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N-PHENOXYACETYLATED GUANOSINE AND ADENOSINE PHOSPHORAMIDITES IN THE SOLID PHASE SYNTHESIS OF OLIGORIBONUCLEOTIDES: SYNTHESIS OF A RIBOZYME SEQUENCE

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Abstract: Phosphoramidites for guanosine and adenosine nucleosides with phenoxyacetyl as N-acyl protecting group were prepared. These nucleoside phosphoramidites, together with previously reported uridine and N-benzoyl cytidine phosphoramidites have been applied to the efficient solid phase synthesis of a trideca and a nonadecaribonucleotide. The later molecule has the sequence corresponding to a ribozyme.

We have recently demonstrated, via the chemical synthesis of a 43-unit oligoribonucleotide corresponding to the 3'-half of $tRNA_f^{Met}$ [1], that the chemical synthesis of oligoribonucleotides is a practical possibility. Synthetic ribonucleotides are thus available for the elucidation of important biochemical processes such as RNA catalysis[2] and RNA splicing[3].

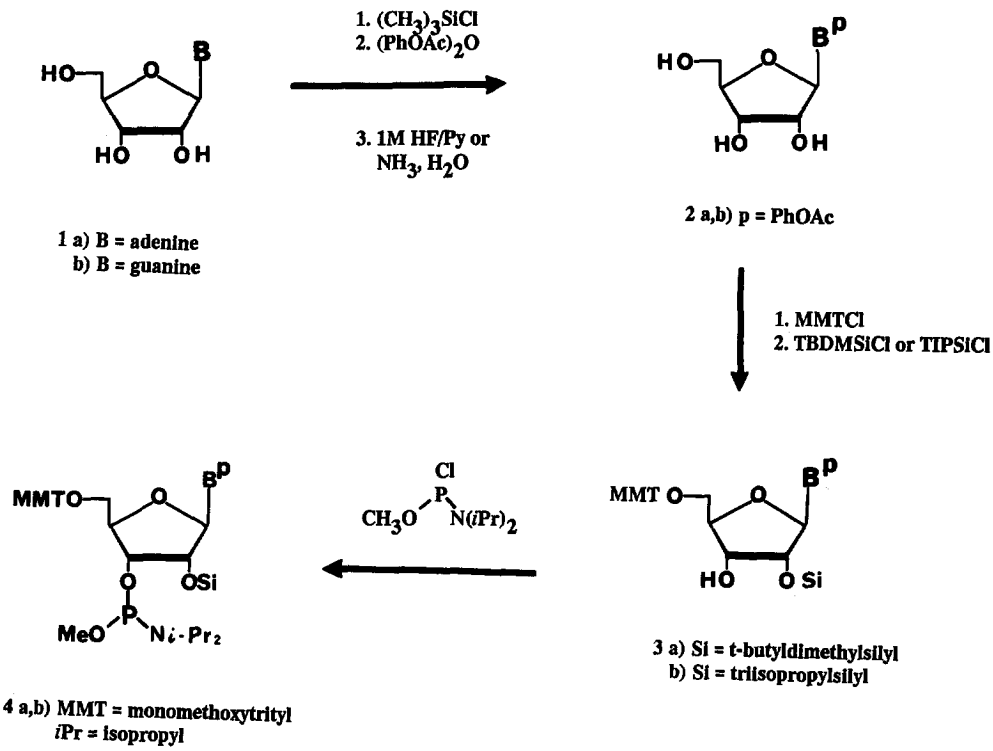
After evaluating the preparation of numerous oligoribonucleotides, we noticed that the number of shorter sequences in the synthetic mixture varied between 5 and 10% of the nucleotide products. Examination of several oligouridylic acid syntheses showed this effect to be related to the severity of the ammonium hydroxide deprotection treatment. A substantial reduction in the number of shorter fragments occurred when this treatment was performed at room temperature (16h) instead of at 50 °C (16h). This milder treatment is sufficient to cleave the sequence from the support and to deacylate N^4-benzoylcytidine units but is insufficient for the deacylation of N^6-benzoyladenosine and N^2-benzoylguanosine. We therefore sought an N-protecting group for adenosine and guanosine which could be removed under these milder conditions.

Recently, the more labile phenoxyacetyl group has been used as an amino protecting group for deoxyadenosine and deoxyguanosine[4]. While this group is stable during the conditions for oligodeoxynucleotide synthesis, it can be removed under much milder conditions than are required for the removal of N-benzoyl groups.

In this communication we report the preparation of N^6-phenoxyacetyladenosine and N^2-phenoxyacetylguanosine and their conversion into N,N-diisopropylmethylphosphoramidites. The solid phase synthesis of two oligoribonucleotides using these new phosphoramidites is also described.

The transient protection method[5] was used to introduce the phenoxyacetyl group (see Scheme). Adenosine (50 mmol, **1a**) was silylated with trimethysilyl chloride (200mmol, 2h) in pyridine. Phenoxyacetic anhydride (100mmol),

Scheme



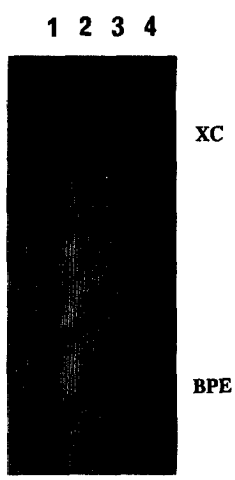


Fig 1. 20% polyacryamide gel electropho-resis/8M urea (visualized by UV shadowing) of purified tridecamer (Lane 1, 5'-CAC UUG ACU AGC C-3') and nonadecamer (Lane 2, 5'-GGC CUG ACU GAU GAG GCG C-3'). Lane 3 is a homouridine pentadecamer and Lane 4 is a homouridine hexamer.

prepared from phenoxyacetyl chloride and phenoxyacetic acid sodium salt[6], was introduced and the reaction was stirred at room temperature. The reaction was stopped and the trimethylsilyl groups were removed by addition of water (50ml) and 1M HF/pyridine complex[7] (200ml, 25min). The mixture was poured into CH_2Cl_2 (800ml) and the organic phase was washed with brine (3x500ml). The solvents were removed at reduced pressure. Recrystalization of the crude mixture from absolute ethanol gave pure N^6-phenoxyacetyladenosine, **2a** (11g, 55%).

In the case of guanosine(70mmol, **1b**), the nucleoside was transiently silylated as described for adenosine. At the end of the silylation, phenoxyacetic anhydride (100mmol) was transferred into the reaction mixture which was then stirred overnight. The solution was cooled (0^oC) and water (50ml), followed after 15min by concentrated ammonia solution (50ml) was added. After 12min the solution was poured into water (1.5l). The aqueous solution was washed with CH_2Cl_2 (2x400ml). Concentration of the aqueous layer initiated crystalization of the product **2b** (16g, 56%).

The N-phenoxyacetylated nucleosides were methoxytritylated and silylated according to standard procedures[8] to give 5'-monomethoxytrityl-N-phenoxyacetyl-2'-*t*-butyldimethylsilyl (triisopropylsilyl for guanosine) nucleosides (**3a-b**). The yields for each step were comparable to those for N-benzoylated derivatives (methoxytritylation: A: 65%, G: 72%; silylation: A: 60%, G: 44%) .

The separation of the N^2-phenoxyacetyl-2'- and 3'-silylated guanosine isomers, by silica gel chromatography (20% ethyl acetate/Ch_2Cl_2), has also been dramatically improved. Isolation of the N^2-phenoxyacetyl-2'-silylated isomer required for nucleotide synthesis, was much easier and less tedious than was the case with the analogous N^2-benzoylated isomers.

The new N-phenoxyacetylated adenosine N,N-diisopropylmethylphosphoramidite (**4a**) was prepared (83% yield) in the manner reported for N-benzoyated derivatives[1]. The preparation of guanosine N,N-diisopropylmethylphosphoramidite (**4b**, 90% yield) was similar except that an additional 0.2 equivalents of 4-dimethylaminopyridine (relative to **3b**) was used. Phosphoramidite **4b** was purified by silica gel chromatagraphy using ethyl acetate/CH_2Cl_2/triethylamine 20/75/5). ^{31}P NMR showed two signals, consistent with the pair of diastereoisomers for each of the two amidites (**4a**: 151.9,150.1 ppm; 4b: 152.3,150.4ppm, downfield from 85% phosphoric acid).

These two new phosphoramidites, together with the previously reported cytidine and uridine phosphoramidites[1] were used in the solid phase synthesis of a tridecaribonucleotide, CACUUGACUAGCC, and a nonadecaribonucleotide, GGCUCGACUGAUGAGGCGC. This latter sequence has been reported to possess catalytic ribozyme activity. These syntheses were carried out on an automated synthesizer (Applied Biosystems Model 381) using a slight variation of the previously reported synthesis cycle[9]. Average coupling yields, determined from trityl cation measurements were 97.7% and 97% respectively.

At the end of chain assembly, the sequence on the solid support was demethylated (thiophenol/dioxane/triethylamine 1:2:2, 30min) and cleaved from the solid support (NH_4OH, 60min). The product was left overnight at room temperature in concentrated ammonia (4ml) to remove the N-acyl protecting groups. This solution was evaporated to dryness and treated with 1M tetrabutylammonium floride (TBAF) in THF (0.5ml) overnight at room temperature to remove the silyl protecting groups. The product was desalted on G-25 Sephadex and purified by preparative polyacryamide gel electreophoresis. The purity of the isolated material is shown in Figure 1.

The purified sequences were treated with snake venom phosphodiesterase and alkaline phosphatase and the digested material was analyzed by HPLC. The correct ratio of bases (with no extra peaks) was observed confirming the absence of base modification in the synthesis and deprotection. The products were also digested with spleen phosphordiesterase. There were no unexpected peaks present, consistent with the internucleotide linkage being exclusively 3'-5'. These results convincingly showed the homogeniety of the final products.

We believe that the phenoxyacetyl group introduces a valuable technical improvement into the solid phase synthesis of oligoribonucleotides since it allows much milder conditions to be employed for the deprotection of the exocyclic amino groups of adenosine and guanosine.

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9. The synthesis cycle was similar to that previously reported[1]: 1)detritylation (5% trichloroacetic acid in dichloromethane, 120sec); 2) washing (acetonitrile, 180sec); 3) base (0.11M in acetonitrile) + tetrazole (0.5M in acetonitrile, 10-15secs); 4) wait (300 sec); 5) capping (0.25M acetic anhydride/0.25M DMAP/collidine in THF, 90sec); 6) oxidation (0.1M I_2 in THF/pyridine/water 7/2/1, 30sec); 7) wash (acetonitrile, 60sec). The yield of individual coupling steps was evaluated by determining the amount of released monomethoxyltrityl cation.

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