (for conditions see fig. 4) showed no difference in profile, so we could conclude that fast deprotection[18] with a 1 M TMG-oximate solution at 70°C because of its

ease and speed was the best way to deprotect an oligonucleotide with internal deoxyinosine residues.

The purified heptamer was desalted on a reversed phase disposable cartridge, and enzymatically degraded to thymidine, thymidine 5'-phosphate and deoxyinosine 5'-phosphate with nuclease P1. Subsequent analysis by HPLC showed no side products. Further treatment with bacterial alkaline phosphatase gave only two products dT and dI (results not shown).

After the incorporation of the O^6-protected deoxyinosine was unambiguously proved, a 23-mer was synthesized as hybridisation probe for the isolation of the gene for a proline-rich protein of rats[19]. The results of this and other probes will be reported later.

Discussion. The 2-(4-nitrophenyl)-ethyl group has been evaluated for protection of the lactam function of 2'-deoxyinosine. Direct introduction of an alkyl group under Mitsunobu conditions on the hypoxanthine lactam function, leads as well to the desired O^6-, as to the unwanted N-1 derivatives. Guanosine on the other hand, yields exclusively the O^6-protected nucleoside under the same conditions. An alternative strategy for protection of 2'-deoxyinosine, reductive deamination of the corresponding guanosine nucleosides with isoamyl nitrite, was also successful. The (4-nitrophenyl)-ethyl alkylated products can be deprotected by the fast deprotection method of oligonucleotides[18], without any side reactions. As shown by multiple incorporation into a heptamer followed by enzymatic degradation, the p-nitrophenyl group is worthwhile to be considered as a protecting group for the synthesis of oligonucleotides containing one or more deoxyinosine residues.

EXPERIMENTAL

Materials and methods. Solvents and reagents were purified as previously described, as well as synthesis of the functionalised support and the protected monomer nucleotides[16]. Triphenylphosphine, diethylazodicarboxylate (DEAD) and 2-(4-nitrophenyl)-ethyl alcohol were purchased from Janssen Chimica. Nuclease P1 and bacterial alkaline phosphatase were bought from Boehringer. M.p.s. were measured using a Büchi-Tottoli apparatus and are uncorrected. I.R. spectra were recorded on a Perkin Elmer Model 580B spectrophotometer. UV measurements were done with a Beckman UV 5230 spectrophotometer. NMR measurements were obtained on a Jeol

FX900 spectrometer with shifts in δ units. Me_4Si was used as internal reference for ^1H NMR and the solvent resonance as reference for ^{13}C measurements (DMSO at 39.6 and $CDCl_3$ at 76.9 p.p.m. from Me_4Si). The carbon with the nitro substituent of the 2-(4-nitrophenyl)-ethyl group is designated as C-para (C_p); C-ortho = C_o; C-meta = C_m. Mass spectra were recorded on a single-focusing AEI MS-12 mass spectrometer (Kratos Ltd., Manchester, U.K.) operated at an accelerating voltage of 8 kV, trap current 100 μA, ionisation energy 70 eV and ion source temperature as described. Relative intensity is included between brackets. Thin layer chromatography was done on Merck Kieselgel 60 F_{254} aluminium sheets and plates were developed in one of the following systems : A: $CHCl_3$-MeOH (9:1); B: $CHCl_3$-MeOH (95:5); C: $CHCl_3$-MeOH (8:2); D: $CHCl_3$-acetone (8:2). Elemental analyses were done at the University of Konstanz.

Mitsunobu reaction on inosine. Inosine (2.68 g, 10 mmol) was coevaporated twice with dry pyridine, and suspended in 40 ml dry pyridine. The suspension was cooled in an ice bath and benzoylchloride (5 ml, 35 mmol) was added over a period of 10 min. After 4 hours reaction at room temperature, TLC showed exclusive formation of the tribenzoylderivative. MeOH was added and the reaction mixture was evaporated in vacuo. The residual oil was taken up in 100 ml $CHCl_3$ and washed with 100 ml $NaHCO_3$ and 2 x 100 ml water. The water phases were extracted once more with $CHCl_3$. After drying of the combined organic extracts, evaporation and coevaporation with dry pyridine, the oily residue was used as such for the next reaction. The compound was dissolved in 100 ml dry dioxane and triphenylphosphine (3,95 g), diethylazodicarboxylate (DEAD, 2.5 ml) and 2-(4-nitrophenyl)-ethyl alcohol (NPE-OH) (2.5 g) were added (all 15 mmol, 1.5 eq.). The mixture warmed up slightly and after 10 min, TLC in $CHCl_3$-MeOH (98:2) showed the formation of 2 new products (Rf=0.18 and 0.43). The reaction was quenched by addition of water and after a few minutes the mixture was evaporated. The residue was taken up in 250 ml MeOH saturated with ammonia and left overnight at room temperature. The next morning TLC revealed 2 main nucleoside products. After evaporation, the mixture was adsorbed on celite and dried in vacuo. The celite mass was put on top of a silica column (70 g), which was eluted with a MeOH gradient in $CHCl_3$ (99:1 to 9:1). The fractions with Rf=0.30 in $CHCl_3$-MeOH (9:1) were pooled and crystallised from a MeOH-H_2O mixture, to yield

2.29 g (5.5 mmol, 55 %) of O^6-[2-(4-nitrophenyl)-ethyl]-inosine 2. An analytical sample was obtained after two crystallisations from the same solvent mixture.

O^6-[2-(4-nitrophenyl)-ethyl]-inosine 2.
M.p.: 105-106°C (MeOH-H_2O), UV: (MeOH) λ_{max} = 253 nm (ε=16700 (4.22)) and 277 nm unchanged at pH 2, 7 and 12; TLC: Rf=0.30 (solvent A); MS: m/e=417 (M^+, weak), 387 (M-30, 0.15 %), 328 (M-89, 0.6 %), 286 (base+2H, 1.5 %), 149 (NO_2-C_6H_4-C_2H_3, 100 %), 136 (NO_2-C_6H_4-CH_2, 7 %), 133 sugar (M-base, 2 %), 119 (149-NO, 23 %), 103 (149-NO_2, 46 %), 91 (C_7H_7, 28 %), 77 (C_6H_5, 65 %), analysis at 140-200°C, IR: maximum at 1585 cm^{-1}. No maxima between 1600 and 1700 cm^{-1} as in inosine (max. at 1695).
NMR: ^1H : (DMSO-d_6) δ =3.31 (t, 2 H, CH_2-ar, J=6.8 Hz), 3.66 (m, 2 H, 2 H-5'), 4.00 (m, 1 H, H-4'), 4.20 (m, 1 H, H-3'), 4.60 (m, 1 H, H-2'), 4.86 (t, 2 H, CH_2-O, J=6.8 Hz), 5.13 (t, 5-'-OH), 5.23 (d, 3'-OH), 5.48 (d, 2'-OH), 6.00 (d, 1 H, H-1', J=6 Hz), 7.64 (d, 2 H, ortho-H, J=8.9 Hz), 8.18 (d, 2 H, meta-H, J=8.9 Hz), 8.54 (s, 1 H, H-2), 8.60 (s, 1 H, H-8) ppm; ^{13}C: (DMSO-d_6) δ =34.5 (CH_2-ar), 61.5 (C-5'), 66.4 (CH_2-O), 70.5 (C-3'), 74.1 (C-2'), 85.9 (C-4'), 88.1 (C-1'), 121.2 (C-5), 123.4 (2 C_m), 130.3 (2 C_o), 142.5 (C-8), 146.4+146.6 (C_x+C_p), 151.6 (C-2), 152.0 (C-4), 160.0 (C-6) ppm;
Anal. Calcd. for $C_{18}H_{19}N_5O_7$.1 1/2 H_2O : C, 48.65; H, 4.99; N, 15.76.
Found : C, 48.74; H, 4.72; N, 15.64.

The fractions with Rf=0.13 were collected and crystallised from MeOH-water to yield 1.004 g (2.4 mmol, 24 %) of 1-[2-(4-nitrophenyl)-ethyl]-inosine 3. An analytical sample was obtained after two recrystallisations from MeOH-H_2O. Yields are an average for 3 syntheses.

1-[2-(4-nitrophenyl)-ethyl]-inosine 3.
M.p.: 220-221°C (MeOH-H_2O); UV: (MeOH) λ_{max} = 253 nm (ε=15050), 273 nm (=14050), unchanged at pH 2, 7 and 12; TLC: Rf=0.13 (solvent A); MS: m/e=417 (M^+, weak), 286 (base+2H, 16 %), 285 (base+H, 4 %), 149 (60 %), 136 (NO_2-C_6H_4-CH_2, 100 %), 133 (sugar, 7 %), 119 (40 %), 103 (28 %), 91 (14 %), 77 (40 %), analysis at 200°C, IR: maxima at 1660, 1600, 1585 cm^{-1};
NMR: ^1H : (DMSO-d_6) δ =3.16 (t, 2 H, CH_2-ar, J=6.8 Hz), 3.51-3.70 (m, 2 H, 2H-5'), 3.96 (m, 1 H, H-4'), 4.07-4.58 (m, 4 H, H-3'+ H-2'+CH_2-N), 5.05 (t, 5'-OH), 5.20 (d, 3'-OH), 5.47 (d, 2'-OH), 5.83 (d, 1 H, H-1', J=5.7 Hz), 7.50 d(2 H, ortho-H, J=8.8 Hz), 8.15 (d, 2 H, meta-H, J=8.8 Hz), 8.24 (s, 1 H, H-2), 8.33 (s, 1 H, H-8) ppm;
^{13}C: (DMSO-d_6) δ =34.7 (CH_2-ar), 46.5 (CH_2-N), 61.5 (C-5'), 70.5 (C-3'), 74.3 (C-2'), 85.9 (C-4'), 87.6 (C-1'), 123.6 (2 C_m), 123.8 (C-5), 130.3 (2 C_o), 139.7 (C-8), 146.3+146.5 (C_x+C_p), 147.6 (C-4), 148.4 (C-2), 156.0 (C-6) ppm.;
Anal. Calcd. for $C_{18}H_{19}N_5O_7$: C, 51.80; H, 4.59; N, 16.78.
Found : C, 51.56; H, 4.46; N, 16.22.

Alkylation of inosine with methanol. 1.97 g (5 mmol) triacetylinosine was alkylated with 0.4 ml (10 mmol) dry MeOH in 50 ml dry dioxane in the presence of 1.5 eq. triphenylphosphine and DEAD. The reaction was complete in 10 min and was quenched like before. Ammonolysis and chromatographic purification on silica gel, afforded after crystallisation from MeOH 974 mg (3.45 mmol, 69 %) of the 1-methylinosine and only 69 mg (0.25 mmol, 5 %) of the O^6-methylinosine derivative.

O^6-methylinosine
M.p.: 138-140°C (lit.[9] 139-142°C). UV: (MeOH) λ_{max} = 248 nm (ε=10950), with shoulders at 252 nm (10700), and 258 nm (7700), unchanged at pH 2, 7 and 12. TLC: Rf=0.40 (solvent C); IR: maxima at 1610 and 1590 cm^{-1}; NMR: ^1H: (DMSO-d_6) δ =3.67 (m, 2 H, 2 H-5'), 4.01 (m, 1 H, H-4'), 4.13 (s, 3 H, CH_3), 4.22 (m, 1 H, H-3'), 4.63 (m, 1 H, H-2'), 5.15 (t, 5'-OH), 5.23 (d, 3'-OH), 5.52 (d, 2'-OH), 6.03 (d, 1 H, H-1', J=6.4 Hz), 8.57 (s, 1 H, H-2), 8.64 (s, 1 H, H-8); ^{13}C: (DMSO-d_6) δ =54.3 (CH_3), 61.5 (C-5'), 70.6 (C-3'), 74.0 (C-2'), 86.0 (C-4'), 88.2 (C-1'), 121.4 (C-5'), 142.6 (C-8), 152.0 (C-4+C-2), 160.7 (C-6) ppm.

1-methylinosine
M.p.: 195-197°C (lit.[8] 200-205°C). UV: (MeOH) λ_{max} = 250 nm (ε=10300), unchanged at pH 2, 7 and 12. TLC: Rf=0.15 (solvent C); IR: maxima at 1690 and 1585 cm^{-1}; NMR: ^1H: (DMSO-d_6) δ =3.52 (s, 3 H, CH_3), 3.70 (m, 2 H, H-5'), 3.97 (m, 2 H, H-3' and H-4'), 4.51 (m, 1 H, H-2'), 5.07 (t, 5'-OH), 5.20 (d, 3'-OH), 5.49 (d, 2'-OH), 5.88 (d, 1 H, H-1', J=6.7 Hz), 8.35 (s, 1 H, H-8), 8.40 (s, 1 H, H-2); ^{13}C: (DMSO-d_6) δ =33.9 (CH_3), 61.5 (C-5'), 70.6 (C-3'), 74.4 (C-2'), 85.9 (C-4'), 87.8 (C-1'), 123.9 (C-5'), 139.5 (C-8), 147.9 (C-4), 149.0 (C-2), 156.8 (C-6) ppm.

5'-monomethoxytrityl-O^6-[2-(4-nitrophenyl)-ethyl]-inosine 4. 1.51 g of O^6-NPE-I 3 (3.4 mmol) was tritylated with 1.26 g (4.1 mmol, 1.2 eq.) monomethoxytritylchloride in 25 ml dry pyridine. The reaction was quenched with MeOH after 4 h, extracted with $CHCl_3$ and purified on silica gel. Precipitation from a concentrated $CHCl_3$ solution in 300 ml hexane gave 2.16 g of the title compound 4 (3.13 mmol, 92 %,) as a white powder.

UV: (MeOH) λ_{max} =234 nm, with shoulders at 252, 260 and 275 nm. TLC: Rf= 0.51 (solvent A); Rf=0.31 (solvent B) NMR: ^1H: ($CDCl_3$) δ =3.29 (t, CH_2-ar) + 3.36 (m, 2 H-5') (4 H), 3.73 (s, 3 H, CH_3-O), 4.40 (m, 2 H, H-3' + H 4'), 4.80 (m, 3 H, H-2' + CH_2-O), 6.01 (d, 1 H, H-1', J=5.5 Hz), 6.74 (d, 2 H, trityl), 7.04-7.53 (m, 14 H, trityl + ortho-H), 8.12 (d, 2 H, meta-H, J=9 Hz), 8.16 (s, 1 H, H-8), 8.41 (s, 1 H, H-2) ppm;
^{13}C: ($CDCl_3$) δ =34.9 (CH_2-ar), 55.0 (CH_3-O), 63.4 (C-5'), 66.6 (CH_2-O), 72.2 (C-3'), 75.4 (C-2'), 85.5 (C-4'), 86.7 (ϕ_3-C), 90.3 (C-1'), 121.6 (C-5), 123.6 (2 C_m), 130.1 (2 C_o), 140.8 (C-8), 145.5 + 146.8 (C_x+C_p), 151.1 + 151.3 (C-2 + C-4), 160.2 (C-6) ppm + trityl signals.

5'-monomethoxytrityl-1-[2-(4-nitrophenyl)-ethyl]-inosine 5. 1.375 g of 3 (3.3 mmol) was tritylated with 1.33 g (4.3 mmol, 1.3 eq.) monomethoxytritylchloride in 30 ml dry pyridine. The reaction was quenched after 5 h by addition of MeOH. Extraction with $CHCl_3$ and chromatographic purification on 60 g silica gel (elution with $CHCl_3$-MeOH-pyridine (97:3:0.1), afforded after precipitation in 350 ml hexane-ether (8:2) 1.800 g of the title compound 5 (2.61 mmol, 79 %) as a white voluminous powder.

UV: (MeOH) λ_{max}=234, 252 and 270 nm, TLC: Rf=0.30 (solvent A); Rf=0.09 (solvent B) NMR: ^{1}H: ($CDCl_3$) δ=3.07 (t, 2H, CH_2-ar), 3.40 (m, 2 H, 2 H-5'), 3.70 (s, 3 H, CH_3-O), 4.05-4.85 (m, 5 H, H-2' + H-3' + H 4' + CH_2-N), 6.00 (d, 1 H, H-1', J=4.8 Hz), 6.74 (d, 2 H, trityl), 7.05-7.48 (m, 14 H, trityl + ortho-H), 7.53 (s, 1H, H-8), 8.01 (d, meta-H, J=8.6 Hz), 8.00 (s, H-2) ppm;
^{13}C: ($CDCl_3$) δ=34.9 (CH_2-ar), 47.8 (CH_2-N), 55.0 (CH_3-O), 63.6 (C-5'), 71.2 (C-3'), 75.0 (C-2'), 84.5 (C-4'), 86.6 (ϕ_3-C), 88.5 (C-1'), 123.8 (C-5 + 2 C_m), 129.5 (2 C_o), 139.0 (C-8), 144.7 (C-4), 146.8 (C_x+C_p), 147.3 (C-2), 156.2 (C-6) ppm + trityl signals.

5'-monomethoxytritylinosine 6 (MeOTr-I) from 4.

Method A: reaction with DBU. 1.38 g of 4 (2 mmol) was dissolved in 50 ml of anhydrous pyridine containing 0.5 M DBU (12.5 eq.). After 4 h no starting material could be detected anymore, but approximately 20 % of the product had reacted to the 1-alkyl derivative 5. The ratio between the different products remained unchanged for 2 days after which the mixture was neutralised by addition of 25 ml of a 1 M acetic acid solution in MeOH-pyridine (9:1). The mixture was concentrated, poured into 150 ml EtOAc, and washed twice with 150 ml water and with 100 ml of a saturated NaCl solution. The water phases were reextracted twice with 100 ml EtOAc. Chromatographic purification and precipitation in hexane-diethyl ether (8:2) afforded 265 mg (0.38 mmol, 19 %) of 5. Further elution of the column afforded 675 mg (1.25 mmol, 62 %) of the title compound 6, which was crystallised from $CHCl_3$-diethyl ether.

M.p.: 175-176°C (from $CHCl_3$-ether); UV: (MeOH) λ_{max} = 235 nm (ε=21400), 250 nm and 270 nm shoulders; TLC: Rf=0.39 (solvent C); NMR: ^{1}H: (DMSO-d_6) δ=3.25 (m, 2 H, 2 H-5'), 3.73 (s, 3 H, CH_3-O), 4.13 (m, 1 H, H-4'), 4.32 (m, 1 H, H-3'), 4.67 (m, 1 H, H-2'), 5.23 (d, 3'-OH), 5.58 (d, 2'-OH), 5.97 (d, 1 H, H-1', J=4.7 Hz), 6.85 (d, 2 H, trityl), 7.11-7.47 (m, 12 H, trityl), 8.02 (s, 1 H, H-2), 8.23 (s, 1 H, H-8) ppm;
^{13}C: (DMSO-d_6) δ =55.4 (CH_3-O), 64.1 (C-5'), 70.5 (C-3'), 73.6 (C-2'), 83.5 (C-4'), 86.2 (ϕ_3-C), 88.4 (C-1'), 124.9 (C-5), 139.2 (C-8), 146.0 (+C-2), 148.6 (C-4), 156.8 C-6) ppm + trityl signals.

Method B: reaction with a 1 M oximate-TMG solution. 700 mg of 4 (1 mmol) was dissolved in 15 ml of a 1 M o-nitrobenzaldoxime - TMG solution in dioxane - water (1:1). The mixture was heated at 70°C in an oilbath. After 4 hours no starting product could be detected anymore, neither alkylation of N-1 took place. The mixture was neutralised with a 1 M acetic acid solution, extracted and purified, as for method A. Crystallisation from $CHCl_3$ - diethylether afforded 416 mg of the title compound 6 (0.77 mmol, 77 %).

5'-monomethoxytritylinosine 6 (MeOTr-I) from 5. 690 mg of 5 (1 mmol) and 1.25 g (10 mmol) p-thiocresol were dissolved in 20 ml anhydrous pyridine containing 0.5 M DBU. After 5 days, workup as for the synthesis of 4 and crystallisation from $CHCl_3$-diethylether yielded 439 mg (0.81 mmol, 81 %) of 6. The first fraction eluted from the column during workup contained thiocresol and [2-(4-nitrophenyl)-ethyl]-(p-tolyl)-thioether. They were resolved by column chromatography with hexane-EtOAc (95:5).

[2-(4-nitrophenyl)-ethyl]-(p-tolyl)-thioether[10]
UV: (MeOH) λ_{max} = 258 nm; ^{1}H NMR: ($CDCl_3$) δ = 2.29 (s, 3 H), 3.05 (m, 4 H), 7.20 (m, 4 H), 7.31 (d, 2 H, ortho-H NPE, J= 8.8 Hz), 8.13 (d, 2 H, meta-H NPE, J=8.8 Hz) ppm.

3',5'-di-O-acetyl-O^6-[4-nitrophenyl)-ethyl]-2'-deoxyguanosine 8. To a mixture of 3.51 g (10 mmol) of 3',5'-di-O-acetyl-2'-deoxyguanosine 7 and 2.505 g (15 mmol) 2-(4-nitrophenyl)-ethyl alcohol in 100 ml dry dioxane was added 3.93 g (15 mmol) triphenylphosphine and 2.5 ml (15 mmol) DEAD. The reaction was stirred overnight at room temperature and heated for two more hours at 60°C.

Quenching with water and evaporation gave an oil which was purified on 200 g of silica gel [elution with an acetone gradient (0 to 20 %) in chloroform]. The fractions with Rf=0.18 (solvent D) were pooled, evaporated and crystallised from $CHCl_3$ - diethylether, yielding 3.402 g (6.8 mmol, 68 %) of the title compound 8.

M.p.: 125°C; UV: (MeOH) λ_{max} = 253 nm (ε=15575), 281 nm (ε=18550); TLC: Rf=0.18 (solvent D); Rf=0.50 (solvent B); MS: m/e=500 (M^+, 23 %), 300 (base+H, 93 %), 201 (sugar, 2 %), 151 (base+H-149, 100 %), 81 (sugar-2 HOAc, 96 %), 43 (acetyl, 52 %), analysis at 170-180°C.
NMR: ^{1}H: ($CDCl_3$) δ =2.08 s + 2.12 (2s, 6 H, $2CH_3$), 2.35-3.10 (m, 2 H, 2 H-2'), 3.26 (t, 2 H, CH_2-ar, J=6.8 Hz), 4.25-4.50 (m, 3 H, 2 H-5' +

H-4'), 4.73 (t, 2 H, CH_2-O, J=6.8 Hz), 4.93 brs (2 H, NH_2), 5.42 (m, 1 H, H-3'), 6.27 (t, 1 H, H-1', J = 6.7 Hz), 7.46 (d, 2 H, ortho-H, J=8.8 Hz), 7.75 (s, 1 H, H-8), 8.15 (d, 2 H, meta-H, J=8.8 Hz) ppm;
^{13}C: ($CDCl_3$) δ =20.5+20.6 (2 CH_3), 34.9 (CH_2-ar), 36.7 (C-2'), 63.5 (C-5'), 66.0 (CH_2-O), 74.3 (C-3'), 82.1 (C-4'), 84.2 (C-1'), 115.7 (C-5), 123.4 (2 C_m), 129,7 (2 C_o), 137.4 (C-8), 145.8 + 146.7 (C_x+C_p), 153.3 (C-4), 159.1 + 160.7 (C-2 + C-6), 170.0 + 170.3 (2 C:O) ppm.

3',5'-di-O-acetyl-O^6-[2-(4-nitrophenyl)-ethyl]-2'-deoxyinosine 9.

Under a N_2 atmosphere 2.5 g (5 mmol) of 8 was brought in reaction with 4 ml (30 mmol) of isoamylnitrite in 60 ml dry THF at 50°C. After 24 hours another 4 ml of isoamylnitrite was added, and the mixture was heated for a further 15 hours till complete disappearance of the starting product. The mixture was evaporated and the remaining oil was purified on 100 g silica gel with an acetone gradient (0 %-30 %) in chloroform. The fractions with Rf=0.27 (solvent D) were pooled and crystallised from $CHCl_3$-diethylether, yielding 1.336 g (2.75 mmol, 55 %) of the title product 9.

M.p: 149-151°C UV: (MeOH) λ_{max} =253 nm (ε=18040), shoulders at 260 and 272 nm; TLC: Rf=0.27 (solvent D), Rf=0.59 (solvent B);
MS: m/e=485 (M^+, 0.7 %), 442 (M-acetyl, 8 %), 201 (sugar, 8 %), 81 (sugar-2 HOAc, 100 %), 43 (acetyl, 40 %), analysis at 170-180°C;
NMR ^{1}H: ($CDCl_3$) δ=2.08 + 2.14 (2s, 6 H, 2 CH_3), 2.50-3.18 m (2 H, 2 H-2'), 3.33 (t, 2 H, CH_2-ar, J=6.7 Hz), 4.25-4.50 (m, 3 H, 2 H-5' + H-4'), 4.86 (t, 2 H, CH_2-O, J=6.7 Hz), 5.45 (m, 1 H, H-3'), 6.15 (dd, 1 H, H-1', width=14 Hz), 7.50 (d, 2 H, ortho-H, J=8.8 Hz), 8.12 (s, 1 H, H-8), 8.16 (d, 2 H, meta-H, J=8.8 Hz), 8.51 (s, 1 H, H-2) ppm;
^{13}C: ($CDCl_3$) δ=20.5 + 20.6 (2 CH_3), 34.9 (CH_2-ar), 37.3 (C-2'), 63.4 (C-5'), 66.3 (CH_2-O), 74.2 (C-3'), 82.5 (C-4'), 84.6 (C-1'), 121.8 (C-5), 123.5 (2 C_m), 129.7 (2 C_o), 140.3 (C-8), 145.5 + 146.7 (C_x+C_p), 151.5 (C-4), 151.9 (C-2), 160.3 (C-6), 169.9 + 170.0 (2 C:O) ppm.

The fractions with Rf=0.08 were treated the same way and yielded 526 mg (1.05 mmol, 21 %) 3',5'-di-O-acetyl-O^6-[2-(4-nitrophenyl)-ethyl]-2'-deoxyxanthosine 11.

M.p.: 150°C ($CHCl_3$-diethyl ether); UV: (MeOH) λ_{max} = 268.5 nm (ε=20000), 245 nm (ε=10560); shoulders at 238 and 284 nm; TLC: Rf=0.08 (solvent D); Rf=0.27 (solvent B);
MS: m/e=501 (M^+, weak), 352 (M-149, weak), 301 (base+H, weak), 201 (sugar, 7 %), 81 (sugar-2 HOAc, 100 %), 43 (acetyl, 66 %), analysis at 170-180°C;
NMR: ^{1}H: ($CDCl_3$/D_2O) δ=2.10 s + 2.14 (2s, 6 H, 2 CH_3), 2.69 (m, 2 H, 2 H-2'), 3.29 (t, 2 H, CH_2-ar, J=6.5 Hz), 4.25-4.50 (m, 3 H, 2 H-5' + H-4'), 4.90 (t, 2 H, CH_2-O, J=6.5 Hz), 5.37 (m, 1 H, H-3'), 6.37 (t, 1 H, H-1', J=6.7 Hz) 7.49 (d, 2 H, ortho-H, J=8.8 Hz), 7.88 (s, 1 H, H-8), 8.15 (d, 2 H, meta-H, J=8.8 Hz) ppm;

^{13}C: ($CDCl_3$) δ=20.5+20.6 (2 CH_3), 34.9 (CH_2-ar), 38.0 (C-2'), 63.3 (C-5'), 67.9 (CH_2-O), 73.6 (C-3'), 82.2 (C-4'), 84.5 (C-1'), 115.0 (C-5), 123.5 (2 C_m), 129,8 (2 C_o), 137.3 (C-8), 145.0 + 146.8 (C_x+C_p), 150.4 (C-4), 159.4 + 161.5 (C-2 + C-6), 170.0 + 170.1 (2 C:O) ppm.

O^6-[2-(4-nitrophenyl)-ethyl]-2'-deoxyinosine 10. 485 mg (1 mmol) of 9 was dissolved in 50 ml of methanol saturated with ammonia and the mixture was left overnight at 4°C. Evaporation in vacuo and repeated coevaporation with xylene left an oil, which was crystallised from $CHCl_3$-toluene, yielding 332 mg (0.83 mmol, 83 %) of dI^{NPE} 10.

M.p: 135-136°C; UV: (MeOH) λ_{max}=253 nm (ε=18780), shoulders at 260 and 272 nm; TLC (solvent A) Rf=0.33; MS: m/e=401 (M^+, weak), 371 (M-30, weak), 312 (M-89, 0.25 %), 285 (base, weak), 136 (NO_2-C_6H_4-CH_2, 100 %), 133 (sugar (M-base), 3 %), analysis at 160-170°C; IR: maximum at 1585 cm^{-1}. No maxima between 1600 and 1700 cm^{-1};
NMR: ^1H: (DMSO-d_6/D_2O) δ =2.20-2.95 (m, 2 H, 2 H-2'), 3.32 (t, 2 H, CH_2-ar, J=6.8 Hz), 3.62 (m, 2 H, 2 H-5'), 3.95 (m, 1 H, H-4'), 4.47 (m, 1 H, H-3'), 4.85 (t, 2 H, CH_2-O, J=6.8 Hz), 6.46 (t, 1 H, H-1', J=6.8 Hz), 7.64 (d, 2 H, ortho-H, J=8.8 Hz), 8.18 (d, 2 H, meta-H, J=8.8 Hz) 8.54 (s, 1 H, H-2), 8.60 (s, 1 H, H-8) ppm;
^{13}C: (DMSO-d_6) δ =34.5 (CH_2-ar), 39.6 (C-2'), 61.8 (C-5'), 66.5 (CH_2-O), 70.9 (C-3'), 84.3 (C-4'), 88.2 (C-1'), 121.3 (C-5), 123.6 (2 C_m), 130.4 (2 C_o), 142.4 (C-8), 146.5 + 146.8 (C_x+C_p), 151.7 (C-2), 151.9 (C-4), 160.1 (C-6) ppm;
Anal. Calcd. for $C_{18}H_{19}N_5O_6$: C, 53.86; H, 4.77; N, 17.45.
Found : C, 53.52; H, 4.80; N, 17.12.

5'-monomethoxytrityl-O^6-[2-(4-nitrophenyl)-ethyl]-2'-deoxyinosine 12. The O^6-protected deoxyinosine 10 (1.097 g, 2.73 mmol) was coevaporated twice with anhydrous pyridine and reacted overnight with 1.2 eq. (1.01 g, 3.28 mmol) monomethoxytritylchloride in 25 ml of dry pyridine. Extraction and chromatographic purification with $CHCl_3$-acetone-pyridine (90:10:0.1), followed by precipitation in 400 ml hexane, yielded 1.424 g (2.11 mmol, 77 %) of 12.

UV: ($CHCl_3$) λ_{max}=240 and 254 nm, shoulders at 262 and 275 nm. TLC (solvent B) Rf=0.36; NMR: ^1H: ($CDCl_3$) δ =2.35-3.00 (m, 2 H,H-2'), 3.34 (m, 4 H, CH_2-ar + 2 H-5'), 3.75 (s, 3 H, CH_3-O), 4.18 (m, 1 H, H-4'), 4.65 (m, 1 H, H-3'), 4.82 (t, 2 H, CH_2-O), 6.46 (t, 1 H, H-1', J=6.5 Hz), 6.77 (d, 2 H, trityl), 7.10-7.55 (m, 14 H, trityl + ortho-H), 8.12 (d, meta-H), 8.07 (s, H-8), 8.41 (s, 1 H, H-2) ppm;
^{13}C: ($CDCl_3$) δ=35.0 (CH_2-ar), 40.0 (C-2'), 55.0 (CH_3-O), 63.7 (C-5'), 66.3 (CH_2-O), 72.3 (C-3'), 84.4 (C-1'), 86.0 (C-4'), 86.7 (ϕ_3-C), 121.7

(C-5), 123.5 (2 C_m), 130.1 (2 C_o), 140.7 (C-8), 145.6 + 146.7 (C_x+C_p), 151.5 + 151.8 (C-2 + C-4), 160.1 (C-6) + trityl signals, 113.1 + 126.9 + 127.7 + 128.1 +129.7 + 135.0 + 143.8 + 158.5) ppm.

Triethylammonium salt of 5'-monomethoxytrityl-3'-(o-chlorophenyl-phosphate)-O^6-[2-(4-nitrophenyl)-ethyl]-2'-deoxyinosine 13. A mixture of 736 µl of 2-chlorophenyl dichlorophosphate (4.5 mmol) and 930 mg (13.5 mmol) of triazole in 15 ml dry pyridine was stirred at RT. After 5 minutes 1.77 g of 12 (2.62 mmol) was added with stirring. 5 minutes later TLC (solvent A) showed the phosphorylated product at the start. The reaction was poured into 15 ml 1 M cold triethylammonium hydrogen carbonate (TEAB) solution and stirred for 15 min at room temperature. More 1 M TEAB was added and the mixture was extracted 3 times with 100 ml $CHCl_3$. The organic phases were washed with 0.1 M TEAB and once more with water. Column chromatography on 70 g silica gel and elution with CH_2Cl_2-EtOH-TEA (95:5:0.5) gave pure 13, which was precipitated from a concentrated CH_2Cl_2 solution with 500 ml hexane-diethylether (8:2). After drying for 24 hours in high vacuum 2.44 g of 13 (2.53 mmol, 97 %) was isolated as a white powder.

UV: ($CHCl_3$) λ_{max} =240 and 252 nm, shoulders at 262 and 275 nm. TLC $CHCl_3$-MeOH-TEA (80:20:2) Rf=0.41, $CHCl_3$-MeOH-TEA (90:10:2) Rf=0.29; NMR: ^1H: unresolved; ^{13}C: ($CDCl_3$) δ =8.3 (3 x $\underline{CH_3}CH_2$-N^+), 34.9 (CH_2-ar), 38.8 brs (C-2'), 45.6 (3 x CH_3-$\underline{CH_2}$-N^+), 54.9 (CH_3-O), 64.0 br (C-5'), 66.3 (CH_2-O), 77.0 (C-3'), 84.6 (C-1'), 85.2 (d, C-4'), 86.5 (ϕ_3-C), 121.5 (C-5), 123.5 (2 C_m), 129.7 (2 C_o), 140.9 (C-8), 145.6 + 146.7 (C_x+C_p), 151.4 (C-2 + C-4), 159.9 (C-6) ppm + trityl and chlorophenyl signals.

Deprotection of the heptamer. After the heptamer had been synthesized on a solid support according to the previous described method,[16] the solid support was washed with CH_2Cl_2 and with diethylether, and divided in two. The first part was treated in a sealed vial with 1 ml 1 M TMG - oximate solution in dioxane-water (1:1) for 18 h at 70°C. After cooling, the support was filtered and washed with dioxane-water (1:1). The filtrate was concentrated and applied to a 10 ml column of Dowex AG 50X8 (pyridinium form). Elution was done with pyridine-water (3:7), and after evaporation the rest was taken up in 5 ml 0.2 M TEAB solution and washed with 5 x 5 ml diethylether. The water phase was evaporated in vacuo and evaporation was repeated twice with 2 ml water. Detritylation was done with 5 ml 80 % acetic acid during 45 min at RT. Acetic acid was removed

in vacuo and the residue was taken up in 0.2 M TEAB and filtered on a disposable C-18 cartridge (Baker - 10 SPE, no. 7020-1) giving the crude deprotected oligonucleotide.

The second part of the solid support was treated with 1 ml 0.5 M TMG - oximate solution at 37°C during 15 h. After filtration, the support was washed and the filtrate was evaporated and coevaporated 3 times with dry pyridine. The rest was taken up in 3 ml of 0.5 M DBU in pyridine for 15 h at RT. After DBU treatment the solution was evaporated and applied to a Dowex AG 50X8 column. Further treatment was done like for the first part of the support.

Enzymatic degradation.

A. Degradation to 5'-monophosphates. To 0.3 A_{260} units oligonucleotide were added 10 µl of a solution with 30 U nuclease P1/ml, 50 µl 30 mM NaOAc pH 5.3 and 10 µl $ZnSO_4$ 10 mM, and the solution was adjusted with sterile, bidistilled water to 150 µl. The mixture was incubated for 30 min on 37°C and was next analysed by HPLC on a Partisil 10 SAX column. Elution: A=0.01 M KH_2PO_4 pH 4.00, gradient isocratic for 5 min with 0 % B, followed by a gradient from 0 to 50 % B in 20 min, 1 ml/min.

B. Degradation to nucleosides. After treatment with nuclease P1, as in A, 150 µl 0.1 M tris(hydroxymethyl)-aminomethane.HCl buffer pH 8.6 and 5 µg bacterial alkaline phosphatase were added to the reaction. The mixture was incubated again at 37°C for 15 min. Analysis was done on a Zorbax C8 column with isocratic elution of 10 % MeOH, 0.1 M TEAA pH 6.8.

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