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PREPARATION OF A DEOXYNUCLEOSIDE THIOPHOSPHORAMIDITE INTERMEDIATE IN THE SYNTHESIS OF NUCLEOSIDE PHOSPHORODITHIOATES

Nasser Farschtschi and David G. Gorenstein*
Department of Chemistry
Purdue University
West Lafayette, Indiana 47907

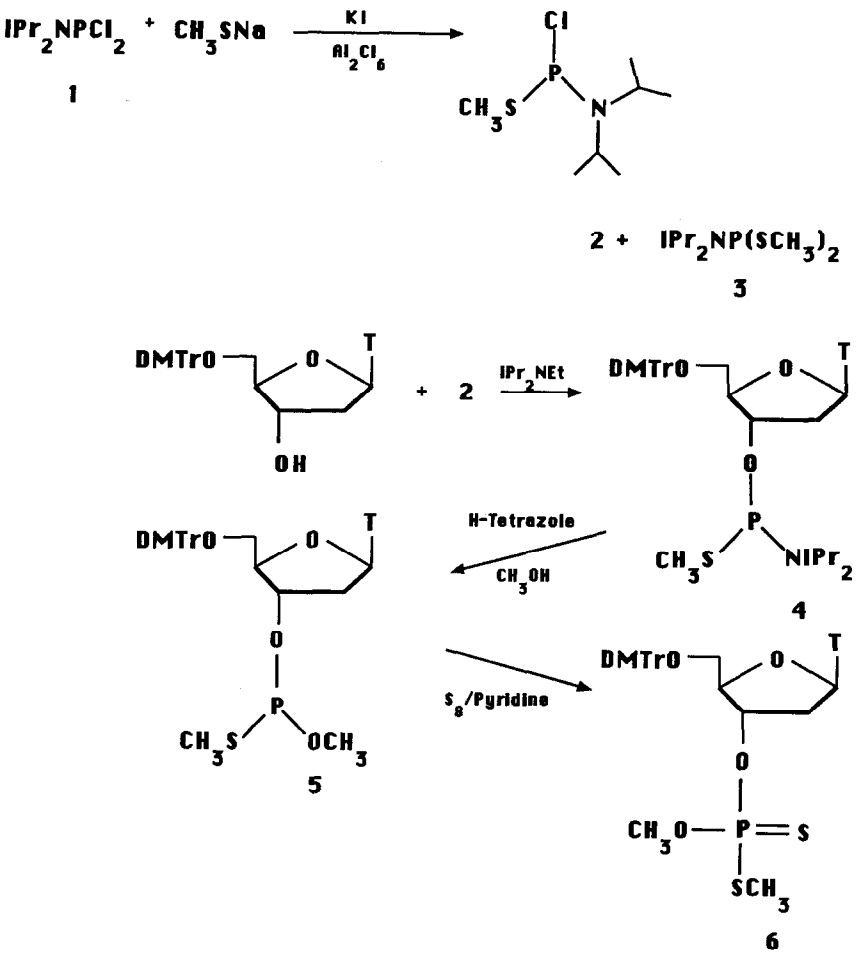
Summary A general synthetic scheme for preparation of thiophosphoramidite intermediates, chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, and 3'-O-deoxythymidine derivatives of N,N-diisopropylaminothiomethoxyphosphine, 4, is described. These intermediates can be used to synthesize dithiophosphate deoxyoligonucleotide analogues.

Oligonucleotides containing diastereomerically pure phosphorothioate linkages have been extensively used to study the enzymatic cleavage of phosphodiester bonds and conformational properties of DNA.[1] Oligonucleotides and various derivatives of oligonucleotides are also known to exhibit antiviral activity.[1] These "antisense oligonucleotide analogue" inhibitors of viral replication and translation have generated considerable current interest. Oligodeoxynucleotides, unfortunately, are susceptible to nuclease digestion[2] and are not stable enough for intravenous or oral administration.

Quite recently Matsukura *et al.*[3] have shown that phosphorothioate analogs of oligonucleotides may be effective "antisense oligonucleotide analogue" inhibitors of HIV replication and are cytopathic to viral infected T cells. However, the introduction of the phosphorothioate function into oligonucleotides leads to the existence of diastereomers (due to the new chiral phosphorus center). They are believed to be less effective *in vitro* than normal oligodeoxynucleotides. Unfortunately, the separation of the R and S-epimers via hplc or dimer couplings of diastereomerically pure dinucleoside phosphorothioate derivatives are often difficult. In this communication we describe the synthesis of key thiophosphoramidite intermediates, 2 and 4 (Scheme 1), which has allowed us to synthesize dithiophosphate deoxyoligonucleotide analogues such as 6. In contrast to the monothiophosphate oligonucleotide analogues, the phosphorus center in the phosphorodithioates is achiral and hence problems associated with mixtures of R and S diastereomers may be avoided.

The overall synthesis of the thiophosphoramidite intermediates, 2 and 4, and deoxythymidine phosphorodithioate, 6, is outlined in Scheme 1. Dichloro-N,N-diisopropylaminophosphine, 1, was synthesized by the reaction of phosphorus trichloride with diisopropylamine (2 equiv).[4] Distillation (56-59° C/0. 5 mm Hg) afforded pure phosphine as shown by ^{31}P NMR (δ ppm in benzene-d_6, downfield relative to external 85% H_3PO_4: 169.0)[4].

Several strategies were investigated for the synthesis of monochloro-N,N-diisopropylaminomercaptophosphine, 2. Dichloro-N,N-diisopropylaminophosphine, 1, in contrast to alkyloxydichlorophosphines, is mildly reactive towards thiols. Several procedures and catalysts were explored to develop optimal reaction conditions for the introduction of the mercapto group into the phosphoramidites. The reaction of dichloro-N,N-diisopropylaminophosphine, 1, with sodium thiomethoxide (1 equiv) was carried out in the present of two catalysts – aluminum trichloride and potassium iodide.



Scheme 1.

The best yield of the intermediate 2 was obtained by the following procedure: a 50 ml addition funnel was charged with a suspension of sodium thiomethoxide (22.8 mmol) and catalytic amount (2.28 mmol) of KI in 40 ml of anhydrous dichloromethane. This suspension was added dropwise over a period of 10 h at -65° C to a magnetically stirred solution of dichloro-N,N-diisopropylaminophosphine, 1 (24.7 mmol + a catalytic amount of $AlCl_3$ (0.5 mmol)) in 20 ml of anhydrous CH_2Cl_2. Generally, after addition of sodium thiomethoxide the resulting suspension was allowed to stir for 3 hr at -35° C, then 5 h at -20° C before stirring for a final 10 h at r.t. The suspension was then vacuum filtered and the sodium chloride salt was washed with 100 ml anhydrous ether. The filtrate was evaporated under a dry nitrogen atmosphere and reduced pressure (100 mm Hg) at room temperature. The purity of the crude residue was checked by ^1H and ^{31}P NMR (in benzene-d_6 solvent: chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, δ ^{31}P 168.0 ppm; N,N-diisopropylaminodithiomethoxyphosphine impurity, 3, δ 118.2 ppm). The chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, is relatively unstable and partially decomposes during purification by distillation, requiring high vacuum (b.p., 55-60° C/0.05 mm Hg). The above synthetic route gave ca. 95% 2 and less

than 5% of 3 and was therefore used for further reaction without purification.

The crude chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, can be stored at −18° C under an inert, dry atmosphere for at least several months without any decomposition. In contrast, the N,N-diisopropylaminodithiomethoxyphosphine, 3, undergoes a Michael-Arbuzov reaction to the extent of roughly 50% after 6 weeks at −18° C. However, a serious problem of all monofunctional phosphitylating agent such as chloro-N,N-dialkylaminomethoxyphosphine[4–6] and chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, is their sensitivity towards hydrolysis and air oxidation which requires careful handling.

Preparation of Deoxynucleoside Thioalkylphosphoramidite, 4. Chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, appears to be a promising reagent for the preparation of deoxynucleoside thioalkylphosphoramidites such as 4. Excess, very reactive, 2 (5.52 mmol) was added to a mixture of diisopropylethylamine (7.4 mmol), 5'-O-(dimethoxytrityl) protected thymidine (1.84 mmol) in 4 ml dichloromethane at r.t. The complete reaction course can be monitored by tlc (silica gel) and by ^{31}P NMR spectroscopy. Excess monochlorophosphine, 2, was consumed by injecting anhydrous methanol (0.2 ml), followed by 2 ml triethylamine in 40 ml ethyl acetate. The results indicate that chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, reacts similarly[7] to the corresponding chloro-N,N-diisopropylaminomethoxyphosphine and it reacts essentially quantitatively with the protected nucleosides in less than 15 m.

After completion of the reaction the mixture was transferred to a separatory funnel and diluted with ethyl acetate. After appropriate aqueous washing steps[7,8] and drying of the organic layer over magnesium sulfate the solvent was evaporated to a foam under reduced pressure. The residue was then taken up in a few ml of dichloromethane and precipitated in 500 ml of ether-n-hexane mixture (−78° C). The crude suspension is > 80% pure N,N-diisopropylamino-3'-O-(5'-O-(dimethoxytrityl)thymidine)thiomethoxyphosphine, 4 (55:45 diastereomeric mixture - the ^{31}P NMR spectrum of 4 shows two signals at 164.85 and 163.14 ppm corresponding to a diastereomeric mixture of the thiophosphoramidite). A small amount of deoxynucleoside methoxyphosphoramidite was found in the product mixture as identified by two ^{31}P signals at 149.80 and 149.14 ppm (1:1 diastereomeric ratio). The latter have identical ^{31}P chemical shifts with that of authentic samples of N,N-diisopropylamino-3'-O-(5'-O-(dimethoxytrityl)thymidine)methoxyphosphine prepared by Barone *et al.*[7] or according to the procedure of McBride and Caruthers.[9] The CI (70ev) MS of 4 shows a prominant pseudo-molecular ion peak at m/z 722 $(M+H)^+$. Additional intense ions are also observed at m/z 692 $(M\text{-}2\times CH_3 + H)^+$, 674 $(M - SCH_3)^+$ and 596 $(M-1 - \text{thyminyl})^+$. ^1H NMR (benzene-d_6, δ ppm) 1.0 (12H, d, $(CH_3)_2CH)_2N$), 1.55 (3H, s, thymine-CH_3), 2.27 (3H, d, CH_3S), 3.30 (6H, s, CH_3O), 3.52 (2H, m, 5',5"), 4.20 (1H, br. s, H4'), 4.78 (1H, br. s, H3'), 6.50 (1H, br. m, H1'), 7.20 (13H, m, aromatic).

In addition to the major peaks assigned to 4, there are some minor ^{31}P peaks at 146.3 ppm and 13.2 ppm, which are assigned to the 3'-3' nucleoside dimer and a phosphoamidous acid, respectively (literature ^{31}P chemical shift for phosphoamidous acids: 13.1 ppm[7]).

Preparation of 3'-Deoxynucleoside Dimethyl thiophosphite, 5. In order to determine the utility of the new monofunctional thiophosphoramidite reagent, 4, in oligonucleotide syntheses, we have initially investigated the condensation of 4 with methanol. Of the various weak acids proposed as potential activating agents by Beaucage and Caruthers[5] 1H-tetrazole appears to be most effective. The reaction was monitored by ^{31}P NMR spectroscopy. Addition of excess methanol and 1H-tetrazole (0.26 mmol) to a benzene solution of 4 (0.035 mmol) at r.t. resulted in the complete disappearance over several hours of the two ^{31}P signals at 164.9 and 163.1 ppm, which are replaced by two new

signals at 193.0, 192.8 ppm (55:45 ratio). These signals are assigned to a R_P and S_P diasteromeric mixture of 3'-O-methoxythiomethoxyphosphine derivative of 5'-O-(dimethoxytrityl)thymidine, 5.

Preliminary Preparation of Dithio Phosphate Analogues. Oxidation of deoxynucleoside methylthiomethylphosphite, 5, by S_8/pyridine generated the dithioate triester analogue, 6 (Scheme 1). The dithioate ester, 6, serves as a model for the synthesis of 3',5'-dideoxynucleotide dithioate analogues. The ^{31}P NMR spectra of the reaction course clearly showed that the sulfurization reaction was rapid. After 10 min. at ambient temperature ca. 5% of unreacted thiophosphite, 5, was still present. The R_P and S_P diastereomeric mixture of dithioate triesters, 6, appears as a set of sharp ^{31}P NMR resonances at 92.62 and 92.11 ppm (benzene-d_6). This is similar to the ^{31}P chemical shift for authentic samples of dimethyl and diethyl dithiophosphoric acids (^{31}P δ (benzene-d_6), 90.05 and 90.67 ppm, respectively). Additional ^{31}P signals are observed at 70.97, 68.97, 68.38 ppm, and a number of peaks in the phosphate ester region around 0.0 ppm the latter presumably arising from desulfurization. The signals around 70 ppm are likely thionophosphate triesters, although they have not been identified. The dithiophosphate triester 6 was relatively stable in various solvents even at elevated temperatures.

We have also found that deoxythymidine thiophosphoramidite, 4, will readily couple with 3'-protected deoxythymidine to yield (after sulfurization) the 3',5'-dideoxythymidine methyl phosphorodithioate as two ^{31}P signals at 92.2 and 92.9 ppm. Demethylation yields the achiral 3',5'-dideoxythymidine phosphorodithioate (Farschtschi and Gorenstein, in preparation). In conclusion then, the present method should provide a general synthetic scheme for preparation of dithiophosphate analogues of deoxynucleotides. The synthesis of dithiophosphate deoxyoligonucleotide analogues on polymer support is also being explored.

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