Stereospecific Grignard-Activated Solid Phase Synthesis of DNA Methylphosphonate Dimers

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Stereoregular R_p or S_p DNA methylphosphonate dimers have been synthesized on a solid phase support. A deprotected 5′-hydroxyl-N^2-isobutanoyldeoxyguanosine 3′-*O*-succinate coupled to high-loaded polyethylene glycol (PEG) coated polystyrene beads (HLP) was activated with a Grignard reagent, *t*-BuMgCl. After activation was complete, a pure diastereoisomer of 5′-(dimethoxytrityl) *N*-benzoyldeoxynucleoside 3′-(*p*-nitrophenyl methylphosphonate) *p*-nitrophenyl ester (R_p or S_p) was added. Coupling of the activated 5′-hydroxyl to the 3′-methylphosphonate ensued, releasing nitrophenol, yielding the R_p or S_p dimer, respectively. The dimers were then cleaved from the solid support, deprotected, and purified, yielding methylphosphonate DNA dimers of defined stereochemistry.

Antisense DNA therapeutics show great potential for gene-specific, nontoxic therapy of a wide variety of diseases.[1] Uncharged oligodeoxynucleoside methylphosphonates are resistant to nucleases, are readily taken up by cells, and specifically inhibit expression of oncogenes or viral genes.[2] However, the racemic methylphosphonate oligomers show limited potency as antisense inhibitors of gene expression.[3] In an animal experiment, bolus administration of 50 mg/kg was necessary to obtain significant reduction of c-*myc* oncogene expression in the circulating cells of a transgenic mice.[4] We have observed that *all-R* oligomers hybridize much more strongly to a complementary target than do the racemic oligomers or *all-S* oligomers.[5] Therefore, it is likely that *all-R* oligomers will display significantly greater potency as antisense or antigene therapeutics than do racemic oligomers.[6]

When Stec and Lesnikowski[7] first published the Grignard coupling method, it was immediately discounted as not being applicable to solid phase synthesis. This opinion arose from the fact that the mixture of a Grignard reagent with an alcohol to be activated was typically a thick, viscous suspension or slurry. The solid supports available at that time were porous silica, controlled pore glass (CPG), or polystyrene beads or particles. The pores in these beads displayed average diameters of 50−100 nm. A successful coupling reaction requires rapid diffusion of the dissolved reactants into the pores of the support, and rapid diffusion of the products back out of the pores. This condition was met by phosphoramidite reactants, but not by Grignard intermediates. Successful synthesis of normal phosphodiester DNA by the phosphoramidite route on polyethylene glycol (PEG) polymers[8] led us to think that most of the benefits of solid phase synthesis could be achieved on a PEG support in solution, without suffering the consequences of slow diffusion of reactants in a Grignard reaction.[9] Although the PEG route was successful, losses of support occurred at each precipitation step. This meant that ultimate yields of oligomers with 10−20 residues would be vanishingly small, despite the success of stereospecific coupling.

To circumvent this problem, we sought a solid support without pores, reacting only on the surface. This would allow retention of the support on a filter, as is standard in solid phase synthesis, but avoid the problem of trying to carry out viscous coupling reactions inside narrow pores. Polystyrene beads covalently bonded with a surface layer of PEG, highly loaded with deoxynucleoside (HLP),[10] met all of these criteria. Stereospecific solid phase synthesis (Scheme 1) then became possible, with the Grignard reagent coupling method,[7] by combining it with PEG as a polymeric support,[9] and extending it to solid phase synthesis on HLP, as described below.

All reagents and anhydrous solvents, of the highest commercially available purity, were purchased from Aldrich or Baker and used without further purification. The S_p and R_p diastereomers of 5′-*O*-(dimethoxytrityl-(DMT))-*N*-benzoyldeoxynucleoside 3′-(*p*-nitrophenyl methylphosphonate) esters of deoxyadenosine (**1a**,**b**) were prepared as described.[9] N^2-Isobutanoyldeoxyguanosine−HLP (**2**)[10] was purchased from Perkin-Elmer/Applied Biosystems (No. 401178, 150 μmol/g). Reaction mixtures were analyzed by thin layer chromatography (TLC) on silica gel 60 F_{254} plates (E. Merck), detected by absorption at 254 nm (Mineralight) or by orange DMT cation following Dische acid spray. Reaction products were purified by normal phase liquid chromatography on Chromatotron (Harrison Research) discs coated with silica 60 PF_{254} (No. 7749, E. Merck) eluted with steps of

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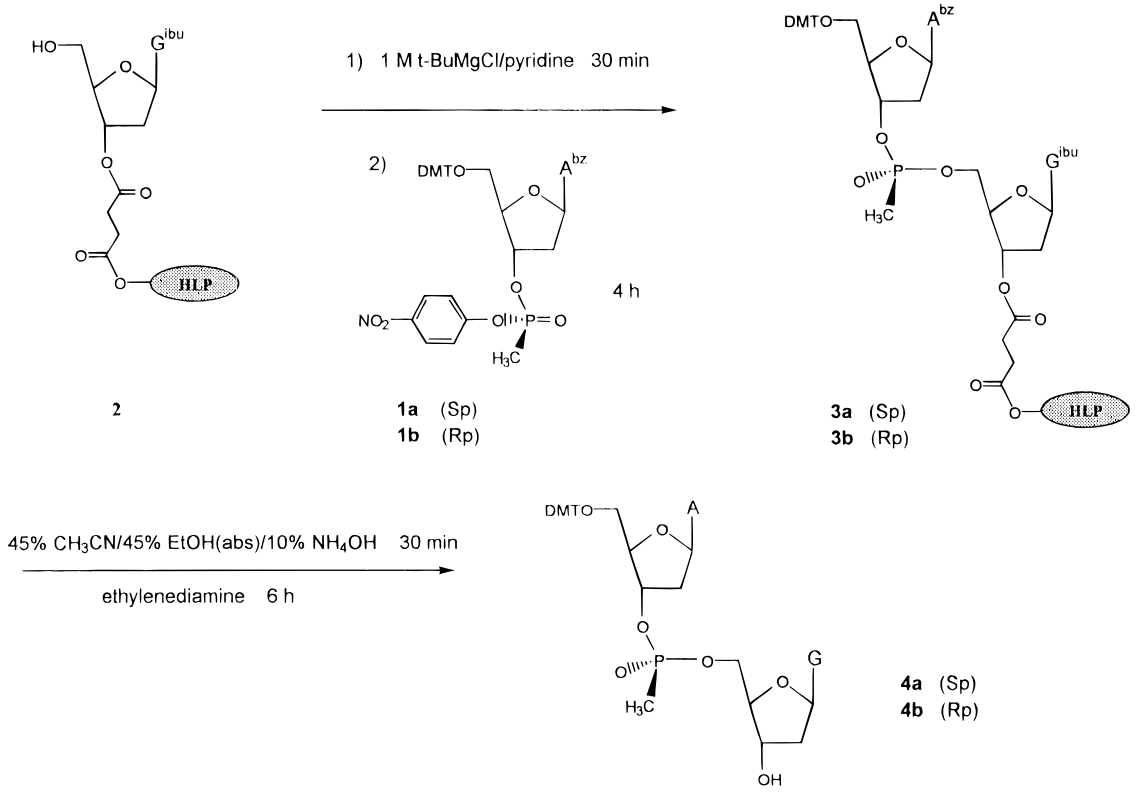
Scheme 1



increasing MeOH in $CHCl_3$, or by high-performance liquid chromatography (HPLC) on an Econosphere silica column (Alltech, 5 μm, 10 mm × 25 cm) eluted at 5 mL/min with a 20 min gradient from 0 to 20% MeOH in $CHCl_3$, delivered by a Waters 600 liquid chromatograph. Matrix-assisted laser desorption−ionization/time of flight (MALDI/TOF) mass spectroscopy[11] from a sinapinic acid matrix was performed on a Hewlett-Packard LDI 1700 spectrometer. Nuclear magnetic resonance spectra in C^2HCl_3, using $(CH_3)_4Si$ as internal standard for ^1H, and dimethyl methylphosphonate (32.4 ppm from H_3PO_4) as external standard for ^{31}P, were obtained on a Bruker AMX 600 MHz spectrometer and compared with chemical shifts previously determined for all 32 dimer diastereomers.[12] Yield of coupling was determined by deprotecting a dried, weighed aliquot of support with 2% Cl_2CHCO_2H in CH_2Cl_2 and then measuring A_{498} of the bright orange DMT cation released, assuming a molar absorptivity of 7.0×10^4.

To prepare the stereospecific dimer DMT-dA(S_p)dG **4a**, HO-dG^{ibu}-HLP **2** (200 mg, 30 μmol) was suspended in 500 μL of anhydrous pyridine, and 150 μL of 1 M *t*-BuMgCl in dry THF (150 μmol, 5 equiv) was added. After 30 min, dA S_p isomer **1a** (45 mg, 52 μmol, 1.7 equiv) in 200 μL of anhydrous pyridine was added. The suspension was rocked for 4 h, until coupling was complete, as indicated by periodic TLC (ethyl acetate/acetone/H_2O, 10:5:1) of the solution phase, which showed that dA S_p isomer **1a** (R_f 0.75) was converted into a new product (R_f 0). The reaction was quenched by washing the support with Et_2O at 0 °C. Colorimetric assays of acid-released DMT cation from 5 mg of support after coupling yielded 155 and 160 μmol/g. Within the experimental limits of this analytical method, this result is indistinguishable from the dG-HLP manufacturer's indicated loading of 150 μmol/g. Thus it appears that coupling was stoichiometric, but within the accuracy of this analytical method applied to a single coupling, the yield could be as low as 95%.

In contrast, attempts to carry out the coupling reaction in THF or CH_3CN were unsuccessful. The HLP beads were not well suspended, coupling did not go to completion, and significant amounts of DMT-dA and DMT-dA methylphosphonate hydrolysis products appeared.

After synthesis, the dimer was cleaved[13] from 50 mg of support with 2 mL of 10% NH_4OH, and 45% EtOH, 45% CH_3CN for 30 min at room temperature, followed by 2 mL of ethylenediamine to remove base protecting groups, leaving the 5′-DMT group in place. The suspension was tumbled for 6 h and then diluted with 60 mL of water, neutralized with approximately 8 mL of 6 N HCl in 10% CH_3CN/H_2O, and the solvents were evaporated under reduced pressure. The beads were suspended in $CHCl_3$, filtered, and rinsed with 100 mL of $CHCl_3$, and the solvents were evaporated under reduced pressure to yield deprotected dimer. TLC ($CHCl_3$/MeOH, 90:10) revealed the desired S_p dimer **4a** (R_f 0.62), a trace of the R_p byproduct **4b** (R_f 0.71), and a trace of incompletely deprotected **4a** (R_f 0.43). HPLC (Figure 1) showed a main peak at 10.3 min, corresponding to the S_p dimer **4a**, a trace of the R_p byproduct **4b** at 9.6 min, and a small peak of incompletely deprotected **4a** at 12.8 min. Neither residual monomer **1a**, which elutes at 5.5 min, nor dG, which elutes at 18 min, was detected. The ratio of the areas under the S_p peak at 10.3 min and the R_p peak at 9.6 min indicated 96.2% S_p fraction, or a diastereomeric

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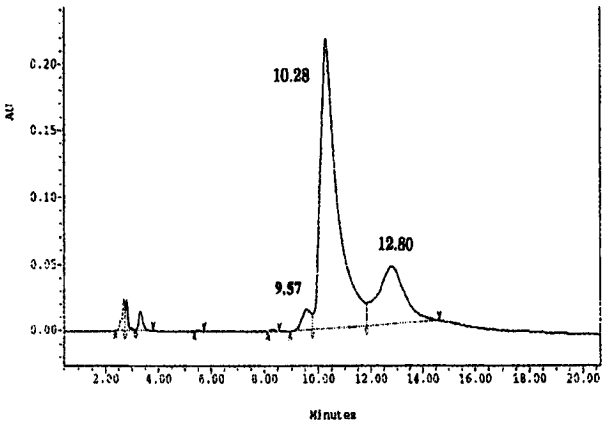

Figure 1. HPLC purification of base-deprotected DMT-dA-(Sp)dG **4a**.

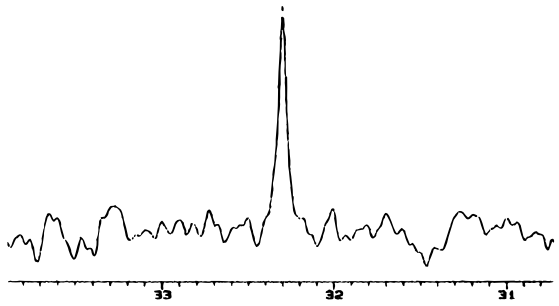

Figure 2. ^{31}P NMR spectrum of base-deprotected DMT-dA(Sp)dG in C^2HCl_3.

ratio of 25.1, implying virtually complete stereospecificity in coupling, as was observed previously on the PEG support in liquid phase.[9]

^{1}H NMR peaks in the purified S_p dimer **4a** fraction were assigned as follows: 1.48 (d, 3H, J_{P-CH3} = 18 Hz, P−CH_3), 2.34 (t, 1H, H2′), 2.39 (m, 1H, H2″), 2.45 (m, 1H, H2′), 2.51 (m, 1H, H2″), 2.79 (m, 1H, H5′), 3.09 (dt, 1H, H5″), 3.22 (dd, 1H, H5′), 3.29 (dd, 1H, H5″), 3.75 (2s, 6H, OCH_3 of DMT), 4.16 (m, 1H, H4′), 4.23 (m, 1H, H4′), 5.28 (m, 1H, H3′), 5.42 (m, 1H, H3′), 6.08 (dd, 1H, H1′), 6.23 (t, 1H, H1′), 6.80−7.35 (m, 13H, aromatic protons of DMT), 7.44 (s, 1H, H8 of dG), 7.68 (s, 1H, H2 of dA), 7.83 (s, 1H, H8 of dA), 9.31 (broad, 1H, NH_2), 9.67 (broad, 1H, NH_2). The ^{31}P NMR spectrum (Figure 2) displayed a single S_p dimer **4a** peak at 32.30 ppm, whereas the R_p dimer peak occurs 0.2 ppm upfield.[9,12] Following DMT removal, the mass spectrum displayed the expected hydrated dimer peak (calculated 596.5; found 588.6) with a small dG peak (calculated 266.2; found 267.5) and a possible broad dA methylphosphonate peak (calculated 347.3) (Figure 3).

The base-deprotected R_p dimer **4b** was prepared similarly, eluting at 9.6 min, displaying R_f 0.71, a single R_p dimer ^{31}P peak at 32.10 ppm, and an ^{1}H spectrum consistent with the previous assignments.[9] Colorimetric assays of acid-released DMT cation from 5 mg of support after coupling yielded 151 and 170 μmol/g. The fully deprotected dimer displayed the appropriate monohydrate mass (calculated 596.5; found 593.3), with a small dG peak (calculated 266.2; found 267.0), and a possible broad dA methylphosphonate peak (calculated 347.3).

Control of backbone stereochemistry represents one of the barriers to development of DNA therapeutics. We have developed a process for synthesizing dimers with either desired methylphosphonate diastereomer, R_p or S_p, on a solid phase support. This process differs from the method of Stec and Lesnikowski[7] in that synthesis was carried out on solid phase beads, suitable for automation, rather than in solution, where each intermediate length DNA must be purified chromatographically before proceeding to another coupling step. This laborious procedure was avoided by solid phase synthesis, which may be helpful for exploiting antisense therapeutic technology.

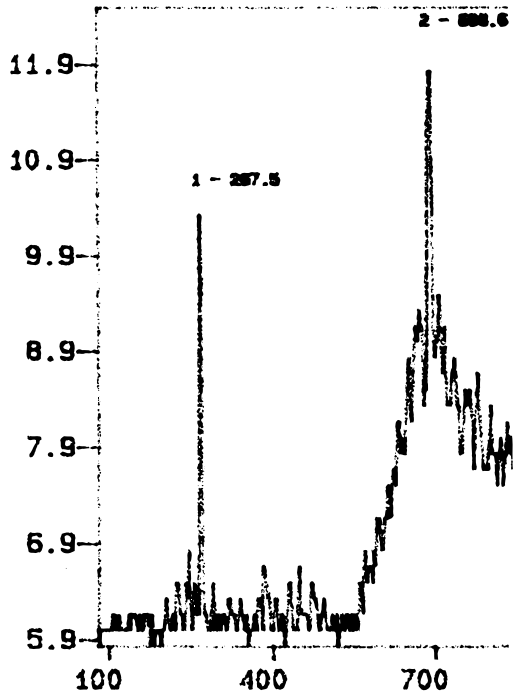

Figure 3. MALDI-TOF MS analysis of fully deprotected dA-(Sp)dG **4a**.

The observation of stereospecific methylphosphonate DNA coupling on HLP does not limit the method to the latter support. The PEG−polystyrene support should be considered only the first example of a nonporous support which allows reactions to occur entirely on the surface of the bead. Highly cross-linked, nonporous polymer beads with a reactive linker on the surface are the only theoretical criteria for supports for this method. The simplest linker for DNA synthesis to date is the long chain alkyl amine. Polymers which are most commonly formulated into cross-linked beads include polystyrene, poly(alkyl methacrylate) (acrylics), and latex. Although we used the same Grignard reagent as Stec and Lesnikowski,[7] *tert*-butylmagnesium chloride, there is no obvious reason for the reaction to be limited to this particular reagent. Finally, the Grignard method does not limit itself to methylphosphonates but may be applied equally well to phosphorothioates, which have been prepared stereospecifically in solution,[14] and to boranophosphonates, which have only been prepared racemically, in solution.[15]

It would be desirable to repeat the cycle as many times as desired, with whatever base is desired at each position, until an oligomer of a particular sequence is completed. Studies are now underway to extend the HLP synthetic route to longer oligomers, and to automate the synthesis. With solid phase stereospecific synthesis under control,

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it will be possible to prepare stereoregular antisense DNA methylphosphonates, and chimeras, for studies of biochemical efficacy, potency in cell culture, and preclinical animal trials.

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