Synthesis of Stereochemically Homogeneous Oligoribonucleoside All-R_P-Phosphorothioates by Combining H-Phosphonate Chemistry and Enzymatic Digestion

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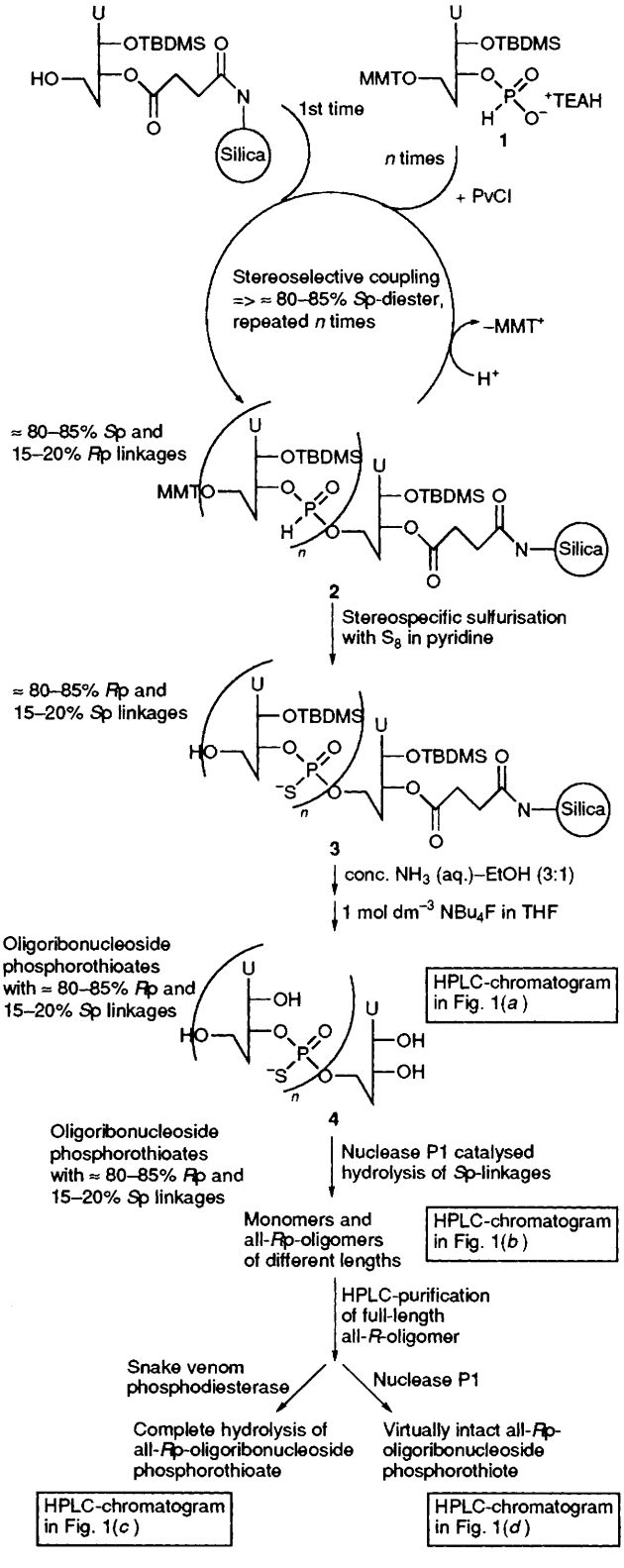
Oligouridine all-R_P-phosphorothioates were synthesized using the H-phosphonate approach followed by sulfurisation with S_8 and treatment with nuclease P1.

The interest in modified oligonucleotides has boomed over the last few years not least because of the potential of 'antisense' oligonucleotides as therapeutic agents for diseases such as cancer and AIDS. The kind of modified oligonucleotide that has been most widely used over the years is when the phosphodiesters are replaced by chiral phosphorothioate linkages. Despite this type of oligomer being one of the most explored it is only recently that it has been possible to obtain homogeneous stereochemistry around the phosphorus centres in chemically synthesised oligodeoxyribonucleoside phosphorothioates.[1] For RNA-fragments even less has been achieved and only dimers and trimers have been made in stereocontrolled reactions.[2,3] Herein we report on a method that so far has produced up to 12 units long, stereochemically homogeneous, oligoribonucleoside all-R_P-phosphorothioates.

We recently reported that condensation of a 2′,3′-*O*-protected nucleoside with a 5′-*O*-protected 2′-*O*-*tert*-butyldimethylsilylribonucleoside 3′-*H*-phosphonate is a stereoselective reaction.[2] About 80–85% of the reaction produces the S_P-diastereoisomer. It was also found that, with elemental sulfur, conversion of the diribonucleoside H-phosphonate to the phosphorothioate is stereospecific.[2] Taking advantage of these two observations we decided to explore the possibility of developing a method for synthesis of oligoribonucleoside phosphorothioates with homogeneous stereochemistry around the phosphorus centres.

When using the H-phosphonate approach to RNA-synthesis[4] oligomers containing H-phosphonate linkages in between each nucleoside are produced. These linkages can be sulfurised to produce oligoribonucleoside phophorothioates.[5] We have synthesizes oligouridine H-phosphonates (**2**, 4-, 8- and 12-mers, n = 3,7,11) using 5′-*O*-(4-monomethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyluridine 3′-H-phosphonate (**1**) (Scheme 1) and a standard protocol for the H-phosphonate approach to RNA-synthesis.[6] After completion of the elongation cycles the oligomers were treated with 0.055 mol dm^{-3} S_8 in pyridine for 15–17 h to produce the support-bound protected oligoribonucleoside phosphorothioate **3**. The oligomers were released from the support with 32% ammonia (aq)–ethanol (3:1) and the *tert*-butyldimethylsilyl (TBDMS) protection was removed with 1 mol dm^{-3} tetrabutylammonium fluoride in THF to produce the fully deprotected oligomers **4**. Postsynthetic procedures involved exchange of tetrabutylammonium ions for sodium and desalting as described previously.[6]

As mentioned above, 80–85% of the H-phosphonate linkages will have the S_P-configuration, and consequently the same amount of R_P-phosphorothioates will be present after sulfurisation. After n number of condensations 2^n stereoisomeric compounds will then be produced but there will be a substantially larger number of R_P-linkages and 20–30% of an 8-mer should have phosphorothioates with only that stereochemistry. To separate the all-R_P-compound from the other isomers is not a trivial task since an 8-mer will consist of 128 compounds which have sufficiently similar physical properties to make their separation most difficult [Fig. 1(*a*)]. Cleavage of all S_P-linkages would leave us with a mixture of oligonucleo-



Scheme 1 Overall procedure for synthesis of oligoribonucleoside all-R_P-phosphorothioates

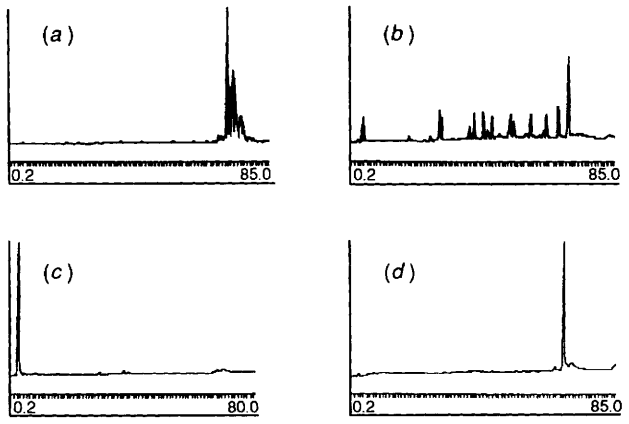


Fig. 1. Reversed-phase (C-18) HPLC profiles of (*a*) An isomeric mixture of oligoribonucleoside phosphorothioates **4** (from synthesis of 8-mer, $n = 7$, prepurified by HPLC to remove minor quantities of shorter fragments); (*b*) **4** treated with Nuclease P1 from *Penicillium citrum* for 24 h;[7] (*c*) isolated oligoribonucleoside all-R_P-phosphorothioate [last eluting compound in (*b*)] treated with snake venom phosphodiesterase (*Crotalus adamanteus*) for 7 h;[8] (*d*) Isolated oligoribonucleoside all-R_P-phosphorothioate retreated with Nuclease P1 for 7 h. Analysis was done using a Supelcosil LC-18 column (3 μm, 4.6 × 150 mm) and a linear gradient of 0–16.5% acetonitrile in 0.1 mol dm^{-3} triethylammonium acetate buffer (pH ≈ 6.5) during 100 min (flow rate = 1 ml min^{-1}). The horizontal scale refers to elution time in minutes.

tides having only R_P-linkages but different number of nucleotide units and thus separable. Fortunately the enzyme Nuclease P1 from *Penicillium citrum* preferentially catalyses the hydrolysis of S_P-phosphorothioate linkages.[7] By treating our isomeric mixture with this enzyme the only diester functions remaining will have the R_P-configuration and most importantly the full-length all-R_P-oligomer could be easily purified since it only had to be separated from shorter fragments [Fig. 1(*b*)]. The last eluting peak integrates as around 20% which is consistent with about 80% coupling selectivity. To check the stereochemistry of the isolated oligomer we subjected it to snake venom phosphodiesterase (SVPD, *Crotalus adamanteus*) an enzyme that catalyses hydrolysis of R_P-phosphorothioates.[8] As can be seen in Fig. 1(*c*) the oligomer was completely cleaved showing that all linkages indeed have the R_P configuration. Redigestion with Nuclease P1 left the oligomer virtually untouched [Fig. 1(*d*)] as it should. This is also evidence that the product contains little if any desulfurised linkages since these would be cleaved even faster than the S_P-phosphorothioate. We can thus produce 8–12 units long stereochemically homogeneous oligouridine all-R_P-phosphorothioates and we hope to increase the selectivity in the near future in order to obtain reasonable quantities of 15–20 mers.

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