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# Synthesis and cellular activity of stereochemically-pure 2'-*O*-(2-methoxyethyl)-phosphorothioate oligonucleotides

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**Stereochemically-pure 2'-O-(2-methoxyethyl)-phosphorothioate (PS-MOE) oligonucleotides were synthesized from new chiral oxazaphospholidine-containing nucleosides. Thermal stability studies showed that the incorporation of *R*p-PS linkages increased RNA-binding affinity. In cells, a full *R*p-PS-MOE splice-switching oligonucleotide targeting part of the ferrochelatase gene was more potent than its *S*p-PS counterpart, but of similar potency to the stereorandom PS-parent sequence.**

Antisense oligonucleotides (ASOs) are an emerging class of therapeutics that bind with high selectivity and affinity to their complementary RNA targets. They have demonstrated useful pharmacological activity after systemic delivery to a number of tissues *in vivo*, and are in clinical development[1]. Clinical ASOs are predominantly chemically modified to improve pharmacokinetic (PK) and pharmacodynamic (PD) properties. All have sulphur substitutions of non-bridging oxygens at each phosphodiester linkage, forming chiral phosphorothioate (PS) groups (*R*p and *S*p diastereomers) (**Fig. 1**; (**1**))[2]. Many are further modified with 2'-*O*-alkylated ribonucleosides, with the most prominent ribose modification being the 2'-*O*-(2-methoxyethyl) (MOE) group (**Fig. 1**; (**2**))[3]. Of note, more than 20 PS MOE-oligonucleotides are currently in clinical testing.

Currently, each PS oligonucleotide in clinical development is a large pool of diastereomers. Each diastereomer has unique structural and biochemical properties, which affect their PK and PD properties. For example, *S*p-PS linkages in an oligoribonucleotide (ORN) reduce its binding affinity with RNA, whereas *R*p-PS linkages are preferentially cleaved by some exonucleases in human plasma[4]. For these reasons and others, the stereocontrolled solid-phase synthesis of modified PS oligonucleotides has been of great interest for decades[5 6], but has seen relatively slow progress[7 8]. This is mainly because the stringent demands of solid-phase synthesis (i.e quantitative yields, limited solvent choice) severely limit the choice of suitable phosphoramidites.

During synthesis, internucleosidic bonds are formed from attack of the 5'-hydroxyl of a support-bound nucleoside at an activated phosphoramidite (**Fig. 1**)[9].

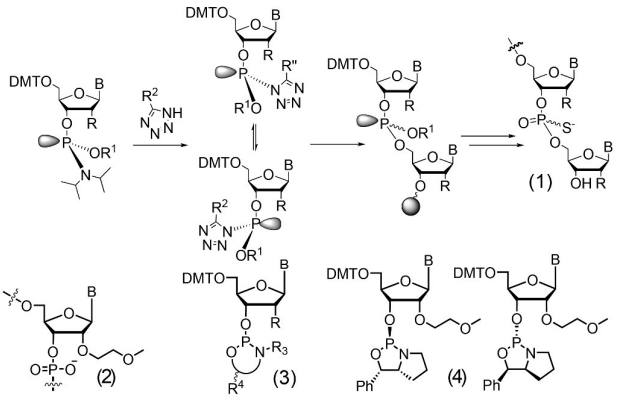


**Fig. 1** Solid-phase oligonucleotide synthesis. Conventional phosphoramidites (R is H, or protected OH; $R^1$ is 2-cyanoethyl) and tetrazole activators ($R^2$ is e.g. ethylthio) couple with epimerization at P to provide (**1**) diastereomeric products. (**2**): MOE nucleotides; B nucleobase. (**3**): generic oxazaphospholidine derived from an aminoalcohol; $R^3$ and $R^4$ are aryl or alkyl substituents. (**4**) Pairs of MOE-OAPs investigated in this study.

Substituted tetrazoles act as weakly acidic and nucleophilic activators during the displacement of diisopropylamine, which is accompanied by epimerization at P from repeated attack of the tetrazole, prior to relatively slow irreversible reaction with the hydroxyl group. Sulfurization proceeds with retention of configuration[10].

An important breakthrough was the introduction of chiral bicyclic oxazaphospholidines (OAP) (**Fig. 1**; (**3**)) combined with a novel activator, e.g. N-(cyanomethyl)-pyrrolidinium triflate (CMPT), thereby providing chiral PS ODNs and PS ORNs stereospecifically, albeit in mediocre yields[11 12 13 14]. With this chemistry the Wada group were able to synthesize 12-nucleotide (nt) long ORNs (12-mers) stereospecifically[8]. This was followed by an account describing the effects of gapmer ASOs consisting of *R*p-or *S*p-PS linked ODN gap regions on target inhibition *in vitro* and *in vivo*[15]. Here, the authors concluded that a mix of *R*p-PS and *S*p-PS stereochemistries in the ASO gap region is needed for optimal activity and nuclease

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stability (whereas flanking PS MOE groups are insensitive to nucleases[16]).

Here, we describe the synthesis and characterization of enantiopure MOE-OAPs (**Fig. 1**; (**4**)) and their application in the solid-phase synthesis of 12-mer splice switching oligonucleotides (SSOs) targeting the ferrochelatase (FECH) pre-mRNA[17], and a gapmer version of the approved drug mipomersen, targeting apolipoprotein B (apo B) mRNA[18].

## Results

Protected 2'-O-(2-methoxyethyl)-ribonucleosides of A[19], 5-methylC[20] (hereafter C) and T[20] were synthesized, as reported (**Table 1** and ESI). The G nucleoside was synthesized in a 5-step sequence from 2-amino-A[21] (see also ESI). The MOE nucleosides were then allowed to react with *in situ*- formed 2-chlorooxazaphospholidine intermediates derived from (*R*)-phenyl((*S*)-pyrrolidin-2-yl)methanol and (*S*)-phenyl((*R*)-pyrrolidin-2-yl)methanol, prepared in turn from L- and D-proline, respectively (see ESI)[15]. We opted to use these chiral auxiliaries because they have been demonstrated by the Wada group to couple stereospecifically independently of substituents at the 2'-position of the ribose, i.e. with 2'-deoxy-[12] as well as 2'-O-protected ribonucleosides[13 14].

**Table 1** Chiral OAPs: synthesis yields and purity

| entry | product | $B^{PRO}$ | yield (%)[b] | $^2J_{PC}$ | *trans*:*cis*[a] |
|---|---|---|---|---|---|
| 1 | (*Sp*)-**4a** | T | 89 | 34.2 | >99:1 |
| 2 | (*Sp*)-**4b** | $^mC^{Bz}$ | 91 | 35.2 | >99:1 |
| 3 | (*Sp*)-**4c** | $A^{Bz}$ | 78 | 34.2 | >99:1 |
| 4 | (*Sp*)-**4d** | $G^{iBu}$ | 49 | 35.2 | >99:1 |
| 5 | (*Rp*)-**4e** | T | 68 | 35.2 | >99:1 |
| 6 | (*Rp*)-**4f** | $^mC^{Bz}$ | 70 | 34.2 | >99:1 |
| 7 | (*Rp*)-**4g** | $A^{Bz}$ | 52 | 35.2 | >99:1 |
| 8 | (*Rp*)-**4h** | $G^{iBu}$ | 40 | 34.2 | >99:1 |

[a]Determined by $^{31}$P NMR and $^2J_{PC}$ coupling constants (see ESI); [b]isolated yields. $B^{PRO}$: nucleobase and protecting group; $^m$C: 5-methylC.

The MOE-OAPs (**4a**-**4h**) were obtained stereoselectively as *trans*-isomers in modest to good yields, as white solids (**Table 1**; ESI). They were rather unstable on silica gel compared to conventional phosphoramidites (**Fig. 1**), especially guanine (*R*p)-**4h**, which was purified on 3-aminopropyl-functionalized silica gel[12]. In general, the *S*p- and pyrimidine OAPs were more stable than their *R*p-analogs and the purine OAPs. *Trans*-*R*p-OAPs and *S*p-OAPs (**4a**-**h**) were configurationally pure, based on the $^{31}$P NMR and the $^2J_{PC}$ coupling constants between P and C-8 (ESI)[12]

Next, we investigated the solid-phase synthesis of PS oligonucleotides on controlled pore glass (CPG) support using **4a-h**. It is known that OAPs couple less efficiently than conventional phosphoramidites due to the increased steric bulk around the phosphorous. This is exacerbated by groups at the 2'-position of the ribose. An investigation of coupling conditions for the preparation of short oligonucleotides was performed to optimize coupling efficiency. N-phenylimidazolium triflate was identified as the best activator for both solution-phase (data not shown) and solid-phase reactions. Coupling efficiencies of up to 97% were obtained (calculated from HPLC chromatograms) by extending coupling times, and using three applications of activated monomers to the reacting solid-phase. The major side reactions (typically <2%) in the synthesis of oligonucleotides were due to minor incorporation of oxygen during the sulfurization or oligonucleotide deprotection, which is a general problem during PS oligonucleotide synthesis[22].

**Table 2** Stereoselectivity in coupling MOE-OAPs

| OAP | N.o. | Oligonucleotide 5'-3'[a] | Stereoselectivity (*Sp*:*Rp*)[b] |
|---|---|---|---|
| **4a** | **5** | $T_{Rp}$-CGTACGT | 4:96 |
| **4b** | **6** | $C_{Rp}$-CGTACGT | 4:96 |
| **4c** | **7** | $A_{Rp}$-CGTACGT | 4:96 |
| **4d** | **8** | $G_{Rp}$-CGTACGT | 2:98 |
| **4a** | **9** | $T_{Rp}$-TGTACGT | 6:94 |
| **4a** | **10** | $T_{Rp}$-AGTACGT | 4:96 |
| **4e** | **11** | $T_{Sp}$-CGTACGT | >99:1 |
| **4f** | **12** | $C_{Sp}$-CGTACGT | >99:1 |
| **4g** | **13** | $A_{Sp}$-CGTACGT | >99:1 |
| **4h** | **14** | $G_{Sp}$-CGTACGT | >99:1 |
| **4g** | **15** | $A_{Sp}$-GGTACGT | >99:1 |

[a]All nucleotides are MOE nucleotides. Nucleosides followed by a subscript "Sp" indicates a 3'-*S*p linkage, nucleosides followed by a subscript "Rp" indicates a 3'-*R*p linkage. All other linkages are phosphodiesters (PO). [b]Stereoselectivities were determined by HPLC-MS (see ESI).

We evaluated the stereoselectivity in coupling of **4a**-**4h** in solid-phase synthesis of a series of 8-mer MOE-oligonucleotides using N-phenylimidazolium triflate. The first 7 nt (6 linkages) were phosphodiester (PO) and were incorporated using conventional phosphoramidites (**Fig. 1**), followed by a standard oxidation step (ESI). A single PS linkage was added at the 5' terminal position, using either an *S*p- or *R*p-OAP (**4a-h**). The oligonucleotides were cleaved from the solid support after incubation with 25% ammonia at 55 ˚C. The crude products with their terminal dimethoxytrityl (DMT) groups were analysed by LCMS (UV area). The two diastereomeric MOE-oligonucleotides migrate differently according to whether *R*p- or *S*p-OAPs were used for their synthesis. We presumed that MOE-OAP coupling proceeded with an inversion of stereochemistry at P similarly to deoxynucleotides and protected ribonucleotides[11]; hence, the faster-eluting peak was tentatively assigned as the *S*p-PS isomer, with the slower-eluting peak as the *R*p-PS diastereomer (See ESI). In the case of PS linkages between natural ODN and ORNs, absolute stereochemistry can be confirmed by stereospecific cleavage with various natural nucleases[23 24]. However, for non-natural nucleotides such as the MOE-nucleotides no stereospecific enzymes have been described.

Calculating ratios of the diastereomers from area under the peaks in the HPLC chromatograms, we determined that the OAP MOE phosphoramidites coupled with very high stereoselectivity: OAPs *S*p-**4b** and *R*p-**4f** yielded oligonucleotides (**6**) and (**12**), with 96% and >99% stereoselectivities, respectively. In order to show that these

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stereoselectivities extended to other sequences, we prepared a full series of 8-mers using all OAPs (**Table 2**; ESI).

Next, we applied these optimized conditions for the synthesis of longer oligonucleotides containing stretches of stereopure PS linkages. We elected to investigate the properties of two classes of oligonucleotide: 20-mer MOE-gapmers corresponding to the approved drug mipomersen[18], in which the MOE wings either carry *R*p-PS or *S*p-PS linkages, and the ODN gap region was composed of a stretch of stereorandom PS ODN; a 12-mer MOE-SSO in *R*p-PS, *S*p-PS and stereorandom PS forms. Oligonucleotides were synthesized and yielded the expected products in high purity. Average coupling yields during the unoptimized syntheses were calculated as approx. 70% (**Table 3**; ESI).

**Table 3** Properties of MOE-oligonucleotides

| N.o. | Name | Sequence[a] | PS stereo-chemistry | Tm (˚C) |
|---|---|---|---|---|
| **16** | Mp | GCCTCAGTCTGCTTCGCACC | *R*p/*S*p | 80.0 |
| **17** | Mp-*R*p | GCCTCAGTCTGCTTCGCACC | 5R-10(R/S)-4R | 81.6 |
| **18** | Mp-*S*p | GCCTCAGTCTGCTTCGCACC | 5S-10(R/S)-4S | 76.9 |
| **19** | Neg Con | CCTTCCCTGAAGGTTCCTCC | *R*p/*S*p | - |
| **20** | FCH | TAGCAGCCTGAG | *R*p/*S*p | 73.9 |
| **21** | FCH-*R*p | TAGCAGCCTGAG | *R*p | 77.2 |
| **22** | FCH-*S*p | TAGCAGCCTGAG | *S*p | 66.8 |
| **23** | FCH-Con | GGACAACGTTCG | Rp/Sp | - |

[a]PS MOE oligonucleotides; underlined nt are DNA (see ESI); the phosphodiester version of compound **20** had a Tm of 79.5 ˚C.

Purity and integrity of the sequences were shown by HPLC-MS (**Fig. S1**; ESI). These oligonucleotides were first hybridized with complementary ORNs to determine their melting temperatures ($T_m$'s) (**Fig. S2**; ESI). Several groups have shown that *R*p-PS ORNs bind more tightly to complementary ORNs than *S*p-PS ORNs[25 13, 15]. This is thought to be due to stronger hydrogen bonds involving the negatively charged *R*p-$S^-$ in an A-form major groove[26]. Oligonucleotides (**17**) and (**21**) showed higher $T_m$'s by approx. 0.2-0.3 ˚C per linkage, relative to the $T_m$'s of the fully stereorandom PS oligonucleotides ((**16**) and (**20**)), respectively, consistent with *R*p-PS nucleotides. In contrast, (**18**) and (**22**) yielded lower $T_m$'s by approx. 0.3-0.6 ˚C per incorporation relative to those of the stereorandom PS parent sequences (**16**) and (**20**), respectively, consistent with *S*p-PS nucleotides. These observations supported our assumption that reaction of the OAPs proceeded with stereochemical inversion[12 15].

Gapmers (**16**), (**17**) and (**18**) were tested for concentration-dependent reduction of apoB mRNA in cultures of Huh7 cells (**Fig. 2a**). The negative control gapmer (**19**) had no effect on apoB mRNA. In contrast, (**16**), (**17**) and (**18**) lowered apoB mRNA levels in a dose dependent manner, with approx. $IC_{50}$ values of 18 ± 2 nM, 22 ± 3 and 25 ± 6, respectively. These $IC_{50}$ values corresponded well with previously reported values (10 to 50 nM) in human HepG2 and Hep3B cell lines and primary human hepatocytes[27]. Hence, the higher $T_m$ of the *R*p-PS isomer over its *S*p-PS counterpart (+4 ˚C) did not impart added inhibitory activity. We cannot rule out that one of the variants would show a superior activity in a mouse model, however it seems likely that the high resistance against nuclease attack offered by a MOE PS linkage will show less dependence on stereochemistry than a PS deoxynucleotide.

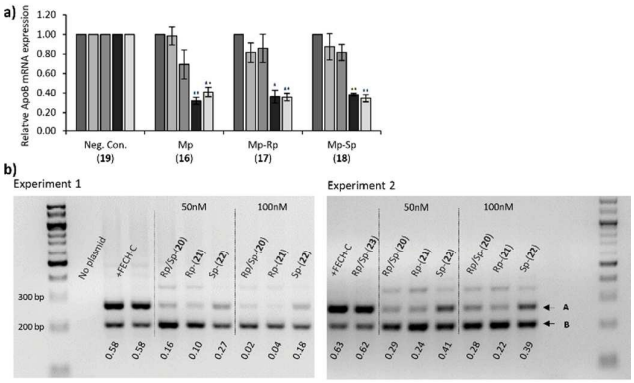


**Fig. 2** Activity of stereochemically-pure or stereorandom-MOE PS oligonucleotides in cells. a) Inhibition of endogenous apoB mRNA in Huh7 cells by PS MOE-gapmers **16**, **17**, and **18** at 0, 7, 18, 45, 112 nM concentrations. b) Splice-switching of a FECH minigene by PS MOE-SSOs **20**, **21**, and **22** in Cos7 cells. A: aberrant splice product; B: correct splice variant.

We turned therefore to SSOs, a class of modified oligonucleotides that have progressed strongly in clinical trials for the treatment of genetic diseases[28]. SSOs are designed not to induce RNA cleavage and so target binding affinity is likely the single most important contributor to their pharmacological activity. Thus, it seemed plausible that a splice-switching mechanism might be a useful application of stereochemically-pure PS MOE-oligonucleotides, where the increased binding affinity of the *R*p-PS nucleotides could be exploited.

Erythropoeitic protoporphyria (EPP) is a rare disease where patients suffer from skin photosensitivity. It is caused by insufficient ferrochelatase enzyme (FECH), which leads to accumulation of protoporphyrin IX in the circulation. EPP only occurs when a mutated allele is combined with an allelic variant in which a polymorphism activates a cryptic 3'-splice site in intron 3[29]. Splicing produces an mRNA with an extra stretch of intronic sequence that contains premature stop codons and induces the degradation of the transcript by nonsense-mediated mRNA decay (NMD). A small screening of stereorandom PS SSOs was performed around the cryptic 3'-splice site and revealed binding sites for MOE-oligonucleotides which corrected aberrant splicing (data not shown). Assays were performed in COS-7 cells expressing a FECH-minigene containing the affected part of the FECH intron. RNA from cells was then amplified by RT-PCR using primers specific for the two splice variants, and quantified by agarose gel electrophoresis. Two bands **A** and **B** corresponding to the aberrantly spliced and correctly spliced RNAs in an approx. 60:40 ratio, respectfully were observed. In order to probe differences in activity of stereochemically pure *R*p- and *S*p-PS SSOs, four 12-mer MOE-oligonucleotides were synthesized and purified (**20**, **21**, **22**, **23**; **Table 3**; ESI) (**Fig. 2b**). Melting temperature analysis showed that the *R*p-PS diastereomer (**21**)

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bound more strongly to a complementary ORN than the Sp diastereomer (**22**) by >10 ˚C, and by 3.3 ˚C compared to the stereorandom PS sequence (**20**). Next, cells were co-transfected with the minigene-expressing plasmid and each of the four 12-mer SSOs, at two concentrations. The scrambled sequence (**23**) had no effect on splicing. After 24 h the stereorandom PS SSO (**20**) switched the aberrant:correct ratio to 0.16 and 0.29 in two independent experiments (**Fig. 2b**). The all *S*p-PS isomer (**22**) was less effective in all cases, whereas the all Rp-PS isomer (**21**) was at least equipotent with, or possibly slightly more potent than (**20**). In contrast to the results from the gapmer oligonucleotides, here the potency of these SSOs clearly followed the trend of the $T_m$'s (**Table 3**).

## Conclusions

The MOE modification is arguably the most important modification in the field[3]. It features in the approved drug mipomersen and many late-stage clinically-tested drugs, including the SSO nusinersen. Here we describe a new synthesis of MOE OAPs and their use in the preparation of *R*p- and *S*p-PS MOE-oligonucleotides. We tested the effects of stereochemically-pure PS MOE-stretches in the wings of mipomersen on apoB mRNA silencing. However, we did not detect any significant difference in silencing between *R*p-PS, *S*p-PS and stereorandom versions of the drug. It is likely that distinct PS stereochemistries in the wings of the ASO do not affect the ability of RNase H to cleave its target, and that the largest contribution to potency derives from the gap region composed of a common stereorandom PS stretch in all three ASOs. These findings complement those made by Wan et al.[15] in which stereochemically-pure gap regions flanked by stereorandom PS wings greatly affected properties of the drug. A different outcome emerged from our use of full PS MOE-SSOs. Here the *R*p-PS stereochemistry was clearly superior to the *S*p-variant, but not significantly better than the stereorandom PS sequence in correcting a pathologically-relevant splicing of *FECH*. It is reasonable to assume that the increased potency of *R*p-PS MOE-oligonucleotide over its *S*p-variant was at least partly due to its increased target binding affinity, although we cannot rule out the influence of other parameters, for example cellular transport. The high activity of the stereorandom PS sequence may be partly attributed to a biased sub-population of the SSO pool which is rich in *R*p-PS stereocenters. Indeed, we recently showed that a pool of PS siRNAs which were enriched in *R*p-PS diastereomers exhibited higher silencing activity[30]. Finally, this study was constrained to homogeneous *R*p- or *S*p-PS sequence compositions, but it is possible that specific combinations will yield improved properties in areas yet to be tested, such as PK (absorption, distribution, metabolism, and excretion). In any case, a shift towards diastereomerically pure drugs would be a welcome advance in the field.

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