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ACYLOXYARYL PRODRUGS OF OLIGONUCLEOSIDE PHOSPHOROTHIOATES

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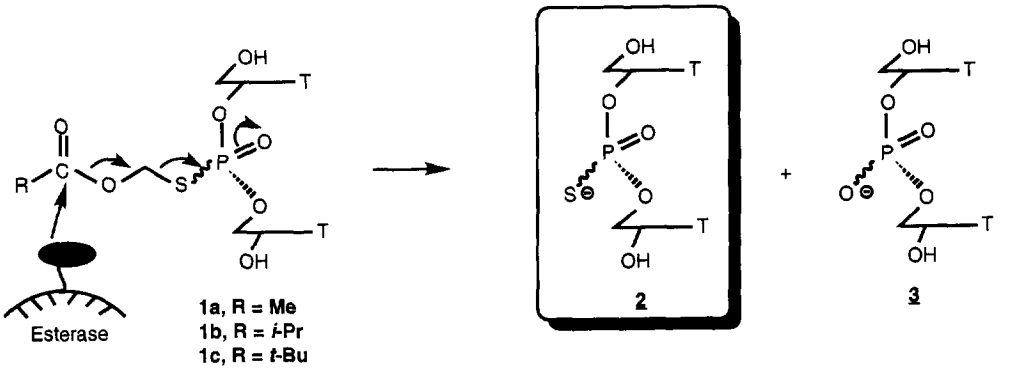
Abstract. Improved design, synthesis, and evaluation of an oligonucleotide prodrug is described.

Chemical derivatizations of drug candidates to produce prodrugs[1] is a fruitful area of research. The concept of using prodrugs has been exploited for several purposes: (a) for tissue-specific targeting, (b) for producing synergistic actions by combining two or more drug moieties, (c) for overcoming the first pass metabolism effect, (d) to serve as a lipophilic carrier for hydrophilic drugs, and (e) to serve as a depot for sustained drug delivery. The prodrug approach has yielded several examples of therapeutically useful "small molecule" entities,[1b] and this concept has been applied to nucleotides and their analogs as well.[1c] Following upon our earlier studies on the use of acyloxyalkyl groups[2a-b] as bioreversible entities for the derivatization of the antiviral trisodium phosphonoformate, we[3a] and others[3b] recently extended this strategy to the emerging field of nucleic acid-based therapeutics.[4] We reported[3a] that bioreversible analogs **1a-c** of oligonucleoside phosphorothioates (PS-oligos) that carry one or more lipophilic acyloxyalkyl ester groups might serve as useful prodrug forms of the parent phosphorothioate **2** (Figure 1). These prodrugs were designed with an intent to meet these specific objectives: (a) to enhance the lipophilicity of the hydrophilic oligonucleotide and consequently the cellular uptake, (b) to mask the negative charges on the backbone of the oligonucleotide so as to lessen the undesirable interactions with other cellular macromolecules, and to reduce polyanion-related side effects, (c) to target tissues by attaching specific ligands, (d) to modulate the pharmacokinetic profile of the parent oligonucleotide, (e) to enhance their resistance to degradation by cellular nucleases, and (f) to develop an

Figure 1



orally bioavailable/sustained delivery dosage form of the oligonucleotide. However, certain limitations related to their solution stability at ambient temperature that were seen with **1a-c** prompted us to undertake an improved prodrug design of **2**. The design, synthesis and evaluation of the new prodrug is detailed in this communication.

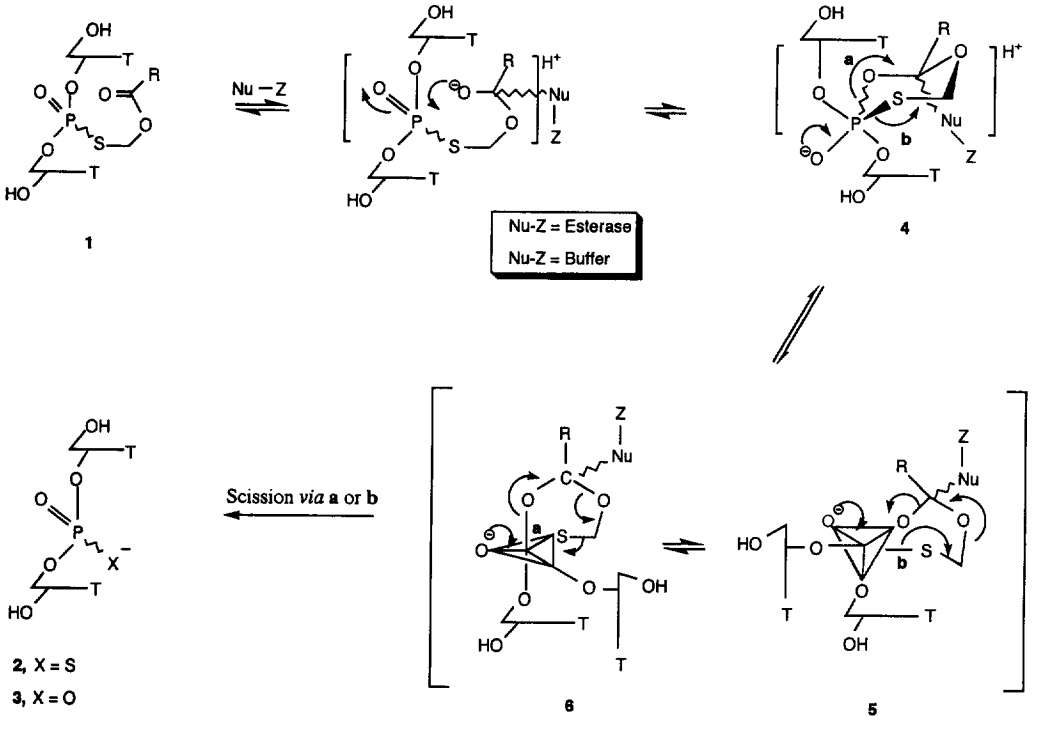
Rationale for the improved design. In each of the *S*-acyloxyalkyl derivatives **1a-c** which we studied earlier (Figure 1), esterase-mediated reversibility to generate the parent phosphorothioate **2** was demonstrated.[3a,5a-b] Interestingly, significant stereodifferentiation in the hydrolytic rates of R_p and S_p **1a-c** was also observed. However, one intriguing observation that emerged from our previous studies was that in the case of esterase-mediated hydrolysis of the hindered dinucleoside analogs (e.g., **1b-c**), small amounts (3-10%) of the desulfurized product **3** had also formed.[5a] Furthermore, although these dinucleoside acyloxyesters remained intact when stored at 0-5 °C as dry powders, they were hydrolyzed upon prolonged storage at ambient temperature in Tris buffer (pH 7.2) (half-life > 5 to 30 days). Unexpectedly, the predominant hydrolytic product in each case was the desulfurized product **3**. To be considered a "true" oligonucleotide prodrug, the formation of the desulfurized product **3** either under enzymatic or non-enzymatic conditions should be minimized.

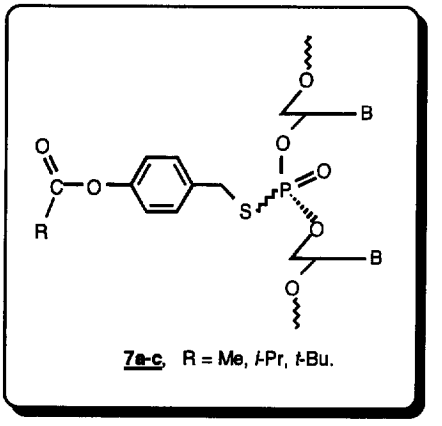
The aforementioned limitations with acyloxyalkyl prodrugs **1a-c** led us to consider an alternate prodrug design that would ensure: (a) greater stability in buffer at ambient temperature, (b) complete esterase-mediated bioreversibility to the parent phosphorothioate from which it was derived, and (c) minimal formation of the desulfurized product upon hydrolysis.

To achieve the design of the prodrug with the criteria laid out as above, it was first of all important to understand the *dichotomy* in product distribution during the hydrolysis of **1** by enzyme and buffer. Amongst the several hydrolytic pathways[6], including the classical phosphotriester hydrolysis mechanism, that could account for the product profile, the one represented in Figure 2 seemed more appealing. Thus, as depicted in Figure 2, the attack of the incoming nucleophile on the electrophilic carbonyl carbon of **1** might be followed by the interception of the resulting oxyanion[7] by the juxtapositioned phosphoryl group to give the cyclic intermediate **4**, in turn leading to the trigonal bipyramidal intermediates **5** and **6** which could interconvert by pseudorotation.[8a] In the presumed intermediate **5**, the *S*-acyloxyalkyl group, the preferred leaving group, is favorably positioned to depart from an apical direction and produce the desulfurized product **3**. However, in the case of the enzyme-mediated hydrolysis, the formation of the preferred intermediate **5** is fortuitously hampered because it would require considerable reorganization of the initially formed enzyme-substrate complex **6**. Consequently, the hydrolysis pathway[8b] is directed to occur *via* **6** to yield **2**. Guided by the postulated mechanism depicted in Figure 2, it was realized that if the formation of the key cyclic intermediate **4** could be precluded, e.g., by using a more rigid analog represented by the general structure **7**, the desulfurization pathway could be suppressed both under enzymatic and non-enzymatic conditions. In **7**, the intervention of the *p*-disubstituted phenyl ring imposes the desired conformational restraint on the PS appendage rendering the formation of **4** geometrically impossible.[9] To test the validity of our improved prodrug design, we chose to study a typical analog **7b**.

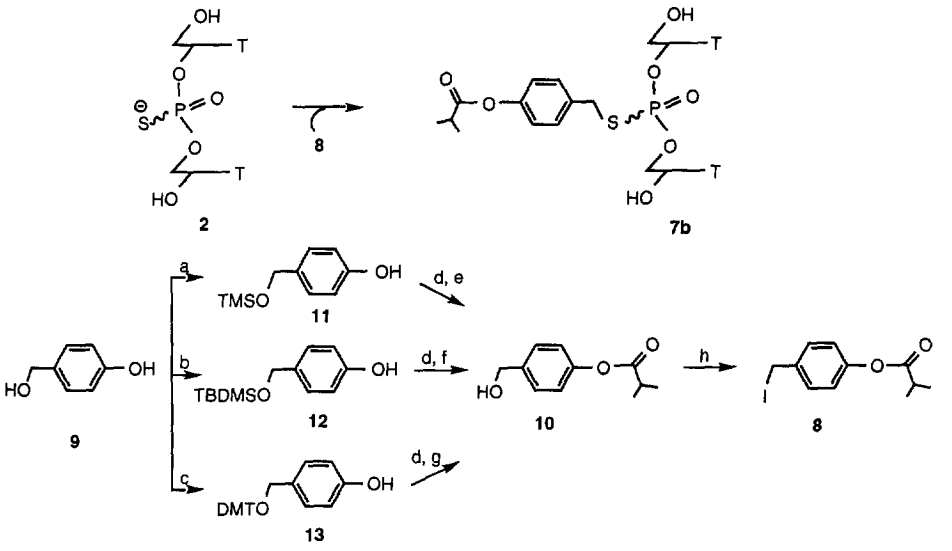
Synthesis of 7b. For the synthesis of **7b**, the requisite alkylating agent **8** was accessed in four steps from 4-hydroxybenzyl alcohol (**9**) *via* the intermediate **10** (Scheme 1). In turn, **10** was obtained from the TMS derivative **11** or from the TBDMS derivative **12** each being derived from **9**. However, attempted preparation of **10** *via* the DMT derivative **13** was not successful. The analog **7b** was synthesized by chemoselective *S*-alkylation of the dinucleoside phosphorothioate R_p/S_p **2** (Scheme 1) with **8**. The resulting diastereomeric

Figure 2


Nu – Z
Nu-Z = Esterase
Nu-Z = Buffer
Scission *via* a or b
1
2, X = S
3, X = O
4
5
6


7a-c, R = Me, *i*-Pr, *t*-Bu.

Scheme 1[@]



[@]Reagents: (a) TMSCl, pyridine; (b) TBDMSCl, pyridine; (c) DMTCl, pyridine; (d) Pyridine, ≻COCl; (e) $MeOH/H_2O$; (f) t-Bu_4NF; (g) Dowex® H^+; (h) $(Ph)_3P/I_2$

mixture of **7b** (R_p/S_p, 52/48) was subjected to reversed-phase HPLC to separate the R_p, S_p diastereomers, lyophilized and stored dry until ready for use.

Bioreversibility studies. To evaluate their stability and bioreversibility, the R_p and S_p diastereomers[5c] of **7b**, either individually or as mixtures, were incubated with human serum. The hydrolysis was monitored by quantifying the product and reactant peaks by reversed-phase HPLC (Figure 3). We observed a slow but stereospecific hydrolytic conversion of the R_p and S_p triesters to the corresponding R_p and S_p **2**. There was no detectable formation of any desulfurized product representing **3**. Nuclease-mediated hydrolytic fission products (e.g., mononucleosides) were also not seen during the assay period. Interestingly, unlike the corresponding R_p and S_p acyloxyalkyl derivatives of **1b** ($t_{1/2}$: R_p **1b** = 23 min; S_p **1b** = 5 min), there was less stereodifferentiation in the rates of hydrolysis between R_p and S_p **7b** ($t_{1/2}$: R_p **7b** = 480 min; S_p **7b** = 780 min).

Stability studies. The solution stabilities of [R_p,S_p]**7b** at 37 °C, in Tris buffer (250 mM, pH 7.4) and glycine buffer (100 mM, pH 2.0) was evaluated by reversed-phase HPLC. The $t_{1/2}$ of hydrolysis, at 37 °C was ~ 25 days at pH 2.0 and ~ 5 days at pH 7.4. Importantly, and unlike the prodrug derivative **1b**, the predominant product of hydrolysis (> 96%) was the desired parent phosphorothioate **2** (data not shown). The presence of **3**, representing desulfurized material was minimal (< 4%). These observations are consistent with the hydrolytic process depicted in Figure 2.

In conclusion, in this model study, we have shown that compared to **1b**, the prodrug **7b** showed significantly improved stability at pH 2.0 and pH 7.4 while at the same time enabling esterase-mediated hydrolysis to regenerate **2**. Using this improved design concept, we have prepared oligonucleotide prodrugs wherein the "pro moiety" is incorporated site-specifically within the oligonucleotide framework. The results of these studies will be reported elsewhere.[10]

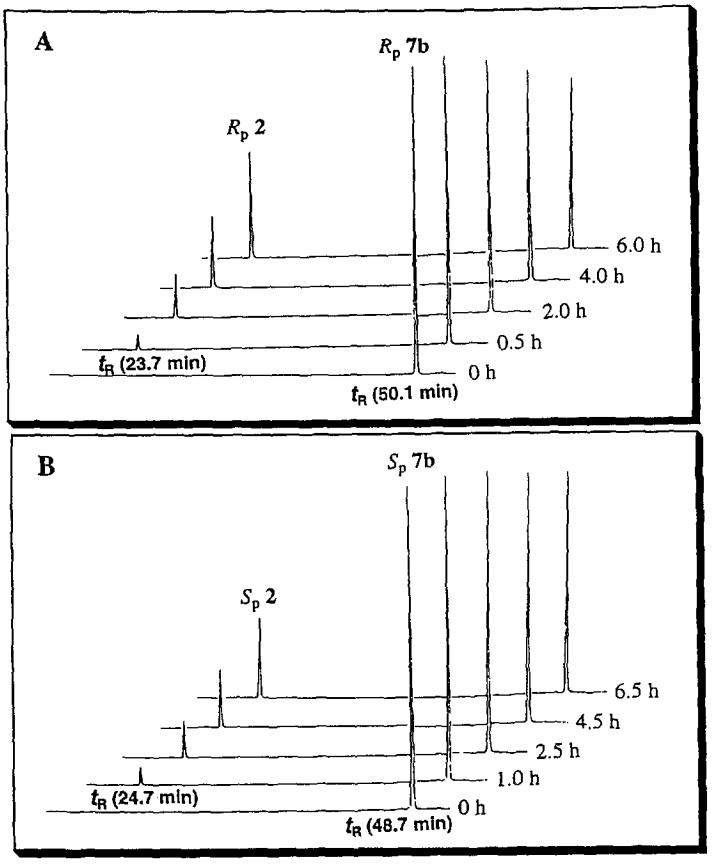


Figure 3. HPLC profiles of the time course of hydrolysis of R_p **7b** (A) and S_p **7b** (B) with human serum.

Experimental:. *(a)* ***Synthesis of 8***

A mixture of triphenyl phosphine (0.28 g, 1.07 mmol), imidazole (0.07 g, 1.07 mmol), iodine (0.27 g, 1.07 mmol), and **10** (0.14 g, 0.71 mmol) was dissolved in diethyl ether (2 mL) and acetonitrile (1 mL). The reaction was stirred for 2 h at ambient temperature and then partitioned between ether (20 mL) and water (5 mL). The aqueous layer was extracted with diethyl ether (3 x 10 mL). The organic layer was dried over Na_2SO_4 and solvent removed in vacuo. The residue was purified by flash column chromatography (silica gel) using hexane/ethyl acetate (90/10) as the mobile phase to give the product **8** in 75% yield.

8: R_f = 0.55 (silica gel, hexane/ethyl acetate, 80/20). ^{1}H NMR ($CDCl_3$) δ 1.3 (6H, d, J = 7 Hz), 2.8 (1H, septet, J = 7 Hz), 4.4 (2H, s), 7.0 (2H, d, J = 9 Hz), 7.4 (2H, d, J = 9 Hz) ppm; ^{13}C NMR (($CDCl_3$) δ 4.6, 18.8, 34.1, 121.8, 129.7, 136.6, 150.3, 175.2 ppm.

(b) ***Synthesis of 7b***. The dinucleoside phosphorothioate **2** was prepared as before. To 50 A_{260} units of **2** in 0.5 mL of Tris buffer (250 mM, pH 7.0) was added the iodocompound **8** (20 mg, 0.06 mmol) in acetonitrile

(0.5 mL) and the reaction mixture incubated in the dark at 37 °C. After 3 to 4 h, the reaction mixture was quenched by the addition of sodium bisulfite (0.5%, 100 μL) and evaporated to dryness in vacuo. The crude **7b** thus obtained was subjected to reversed-phase HPLC as described previously. The solvent was concentrated to a small volume and lyophilized dry to give 42 A_{260} units of **7b**.

References and Notes

1. For reviews see: (a) Bundgaard, H. In *Bioreversible Carriers in Drug Design. Theory and Application* Roche, E. B., Ed.; Pergamon: New York, NY., 1987, pp 13-94; (b) Balant, L. P.; Doelker, E. In *Burger's Medicinal Chemistry and Drug Discovery*, Wolff, M. E., Ed.; John Wiley & Sons: New York, NY., 1995; Vol. 1, pp 949-982; (c) For recent examples see: (i) Manouilov, K. K.; Fedorov, I. I.; Boudinot, F. D.; White, C. A.; Kotra, L. P.; Schinazi, R. F.; Hong, C.; Chu, C. K. *Antiviral Chem. Chemother.* **1995**, *6*, 230; (ii) Mitchell, A. G.; Thomson, W.; Nicholls, D.; Irwin, W. J.; Freeman, S. *J. Chem. Soc. Perkin Trans.* 1 **1992**, 2345.
2. (a) Iyer, R. P.; Phillips, L. R.; Biddle, J. A.; Thakker, D. R.; Egan, W.; Aoki, S.; Mitsuya, H. *Tetrahedron Lett.* **1989**, *30*, 7144; (b) Iyer, R. P.; Boal, J.; Phillips, L. R.; Thakker, D. R.; Egan, W. *J. Pharm. Sci.* **1994**, *83*, 1269.
3. (a) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2471; (b) Barber, I.; Rayner, B.; Imbach, J.-L. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 563.
4. For reviews see: (a) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 544; (b) Agrawal, S.; Iyer, R. P. *Curr. Op. Biotech.* **1995**, *6*, 12.
5. (a) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Chem.* **1995**, *23*, 1; (b) Iyer, R. P.; Yu, D.; Ho, N.-H.; Agrawal, S. *Synth. Commun.* **1995**, *25*, 2739; (c) Configurations were assigned as described.[5a]
6. For a discussion on phosphate ester hydrolysis see: (a) Cox, Jr., J. R.; Ramsay, O. B. *Chem. Rev.* **1964**, *64*, 317; (b) Dugas, H.; Penney, C. *Bioorganic Chemistry: A Chemical Approach to Enzyme Action;* Springer-Verlag: New York, 1981; pp 93-135.
7. A similar explanation in conjunction with the phenomenon of "pseudorotation"[8a] was first advanced to explain the product distribution during the hydrolysis of dimethyl phosphoacetoin, see: Frank, D. S.; Usher, D. A. *J. Am. Chem. Soc.* **1967**, *89*, 6360.
8. (a) The concept of pseudorotation (along with the associated "preference rules") is well known, and is used to explain the observed rate enhancement, as well as, the unusual product profiles observed during the hydrolysis of five-membered cyclic phosphates, phosphinates and phostonates, compared to their acyclic counterparts. For excellent reviews on this subject, see: (i) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70; (ii) Singleton Jr., R. *J. Chem. Ed.* **1973**, *50*, 538; (b) In the case of the prodrugs **1a-c**, although the suggested hydrolytic pathway involves the formation of six-membered ring intermediates, the concept of pseudorotation is being invoked only to account for the observed dichotomy in product distribution during their hydrolysis under enzymatic and non-enzymatic conditions.
9. Mitchell et al., have used the acyloxyaryl moiety for prodrug derivatization of phosphonoacetic acid; see ref 1c (ii).
10. Manuscript in preparation.

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