Pergamon

Tetrahedron Letters, Vol. 35, No. 51, pp. 9525-9528, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $7.00+0.00

0040-4039(94)02207-0

Stereospecific Grignard Activated Coupling of a Deoxynucleoside Methylphosphonate on a Polyethylene Glycol Support

Christine Le Bec and Eric Wickstrom*

Department of Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 USA

Abstract: Stereospecific synthesis of Rp and Sp methylphosphonate diastereoisomers of d(A*p*G) was carried out on a polymeric support, polyethylene glycol (PEG), by the Grignard reagent solution route described by Stec. The coupling yield on PEG was higher than that observed in solution, and precipitation of the PEG at each step allowed easy separation of oligomer product from soluble reactants at each step.

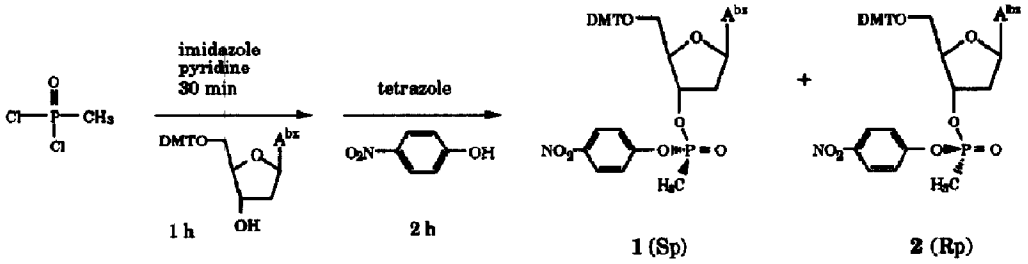
Oligodeoxynucleoside methylphosphonates (MP) display significant potential for antisense DNA therapy.[1] Spectroscopic, thermodynamic, and theoretical analyses imply that their potency as antisense inhibitors might be elevated if all of the chiral phosphorus linkages were coupled in the Rp, or equatorial, configuration.[2] Unfortunately, current methods for automatic synthesis of MP oligomers, or any other chiral modified phosphate, yield a racemic mixture.[3] However, Stec and his colleagues have described a pentavalent route to synthesize stereoregular MP linkages in solution by coupling with a Grignard reagent.[4] This procedure has been used to synthesize stereospecifically the four different homodimers, trithymidylate, and tetrathymidylate, but coupling yields were never greater than 65%, and each step requires chromatographic purification of the MP product. Nevertheless, when two stereoregular MP tetrathymidylates were joined racemically to yield MP octathymidylates with only one central racemic linkage, it was observed that the Rp-enriched MP octamer hybridized to poly(dA) more strongly than the normal phosphodiester octamer, or the racemic MP octamer, or the Sp-enriched MP octamer.[5]

Recently, it has been found that a MP heptamer of heterogeneous sequence, d(C*p*C*p*A*p*A*p*C*p*C*p*A), when prepared exclusively with Rp linkages, hybridized much more strongly to complementary DNA than did the normal phosphodiester heptamer, or the racemic MP heptamer, or the all-Sp heptamer.[6] These stereoregular sequences were prepared by racemic couplings, followed by chromatographic separation of the diastereoisomers at each step.[7] Unfortunately, this laborious method has such low yields that it cannot be applied for a routine synthesis of MP oligomers.

While the Grignard route[4] looked attractive, it seemed unlikely that the reactants could diffuse easily into and out of the pores of a controlled pore glass or porous polystyrene support, in view of the high viscosity of the Grignard-activated solution. However, Bonora, et al.[8] recently reported phosphodiester synthesis in which the 3' deoxynucleoside was linked to polyethylene glycol monomethyl ether (PEG) as a soluble polymeric support. This allowed quantitative coupling in a homogenous solution, while allowing separation of the growing oligomer from excess reactants by precipitation of the support at each step in the synthesis. Hence, we attempted a stereospecific synthesis of a MP heterodimer by the Grignard route[4] on a PEG support.[8]

The first step was to synthesize and purify each diastereoisomer of the protected-deoxynucleoside-3'-(4-nitrophenyl)methylphosphonate which will then be coupled with the 5'-OH of the PEG-bound oligomer, after

activation by the Grignard reagent (Scheme 1). 5'-O-Dimethoxytrityl (DMT)-N-benzoyl-deoxyadenosine (1 eq) was phosphorylated in pyridine by methylphosphonic dichloride (1 eq), preactivated with imidazole (5 eq) and after 60 min, tetrazole (3.5 eq) and 4-nitrophenol (1.1 eq) were added to produce the racemic mixture of **1** and **2**.[9] After separation by liquid chromatography in CH_2Cl_2/MeOH (97:3) on silica gel, the faster eluting compound was assigned to the Sp-isomer **1** (32% yield) and the slower eluting compound to the Rp-isomer **2** (25% yield), as described.[4]

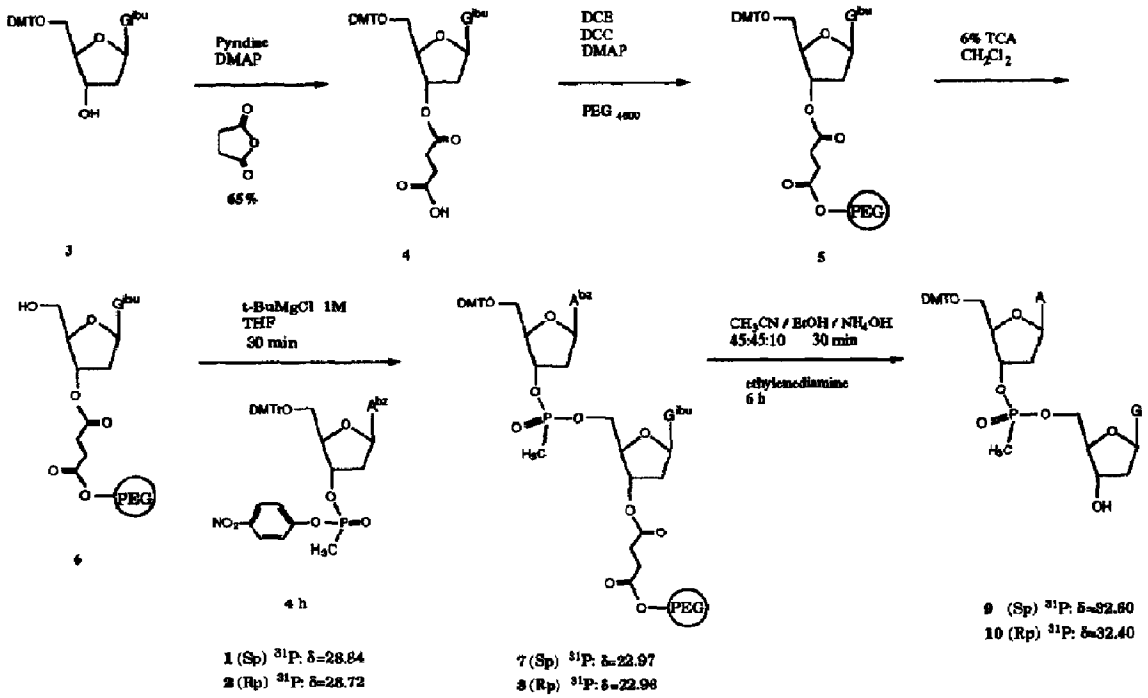


Scheme 1

For Sp-isomer **1**, TLC on silica gel developed with CH_2Cl_2/MeOH (92.5:7.5) showed Rf=0.69, analytical silica gel HPLC with a 20 min gradient of 0-10% MeOH in $CHCl_3$ showed Rt=6.35 min, and ^{31}P NMR ($CDCl_3$) showed a shift of 28.84 ppm. For Rp-isomer **2**, TLC showed Rf = 0.61, HPLC showed Rt = 7.67 min, ^{31}P NMR showed a shift of 28.72 ppm, and ^1H NMR ($CDCl_3$) yielded peaks at 1.82 (d, 3H, P-CH_3); 2.85 (dd, 1H, H2'); 3.15 (d, 1H, H2"); 3.33 (dd, 1H, H5'); 3.47 (dd, 1H, H5"); 3.80 (2s, 6H, OCH_3); 4.36 (m, 1H, H4'); 5.33 (m, 1H, H3'); 6.58 (t, 1H, H1'); 6.79-7.64 (m, 13H, aromatic protons of DMT); 8.06 (d, 2H, protons of 4-nitrophenyl); 8.18 (d, 2H, protons of 4-nitrophenyl); 8.74 (s, 1H, H8); 9.23 (s, 1H, H2).

The 3'-hydroxyl of 5'-DMT-2'-deoxyguanosine **3** was anchored to the PEG support via a succinyl linker according to the method described for oligonucleotide synthesis (Scheme 2).[8] Reaction of **3** (1 eq) with succinic anhydride (2.1 eq) in presence of dimethylaminopyridine (DMAP) (1 eq) in pyridine produced **4** in 65% yield. Then, the succinyl derivative **4** with dicyclohexylcarbodiimide (0.5 eq) and DMAP (0.5 eq) in 1,2-dichloroethane and coupled onto the PEG_{4600} support (0.4 eq of free OH-groups). Excess **4** was eliminated by precipitation of the support with Et_2O and absolute EtOH. Reaction yield was determined by measuring DMT absorption (504 nm); the loading of PEG-bound deoxyguanosine **5** corresponded to 140 μmol/g. The unreacted hydroxyl groups of the resin were acetylated with 10% Ac_2O, 10% 2,6-lutidine and 10% N-methylimidazole in CH_3CN. Finally, the DMT protecting group of **5** was removed at 0°C with 6% trichloroacetic acid in CH_2Cl_2.

To achieve stereospecific coupling, the 5'-OH of deoxyguanosine linked to the PEG support **6** (1 eq) in pyridine was activated with 4 eq of t-BuMgCl in THF. After 30 min, Sp-isomer **1** or Rp-isomer **2** (1.05 eq) in THF was added. Coupling was monitored by TLC on silica developed with EtOAc/acetone/H_2O (10:5:1). After 3 hours, the reaction yielded DMT-dA^{bz}pdG^{ibu}-PEG **7** (Sp) or **8** (Rp) (Rf=0), respectively. After 4 hours, the support was precipitated with a two-fold excess of Et_2O at 0°C, filtered, and rinsed with Et_2O to give a yellow gum, which was dissolved in $CDCl_3$ and analyzed by ^{31}P NMR (Fig. 1). The fully protected d(ApG)-PEG Sp-isomer **7** displayed a peak at 22.97 ppm, while the Rp-isomer **8** showed a peak at 22.96 ppm.



Scheme 2

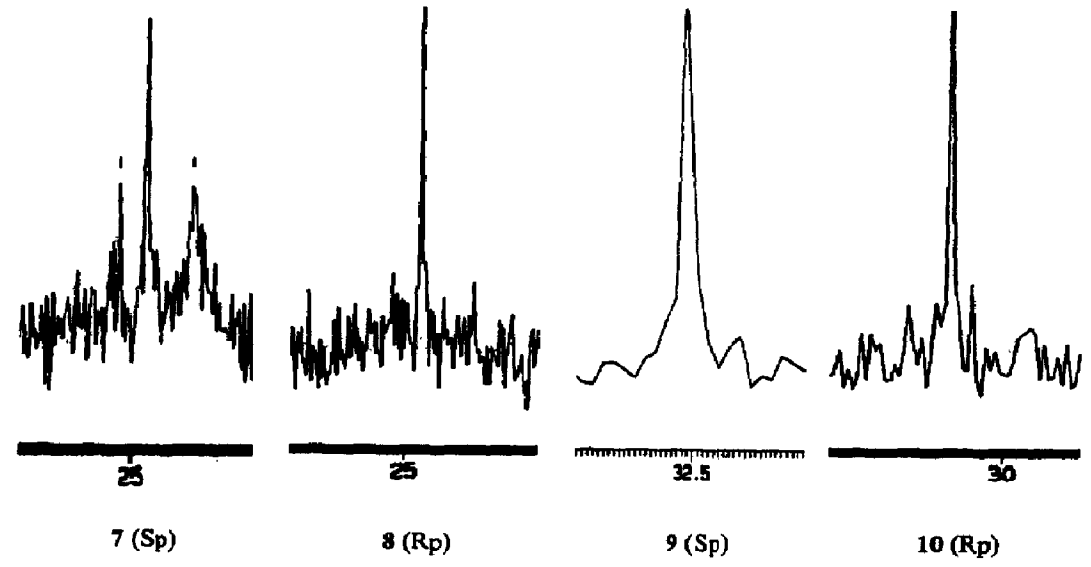


Figure 1: ^{31}P NMR of d(A*p*G) before deprotection **7** (Sp) and **8** (Rp), and after deprotection **9** (Sp) and **10** (Rp). The spectra were measured in $CDCl_3$ using dimethyl methylphosphonate as external standard (32.4 ppm from H_3PO_4).

To confirm the stereospecificity of the coupling reactions, the products **7** and **8** were characterized after cleavage from the support with dilute ammonium hydroxide and removal of the protecting groups by treatment with ethylenediamine for 6 hours.[10] The resulting products were analyzed by silica gel HPLC with a 20 min gradient of 0-20% MeOH in $CHCl_3$ and ^{31}P NMR (Fig. 1). d(A*p*G) Sp-isomer **9** displayed Rt = 8.20 min, and a chemical shift of 32.60 ppm; the Rp-isomer **10** showed Rt = 7.40 min, and a ^{31}P shift of 32.40 ppm.

The yield of the coupling was determined by measuring the DMT absorption at 504 nm (100 μmol/g, 71%), while the yield of multiple precipitations of PEG was 85%. The reaction was carried out in solvents other than THF, such as CH_3CN, DMF, and pyridine. In the latter three solvents, coupling was slow, with very little yield by 3 hours, and concomitant racemization of the monomer. Therefore, the degree of stereospecificity was very dependent upon the solvent used. It has already been pointed out that the coupling reaction must be fast to avoid P-epimerization of the monomer, which results in racemization of the dimer.[11]

Easy purification, stereospecific coupling and high yields are fundamental necessities for synthesis of therapeutically useful MP oligomers. PEG-supported Grignard-activated coupling can be repeated until the required length of the MP oligomer with defined stereochemistry is obtained. It would be desirable to improve the precipitation and coupling yields further. Despite the difficulties presented by viscosity in this route, it would be even better to identify a solid support which would allow stereospecific automated solid phase synthesis.

Acknowledgments: We thank Dr. Jason Rife for useful discussions. This work was supported by US NIH grant CA60139 to E. W.

References:

1. Miller, P.S., *Oligodeoxynucleotides: Antisense inhibitors of gene expression*, Cohen, J. S. (ed), CRC Press, Boca Raton FL, **1989**, pp. 79-95.
2. Wickstrom, E. *Gene Regulation: Biology of Antisense RNA and DNA*, Erickson, R. P., and Izant, J. G., (eds.), Raven Press, New York, **1992**, pp. 119-132.
3. Knorre, D. G.; Vlassov, V. V.; Zarytova, V. F.; Lebedev, A. V. and Fedorova, O.S. *Design and Targeted Reactions of Oligonucleotide Derivatives*, CRC Press, Boca Raton FL, **1994**.
4. Stec, W. J. and Lesnikowski, Z. J. *Methods in Molecular Biology*, Vol. 20, *Protocols for Oligonucleotides and Their Analogs, Synthesis and Properties*, Agrawal S. (ed.), Humana Press, Totowa NJ, **1993**, *Vol. 20*, pp 285-313.
5. Lesnikowski, Z. J., Jaworska, M., and Stec, W. J. *Nucleic Acids Res.*, **1990**, *18*, 2109-2115.
6. Vyazovkina, E. V.; Savchenko, E. V.; Lokhov, S. G.; Engels, J. W.; Wickstrom, E. and Lebedev, A. V. *Nucleic Acid Res.* **1994**, *22*, 2404 -2409.
7. Vyazovkina, E. V.; Rife, J. P.; Lebedev, A. V.; Wickstrom, E. *Nucleic Acid Res.* **1993**, *21*, 5957-5963.
8. Bonora, G. M.; Biancotto, G.; Maffini, M. and Scremin, C. L. *Nucleic Acids Res.* **1993**, *21*, 1213-1217.
9. Miller, P.S.; Agris, C. H.; Blandin, M.; Murakami, A.; Reddy, P. M.; Spitz, S.A.; Ts'o, P. O. P. *Nucleic Acid Res.* **1983**, *11*, 5189-5204.
10. Hogrefe, R. I.; Reynolds, M. A.; Vaghefi, M. M.; Young, K. M.; Riley, T. A.; Klem, R. E. and Arnold, L. J. Jr. *Methods in Molecular Biology*, Vol. 20, *Protocols for Oligonucleotides and Their Analogs, Synthesis and Properties*, Agrawal S. (ed.) Humana Press, Totowa NJ, **1993**, *Vol. 20*, pp 143-164.
11. Lesnikowski, Z. J.; Wolkanin, P. J.; Stec, W. J. *Tetrahedron Lett.*, **1987**, *28*, 5535-5538.

(*Received in USA* 31 *August* 1994; *revised* 2 *November* 1994; *accepted* 3 *November* 1994)