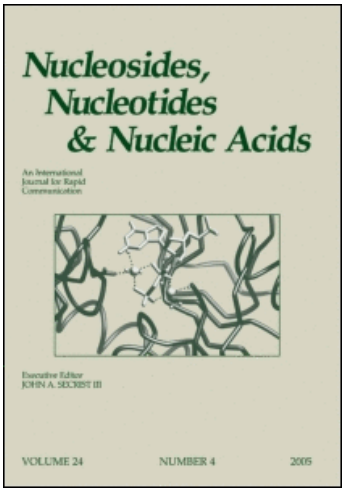

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ALLYL PROTECTION IN THE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATES†

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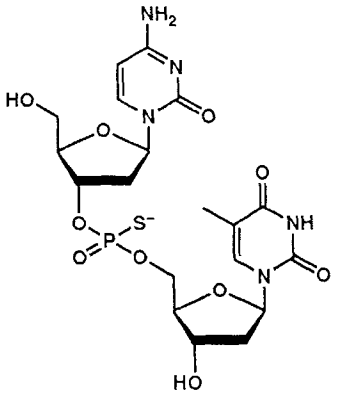
Abstract: Allyl and allyloxycarbonyl groups serve as useful protectors for internucleotide linkages and nucleoside bases, respectively, in the synthesis of partially or fully phosphorothioate-modified oligodeoxyribonucleotides.

Potent inhibition of HIV replication by certain oligodeoxyribonucleotide phosphorothioates[1)] has motivated chemists to develop efficient syntheses.[2)] Although mild deprotection conditions are crucial for attaining high synthetic efficiency, existing methods do not satisfy this requirement.[1-3)] The procedures usually use acyl protectors for nucleoside bases as the strong basic conditions needed for their removal often causes hydrolytic cleavage of the phosphate and phosphorothioate linkages. We describe here a convenient synthesis of phosphorothioate-incorporated oligodeoxyribonucleotides. The key procedure is the allylic protection aided by organotransition metal chemistry which can be used safely for sulfur-containing compounds.[4)]

†This paper is dedicated to Dr. Morio Ikehara, Emeritus Professor of Osaka University, in celebration of the 70th anniversary of his birth.

First, we examined the utility of the allyl protection in the solution-phase synthesis of the dinucleoside phosphorothioate **1**. The dimeric compound **2** (diastereomers) was prepared in 75% overall yield by 1*H*-tetrazole-promoted condensation of the allyl- and allyloxycarbonyl (AOC)-protected 2'-deoxycytidine 3'-phosphoramidite **3b**[5] and 5'-O-unprotected thymidine **4**, thioation of the resulting phosphite by S8 in the presence of pyridine,[3a] and detritylation using dichloroacetic acid. Brief treatment of **2** in dichloromethane with a mixture of 5 mol %/allyl of $Pd[P(C_6H_5)_3]_4$ and 3 mol %/allyl of $P(C_6H_5)_3$ in the presence of excess diethylammonium hydrogencarbonate[6] deblocked



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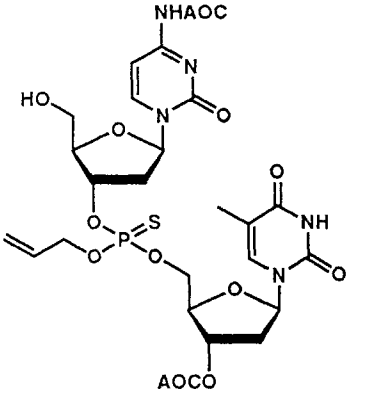

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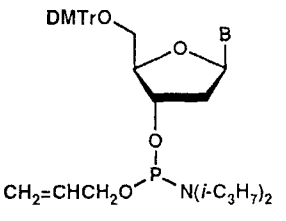
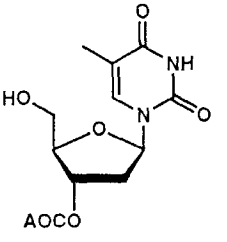


3a, B = Ad^{AOC}
3b, B = Cy^{AOC}
3c, B = Th



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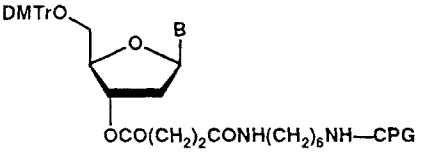


6a, B = Ad^{AOC}
6b, B = Cy^{AOC}
6c, B = Th

simultaneously the allyl and AOC protection[5)-7)] to afford quantitatively the desired phosphorothioate **1** as a ca. 1:1 diastereomeric mixture. The deprotection did not cleave the phosphorothioate bond. The crude product had an extremely high purity as shown by

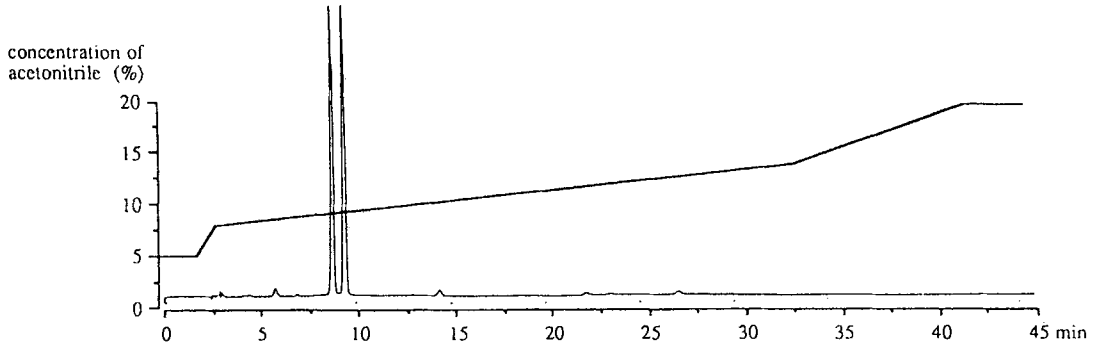
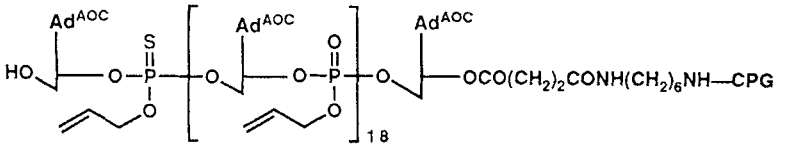


FIGURE 1. HPLC profile of the crude dinucleoside phosphorothioate **6** [column = ODS-7 (4.6-mm diameter, 250-mm length); flow rate = 1 mL/min; temperature = 40 °C].

HPLC analysis of Figure 1. The structure of **1** was confirmed by the ^{1}H- and ^{31}P-NMR spectra.

Allylic protection can be used effectively in the solid-phase synthesis of phosphorothioate-incorporated oligodeoxyribonucleotides via the phosphoramidite method. For instance, the monophosphorothioate-modified deoxyadenylyl homo-20mer, $5'Ap_S(Ap)_{18}A3'$ (**5**), where p_S refers to phosphorothioate, was prepared from the deoxyadenosine (**6a**) covalently attached to a controlled pore glass (CPG) support. Chain elongation was achieved by repetition of the following four reactions, (1) 1*H*-tetrazole promoted phosphitylation of the 5'-hydroxyl group with the phosphoramidite **3a**, (2) capping of the unreacted hydroxyl with acetic anhydride in the presence of 2,6-lutidine, (3) oxidation of the phosphite to the phosphate with *tert*-butyl hydroperoxide,[8] and (4) detritylation with dichloroacetic acid. After the final coupling, the phosphite was thioated by tetraethylthiuram disulfide (TETD),[9] to give the solidly-anchored, protected deoxyadenylyl homo-20mer **7** in 97% overall yield (trityl assay). The allyl and AOC protectors were removed while *on the solid support* by the $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$ catalyzed reaction in the presence of butylammonium formate, and finally, the deoxyadenylyl 20mer **5** was detached from the CPG support by treatment with conc ammonia. According to the HPLC analysis, no cleavage of the

internucleotide linkages had taken place in these operations. There was little palladium residue in the product as proved by atomic absorption spectroscopy. Partially and fully



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phosphorothioate-modified oligodeoxyribonucleotides, $^{5'}Tp_S(Tp)_{18}T^{3'}$, $^{5'}Cp_S(Cp)_{18}C^{3'}$, and $^{5'}(Cp_S)_{19}C^{3'}$, were prepared in a similar manner in 90, >99, and 77% overall coupling yields, respectively, starting from the CPG-bound nucleoside **6b** or **6c** using the phosphoramidite monomer **3b** or **3c**.

We have realized a convenient solid-phase synthesis of partially and fully phosphorothioate-incorporated oligodeoxyribonucleotides. The success is obviously based on the use of allylic protection.[7] The present synthesis has the advantage of mild deprotection conditions as well as operational simplicity in isolation of the oligodeoxyribonucleotides. The latter arises from the removal of all the protectors on the solid support.

Experimental Section

General. General experimental conditions and spectroscopic instrumentation are as described.[5] Chromatography was achieved on a column of E. Merck Kieselgel 60 (70—230 mesh) using the stated solvent(s) as eluent. The phosphoramidites **3a—3c** were prepared according to the known procedure.[5]

Synthesis of the Protected Dinucleoside Phosphorothioate 2. A mixture of the 5'-O-unprotected thymidine **4** (310 mg, 0.95 mmol), the cytidine 3'-phosphoramidite **3b** (1.24 g, 1.48 mmol), and 1*H*-tetrazole (536 mg, 7.65 mmol) in acetonitrile (13 mL) containing THF (1 mL) was stirred under argon at room temperature

for 2 h. The reaction mixture was poured into a sodium hydrogencarbonate-saturated solution (50 mL) and extracted with dichloromethane (20 mL x 2). The combined organic extracts were washed with brine (50 mL x 2). After drying the organic solution was concentrated to give a viscous oil, which was subjected to short column chromatography on silica gel with a 5:1 mixture of ethyl acetate and hexane containing a small amount of triethylamine as an eluent to afford a colorless foam (959 mg). To a solution of the foamy product (861 mg) in pyridine (4 mL) was added a 0.6 M solution of S_8 in a 3:4 mixture of pyridine and carbon disulfide (14 mL, 8.3 mmol) and the resulting mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated in vacuo and the resulting residue was treated with ethyl acetate (10 mL). The insoluble material was removed by filtration and the filtrate was concentrated to give a foam. This crude product was chromatographed on a short silica gel column using a 5:1 mixture of ethyl acetate and hexane containing a trace amount of triethylamine to afford yellow foam (749 mg). The chromatographed material (600 mg) was dissolved in dichloromethane (15 mL) and dichloroacetic acid (1.0 mL, 1.5 g, 12 mmol) was added. After stirring at room temperature for 10 min, the reaction mixture was poured into a sodium hydrogencarbonate-saturated solution (100 mL) and extracted with dichloromethane (50 mL x 2). The collected organic extracts were washed with a sodium hydrogencarbonate solution (50 mL) and brine (50 mL x 2), dried, and evaporated. The residue was subjected to silica gel column chromatography using a 5:1 ethyl acetate–hexane mixture followed by a 1:10 methanol–dichloromethane mixture as eluents to afford a diastereomeric mixture of the protected dinucleoside phosphorothioate **2** (408 mg, 75% overall yield). ^{1}H NMR ($CDCl_3$) δ 1.94 and 1.95 (two s's, 3 H, CH_3), 2.25—2.71 (m, 4 H, 4 H-2'), 3.55 (br s, 1 H, 5'-OH), 3.77—4.01 (m, 2 H, 2 H-5' of Cy), 4.23—4.45 (m, 5 H, 2 H-5' of Th, H-4', and CH_2=CHC*H*$_2$), 4.52—4.76 (m, 6 H, H-3', H-4', and 2 CH_2=CHC*H*$_2$), 5.14—5.46 (m, 7 H, H-3' and 3 C*H*$_2$=CHCH$_2$), 5.83—6.02 (m, 3 H, 3 CH_2=C*H*CH$_2$), 6.21 (t, 1 H, J = 6.9 Hz, H-1'), 6.29—6.40 (m, 1 H, H-1'), 7.24 (d, 1 H, J = 7.4 Hz, H-5 of Cy), 7.41 and 7.42 (two s's, 1 H, H-6 of Th), 8.22 (d, 1 H, J = 7.4 Hz, H-6 of Cy), 8.22

(br s, 1 H, NHCO), 9.33 ppm (br s, 1 H, NHCO); ^{31}P NMR ($CDCl_3$) δ 68.3 and 68.6 ppm.

Deprotection of 2 Affording dCp_sT (1). To a heterogeneous mixture of **2** (127 mg, 159 μmol) and diethylammonium hydrogencarbonate (404 mg, 2.99 mmol) in dichloromethane (3 mL) was added with stirring a solution of $Pd[P(C_6H_5)_3]_4$ (42.7 mg, 37.0 μmol) and $P(C_6H_5)_3$ (7.7 mg, 29.4 μmol) in dichloromethane (5 mL) and stirring was continued at room temperature for 1 h. The reaction was quenched with water (15 mL) and the resulting aqueous suspension was washed with dichloromethane (30 mL x 2). The organic layers were extracted with water (20 mL x 2). The combined aqueous extracts were diluted with ethanol (150 mL) and concentrated at 60 °C under reduced pressure. The resulting viscous residue was dissolved in 95% ethanol (2 mL) and the solution was subjected to column chromatography on cellulose (25 g). Elution with 95% ethanol gave diethylammonium salts of **1** (98 mg, 99% yield) as a pale yellow amorphous solid. ^{1}H NMR (D_2O) δ 1.19 [t, 6 H, J = 6.9 Hz, $(CH_3CH_2)_2NH_2^+$], 1.92 and 1.95 (two s's, 3 H, CH_3 of Th), 2.31—2.43 (m, 3 H, 3 H-2'), 2.60 (m, 1 H, H-2'), 3.66 [q, 4 H, J = 6.9 Hz, $(CH_3CH_2)_2NH_2^+$], 3.73—3.94 (m, 2 H, 2 H-5'), 4.08—4.26 (m, 4 H, 2 H-4' and 2 H-5'), 4.59 (m, 1 H, H-3' of Th), 4.87 (m, 1 H, H-3' of Cy), 5.99 and 6.00 (two d's, 1 H, J = 7.6 Hz, H-5 of Cy), 6.19 (m, 1 H, H-1'), 6.32 (t, 1 H, J = 6.6 Hz, H-1'), 7.73 (br s, 1 H, H-6 of Th), 7.84 ppm (d, 1 H, J = 7.6 Hz, H-6 of Cy); ^{31}P NMR (D_2O) δ 55.6 and 56.2 ppm.

Preparation of Protected Deoxyadenosine 6a and Deoxycytidine 6c Attached to CPG Support. To a solution of N^6-allyloxycarbonyl-5'-*O*-*p*,*p*'-dimethoxytrityl-2'-deoxyadenosine (328 mg, 514 μmol) in pyridine (5 mL) were added succinic anhydride (86.2 mg, 861 μmol) and 4-dimethylaminopyridine (96.5 mg, 790 μmol). The mixture was stirred at room temperature for 12 h and then evaporated in vacuo. The resulting residual oil was dissolved in dichloromethane (100 mL) and the solution was washed with an aqueous solution saturated with ammonium chloride (50 mL x 2) and brine (50 mL x 2). Concentration of the organic solution gave a viscous liquid,

which was subjected to silica gel column chromatography with a 5:1 mixture of ethyl acetate and hexane followed by a 1:10 mixture of methanol and dichloromethane containing a small amount of triethylamine to give triethylammonium N^6-allyloxycarbonyl-5'-*O*-*p*,*p*'-dimethoxytrityl-2'-deoxyadenosine 3'-succinate (382 mg, 89% yield). IR (KBr) 3430, 2940, 1740, 1610, 1510, 1250, 1210 cm^{-1}; UV (CH_3OH) 268 (ε 19600) and 236 (24200) nm; ^1H NMR ($CDCl_3$) δ 1.29 [t, 9 H, *J* = 7.3 Hz, ($CH_3CH_2)_3NH^+$], 2.58—2.69 (m, 5 H, CH_2CH_2 and H-2'), 2.83—3.08 [m, 7 H, ($CH_3CH_2)_3NH^+$ and H-2'], 3.43 (d, 2 H, *J* = 3.6 Hz, 2 H-5'), 3.77 (s, 6 H, 2 CH_3O of DMTr), 4.29—4.38 (m, 1 H, H-4'), 4.76 (dt, 2 H, *J* = 5.9 and 1.0 Hz, CH_2=CHCH_2), 5.29 (dd, 1 H, *J* = 1.3 and 10.6 Hz, *cis*-CH_2=$CHCH_2$), 5.42 (ddd, 1 H, *J* = 1.7, 3.0, and 17.2 Hz, *trans*-CH_2=$CHCH_2$), 5.53—5.55 (m, 1 H, H-3'), 6.00 (ddt, 1 H, *J* = 10.6, 17.2, and 5.9 Hz, CH_2=C*H*CH_2), 6.48 (dd, 1 H, J = 5.6 and 8.6 Hz, H-1'), 6.73—6.82 (m, 4 H, ortho protons to CH_3O of DMTr), 7.18—7.41 (m, 9 H, aromatic protons of DMTr), 8.13 (s, 1 H, H-2), 8.68 ppm (s, 1 H, H-8).

A mixture of the protected deoxyadenosine 3'-succinate (233 mg, 278 μmol), 1-hydroxybenzotriazole (94.6 mg, 700 μmol), DCC (71.9 mg, 349 μmol), and CPG (1.30 g) was shaken until the supernatant became homogeneous. The reaction mixture was allowed to stand at 30 °C for 2 days. The solid was collected by filtration and washed with methanol (50 mL) followed by ethyl acetate (50 mL), dried in vacuo, and then treated with phenyl isocyanate (1.3 mL) in pyridine (6 mL) at 30 °C for 3 h. The reaction mixture was passed through a glass filter. The collected solid was washed with methanol (50 mL) and hot ethyl acetate (150 mL) and then dried in vacuo at room temperature. The usual trityl assay indicated that the solid contained 26.4 μmol/g of the protected deoxyadenosine.

In a similar way, 21.0 μmol/g of the CPG-anchored deoxycytidine **6b** was prepared.

Typical Procedure for Solid-Phase Synthesis of Oligodeoxyribonucleotide Phosphorothioates. The protected oligodeoxyribonucleotide phosphorothioates bound on the CPG support was prepared on a 1-μmol scale according to

the procedure described previously,[3] where *tert*-butyl hydroperoxide and TETD were employed for the oxidation and thioation of the phosphites, respectively. After washing with THF (1.0 mL) and drying in vacuo, the CPG beads were added to a solution of $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$ (100 mg, 96.6 μmol, 2.5 equiv/allyl) and $P(C_6H_5)_3$ (256 mg, 0.97 mmol, 25 equiv/allyl) in a 1.2 M butylammonium formate–THF solution (4.9 mL, 150 equiv/allyl), and the mixture was allowed to stand at 50 °C for 1 h. After the supernatant fluid was decanted, the glass beads were washed successively with THF (1 mL x 2), acetone, (1 mL x 2), a 0.1 M sodium *N*,*N*-diethyldithiocarbamate (ddtc) aqueous solution (1 mL, 15 min), acetone (1 mL x 3), and water (1 mL x 2). The ddtc washing was repeated once more. The CPG supports were treated with concentrated ammonia (1.5 mL) at 25 °C for 30 min. The CPG beads were removed by decantation and washed with water (1.5 mL). The supernatant fluids and washings were combined and concentrated under reduced pressure to give the oligodeoxyribonucleotide phosphorothioate.

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