Selective O-phosphitilation with nucleoside phosphoramidite reagents

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ABSTRACT

In contrast to tetrazole, pyridine hydrochloride/imidazole converts nucleoside phosphoramidites to intermediates that show a high preference for phosphitilating hydroxyl groups relative to nucleoside amino groups. Use of this activating agent and incorporation of a pyridine hydrochloride/aniline wash step in the synthetic cycles permit synthesis of mixed base twenty-mer oligonucleotides from nucleoside reagents containing unprotected amino groups. This approach should be useful for the synthesis of oligonucleotide analogues containing substituents sensitive to reagents used in conventional deblocking steps. Pyridine hydrochloride itself is an effective reagent for activating nucleoside methylphosphoramidites and ribonucleoside phosphoramidites, as well as deoxyribonucleoside phosphoramidites, when high O/N selectivity is not needed.

INTRODUCTION

Phosphitilating and phosphorylating agents generally attack the amino as well as the hydroxyl groups of nucleosides. In conventional procedures for synthesizing oligonucleotides, selective reaction at oxygen is achieved by protecting the amino groups during chain elongation.

Recently we described a procedure for synthesizing oligonucleotides in the ten-mer range from amidite synthons lacking N-protecting groups¹. Selectivity was achieved by cleaving undesired P(III)-N bonds, formed by indiscriminate phosphitilation, under conditions that left P(III)-O bonds intact. This method holds promise for syntheses of short, modified oligonucleotides possessing substituents sensitive to reagents used in removing N-protecting groups; however, it suffers the disadvantage that considerable amounts of nucleoside amidite reagents are consumed by non-productive phosphitilation at the amino groups.

We report in the present paper conditions for activating nucleoside phosphoramidites that lead to preferential phosphitilation of hydroxyl groups in the presence of free amino groups. Buttressed by the chemistry for selective P-N cleavage,

this method enables synthesis of oligomers in the twenty-mer range from nucleoside reagent **1a,c,g,t**. In pursuing this work we first surveyed several weak acids as potential activating reagents, using the coupling of nucleoside phosphoramidites **1a,c,g,t**, **2**, and **3** to thymidine anchored to a controlled pore glass support (dT-cpg) to monitor reactivity; then the optimum conditions were used in synthesizing two oligodeoxyribonucleotides, d-(C)₁₃T and d-ACACCAATTCTGAAAATGG.

EXPERIMENTAL SECTION

General methods and reagents

5'-O-Dimethoxytrityldeoxyadenosine (DMT-dA), 5'-O-dimethoxytrityldeoxyguanosine (DMT-dG), and phosphoramidite **1t** were purchased from Sigma. Phosphoramidites **2** and **3** were purchased from ABN and Glen Research, respectively. 5'-O-Dimethoxytrityldeoxycytidine (DMT-dC) was prepared by hydrolysis of the N-benzoyl derivative and by direct dimethoxytritylation of deoxycytidine.² Standard solutions of pyridine hydrochloride (Aldrich) and imidazole were prepared by dissolving commercial samples in dry acetonitrile and redrying over 4A molecular sieves for 24 hours or more. Acetonitrile was dried over calcium hydride. Nucleases were purchased from Boehringer Mannheim.

HPLC was carried out on a Hewlett Packard 1090 chromatograph (for experiments A and B, Figure 1) or a Dionex chromatograph (all other experiments), using a Hypersil column (4.6×200 mm, 5μ ODS) with a 1%/min gradient of acetonitrile in aqueous 0.03 M triethylammonium acetate (TEAA), pH 7.0, for reversed phase chromatography, and a Dionex OmniPak

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column (4×250 mm, NA 100) with a 2%/min gradient of 1.0 M aqueous sodium chloride in 0.03 M TEAA buffer, pH 7.0, for ion exchange chromatography.

Silica gel plates (0.25 mm, fluorescent indicator, Aldrich, prerun in dichloromethane/triethylamine, 98/2 v/v, and dried in air) were used for analytical TLC. The solvent system was dichloromethane/methanol/triethylamine 95/5/1, v/v/v.

Gel electrophoresis was carried out in 0.4 mm 20% polyacrylamide-5% bis-acrylamide gels with TB×1 buffer, pH 8.2. Xylene cyanol and bromophenol blue were used for reference. Oligonucleotides were visualized by staining with 0.01% methylene blue in water.

For enzymatic hydrolyses, 0.25–0.5 A_{260} units of oligonucleotide, 0.15 units of snake venom phosphodiesterase and 4 units of alkaline phosphatase were incubated in 150 μ L buffer (32 mM Tris·HCl, 12 mM MgCl₂) at pH 8.2 for 7 h. The solution was then heated rapidly to 80°C to deactivate the enzymes, diluted with water, and analyzed by RP HPLC (gradient B).

FAB mass spectra were obtained on a VG-70-250SE mass spectrometer by Dr. Doris Hung. NMR spectra were run on a Varian XL400 instrument with acetonitrile- d_3 as solvent and 85% phosphoric acid as an external standard.

DMT-nucleoside phosphoramidites (1a,c,g)

The procedure used by Barone et al.³ to prepare N-acylnucleoside phosphoramidite reagents was adapted to the synthesis of the free amino derivatives. To a solution of the 5'-O-DMT-nucleoside (1 mmol) and diisopropylamine/tetrazole (1 mmol for DMT-dA and DMT-dG; 1.5 mmol for DMT-dC) in dry dichloromethane (10 ml) under argon was added bis-(diisopropylamino)methoxyphosphine (1.2 mmol for DMT-dA and DMT-dG; 1.8 mmol for DMT-dC). After 1.5 h (TLC indicated complete reaction of the nucleoside) the mixture was poured into saturated aqueous sodium bicarbonate. The organic layer was washed 4 times with saturated aqueous sodium chloride, dried (sodium sulfate), and evaporated. The foamy white solid was dissolved in dichloromethane (5 ml) and precipitated in pentane (0.8 l, -70°C), and the precipitate was collected, washed with cold pentane, and stored in a vacuum desiccator; yields of 1a,b,c, 85–90%; ³¹P NMR (C₅D₅N) ppm, 1a (152.17, 151.96), 1c (152.21, 152.92), 1g (152.15, 152.19); FAB positive ion mass spectrum, m/z, 1a (715), 1c (691), 1g 731; R_f (TLC) 1a (0.70), 1c (0.60), 1g (0.65). These products served as well for synthesizing oligonucleotides as samples that were further subjected to column chromatography on silica gel (70–230 mesh, 60 Å, preequilibrated with methylene chloride/triethylamine, 98/2 v/v, and developed with a 0 to 5% gradient of methanol in methylene chloride/triethylamine, 98/2 v/v).

Synthesis of dinucleoside monophosphates

Except where noted, the reactions were carried out with DMT-dT-succinyl-cpg (MilliGen/Biosearch; 1 μ mol of DMT-dT) in a Hamilton gas tight 1 ml syringe equipped with a plug of glass wool at the base. Standard methods were used in the synthesis of 1a,c,g,t (de-tritylation with DCA, oxidation with iodine/water, cleavage with concentrated ammonium hydroxide, etc.). For the condensation step, a nucleoside phosphoramidite reagent (0.1 M in acetonitrile, 100 μ L) was drawn into the syringe, followed by the specified activating agent (100 μ L of solution in acetonitrile; see Figures 1 and 2). In the case of 2 and 3, and the corresponding control reactions involving tetrazole activation, oxidation was

Figure 1. Reversed phase HPLC profiles of products from preparation of d-CT with activation of 1c by solutions (acetonitrile, 100 μ M) of (A) tetrazole, 0.5 M; (B) pyridine hydrochloride, 0.5 M; (C) pyridine hydrochloride, 0.5 M, and imidazole, 1 M; (D) pyridine hydrochloride, 0.5 M, and imidazole, 1 M, but with the condensation reaction followed by washing with pyridine hydrochloride/aniline (0.1 M each). A Hewlett Packard chromatograph was used for A and B, and a Dionex chromatograph for C and D. The highest peak corresponds to d-CT in each case.

carried out with 0.1 M iodine in pyridine/acetic acid, 9/1 v/v (1 min), and products were cleaved from the support with ammonium hydroxide/ethanol 3/1 v/v (30 min, 55°C). DMT-dT-oxalyl-cpg⁴ (0.1 μ mole DMT-dT) was used as a solid support in the reaction of 3, and the condensation time was 1 minute.

Synthesis of d-CCCCCCCCCCCCCT (4) and d-ACACCCAATTCTGAAATGG (5)

The syringe technique was used, starting with 0.5 μ mole of DMT-nucleoside. For a given cycle the chemical steps, reagents, and reaction times were: (a) detritylation, 3% dichloroacetic acid in dichloromethane, 1.5 min; (b) coupling, amidite reagent (1a, 1c, 1g, or 1t, 0.1 M in acetonitrile, 100 μ L) plus activator (1 M pyridine hydrochloride and 2 M imidazole in acetonitrile, 100 μ L), 5 min; (c) wash to cleave any P(III)-N bonds, 0.1 M pyridine hydrochloride and 0.1 M aniline in acetonitrile, 3 min; (d) oxidation, 0.1 M iodine in pyridine-tetrahydrofuran-water, 10/10/1, v/v/v, 1 min. On completion of the cycles the support bound polymers were deprotected (3% dichloroacetic acid) and cleaved from the support with concentrated ammonium hydroxide. The oligonucleotides were isolated by ion exchange HPLC (Figure 3).

RESULTS AND DISCUSSION

Activation of deoxyribonucleoside amidite reagents

Since cytosyl is the most reactive of the common nucleoside bases toward phosphitylating agents,¹ the synthesis of d-CT was selected to screen weakly acidic agents as activators for nucleoside phosphoramidites. In each case 1c was activated in the presence of thymidine anchored through the 3' oxygen atom to a controlled pore glass support. Following condensation, oxidation, and detritylation, the products were cleaved from the support with

Figure 2. ^{31}P spectra of products formed on successive addition of excess reagents to **1t** in acetonitrile- d_3 .

Figure 3. Ion exchange HPLC profiles of crude products from preparation of (A) $\text{d-C}_{13}\text{T}$, and (B) $\text{d-ACACCCAATTCTGAAAATGG}$.

ammonium hydroxide. HPLC profiles for several of the product mixtures are shown in Figure 1.

Activation with tetrazole, the agent commonly used in syntheses with N-protected derivatives, afforded a complex mixture. (Figure 1A). The compounds eluting more slowly than d-CT reflect phosphitilation at the NH_2 group. As previously reported,¹ these products may be largely eliminated by treating the solid supported derivatives with pyridine hydrochloride/aniline prior to oxidation.

A much cleaner profile was obtained when pyridine hydrochloride was used in place of tetrazole as the activating reagent (Figure 1B), and further improvement was realized when imidazole was added to the pyridine hydrochloride solution prior to the activation step (Figure 1C). Over 80% of the crude nucleotide product from the latter reaction was dCT. We further

Figure 4. Reversed phase HPLC profiles for crude products from preparation of $(2'\text{OSiMe}_2\text{tBu})\text{rUdT}$ with activation of **3** by (A) pyridine hydrochloride, and (B) tetrazole. The peaks at 30 min., 11 min., and ~3 min. represent $(2'\text{OSiMe}_2\text{tBu})\text{rUdT}$, thymidine, and non-nucleotidic material, respectively.

found that **1a**, **1g**, and **1t** react rapidly with dT-cpg under these conditions, yielding the expected dinucleoside phosphates with fewer side products than **1c**. These experiments indicate a high preference for phosphitilation at the hydroxyl group relative to the amino group for nucleoside phosphoramidite reagents activated by pyridine hydrochloride/imidazole.

Some insight concerning the intermediates in the phosphitilation reactions is provided by the ^{31}P NMR spectra in Figure 2. On addition of excess pyridine hydrochloride to **1t** in acetonitrile- d_3 , a portion of the phosphoramidite (δ 146.2, 146.5 ppm) was converted to a product with a resonance peak at 165.1 ppm,

characteristic for a phosphoromonochloridite. This mixture reacted quantitatively with methanol to give a phosphite (δ 142.2 ppm) and with imidazole to give an imidazolide (δ 129.8 ppm), which likewise afforded a phosphite (δ 142.2 ppm) on treatment with methanol. These data are consistent with the view (a) that a mixture of a nucleoside phosphoromonochloridite and phosphoramidite is formed when the amidite reagent is treated with pyridine hydrochloride, (b) that these P(III) derivatives equilibrate readily, though slowly on the NMR time scale, and (c) that the reactive intermediate is a P(III)-monochloridite when the amidite is activated by pyridine hydrochloride and a P(III)-imidazolide when the amidite is activated by pyridine hydrochloride/imidazole.

Pyridine hydrochloride/triazole behaved like pyridine hydrochloride/imidazole as an activator of 1c in the synthesis of d-CT. Pyridine hydrochloride/aniline was much less effective; only ~60% of the dT-cpg reacted during a five minute exposure to the phosphitilating mixture. Fourrey and Varenne used N-methylanilinium trifluoroacetate to activate nucleoside phosphoramidite reagents.⁵ In our hands this reagent was not very satisfactory for couplings involving unprotected bases. Only ~70% of the nucleotide material obtained on HPLC of the products from the reaction with 1a corresponded to d-CT. It appeared that the activated phosphoramidite functioned as an acylating as well as a phosphitilating agent in this system.

Synthesis of oligodeoxyribonucleotides

On the basis of the dimer studies, we selected pyridine hydrochloride/imidazole as an acidic activating agent in synthesizing oligomers with unprotected bases. A pyridine/aniline wash step¹ was also included in the general protocol to cleave any P(III)-N bonds that form. As shown by the HPLC profile in Figure 1D, two of the side products in the synthesis of d-CT (material eluting at ~22 min in Figure 1C; presumably products of phosphitilation at the amino group) were eliminated by this treatment.

The utility of this coupling procedure was examined by preparing d-(C)₁₃T (4) and d-ACACCCAATTCTGAAAATGG (5). Coupling yields averaged 97% in each cycle as judged by the DMT+ test. The HPLC profile of the crude reaction mixture from the preparation of d-(C)₁₃T (Figure 3A) shows a high conversion to the desired oligonucleotide. For the mixed base 20-mer, substantial amounts of faster eluting products were obtained (Figure 3B). Although the procedure was less efficient here than one utilizing nucleosides with base protecting groups, good quality 20-mer was recovered (18 A₂₆₀ units from 0.5 μ mol DMT-dT-cpg, 17%). The oligonucleotides were characterized by reversed phase HPLC, polyacrylamide gel electrophoresis (R_m relative to bromophenol blue: 0.8 for 4, 0.66 for 5), and enzymatic hydrolysis to the nucleosides (dC and dT from 4, and dA, dC, dG, and dT from 5, in the appropriate mole ratios).

Activation of other nucleoside amidite reagents

To see whether pyridine hydrochloride and imidazole might also be useful in activating amidites for synthesis of oligonucleoside methyl phosphonates and oligoribonucleotides, we examined the reactions of 2 and 3 with dT-cpg. Following condensation, the dinucleoside derivatives were worked up in the standard manner and analyzed by reversed phase HPLC.

For activation of amidite 2 with tetrazole (2 minute reaction time), with pyridine hydrochloride (2 minute), and with pyridine

hydrochloride/imidazole (5 minute), the conversion of thymidine to d-T[OP(O)(Me)O]T (obtained as two well resolved peaks, corresponding to the expected diastereoisomers) was 95%, 99%, and 86%, respectively. The remainder was principally thymidine in each case. These results show that pyridine hydrochloride serves as well as tetrazole in activating the methyl phosphonoamidite reagent. Indeed, the intermediate appears somewhat more reactive than that generated by tetrazole. On the other hand, the phosphitilating agent produced in the imidazole reaction (probably a methylphosphonoimidazolide) was much less reactive. The imidazole reagent therefore does not look attractive for use in routine syntheses of methyl phosphonate derivatives.

Profiles for the products of reaction of 3 with dT-cpg are shown in Figure 4. Conversion to the dimer was essentially complete within 1 minute (4A) for the reaction involving pyridine hydrochloride. Under comparable conditions, some thymidine remained in the system utilizing tetrazole (4B). The high activity of the phosphorochloridite generated in the reaction with pyridine hydrochloride was also demonstrated by ³¹P NMR spectral data for a reaction of 3 (0.05 M, δ 154.55, 155.03 ppm) with 3'-levulinyldeoxythymidine (0.06 M) in acetonitrile-d₃. In the presence of pyridine hydrochloride (0.25 M) the amidite was completely converted to the phosphite (δ 144.7 ppm) in less than 1 minute at room temperature. These reactions therefore demonstrate that pyridine hydrochloride should prove useful in activating phosphoramidites for the synthesis of oligoribonucleotides as well as oligodeoxyribonucleotides and methyl phosphonates.

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