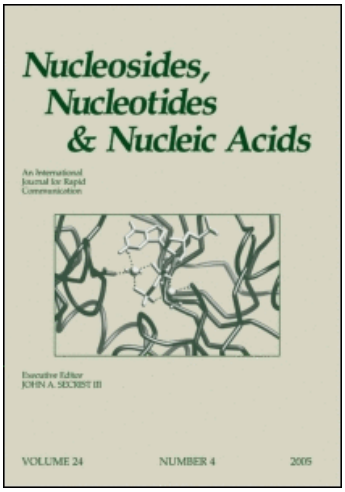

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Angeles Canol[a]; Myron F. Goodman[b]; Ramon Eritja[a]
[a] Dpt. of Molecular Genetics, CID-CSIC, Barcelona, Spain [b] Dpt. of Biological Sciences, University of Sourthern California, Los Angeles, California, USA

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SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES CONTAINING 2,6-DIAMINOPURINE.

Angeles Cano[1], Myron F. Goodman[2] and Ramon Eritja*[1].

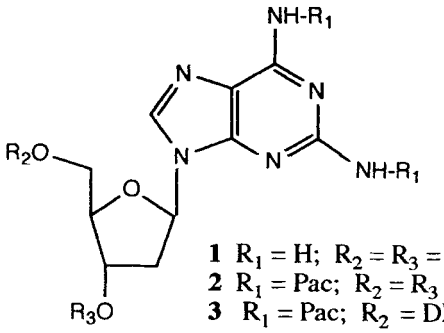
[1]Dpt. of Molecular Genetics. CID-CSIC. Jordi Girona 18-26. 08034 Barcelona. Spain.
[2] Dpt. of Biological Sciences, University of Sourthern California. Los Angeles, California 90089-1340. USA.

Abstract: The preparation of a new protected derivative of 2,6-diaminopurine 2'-deoxyriboside carrying two phenoxyacetyl groups is described. The new derivative is useful to prepare oligonucleotides containing 2,6-diaminopurine and it is deprotected at the same time as the standard protecting groups of the natural bases.

Oligonucleotides having 2,6-diaminopurine (**1**, 2-aminoadenine, D) substituting adenine in a predeterminate site are being used for a variety of applications including improved hybridization probes[1-3], DNA structural studies and for the elucidation of DNA-protein interactions[4]. For the preparation of such modified oligonucleotides the use of the N-2-isobutyryl-N-6-benzoyl derivative of 2,6-diaminopurine was first described[4]. This compound was found to be deprotected very slowly with ammonia. Replacing the benzoyl group in position 6 by N-methylpyrrolidine[3] or N,N-dibutylformamidine[2] groups increases the lability to ammonia but long treatments (2-3 days) are still needed for the complete deprotection and the preparation of these derivatives is not simple.

Recently a more labile phenoxyacetyl (Pac) group has been reported[5] and a one-pot conversion from dG to 2,6-diaminopurine 2'-deoxyriboside (**1**) has been described[6]. Also, the phenoxyacetyl group has been used to protect 2'-allyl-2,6-diaminopurine riboside[7]. In the present communication we would like to describe the preparation of protected 2,6-diaminopurine with the Pac groups. The new derivative is much more labile to ammonia than the previously described derivatives and it could be prepared in a much shorter protocol.

This paper is dedicated to the memory of Professor Roland K. Robins.



1 $R_1 = H$; $R_2 = R_3 = H$
2 R_1 = Pac; $R_2 = R_3 = H$
3 R_1 = Pac; R_2 = DMT; R_3 =H

DMT : dimethoxytrityl
Pac : phenoxyacetyl

RESULTS AND DISCUSSION

Compound **1** was prepared directly from unprotected dG following the method described in ref. 6. The protection of the amino groups with the phenoxyacetyl group was performed using both peracylation and transient protection methods. In the peracylation method the nucleoside was reacted with six equivalents of phenoxyacetyl chloride in pyridine and the resulting product was hydrolyzed with a triethylamine/ pyridine/ water (1:1:3) solution as described in ref. 5. Hydrolysis of the ester functions took 2 hours instead of the 15 minutes described for the natural nucleosides. The desired product was isolated by silica gel column chromatography presenting a single peak in analytical reverse-phase HPLC but, by ^{1}H-NMR, it was observed that the product was partially contaminated with triethylammonium phenoxyacetate. Mass spectrometry gave the expected molecular ion (M+ 534). In the transient protection method, the nucleoside was silylated with trimethylsilyl chloride (5 mmol) in pyridine at 0°C (30 min) followed by a treatment with 2,4,5-trichlorophenyl phenoxyacetate at 40°C overnight. After hydrolysis of the trimethylsilyl groups with dilute ammonia and following the work-up described for 2-aminopurine in ref. 8, the desired protected nucleoside was obtained in 40% yield and it was identical by TLC, HPLC and UV spectrometry to the sample obtained by the peracylation method. In both peracylation and the transient protection methods, compound **2** was obtained in similar yields, but the transient protection method is preferred because cleaner reaction mixtures were obtained. Finally, when phenoxyacetyl chloride was used as acylating agent in the transient protection method no product was obtained. A similar result has been observed in the protection of 2-aminopurine 2'-deoxyriboside, where an acid chloride was shown to produce depurination of the transiently protected nucleoside[8].

The deprotection of the N^2, N^6-diphenoxyacetyl derivative (**2**) in concentrated ammonia was studied at 1.9 mM concentration. Complete deprotection was observed after 2 hours at

60°C. Only 2,6-diaminopurine 2'-deoxyriboside (**1**) was observed as product by analytical HPLC. When a less concentrated ammonia solution was used, the deprotection was slower and an intermediate product was observed. This product was formed rapidly but it was slowly converted to compound **1** (most probably the intermediate had a phenoxyacetyl group at position 2[3]). Also compound **2** was stable in concentrated ammonia at room temperature at least for 5 hours. These results show that compound **2** is more resistant to ammonia deprotection than the similar derivatives of the natural bases but still, the new 2,6-diaminopurine derivative is much more labile to ammonia than the previously described derivatives[1-4].

Compound **2** was protected with DMT-Cl and pyridine according to a standard procedure[9] and the resulting DMT derivative was reacted with 2-cyanoethoxy N,N-diisopropylaminochlorophosphine[9] to obtain the phosphoramidite derivative needed for oligonucleotide synthesis. CPG was also functionalized with compound **3** using the standard succinyl linkage[9]. The following sequences were prepared using the standard protocols of an automatic DNA synthesizer :

A: 5' CTG CD 3'

B: 5' GCC GGD TCT ACA CG 3'

C: 5' CGT CTA GDT CCG GC 3' where D represents 2,6-diaminopurine

Standard ammonia deprotection conditions were used. Oligonucleotides were purified by OPC followed by HPLC and the purified products were treated with snake venom phosphodiesterase and alkaline phosphatase. As it can be seen in FIGURE 1, a correct nucleoside composition was found, showing that the deprotection of the new 2,6-diaminopurine derivative was complete.

In conclusion, we have shown the preparation of a new protected derivative of 2,6-diaminopurine 2'-deoxyriboside carrying two phenoxyacetyl groups. The preparation of this product is shorter and easier than previously described 2,6-diaminopurine derivatives[1-4]. The new derivative is useful to prepare oligonucleotides containing 2,6-diaminopurine and it is deprotected at the same time as the standard protecting groups of the natural bases.

EXPERIMENTAL PART

Abbreviations used : A_{260}: absorbance at 260 nm, Bz: benzoyl, CPG: controlled-pore glass, DMF: N,N-dimethylformamide, DCM: dichloromethane, DMAP: N,N-dimethylaminopyridine, DMT : dimethoxytrityl, ibu: isobutyryl, OPC: oligonucleotide purification cartridges, Pac : phenoxyacetyl, LCAA-CPG : long chain aminoalkyl-controlled pore glass. MeOH : methanol.

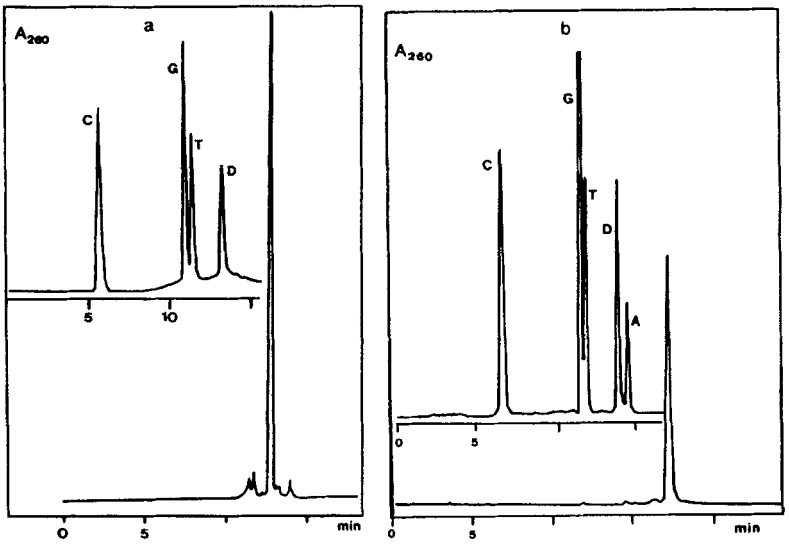


FIGURE 1. HPLC profile of a) pentanucleotide A: 5' CTGCD 3'and b) 14 mer C: 5' CGT CTA GDT CCG GC 3'. The inserts show the enzymatic digestion of purified oligonucleotides. In both cases a C-18 Nucleosil (10 μm) column (250 x 4 mm) with a 5-25% gradient of acetonitrile in 10 mM triethylammonium acetate over 20 min was used.

9-(ß-D-2-Deoxyribofuranosyl-2,6-diaminopurine (**1**) was prepared as described in ref. 6, with the exception that silica gel purification was used instead of preparative reverse-phase HPLC purification.

2,4,5-Trichlorophenyl phenoxyacetate.

2,4,5-Trichlorophenol (4 g, 20 mmol) was dissolved in 20 ml of a 1:1 mixture of DCM and pyridine and the solution was cooled with an ice bath. 2 ml of phenoxyacetyl chloride (20 mmol) were added and the mixture was stirred for 30 minutes at room temperature. The solvents were evaporated and the residue was dissolved in DCM (60 ml), washed with water (3 x 60 ml), a saturated solution of sodium bicarbonate (3 x 60 ml) and again with water (3 x 60 ml). The organic phase was dried with anhydrous sodium sulphate and concentrated to dryness giving 5.4 g of an oil that was used without further purification (yield 82%). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ ppm : 7.57 (s, 1H), 7.31 (s, 1H), 6.9-7.4 (m, 5H), 4.94 (s, 2H).

N^2, N^6-bis(phenoxyacetyl)-2,6-diamino-9-ß-D-2 -deoxyribofuranosyl) purine (2).

a) *Peracylation method.*

Compound **1** (4 mmol) was dried by coevaporation with pyridine. The residue was dissolved in 20 ml of pyridine, cooled with an ice bath and phenoxyacetyl chloride (3.5 ml, 25 mmol) was added dropwise with magnetic stirring. When the addition was finished, the reaction mixture was allowed to warm to room temperature and stirring was maintained for 90 minutes. The reaction was stopped by adding water (3.3 ml) and the reaction mixture was diluted with 75 ml of chloroform. The organic phase was washed with a saturated aqueous sodium bicarbonate (3 x 50 ml) and water, dried with anhydrous sodium sulphate and concentrated to dryness.

The residue was treated with a 20:20:60 mixture of triethylamine, pyridine and water at room temperature. The hydrolysis of the ester groups was followed by TLC (10% MeOH-DCM) showing different intermediates until the formation of a product that had a higher Rf than the starting compound **1**. The optimal time for the hydrolysis was estimated to be around 2-3 hours. The reaction mixture was concentrated to dryness and the residue was purified by silica gel column chromatography with a 0-20% MeOH gradient in DCM, yielding 1.1 g (2 mmol, 50% yield) of a product that was homogenous by TLC and HPLC, but contaminated with triethylammonium phenoxyacetate as seen by ^{1}H-NMR. TLC (10%MeOH/ DCM) R_f= 0.37. HPLC, retention time: 27.1 min (see conditions in FIGURE 2). UV (H_2O/MeOH 1:1)λ_{max} = 268 nm. Mass spectrometry (electron impact) M^+= 534.

b) *Transient protection method.*

Compound **1** (1.5 g, 5.5 mmol) was dried by coevaporation with pyridine. The residue was dissolved in 25 ml of pyridine and trimethylsilyl chloride (3.4 ml, 26 mmol) was added. After 15 minutes of magnetic stirring at room temperature 5.4 grs of 2,4,5-trichlorophenyl phenoxyacetate dissolved in 15 ml of pyridine were added and the reaction was kept at 40 °C for 16 hours. Afterwards, the reaction mixture was cooled with an ice bath and 4.2 ml of water were added. After 5 minutes, 6 ml of concentrated aqueous ammonia were added and the mixture was concentrated to dryness. The product was purified by silica gel column chromatography as described above giving 1.2 g (40% yield) of compound **2** that had the same UV, TLC, and HPLC characteristics as the product obtained by the peracylation method.

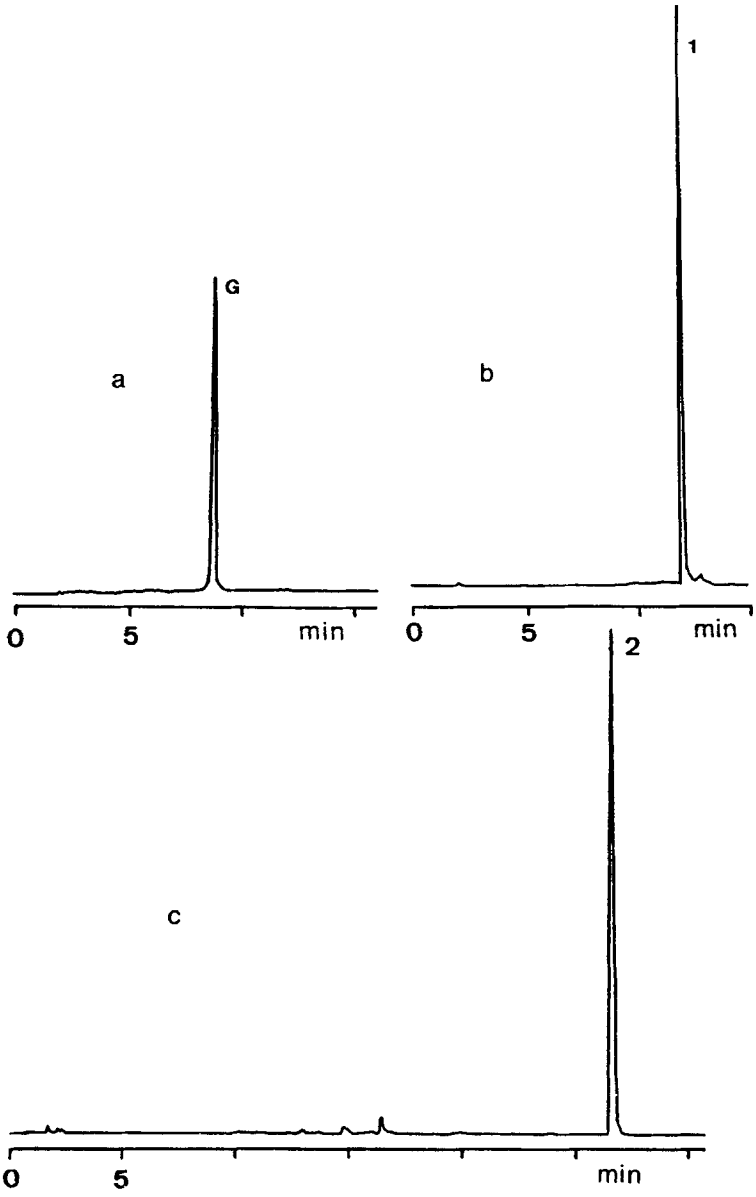


FIGURE 2. Analytical HPLC profile of a) 2'-deoxyguanosine, b) compound **1**, and c) compound **2**. HPLC conditions : Column C-18 Nucleosil 120 (10 μm), dimensions 250 x 4 mm, flow rate 1 ml/ min, solvent A: 10 mM triethylammonium acetate, solvent B: acetonitrile/ water (1:1). In figures a) and b) a 5 to 50 %B gradient was used over 20 minutes. In figure c) a 0 to 100% B gradient was used over 40 minutes.

Hydrolysis studies.

At 60 °C.

Aliquots of approx. 1 mg (1.9 μmol) of compound **2** were dissolved in 1 ml (1.9 mM) of concentrated ammonia (32%) in screw-cap vials. The vials were closed tightly and heated at 60 °C. Vials were removed at fixed intervals, cooled with ice and the ammonia was evaporated. The residue was dissolved in MeOH/ H_2O (1:1) and the solution analyzed by HPLC (HPLC conditions similar to the conditions described in FIGURE 2). Deprotection was complete at 2 hours. When less concentrated ammonia was used the formation of an intermediate product (presumably the product of the hydrolysis of one Pac group) was observed. Retention times : compound **2**: 26 min, intermediate product: 16 min, and compound **1**: 14 min.

At room temperature.

In order to check if the Pac groups were affected by ammonia at room temperature during the hydrolysis of the trimethylsilyl groups, compound **2** (1 mg) was treated with concentrated aqueous ammonia (0.1 ml) at room temperature. The reaction was stopped by addition of 0.1 ml of acetic acid at 8 different times ranging from 2 min until 5 hours. The solutions were concentrated to dryness and the products analyzed by HPLC. No hydrolysis of the Pac groups was observed under these conditions.

5'-O-Dimethoxytrityl-N^2, N^6-bis(phenoxyacetyl)-2,6-diaminopurine 2'-deoxyriboside (3).

Compound **2** (1.1 g, 2.0 mmol) was coevaporated twice with pyridine. The residue was dissolved in pyridine (35 ml), 0.94 g of dimethoxytrityl chloride (2.7 mmol) was added and the mixture was stirred at room temperature. After four hours 5 ml of methanol were added and the solvents evaporated. The residue was dissolved in chloroform and the solution was washed twice with a saturated aqueous solution of sodium bicarbonate and twice with a saturated aqueous sodium chloride. The organic phase was dried and concentrated to dryness. The residue was purified by silica gel column chromatography eluted with a 0-10% MeOH in DCM. Yield 0.43 g (26%). TLC: R_f 0.54 (10%MeOH/DCM). ^{1}H-NMR (d_6-acetone, 200 MHz) δ (ppm): 8.15 (s, 1H, H-8), 6.7-7.5 (m, 19H, H arom.), 6.5 (t, 1H, H-1'), 5.52 (s, 4H, CH_2 Pac), 5.2 (s, 1H, OH), 4.7 (m, 1H, H-3'), 4.2 (m, 1H, H-4'), 3.65 (m, 8H, H-5', O-CH_3 DMT), 2.4-2.5 (m, 2H, H-2').

5'-O-Dimethoxytrityl-N^2, N^6-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deoxyriboside 3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite.

Compound **3** (0.15 g, 0.18 mmol) was dried by coevaporation of dry acetonitrile. The residue was dissolved in 0.5 ml of dry DCM and 0.12 ml of ethyldiisopropylamine (0.71 mmol) and 64 mg (0.27 mmol) of chloro-N,N-diisopropyl-2-cyanoethoxy phosphine were added under argon atmosphere. After 1 hour, 0.1 ml of MeOH was added, the reaction mixture was diluted with dichloromethane (20 ml) and transferred to a separatory funnel. The solution was washed with a saturated sodium bicarbonate solution and with a saturated solution of NaCl. The organic phase was dried and the product was purified by silica gel column chromatography eluted with a 10% triethylamine solution in DCM. Yield 90 mg (44%). TLC : two spots Rf 0.9 (ethyl acetate/DCM/ triethylamine 45:45:10). ^{31}P-NMR (Cl_3CD, 121 MHz): 149.2 and 149.4 ppm.

5'-O-Dimethoxytrityl-N^2, N^6-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deoxyriboside 3'-O-succinate.

Compound **3** (0.24 g, 0.28 mmol) was treated with succinic anhydride (43 mg, 0.43 mmol) and DMAP (52 mg, 0.43 mmol) in DCM. After 16 hours the reaction mixture was diluted with DCM and washed with 0.1 M monosodium phosphate aqueous solution and water. The organic phase was dried and evaporated to dryness. The residue was dissolved with DCM and the solution was added to pentane. The precipitated white product was collected. Yield 160 mg (60%).

Preparation of polymeric support with DMT, Pac protected 2,6-diaminopurine 2'-deoxyriboside.

80 mg (0.085 mmol) of the succinate prepared above were treated with dicyclohexylcarbodiimide (14 mg, 0.085 mmol), and 1-hydroxybenzotriazole (14 mg, 80% purity, 0.085 mmol) in DMF at 0 °C. After 10 minutes the solution was added to 0.8 g of LCAA-CPG support (70 μmol/ g) and 10 mg of DMAP (0.085 mmol) were added. After 16 hours at room temperature, the resin was filtered and washed with DMF, DCM and acetonitrile. The unreacted amino groups were capped with 10% acetic anhydride in pyridine. Loading was 15 μmol of DMT group per g.

Oligonucleotide synthesis.

Oligodeoxynucleotides were synthesized on controlled pore glass (0.2 μmol) by succesive additions of the appropriate phosphoramidites (DMT-A^{Bz}, C^{Bz}, G^{ibu}, T- 2-cyanoethyl-N,N-diisopropyl phosphoramidites) in an automated DNA synthesizer. No

difference on the incorporation yield were observed when the 2,6-diaminopurine phosphoramidite was used. At the end of the syntheses, the DMT-oligonucleotide-solid supports were treated overnight with concentrated ammonia (32%) at 60 °C. The resulting DMT-oligonucleotides were purified by OPC. The homogeneity of the purified oligomers was checked by analytical HPLC and the nucleoside composition was determined by snake venom phosphodiesterase, alkaline phosphatase digestion followed by HPLC analysis[10].

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