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Solid-phase synthesis of oligo-2-pyrimidinone-2′-deoxyribonucleotides and oligo-2-pyrimidinone-2′-deoxyriboside methylphosphonates

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ABSTRACT

A synthetic method was developed for the synthesis of oligodeoxyribonucleotides and oligodeoxyribonucleoside methylphosphonates comprised exclusively of the fluorescent 2-pyrimidinone base for the first time. The method utilized the solid-phase 2-cyanoethyl-phosphoramidite and methylphosphonamidite chemistry for internucleotide couplings and a base-labile oxalyl linkage to anchor the oligomers onto the CPG support. Cleavage of the oligomers from the support was effected by a short treatment of the support with 5% ammonium hydroxide in methanol at room temperature, without any degradation of the base-sensitive 2-pyrimidinone residues or the base-sensitive methylphosphonate backbone.

INTRODUCTION

Oligonucleotides and oligonucleotide analogs conjugated with fluorescent dyes have been widely used in various biochemical fields and biomedical sciences (1), including the study of cellular and tissue uptake and intracellular distribution of oligonucleotide-based therapeutics (2,3). As part of our ongoing effort in developing novel strategies for targeted cellular delivery of antisense/anti-code therapeutic agents (4–6), we have been interested in synthesizing fluorescent oligonucleotides and oligonucleoside methylphosphonates whose structures are as close as possible to those of the oligomers containing the four normal nucleobases. To this end, we have chosen to incorporate 2-aminopurine, a fluorescent purine analog, and 2-pyrimidinone, a fluorescent pyrimidine analog, into oligonucleoside methylphosphonates, without the use of the conventional bulky fluorescent groups. These base analogs also do not form Watson–Crick base pair duplexes with cellular nucleic acids. We have recently succeeded in the solid-phase synthesis of methylphosphonate oligomers containing multiple 2-aminopurine bases (7). We wish to report here the solid-phase synthesis of both oligodeoxyribonucleotide and oligodeoxyribonucleoside methylphosphonate oligomers containing exclusively the fluorescent 2-pyrimidinone base.

Although 2-pyrimidinone and its nucleosides are well known and their syntheses are well documented (8–11), their incorporation into oligonucleotides has been a challenge to the synthetic chemists. The difficulties arise from the sensitivity of this heterocycle to the alkaline conditions used in oligonucleotide synthesis. Degradation of the heterocycle occurs readily at the final oligonucleotide deprotection stage, a treatment required to remove the normal base-protecting groups and to release the oligomers from the solid support. Consequently, it had been only possible from these syntheses to obtain oligodeoxyribonucleotides (12–17) or oligoribonucleotides (18) containing a single 2-pyrimidinone residue, whether by solution-phase method (12–14) or by solid-phase method (15–18). The maximum fluorescence emission intensity of these oligomers were thus limited to a single 2-pyrimidinone base incorporated. Furthermore, there has been no report to date on the incorporation of 2-pyrimidinone base into other type of oligomer backbones.

Oligomers containing a single 2-pyrimidinone residue are unlikely to be useful in the cellular and tissue uptake studies or in the intracellular distribution studies. This is due to the fact that the 2-pyrimidinone base is relatively weak in fluorescence as compared with most of the commonly used bulky chromophores (15–18) and that the levels of cellular uptake of oligonucleotides and their analogs are usually low (5,6). It is therefore highly desirable to have as many as possible 2-pyrimidinone residues in an oligomer molecule. We report in this article a solid phase synthesis method which for the first time allowed the successful incorporation of multiple 2-pyrimidinone residues into the oligomers consisting of either phosphodiester backbone or the base-sensitive methylphosphonate backbone. These methylphosphonate oligomers and phosphodiester oligomers contain exclusively the 2-pyrimidinone bases. Our laboratory is currently using these fluorescent oligomers, together with the oligomers containing multiple fluorescent 2-aminopurine bases, in studies of cellular and tissue uptake, as well as the intracellular distribution of these oligomers. These studies provide the foundation for the antisense/anticode drug development.

RESULTS AND DISCUSSION

Synthesis of 2-pyrimidinone-2′-deoxyriboside 3′-*O*-phosphoramidite (3) and 3′-*O*-methylphosphonamidite (4)

We used solid-phase 2-cyanoethylphosphoramidite (19) and methylphosphonamidite (20) chemistry to incorporate 2-pyrimidinone into oligodeoxyribonucleotides and oligodeoxyribonucleoside methylphosphonates. The required synthons, 5-*O*-pixyl-2- pyrimidi-

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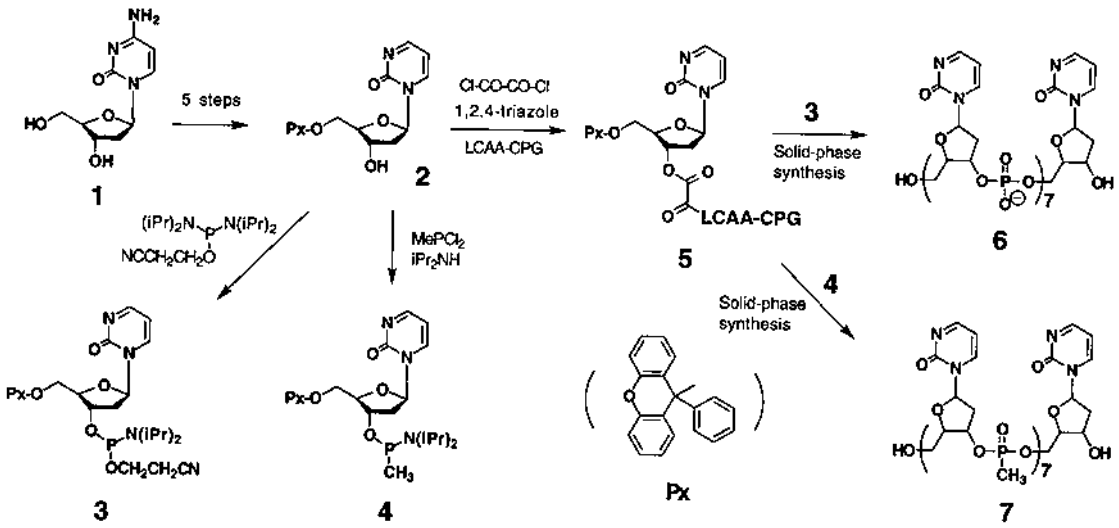

Figure 1. Synthetic scheme for the oligo-2-pyrimidinone-2′-deoxyribonucleotides and oligo-2-pyrimidinone-2′-deoxyriboside methylphosphonates.

none-2′-deoxyriboside-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (**3**) and 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside-3′-*O*-(*N*,*N*-diisopropyl)methylphosphonamidite (**4**), were prepared from a common nucleoside precursor 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside (**2**) as shown in Figure 1. Compound **2** was synthesized by converting the 4-amino group of 2′-deoxycytidine (**1**) to an aromatic hydrogen, based on a previous procedure (15) with several modifications (see Experimental section for details). It was rigorously purified before it was converted into the corresponding synthons and used in the synthesis of 2-pyrimidinone-CPG (5). Conversion of compound **2** into phosphoramidite **3** was achieved by the phosphitylation reaction with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of diisopropylammonium tetrazolide (21). The methylphosphonamidite (**4**) was synthesized from compound **2** by the reactions involving methyldichlorophosphine and diisopropylamine, using a modified phosphitylation procedure based on those of Agrawal and Goodchild (22). The synthons were purified by repeated hexane precipitation. Their identity and purity were confirmed by ^{1}H and ^{31}P NMR. Solid-phase synthesis study revealed step-wise coupling efficiencies of >98% for both synthons.

Attempted solid-phase synthesis of oligo-2-pyrimidinone-2′-deoxyriboside methylphosphonates on succinyl-CPG support

The conventional nucleoside succinyl-CPG supports have been used previously in the solid-phase incorporation of a single 2-pyrimidinone nucleoside into oligodeoxyribonucleotide (15) and oligoribonucleotide (18) sequences containing mixed bases. Final deprotections of the 2-pyrimidinone containing oligomers were performed by the treatment of the support with either concentrated ammonium hydroxide at elevated temperatures (15) or ammonia-saturated methanol at 30°C for 24 h (18) in order to cleave the oligomers from the CPG and to remove the base protecting groups (benzoyl and isobutyryl groups) and the phosphate protecting group (2-cyanoethyl group). Both deprotecting procedures caused significant degradation (>30% when deprotected by ammonium hydroxide) (15) of the incorporated 2-pyrimidinone base. The degradation had been explained by the susceptibility of the C5=C6 double bond of the 2-pyrimidinone base to the nucleophilic attack by the ammonia (16), which was in agreement with the observation that the 2-pyrimidinone derivative was more sensitive to ammonia than the 5-methyl-2-pyrimidinone derivative (17). Improved synthesis of 2-pyrimidinone-containing oligonucleotides was also reported (though without experimental details) by using more base-labile amino protecting groups and by performing the final deprotection at room temperature overnight using concentrated ammonium hydroxide (17).

A final deprotection condition causing significant degradation of the 2-pyrimidinone residue, although practical for the deprotection of oligonucleotides containing a single 2-pyrimidinone base, is unlikely to be useful for the synthesis of multiple 2-pyrimidinone containing oligomers. This is because the ratio of undegraded full-length product would diminish dramatically as the number of 2-pyrimidinone bases increases in an oligomer. However, for the oligomers containing only 2-pyrimidinone bases or other bases requiring no base-protecting groups for their incorporation, such as thymine, the final deprotection could be performed under much milder conditions than those required to deprotect the oligomers bearing base-protecting groups, therefore, the degree of 2-pyrimidinone degradation could be reduced significantly. In such cases, the lowest level of 2-pyrimidinone degradation is then determined by the strength of the conditions necessary only to deprotect the cyanoethyl group on the phosphate and to cleave the oligomers from the solid support.

To investigate the possibility of using the commercially available succinyl-CPG support in the synthesis of methylphosphonate oligomers containing multiple 2-pyrimidinone bases involving no base protecting groups, we conducted a solid-phase synthesis of a 17mer methylphosphonate oligomer containing sixteen 2-pyrimidinone residues. The synthesis utilized the methylphosphonate synthon **4** and the commercial dT-succinyl-CPG support. The final deprotection of the oligomer, which involves only the cleavage of the succinyl linkage, was studied under several conditions.

The succinyl CPG bearing the 17mer methylphosphonate was thus treated with freshly prepared saturated ammonia in anhydrous

methanol at room temperature. This reagent has been used previously to deprotect oligonucleoside methylphosphonates (23) and oligoribonucleotides containing a single 2-pyrimidinone residue (18). Yield of CPG cleavage was found to be 35% in 3.5 h and reached 95% in 24 h. The UV spectra of the product mixture from 3.5 h treatment showed an intense band ~284 nm due to the degraded 2-pyrimidinone residue besides the expected band at 303 nm for the intact 2-pyrimidinone base (15). The ratio of A284 to A303 nm was ~5:4. After 24 h of treatment, the 303 nm band was nearly unobservable and the spectra showed only the 284 nm band, and the product mixture was very weak in fluorescence. Reversed-phase HPLC profile (at both 285 and 303 nm) of the product from 3.5 h of treatment looked similar to those of the failure sequences from the solid-phase synthesis of unmodified methylphosphonate oligomers, showing no major peaks corresponding to the full-length sequences. The profile for the product obtained from 24 h treatment showed only a fast eluting species with no presence of any oligomer. These analyses indicated that degradation of the 2-pyrimidinone residue occurred at about the same rate as the cleavage of the oligomer-linked succinyl CPG support under the methanolic ammonia condition. Treatment with concentrated ammonium hydroxide at room temperature for 1 h was enough to completely release the oligomer from the CPG support, but it also caused ~30% degradation of the 2-pyrimidinone nucleoside. The sensitivity of methylphosphonate backbone to concentrated ammonium hydroxide also precludes this type of treatment (24). When the oligomer-containing CPG was treated with 50% ethylenediamine in acetonitrile/ethanol/water (47.5/47.5/5) (the Genta one pot reagent for methylphosphonate deprotection) (24), complete CPG cleavage occurred in 40 min. However, this treatment produced a highly UV absorbing, but weak fluorescent product mixture, with maximum absorbing wavelength at 298 nm, instead of at 303 nm. Reversed phase HPLC revealed a fast eluting strong UV absorbing species, but with little sign of the presence of any oligomers.

The above results suggested that the sensitivity to basic treatment of the 2-pyrimidinone residue in the oligomer was too close to that of the oligomer-linked succinyl-CPG linkage to allow one to selectively cleave the succinyl linkage without significant damage to the 2-pyrimidinone residue, which was critical to the synthesis of oligomers containing multiple 2-pyrimidinone residues. To circumvent the problem, the ideal choice would be to use a CPG linkage which is more base labile than the succinyl linkage, for example, the oxalyl linkage (25).

Studies of oxalyl-CPG cleavage conditions without degradation of 2-pyrimidinone base and methylphosphonate backbone

The use of oxalyl CPG in the solid-phase synthesis of base-sensitive oligonucleotide analogs was first reported by Alul *et al.* (25). That the oxalyl linkage is more base labile than the succinyl linkage was demonstrated under several weak alkaline conditions. For example, oligomers linked via an oxalyl linkage to the CPG support were released quantitatively by the treatment with 5% ammonium hydroxide in methanol at room temperature for a few minutes, whereas <5% of succinyl CPG cleavage had occurred by the same treatment (25).

To investigate the conditions which could be used to release the methylphosphonate oligomers from the oxalyl CPG without damage to the 2-pyrimidinone residue and the methylphosphonate backbone, we synthesized an oligodeoxythymidine methylphosphonate 8mer on the oxalyl CPG support, using the commercial DMT-dT methylphosphonamidite and the DMT-dT-oxalyl-LCAA-CPG prepared as described previously (25). Cleavage of the oligomer from the CPG support was studied under several basic conditions. The Genta one pot reagent (24) was found to give the fastest oxalyl CPG cleavage, releasing 98% of oligomer in <2.5 min. This amount of CPG cleavage was also obtained by treatment with 5% ammonium hydroxide in methanol at room temperature for 5–10 min. Treatment with triethylamine/methanol (1/1) gave slower CPG cleavage, but 98% cleavage could still be obtained in 1 h. The methylphosphonate backbone remained intact after all of these treatments, as revealed by reversed-phase HPLC analysis. No degradation of the backbone was detected even after 1 day of treatment with 5% ammonium hydroxide in methanol or 1/1 triethylamine/methanol.

The stability of 2-pyrimidinone residue in 5% ammonium hydroxide in methanol was then studied by the treatment of the free 2-pyrimidinone nucleoside with this reagent for at least 1 h at room temperature. Samples from the treatment were found to be essentially identical to the untreated nucleoside as indicated by their UV spectra, HPLC analysis and fluorescence measurements, indicating that the 2-pyrimidinone residue is stable in 5% ammonium hydroxide in methanol for at least 1 h. This reagent was then chosen as the standard condition in this study for cleaving 2-pyrimidinone-containing oligomers from the oxalyl CPG support.

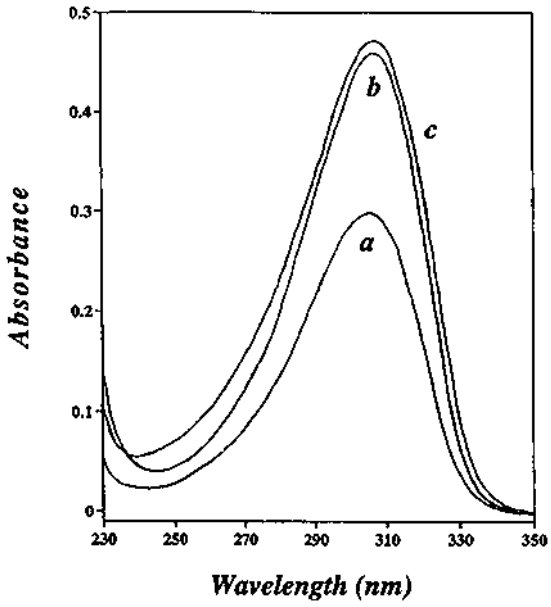

Figure 2. UV absorption spectrum of (**a**) 2-pyrimidinone-2′-deoxyriboside, (**b**) oligo-2-pyrimidinone-2′-deoxyribonucleotide 8mer (**6**), and (**c**) oligo-2-pyrimidinone-2′-deoxyriboside methylphosphonate 8mer (**7**). Solvent: 10% ACN in 10 mM sodium phosphate, pH 7. Sample concentrations are arbitrary.

Solid-phase synthesis of oligo-2-pyrimidinone-2′-deoxyribonucleotides on oxalyl-CPG support

The oxalyl CPG (**5**) was then used together with the phosphoramidite (**3**) in the automated solid-phase synthesis of an 8mer oligo-2-pyrimidinone-2′-deoxyribonucleotide (**6**) by standard

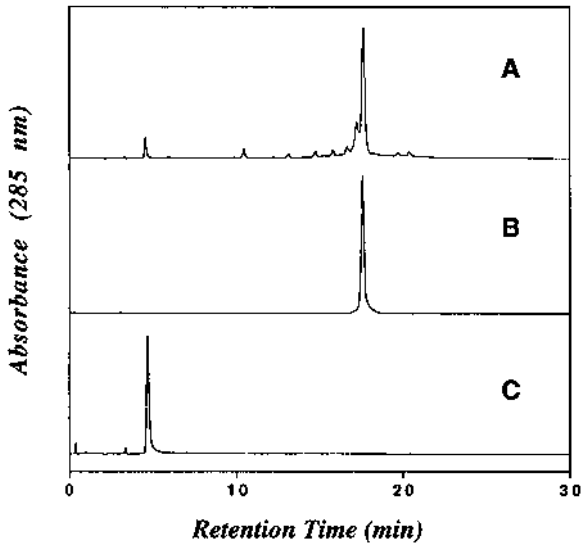

Figure 3. Reversed-phase HPLC analysis of oligo-2-pyrimidinone-2′-deoxyribonucleotide 8mer (**6**). Column: Microsorb 250 × 4.6 mm. Detection: A285 nm. Elution: linear 2–15% ACN gradient in 50 mM sodium phosphate, pH 7. (**A**) Crude **6**. (**B**) HPLC purified **6**. (**C**) HPLC purified **6** digested with phosphodiesterase and alkaline phosphatase.

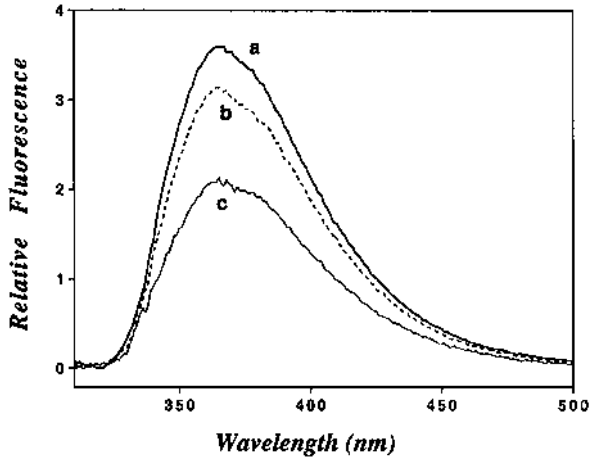

Figure 4. Fluorescence emission spectrum of (**a**) 2-pyrimidinone-2′-deoxyribonucleoside, (**b**) oligo-2-pyrimidinone-2′-deoxyribonucleotide 8mer (**6**), and (**c**) oligo-2-pyrimidinone-2′-deoxyriboside methylphosphonate 8mer (**7**). Solvent: 10% ACN in 10 mM sodium phosphate, pH 7. Excitation: 303 nm. Each sample contains 8 µM 2-pyrimidinone-2′-deoxyriboside residues.

2-cyanoethylphosphoramidite chemistry (19). Stepwise coupling efficiencies were found to be >98%. After the last step of deblocking of the 5′-pixyl group (2.5% dichloroacetic acid in dichloromethane), the support was treated with 5% ammonium hydroxide in methanol at room temperature. The oligomer was found to be cleaved from the support within 10 min of treatment, but longer treatment was necessary in order to completely remove the cyanoethyl group from the phosphate. Therefore, the support was normally treated with the reagent for a total of 70 min. The supernatants were evaporated to dryness under reduced pressure and the crude oligomer obtained was dissolved in 50 mM sodium phosphate at pH 7. The UV spectrum of the crude oligomer was almost indistinguishable from that of the 2-pyrimidinone-2′-deoxyriboside (Fig. 2a), showing the same shape and the same maximum absorption at 303 nm. Reversed-phase HPLC profile of the crude oligomer (at both 285 and 303 nm) showed a major peak corresponding to the full-length product together with seven small earlier eluting peaks due to the failure sequences (Fig. 3A). These analyses indicated that no degradation of the 2-pyrimidinone residue had occurred during the solid-phase assembly and at the final deprotection stage. The crude oligomer was then subjected to preparative reversed phase HPLC purification and the full-length product was isolated, desalted, and analyzed. Its purity was confirmed by UV spectral analysis (Fig. 2b) and analytical HPLC (Fig. 3B). The oligomer was then digested to the nucleoside level with phosphodiesterase and alkaline phosphatase. Reversed-phase HPLC analysis of the digest at both 285 and 303 nm showed the presence of a single earlier eluting peak which co-eluted with the standard nucleoside 2-pyrimidinone-2′-deoxyriboside upon co-injection (Fig. 3C), indicating that no modification to the 2-pyrimidinone residue had occurred. The extinction coefficient of the oligomer at 303 nm (50 100 $M^{-1}cm^{-1}$) was then derived from the hyperchromicity upon enzymatic digestion, using 6470 $M^{-1}cm^{-1}$ as the extinction coefficient for 2-pyrimidinone-2′-deoxyriboside (15). The fluorescence excitation and emission spectra of the oligomer before and after digestion were all similar to those of the free nucleoside, showing excitation maxima at 303 nm and emission maxima at 365–366 nm (18). Comparison of the emission intensities at 365 nm (excitation 303 nm) revealed that a 2-pyrimidinone residue in the oligomer was ~87% as fluorescent as the free nucleoside (Fig. 4, spectra a and b). The observation that the oligomer fluorescence was quenched by only 10% is in agreement with the expectation in regard to the weak stacking interactions between the pyrimidine bases. The weak stacking interactions were also suggested by the small hyperchromicity (4.7%) obtained upon enzymatic digestion of the oligomer. The dependence of fluorescence emission on base stacking interaction has been used to study the structures of a DNA duplex containing a single 2-pyrimidinone substitution using time-resolved fluorescence spectroscopy (26).

Solid-phase synthesis of oligo-2-pyrimidinone-2′- deoxyriboside methylphosphonate on oxalyl-CPG support

An 8mer oligo-2-pyrimidinone-2′-deoxyriboside methylphosphonate (**7**) was also synthesized on the oxalyl CPG support by the automated solid-phase phosphonamidite chemistry (20), using the methylphosphonamidite (**4**) and the oxalyl CPG (**5**). Stepwise coupling efficiencies were found to be >98%. The 5′-depixylated oligomer was cleaved from the support by treatment with 5% ammonium hydroxide in methanol at room temperature for ~10 min. After evaporating the supernatants under reduced pressure, the crude oligomer was dissolved in 50 mM sodium phosphate at pH 7 containing 10% acetonitrile. The UV spectrum of the crude oligomer was found to be essentially similar to that of the free nucleoside, indicating that no degradation of the 2-pyrimidinone residue had occurred. Figure 5A shows the analytical HPLC profile of the crude oligomer. The full-length product was then isolated by preparative reversed phase HPLC and the purified oligomer was analyzed by UV (Fig. 2c) and analytical HPLC (Fig. 5B). Its molecular weight was confirmed

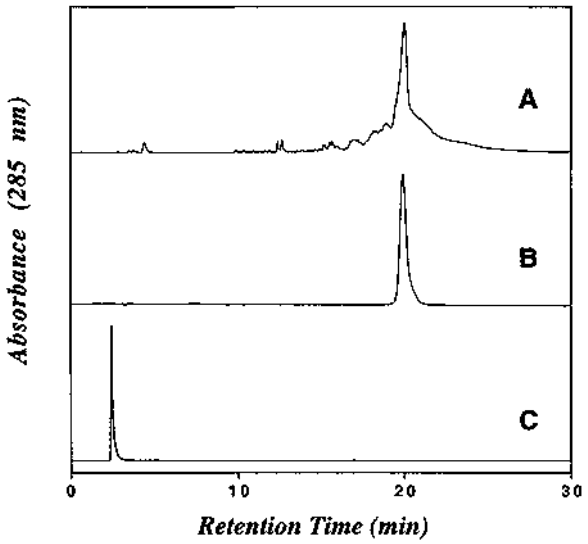

Figure 5. Reversed-phase HPLC analysis of oligo-2-pyrimidinone-2′-deoxyribonucleoside methylphosphonate 8mer (**7**). Column: Microsorb 250 × 4.6 mm. Detection: A285 nm. Elution: linear 2–30% ACN gradient in 50 mM sodium phosphate, pH 7. (**A**) Crude **7**. (**B**) HPLC purified **7**. (**C**) HPLC purified **7** digested with 0.1 M HCl.

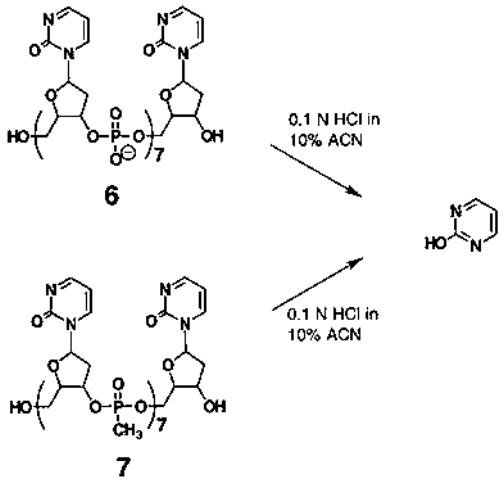

Figure 6. Depyrimidination of oligo-2-pyrimidinone-2′-deoxyribonucleotides and oligo-2-pyrimidinone-2′-deoxyriboside methylphosphonates by 0.1 M HCl.

by pneumatically assisted electrospray mass spectral analysis. Calculated: 2117.6; found 2118.0.

To determine the extinction coefficient, the 8mer oligo-2-pyrimidinone-2′-deoxyriboside methylphosphonate (**7**) was treated with 0.1 M HCl containing 10% acetonitrile at room temperature for 2 days. This treatment caused depyrimidination of the 2-pyrimidinone residues in both the methylphosphonate oligomer and the phosphodiester oligomer (27), as shown in Figure 6. Completeness of digestion and formation of 2-hydroxypyrimidine as the sole UV-absorbing species was confirmed by reversed phase HPLC analysis, using the commercial 2-hydroxypyrimidine as the standard (Fig. 5C). Extinction coefficients of 2-hydroxypyrimidine was pre-determined to be 5220 $M^{-1}cm^{-1}$. The extinction coefficient of the oligomer **7** at 303 nm (47 200 $M^{-1}cm^{-1}$) was thus calculated from the absorbance of the oligomer before digestion and the concentration of 2-hydroxypyrimidine formed after acid digestion. Digestion of the phosphodiester oligomer **6** gave a 303 nm extinction coefficient of 51 100 $M^{-1}cm^{-1}$, which was in agreement (within 98%) with the value obtained by enzymatic digestion. The piperidine digestion method (28), a standard digestion method for methylphosphonate oligomers containing the normal bases, was found to be unsuitable in this study, since it caused fast degradation of the 2-pyrimidinone residues.

The fluorescence excitation and emission spectra of oligomer **7** were also similar to the free nucleoside. A 2-pyrimidinone residue in this oligomer was ~70% as fluorescent as the free nucleoside, as judged by the 365 nm emission intensities (Fig. 4c).

We have described the successful syntheses of both oligodeoxyribonucleotide and oligodeoxyribonucleoside methylphosphonate oligomers containing exclusively the fluorescent 2-pyrimidinone base. One might be interested in incorporating multiple 2-pyrimidinone base into mixed sequences. Such syntheses will require the use of a very base-labile amino protecting group for the protection of the other nucleobases together with the use of the base-labile oxalyl CPG support. We believe that the 9-fluorenylmethoxycarbonyl(Fmoc)-protected nucleotide synthons can be used in conjunction with the oxalyl CPG supports for these syntheses. It had been shown that Fmoc can be deprotected by the treatment with a mixture of diisopropylamine in methanol (29,30), and we have found that the 2-pyrimidinone nucleoside and its oligomers are stable after this treatment.

EXPERIMENTAL

Materials and Methods

2′-Deoxycytidine monohydrochloride was purchased from Chem-Impex International (Wood Dale, IL). 2-Hydroxypyrimidine hydrochloride, *tert*-butyldimethylsilyl chloride, 9-chloro-9-phenylxanthene (pixyl chloride), oxalyl chloride, imidazole, 4-dimethylaminopyridine, anhydrous 1,4-dioxane, anhydrous tetrahydrofuran (THF), anhydrous hydrazine, sodium bisulfite, silver (I) oxide, tetrabutylammonium fluoride (1.0 M solution in THF), anhydrous pyridine, anhydrous dichloromethane, anhydrous diisopropylamine, anhydrous *N*,*N*-diisopropylethylamine, 1,2,4-triazole, 1H-tetrazole were from Aldrich (Milwaukee, WI). Methyldichlorophosphine was purchased from Alfa Aesar (Ward Hill, MA). Long chain aminoalkyl controlled pore glass (LCAA CPG) was from Sigma (St Louis, MO). Phosphodiesterase from *Crotalus durissus* was purchased from Boehringer Mannheim (Indianapolis, IN). Shrimp alkaline phosphatase was purchased from USB (Cleveland, OH). Liquid chromatography was performed on columns packed with silica gel 60 (230–400 mesh) from Aldrich. Analytical TLC was performed on aluminum sheets coated with silica gel 60 F254 from EM Science (Gibbstown, NJ). DNA synthesis reagents were from Glen Research (Sterling, VA).

Nuclear magnetic resonance spectra were recorded on a Bruker AMX300 spectrometer at ambient temperatures. Chemical shifts were measured relative to solvent signals as internal standards and expressed in p.p.m. relative to tetramethylsilane. The exchangeable protons were confirmed by adding either deuterium oxide or deuteriated methanol. ^{31}P NMR were recorded in CD_3CN. Chemical shifts were obtained from the proton-decoupled experiments and were expressed relative to the external standard, 85% phosphoric acid. Pneumatically assisted electrospray mass

spectra of oligonucleoside methylphosphonates were recorded on a Perkin Elmer SCIEX ATI3 instrument at Scripps Research Institute Mass Spectrometry Facility, La Jolla, CA. Fluorescence emission and excitation spectra were recorded on an Aminco•Bowman Series 2 luminescence spectrometer at 20°C. Samples of known concentration were diluted so that the 303 nm absorbances were <0.06. All fluorescence spectra were instrument corrected.

5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside (2)

This compound was synthesized from 2′-deoxycytidine monohydrochloride (10.0 g, 37.9 mmol) by converting the 4-amino group of cytosine into an aromatic proton, using a multi-step synthesis scheme published previously (15). Some of the procedures were modified to provide products in higher yield and higher purity. Overall yield from 2′-deoxycytidine: 4.64 g (9.91 mmol), 26%. Modifications to the procedures are as outlined below:

(i) Ethyl acetate/hexane solvent system (instead of methanol/dichloromethane) was used for the purification of 3′,5′-bis(*tert*-butyldimethylsilyl)-2-pyrimidinone-2′-deoxyriboside on silica gel column. The product was obtained in higher yield and purity.

(ii) When using tetrabutylammonium fluoride to remove the *tert*-butyldimethylsilyl group from 3′,5′-bis(*tert*-butyldimethylsilyl)-2-pyrimidinone-2′-deoxyriboside, the reaction was quenched by adding an aqueous buffer containing the necessary amounts of equimolar pyridine and pyridine hydrochloride, instead of just pure water. This gave rise to a neutral mixture, thereby preventing the 2-pyrimidinone nucleoside from degradation under basic conditions. A small portion of the crude 2-pyrimidinone-2′-deoxyriboside was purified by preparative reversed-phase HPLC to afford ~20 mg of pure materials which served as the nucleoside standard for various studies in this work. The rest of the crude materials were used directly in the next step reaction with pixyl chloride.

(iii) 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside (**2**) was rigorously purified by repeated silica gel column chromatography. The first column used methanol/dichloromethane as the elution solvent to remove most of the impurities. The second column used ethyl acetate followed by 2% methanol in ethyl acetate as the elution solvents to remove the remaining two impurities, which on TLC co-migrated with **2** when developed in methanol/dichloromethane, but appeared as two higher moving spots when developed in ethyl acetate.

^{1}H NMR($CDCl_3$): δ 8.54(dd, 1H, H4), 8.43(dd, J = 2.8 and 6.7 Hz, H6), 7.0–7.4(m, pixyl), 6.25(dd, J = 4.2 and 6.7 Hz, 1H, H5), 6.21(t, J = 5.9 Hz, 1H, H1′), 4.41(m, 1H, H3′), 4.13(m, 1H, H4′), 3.56(bs, 1H, 3′-OH), 3.30(dd, J = 2.9 and 10.7 Hz, 1H) and 3.14(dd, J = 3.4 and 10.7 Hz, 1H) (H5′/H5″), 2.82(ddd, J = 4.9, 6.0 and 13.8 Hz, 1H, H2″), 2.28(dt, J = 5.9 and 13.8 Hz, 1H, H2″).

5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (3)

This compound was prepared by the reaction of 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside (**2**, 469 mg, 1 mmol) with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (500 mg, 1.66 mmol) in the presence of diisopropylammonium 1H-tetrazolide (250 mg, 1.46 mmol), according to a known procedure (21) in a reaction time of 1.5 h. The compound was purified by dissolving the crude product in 5 ml toluene and then precipitated from 200 ml vigorously stirring hexanes at room temperature. The precipitates were redissolved in toluene and were reprecipitated similarly to afford 460 mg (0.69 mmol) pure **3** as white amorphous solid. Yield: 69%. ^{1}H NMR (CD_3CN): δ 8.48–8.53(m, 1H, H4), 8.30(dd, 0.5H) and 8.23(dd, 0.5H) (J = 2.8 and 6.7 Hz, H6), 7.0–7.4(m, pixyl), 6.24–6.30(m, 1H, H5), 6.02–6.09(m, 1H, H1′), 4.42–4.56(m, 1H, H3′), 4.12–4.20(m, 1H, H4′), 3.45–3.85(m, 4H, $POCH_2$ and PNCH), 3.08–3.30(m, 2H, H5′/H5″), 2.65–2.78(m, 1H, H2′), 2.62(t, 1H) and 2.49(t, 1H) (J = 6 Hz, CH_2CN), 2.24–2.36(m, 1H, H2″), 0.98–1.17(2dd, 12H, iPr CH_3). ^{31}P NMR (CD_3CN): δ 152.8 p.p.m.

5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside-3′-*O*-(*N*,*N*-diisopropyl)methylphosphonamidite (4)

To a stirring solution of methyldichlorophosphine (290 µl, 2.31 mmol) in 10 ml anhydrous dichloromethane under the protection of nitrogen, was slowly injected 647 µl (4.64 mmol) of diisopropylamine. The stirring was continued for 1 h at room temperature. The solution was slowly transferred, through a stainless steel cannula via nitrogen pressure, to another stirring solution containing 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside (**2**, 361 mg, 0.77 mmol), diisopropylethylamine (886 µl, 5.08 mmol) and dichloromethane (10 ml). The reaction went to completion in 1.5 h as revealed by TLC analysis. The excess amount of chlorophosphine was then quenched by the addition of 2 ml anhydrous methanol and the stirring was continued for an additional 5 min. The mixture was then diluted with 100 ml dichloromethane and washed twice with 5% aqueous sodium bicarbonate and once with water. The organic phase was dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml toluene, and precipitated by adding small drops of the toluene solution to 150 ml vigorously stirring hexanes at room temperature. The red-brown precipitates were filtered off and were discarded. The hexane mother containing most of the synthon was concentrated to 20 ml under reduced pressure and was refrigerated overnight to obtain off-yellow precipitates, which were dried under vacuum to give 325 mg synthon **4**. Yield: 69%. ^{1}H NMR (CD_3CN): δ 8.48–8.53(m, 1H, H4), 8.31(dd, 0.5H) and 8.13(dd, 0.5H) (J = 2.8 and 6.7 Hz, H6), 7.02–7.42(m, pixyl), 6.31(dd, 0.5H) and 6.27(dd, 0.5H) (J = 4.1 and 6.7 Hz, H5), 6.01 and 6.02(2t overlap, 1H, H1′), 4.33–4.43(m, 0.5H) and 4.17–4.27(m, 0.5H) (H3′), 4.07–4.14(m, 1H, H4′), 3.3–3.6(m, 2H, PNCH), 3.24(dd, J = 3.1 and 10.7 Hz, 0.5H) and 3.16(dd, J = 3.2 and 10.6 Hz, 0.5H) and 3.10(dd, J = 3.6 and 10.7 Hz, 0.5H) and 3.04(dd, J = 4.4 and 10.6 Hz, 0.5H) (H5′/H5″), 2.66–2.76(m, 0.5H) and 2.50–2.62(m, 0.5H) (H2′), 2.1–2.3 (m, 1H, H2″), 0.92–1.18(m, 15H, P-CH_3 and iPr CH_3). ^{31}P NMR (CD_3CN): δ 124.2 and 125.8 p.p.m.

5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside-3′-*O*-oxalyl-LCAA-CPG (5)

To a mixture containing 193 mg 1,2,4-triazole (2.75 mmol), 4.5 ml anhydrous acetonitrile and 0.25 ml anhydrous pyridine under the protection of nitrogen, was slowly injected a 10% solution of oxalyl chloride in acetonitrile (500 µl, 0.575 mmol). After stirring for ~10 min at room temperature, another solution containing 270 mg 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside (**2**, 0.575 mmol), 2.5 ml dry acetonitrile and 1.25 ml pyridine was slowly injected into the reaction mixture. Stirring was continued for an additional hour. The reaction solution was then mixed with 1 g LCAA-CPG (pre-activated with trichloroacetic acid) (31) in a

small round-bottomed flask and the flask was gently rotated for ~30 min. The liquid was filtered off, and the CPG was washed successively with dry acetonitrile, dry methanol and dry acetonitrile. Unreacted amino groups on the CPG were then capped by the treatment of the CPG for 10 min with a 10 ml solution containing equal volumes of cap A (0.3 M 4-dimethylaminopyridine/THF) and cap B (0.6 M acetic anhydride/THF). After filtration, the CPG was washed with pyridine and dry acetonitrile and dried in vacuum overnight. To determine the nucleoside loading on the CPG, an accurately weighed portion of the CPG (11.0 mg) was mixed with 20 ml 5% dichloroacetic acid in dichloromethane and the absorbance of the solution at 372 nm was measured (A372 = 0.582, extinction coefficient for the pixyl group at 372 nm: 36 600 $M^{-1}cm^{-1}$). The calculated nucleoside loading was 29 μmol/g. To characterize the CPG for its 2-pyrimidinone integrity, a portion of the CPG was depixylated by a brief treatment with 2.5% dichloroacetic acid in dichloromethane. The reagent was removed and the CPG was washed with dichloromethane and dried. The depixylated CPG was then treated with 5% ammonium hydroxide in methanol for 10 min. The resulting solution containing the liberated nucleoside was evaporated *in vacuo* and the residue was dissolved in 50 mM sodium phosphate buffer at pH 7 for UV, HPLC, and fluorescence measurements. The UV and fluorescence spectra were identical to those of 2-pyrimidinone-2′-deoxyriboside. HPLC (C18-RP) analysis showed a single peak which co-eluted with the nucleoside standard 2-pyrimidinone-2′-deoxyriboside.

Solid-phase synthesis of 2-pyrimidinone-containing oligodeoxyribonucleotides and methylphosphonates

The 2-pyrimidinone-containing oligodeoxyribonucleotides and oligodeoxyribonucleoside methylphosphonates were synthesized by the solid-phase 2-cyanoethylphosphoramidite and methylphosphonamidite chemistry on ABI 392-5 DNA synthesizer on 1 μmol scale, using the standard solid-phase 2-cyanoethylphosphoramidite program and methylphosphonamidite program. Solutions in anhydrous acetonitrile containing 0.1 M 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (**3**) or 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside-3′-*O*-(*N*,*N*-diisopropyl)methylphosphonamidite (**4**) were used for the solid-phase couplings. When 5′-*O*-pixyl-2-pyrimidinone-2′-deoxyriboside-3′-*O*-oxalyl-LCAA-CPG (5) was used as the solid support for oligomer assembly, a pre-capping step was performed to the support before initiating the solid-phase synthesis. Deblocking of the 5′-pixyl group was effected by the use of 2.5% dichloroacetic acid in dichloromethane for the specified duration of time as determined by the synthesis programs. The deblocking fractions were collected and were assayed for the released pixyl group to assess the step-wise coupling efficiency. Average coupling efficiencies for both synthon **3** and **4** were found to be >98%. Cleavage of the 2-pyrimidinone-containing oligomers (5′-Px-off) from the oxalyl-CPG was achieved by a short treatment (10 min) of the support with 5% ammonium hydroxide in methanol at room temperature. Average yield of CPG cleavage was 98%. For the deprotection of the 2-pyrimidinone-containing oligodeoxyribonucleotides, a treatment of 70 min was used instead in order to fully deprotect the cyanoethyl groups on the phosphate. The supernatants obtained from the CPG cleavage and the oligomer deprotection reactions were evaporated to dryness under reduced pressure and the crude oligomers were dissolved in 50 mM sodium phosphate buffer at pH 7 (for the phosphodiester oligomers) or 50 mM sodium phosphate buffer at pH 7 containing 10% acetonitrile (for the methylphosphonate oligomers). The crude oligomer solutions were then subjected to UV and reversed-phase HPLC analysis (Rainin Microsorb C18 4.6 × 250 mm, Woburn, MA) and were purified by preparative reversed phase HPLC (Rainin Microsorb C18 10 × 250 mm). The columns were eluted with linear gradients of acetonitrile in 50 mM sodium phosphate, pH 7. Preparative HPLC fractions containing the pure full-length products were pooled, diluted with 50 mM sodium phosphate, pH 7, and then desalted on a C18 guard column, eluting with 50% acetonitrile/water. The desalted oligomer solutions were diluted to 20% acetonitrile/water and stored in the freezer at –70°C until use.

Enzymatic digestion of the 8mer oligo-2-pyrimidinone-2′-deoxyribonucleotides (6)

The HPLC purified 8mer oligo-2-pyrimidinone-2′-deoxyribonucleotides (**6**, 0.277 OD_{303} unit) was dissolved in 96 μl buffer containing 50 mM Tris, 2 mM $MgCl_2$ at pH 8.2. To the solution was added 2 μl phosphodiesterase and 5 μl alkaline phosphatase and the mixture was incubated at 37°C overnight. The mixture was neutralized to pH 7 and diluted to 1 ml with distilled water. The sample solution was then subjected to HPLC analysis and UV and fluorescence measurements. To determine the extinction coefficients of the oligomer, the above digestion and measurements were performed in triplicates. Extinction coefficient of the oligomer **6** at 303 nm was calculated to be 50 100 $M^{-1}cm^{-1}$, using the equation:

$$\varepsilon(\text{oligo}) = 8 \times \varepsilon(\text{dK}) \times A(\text{oligo})/A(\text{oligo+E})$$

where A(oligo) and A(oligo+E) represent the 303 nm absorbances of the undigested and the digested oligomer samples, respectively, ε(oligo) and ε(dK) represent the 303 nm extinction coefficients for the oligomer and 2-pyrimidinone-2′-deoxyriboside [ε(dK) = 6470 $M^{-1}cm^{-1}$) (15).

Digestion of 2-pyrimidinone-containing oligomers with 0.1 N HCl

A portion of a stock solution containing the methylphosphonate oligomer (**7**) was dried and treated with 200 μl of 0.1 M HCl containing 10% acetonitrile at room temperature for 2 days. The solution was neutralized with 0.1 M NaOH and brought to 1 ml so that the final digested sample contained 35 mM sodium phosphate, 20 mM NaCl, and 10% acetonitrile, pH 7. Absorbances at 303 nm were then measured for the digested and undigested samples in the same buffer system. Digestion and measurements were performed in triplicate to reduce experimental errors. Completion of digestion was confirmed by HPLC analysis using 2-hydroxypyrimidine as the standard. Digestion of the phosphodiester oligomer (**6**) was performed in a similar manner. The extinction coefficients of the two oligomers were then calculated using the equation:

$$\varepsilon(\text{oligo}) = 8 \times \varepsilon(\text{K}) \times A(\text{oligo})/A(\text{oligo+HCl})$$

where A(oligo) and A(oligo+HCl) represent the 303 nm absorbances of the undigested and digested oligomers, respectively, ε(oligo) and ε(K) represent the 303 nm extinction coefficients of the oligomers and 2-hydroxypyrimidine. [ε(K) in this solvent system was pre-determined to be 5220 $M^{-1}cm^{-1}$]. This method gave extinction coefficients for both the phosphodiester oligomer

(**6**, 51 100 $M^{-1}cm^{-1}$) and the methylphosphonate oligomer (**7**, 47 200 $M^{-1}cm^{-1}$).

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