Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 7, pp. 871-876, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00119-4

BIOREVERSIBLE OLIGONUCLEOTIDE CONJUGATES BY SITE-SPECIFIC DERIVATIZATION

Radhakrishnan P. Iyer, Nan-hui Ho, Dong Yu, and Sudhir Agrawal

Hybridon Inc., One Innovation Drive, Worcester, MA 01605

Abstract. Oligonucleotide analogs which are site-specifically derivatized with *O*-acyloxyaryl groups were prepared using phosphoramidite chemistry. Upon their incubation with *pig liver esterase (PLE)* or *chymotrypsin*, the analogs were shown to revert to the parent compound. © 1997 Elsevier Science Ltd.

Oligonucleotide conjugates[1] are expected to have a number of applications in therapeutics and diagnostics. Ideally, in antisense therapeutics,[2] such oligo conjugates can be used (a) for enhancing cellular uptake, (b) for tissue-specific targeting, (c) for topical (e.g., transdermal), as well as, oral delivery of oligonucleotides, (d) as a reservoir for controlled/sustained delivery of the oligonucleotide at the target, (e) as a handle for modulating the biophysical, biochemical, and biological properties of oligonucleotides, and (f) for reducing polyanion-associated side effects of oligonucleotides. However, in certain cases, the covalent attachment of ligands to an oligonucleotide reduces the affinity of the resulting conjugate for its intended target (as a measure of T_m) compared to the parent due to local or global distortion of duplex structures.[1] Furthermore, in the context of antisense activity, the conjugates might sterically block the action of RNase H which cleaves the RNA segment of the DNA/RNA duplex. Thus, conjugation with a ligand might compromise the antisense potential of an oligonucleotide. An alternate approach is to design a bioreversible conjugate, wherein the oligonucleotide can be released free from the ligand by the action of specific cellular enzymes. Thus, the natural affinity and selectivity of the antisense oligonucleotide for the target is restored without interference from the conjugating ligand.

We have recently reported[3a] *S*-(acyloxyaryl) dinucleoside phosphorothioate **1** as an example of a bioreversible prodrug conjugate (Figure 1), and showed that *in vitro*, *esterase*-mediated hydrolysis converted **1** to the parent oligonucleotide **1a**. The synthetic approach to the aforementioned prodrugs relied upon post-

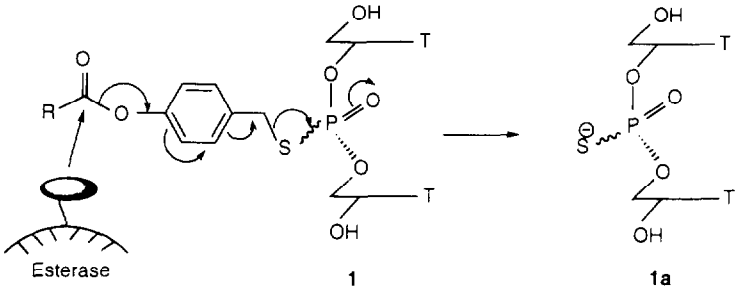


Figure 1. Esterase-mediated hydrolysis of the acyloxyaryl derivative of a dinucleotide.

synthetic *albeit* chemoselective reaction of a phosphorothioate oligonucleotide with haloalkyl esters. Using this methodology, the phosphorothioate backbone of an oligonucleotide could be uniformly modified.[3b,c] In

continuation of these studies, we have investigated an alternate approach, which would allow the conjugate to be introduced site-specifically in an oligonucleotide chain to produce *O*-acyloxyaryl phosphorothioate analogs (e. g. **2**, Figure 2) by solid-phase synthesis. The problem here was to define an "R" group in the acyloxyaryl functionality which will: (a) withstand the rigors of solid-phase oligonucleotide synthesis, (b) be stable to the deprotection and cleavage conditions, following the chain assembly of the oligonucleotide on the solid support, and (c) undergo *esterase*-mediated conversion to the parent oligonucleotide.

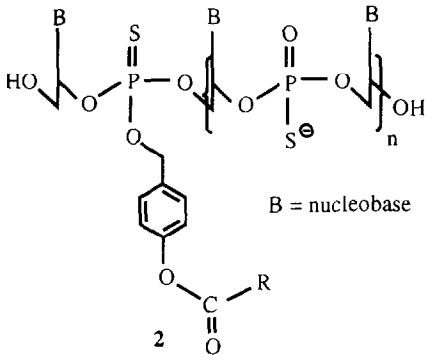
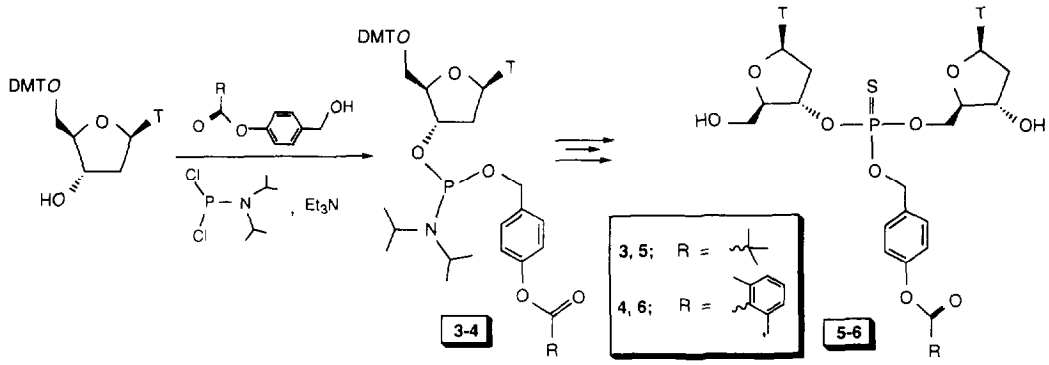


Figure 2. General structure of a bioreversible oligonucleotide conjugate

Guided by our earlier studies,[1a,b] we chose the sterically hindered *tert*-butyl, and 2,6-dimethylphenyl as the "R" groups in order to minimize the potential hydrolytic cleavage of the oligonucleotide conjugate during the implementation of the fast deprotection protocols[4] under non-aqueous base conditions.[5]

The requisite acyloxyarylphosphoramidites **3-4** were prepared[6] by a one pot reaction of diisopropylamino phosphorous dichloride with 5'-*O*-dimethoxytrityl nucleoside and the appropriate 4-acyloxybenzyl alcohols.

Scheme 1

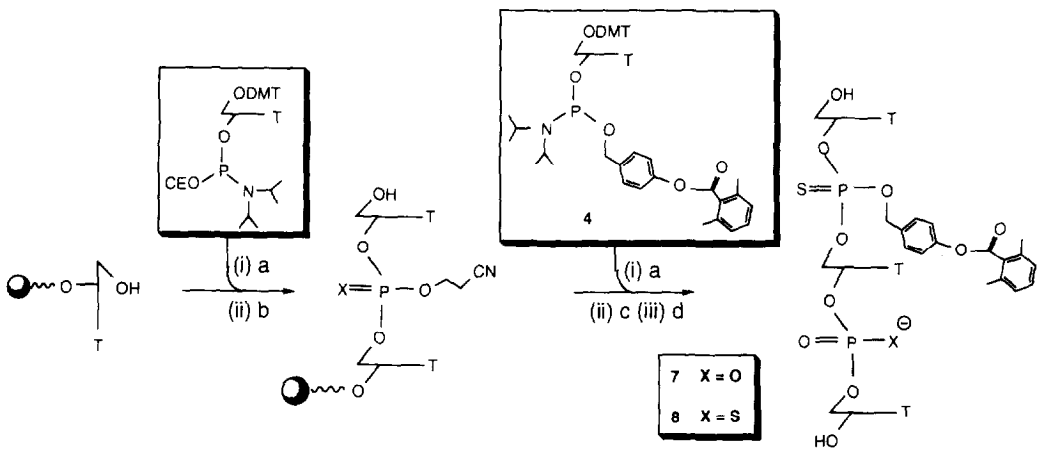


In initial model studies, these monomers were employed in the synthesis of T-T dinucleoside phosphorothioate conjugates **5** and **6** using solid-phase phosphoramidite chemistry[7] on a 1 μm scale using T-nucleoside anchored to the controlled-pore-glass support (CPG) *via* succinyl linkage. Following coupling, the

internucleotidic phosphite linkage was sulfurized with 3*H*-1,2-benzodithiole-3-one-1,1-dioxide.[8] After removal of the 5'-*O*-DMT group, the dinucleotide was cleaved from the support using K_2CO_3/MeOH (0.05 M, rt, 1 h). The analogs **5** and **6** were obtained as a pair of R_p and S_p diastereomers[9] by reversed-phase HPLC. The analysis of crude **5**, a pair of (R_p,S_p) diastereomers (t_R, 57.8, and 59.5 min) revealed that it had suffered hydrolysis (ca. 50%) to the corresponding phosphorothioates **1a** (t_R, 28.1 and 30.0 min) during the cleavage procedure whereas the more sterically hindered analog (R_p,S_p) **6** (t_R, 62.0 and 63.1 min) had fully survived the cleavage conditions. In the event, incubation of the (R_p,S_p) mixture of each of the analogs **5**, and **6** with either *PLE or chymotrypsin*, resulted in their conversion to the dinucleoside phosphorothioate **1a** (data not shown).

Based on these model studies, we chose the phosphoramidite **4** for the synthesis of the trinucleoside analogs **7** and **8**. For the synthesis of **7**, the PO linkage was generated using *tert*-butylhydroperoxide as the oxidant (Scheme 2). Following removal of the β-cyanoethyl phosphate protecting group, and cleavage from the support using K_2CO_3 (0.05 M, MeOH), the analogs were isolated by reversed-phase HPLC.

Scheme 2. Synthesis of trinucleoside conjugates **7** and **8**[@]



[@] (a) 1*H*-Tetrazole; (b) *tert*-butyl hydroperoxide or 3*H*-benzodithiole-3-one 1,1-dioxide (3*H*-BD); (c) 3*H*-BD; (d) K_2CO_3/MeOH (0.05 M).

The analogs **7** and **8** were soluble in aqueous buffers. For evaluation of their stability at different pH conditions, aliquots of the analogs stored in buffers (pH 2.5, and pH 7.4, rt), were analyzed periodically for 48 h by reversed-phase HPLC. Both analogs **7** and **8** remained stable under these conditions (>95% intact material). The (R_p,S_p) mixture of **7** and **8** was also subjected to bioreversibility studies using *PLE* and *chymotrypsin*. Table 1 shows the half-lives of hydrolysis ($t_{1/2}$). In the case of **7**, the R_p isomer (t_R, 57.1) was hydrolyzed more rapidly (*esterase*, $t_{1/2}$, 400 min; *chymotrypsin*, $t_{1/2}$, 325 min) compared to the S_p isomer (t_R, 56.5) (*esterase*, $t_{1/2}$, 650 min; *chymotrypsin*, $t_{1/2}$, 360 min) (Figure 3). Furthermore, each isomer conjugate of **7**

Table 1[@]

#	t_R (min)	$t_{1/2}$ *(chymotrypsin)* (min)	$t_{1/2}$ *(esterase)* (min)
(S_p) **7**	56.5	360	650
(R_p) **7**	57.1	325	400

[@]Approximately 0.4 A_{260} units of the conjugate **7** was incubated with 2 μL of *α-chymotrypsin* (type I, bovine pancreas, Sigma) (4 units), or PLE (suspension in 3.2 M $(NH_4)_2SO_4$, Sigma) in Tris-HCl buffer (20 μL, 100 mM, pH 7.0) at 37 °C. Aliquots of the incubate were periodically analyzed by reversed-phase HPLC[3a] (see Figure 3).

was found to be stereospecifically hydrolyzed to the corresponding trinucleoside diester **7a**. Similarly, incubation of the stereoisomeric mixture of the analog **8** with *esterase* or *chymotrypsin* resulted in its conversion to the corresponding trinucleoside diester **8a** (Figure 4).

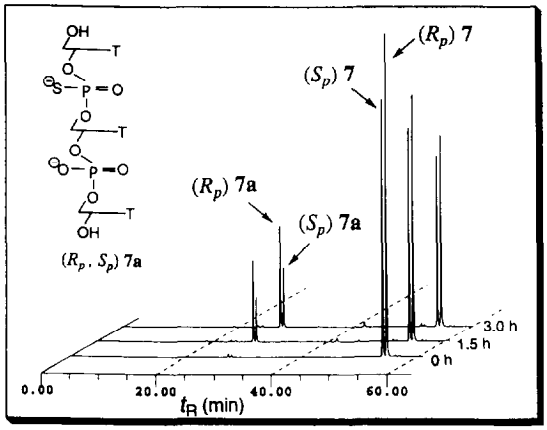


Figure 3. HPLC profiles depicting the time-course of hydrolysis of (R_p, S_p) **7** by *α-chymotrypsin*.

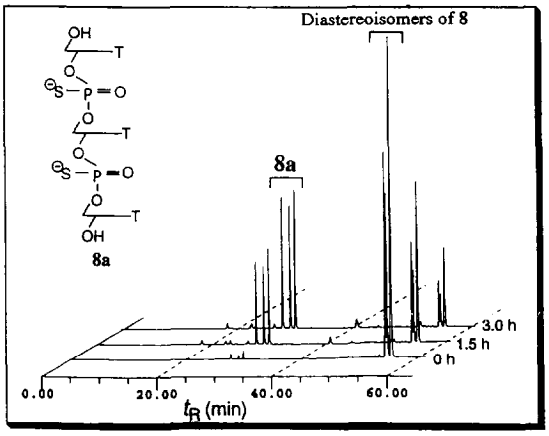


Figure 4. HPLC profiles depicting the time-course of hydrolysis of the stereoisomeric mixture of trinucleoside conjugate **8** by *α-chymotrypsin*.

The facile *esterase*-mediated hydrolysis of these sterically hindered esters can be rationalized on the basis of the model proposed by Jones et al.,[10a-c] for ester hydrolyis by PLE in which the substrate is positioned in the

most favorable conformation in four binding pockets: large H_L and small H_B (hydrophobic sites), as well as, front and back polar binding sites (P_F and P_B). Also, the faster rates of hydrolysis of **7**, and **8** by *chymotryspin* compared to those by PLE may be due to the fact that the active site of the former readily accommodates an aromatic residue at the carboxy terminus of an ester substrate as in the case of **7**, and **8**.

We next incorporated the phosphoramidite **4**, site-specifically at a defined position at the 5'-end of a 15-mer oligonucleotide. The oligonucleotide conjugate **9** was synthesized on a 1μM scale using *N*-pent-4-enoyl (*PNT*) nucleoside phosphoramidites.[4d,11] The use of the *PNT* nucleosides allowed the complete removal of the *PNT*, the β-cyanoethyl protecting groups, and cleavage from the support under mild conditions using K_2CO_3/MeOH (0.05 M, rt, 8 h).[4d,12] The conjugate **9** was characterized by ^{31}P NMR (Figure 5, Panel A). In polyacrylamide

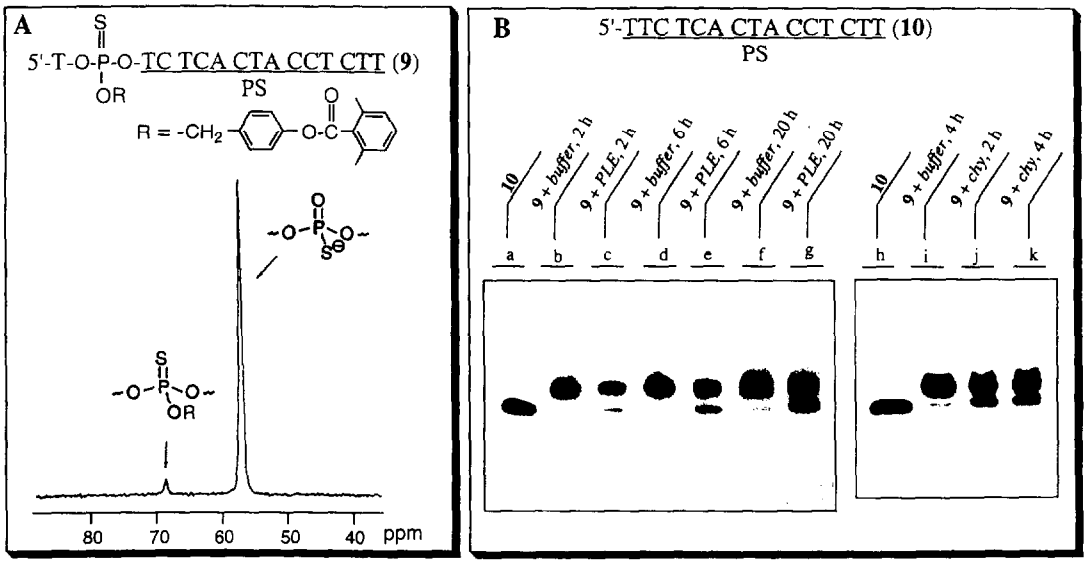


Figure 5. Panel A. ^{31}P NMR spectrum of oligo conjugate **9**. **Panel B**. PAGE (20%) profile showing the time course of bioreversibility of **9** to **10** following incubation with *chymotrypsin (chy) and PLE*.

gel electrophoresis (PAGE), the mobility of **9** was slower than that of the parent **10**. Upon incubation with *chymotrypsin*, **9** was found to undergo enzyme-mediated conversion to the parent oligonucleotide **10** (Figure 5, Panel B).

In conclusion, we have prepared bioreversible conjugates site-specifically appended to the backbone of an oligonucleotide. Because the gastrointestinal tract is rich in hydrolytic enzymes such as chymotrypsin, the conjugates described herein may be useful as orally bioavailable prodrug forms of antisense oligonucleotides. Furthermore, the herein described strategy, with appropriate modifications, is being applied to the synthesis of other oligonucleotide conjugates where the "promoiety" contains carriers, or receptor-specific molecules, for enhancing cellular uptake, and for facilitating tissue-specific targeting. The evaluation of biological activity of the oligoconjugates using *in vitro* and *in vivo* models, as well as, the study of their pharmacokinetics and metabolism are underway. The results of these studies will be reported elsewhere.

References and notes

1. For a recent review see: Fidanza, J. A.; Ozaki, H.; McLaughlin, L. W. In *Protocols for Oligonucleotide Conjugates*, Agrawal, S. Ed.; Humana Press, New Jersey, 1994, pp. 121-143; For applications of phosphoramidite chemistry in the preparation of oligonucleotide conjugates, see: Beaucage, S. L.; Iyer, R. P. *Tetrahedron*, **1993**, 49, 1925.
2. For selected reviews see: (a) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 544; (b) Thuong, N. T.; Helene, C. *Angew. Chem. Intl. Ed. Engl.* **1993**, *32*, 666; (c) Agrawal, S., Iyer, R. P. *Curr. Op. Biotech.* **1995**, *32*, 12.
3. (a) Iyer, R. P.; Yu, D.; Devlin, T.; Ho, N.-H.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1917; (b) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2471; (c) Barber, I.; Rayner, B.; Imbach, J. -L. *Bioorg. Med.Chem.* Lett. **1995**, *5*, 563.
4. (a) Sinha, N. D.; Davis, P.; Usman, N.; Perez, J.; Hodge, R.; Kremsky, J.; Casale, R. *Biochimie* **1993**, *75*, 13, and references therein; (b) Reddy, M. P.; Hanna, N. B.; Farooqui, F. *Tetrahedron Lett.* **1994**, *35*, 4311; (c) Boal, J. H.; Wilk, A.; Harindranath, N., Max, E. E.; Kempe, T.; Beaucage, S. L. *Nucl. Acids Res.* **1996**, *24*, 3115, and references therein; (d) For a recent study of fast deprotection using *PNT* nucleosides, see: Iyer, R. P.; Yu, D.; Habus, I.; Ho, N. -H.; Johnson, S.; Devlin, T.; Jiang, Z.; Zhou, W.; Xie, J.; Agrawal, S. *Tetrahedron*, **1997**, (in press).
5. For examples, see: (a) Kuijpers, W. H. A.; Huskens, J.; Koole, L. H.; van Boeckel, C. A. A. *Nucl. Acids Res.* **1990**, *18*, 5197; (b) Also see references 4a-d.
6. Typical spectral data: phosphoramidite **4**, ^{31}P NMR ($CDCl_3$), δ, 145.79, 145. 26 ppm; ^{1}H NMR ($CDCl_3$), δ, 7.5-6.7 (m, 20H, Ar-H), 7.6 (s, 1H, H-6), 6.4 (dd, 1H, 1'H), 4.7 (m, 1H, 3'H), 4.2 (m, 1H, 4'H), 3.8 (s, 3H, -O$\underline{CH_3}$), 3.75 (s, 3H, -O$\underline{CH_3}$), 3.6 (m, 2H, C$\underline{H}$(CH_3)$_2$, 3.4 (m, 2H, 5'H), 2.5 (s, 6H, Ar-$\underline{CH_3}$), 2.4 (m, 2H, 2'H), 1.2-1 (m, 12 H, CH($\underline{CH_3}$)$_2$ ppm.
7. (a) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859; (b) For a review, see: Beaucage, S. L.; Iyer, R. P. *Tetrahedron*, **1993**, *49*, 1925; (c) The alternative approach to **5**, and **6** using the corresponding acyloxyalcohols in conjunction with *H*-phosphonate chemistry[7d] was not successful; (d) Garegg, P. J.; Regberg, T.; Stawinski, J.; Stromberg, R. *Chem. Scr.* **1985**, *25*, 280.
8. Iyer, R. P.; Regan, J. B.; Egan, W.; Beaucage, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 1253.
9. For the assignment of absolute configuration, the individual R_p and S_p isomers, were treated with *chymotrypsin* to generate the parent trinucleoside. The trinucleoside was then digested sequentially with *snake venom phosphodiesterase*, and *alkaline phosphatase* to give the corresponding dinucleoside phosphorothioates which was analyzed by reversed-phase HPLC. Absolute configurations were assigned by comparison of retention times with authentic samples of known configuration (see reference 3a).
10. (a) Toone, E. J.; Werth, M. J.; Jones, J. B. *J. Am. Chem. Soc.* **1990**, *112*, 4946, and references therein; (b) For a recent application of this model, see: Bugg, T. D. H.; Lewin, A. M.; Catlin, E. R. *J. Chem. Ed.* **1997**, *74*, 105; (c) Interestingly, simple 2,6-dimethylbenzoyl esters of terbutaline® did not biorevert to terbutaline; see: Svensson, L. -Å.; Tunek, A. *Drug. Metab. Rev.* **1988**, *19*, 165.
11. Iyer, R. P.; Yu, D.; Ho, N.-H.; Devlin, T.; Agrawal, S. *J. Org. Chem.* **1995**, *60*, 8132
12. Alternatively, the use of oxalyl-linked CPG expedited the cleavage of the oligo from the support; See: Alul, R. H.; Singman, C. N.; Zhang, G.; Letsinger, R. L. *Nucl. Acids Res.* **1991**, *19*, 1527.

(*Received in USA* 23 *January* 1997; *accepted* 26 *February* 1997)