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Phosphorodithioates: synthesis and evaluation of new haptens for the generation of antibody acyl transferases

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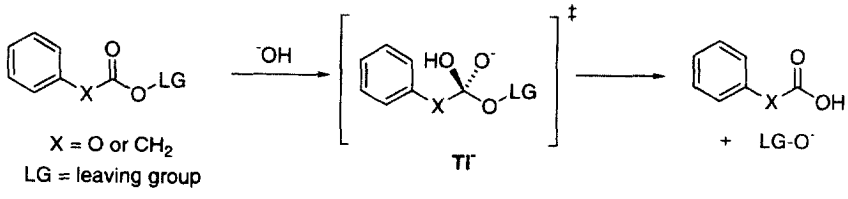
Abstract

An extension of the transition state analogue (TSA) hapten approach for the elicitation of catalytic antibodies for acyl-transfer reactions is described. It is based on the enlistment of phosphorodithioate **1** as a stable mimic of the putative tetrahedral intermediate (TI^-) formed during the base-catalyzed hydrolysis of bisaryl carbonate **2**. Six members of a library of 25 monoclonal antibodies elicited to **1** catalyze the hydrolysis of **2**. The most efficient catalyst, 48F10, exhibits Michaelis–Menten kinetics [K_m(**2**)=686 μM, k_{cat}(**2**)=2.7 min^{-1}, k_{cat}(**2**)/k_{uncat}(**2**)=3×10^4] and is one of the most active carbonate hydrolyzing antibodies yet reported. This report highlights the utility of haptens that incorporate substitution of the non-bridging phosphorus(V) oxygen atoms present in the more classical TSA approaches with sulfur, to elicit efficient catalysts for acyl-transfer processes.

Keywords: antibodies; catalysis; bisaryl carbonate hydrolysis; transition state analogues; phosphorodithioate.

Since the emergence of catalytic antibodies,[1] acyl-transfer reactions have remained a major focus in this field, primarily as a testing ground for new hapten strategies. Such reactions that involve good leaving groups proceed via rate-determining attack of the nucleophile to form a tetrahedral intermediate (TI^-) with subsequent expulsion of the leaving group, a fact supported by recent computational and ab initio analyses for the hydrolysis of both alkyl and aryl esters (Scheme 1).[2]



Scheme 1. Base-catalyzed acyl-transfer reactions proceed via the putative anionic tetrahedral intermediate (TI^-)

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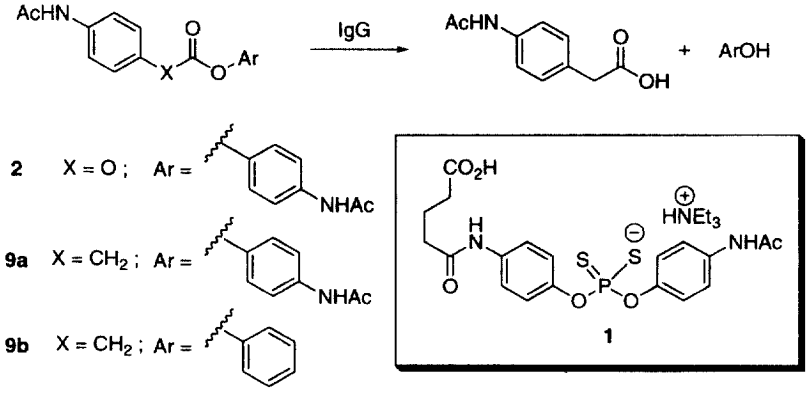
Thus, for the generation of antibodies that catalyze the hydrolysis of aryl esters, alkyl esters, carbonates and anilides, the design of the hapten has focused on facilitating nucleophile attack.[3] The tetrahedral mimics used for this purpose initially deployed phosphorus(V) systems as transition state analogues (TSAs). Many of the possible species containing an ionized P–OH group have been included: phosphate diesters,[1b] phosphonate monoesters,[4] phosphorothioate diesters,[5a] phosphonamidates[5b] etc. Other tetrahedral systems have included sulfones,[6] secondary alcohols[7] and α-difluoroketones.[8] Despite the success of the TSA strategy, recent ab initio investigations[2] have revealed that the lack of congruency between the TSAs and the TI^{-} (and the transition-states which flank it) may be limiting further development of this approach.[2b,9] Therefore, there is considerable effort being expended to improve the scope and efficiency of antibody catalysts by the incorporation of novel TSA designs.

Based on the calculations of Teraishi[2a] and Houk,[2b] we rationalize that phosphorodithioates such as **1**, which possess two non-bridging sulfur atoms, may be superior to the phosphoryl–oxygen containing hapten systems vide supra because the polarization of the P=S bond is coupled with longer bond-lengths; ionized $P–S^{-}$=1.98 Å (Scheme 2).[10] Therefore the phosphorodithioates possess bond lengths more comparable (0.1[2a]–0.3[2b] Å shorter) to the C···O bonds forming and breaking in the transition states than do the phosphate-containing haptens (0.6[2a]–0.8[2b] Å shorter). Antibodies elicited to phosphorodithioates, therefore, may be able to bind, and hence stabilize the transition states for this reaction more efficiently than antibodies elicited to their phosphate analogues and consequently be more powerful catalysts.

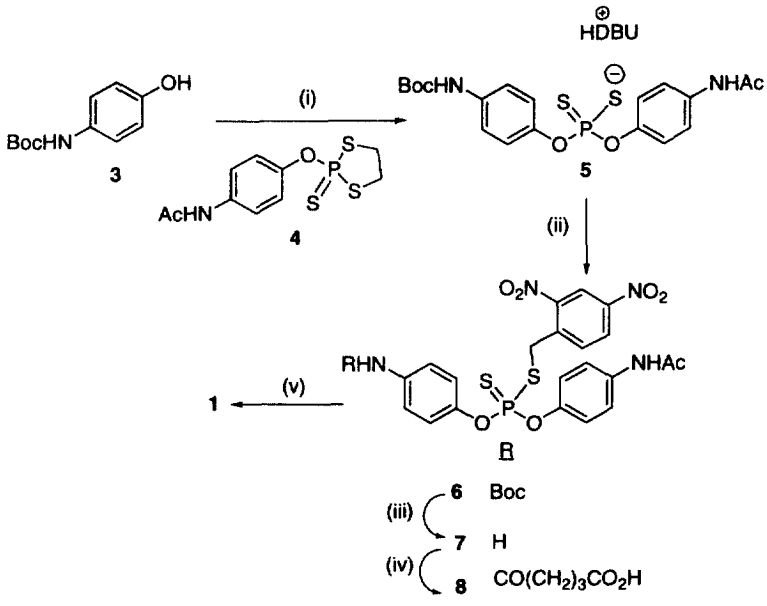


Scheme 2. Phosphorodithioate **1** deployed as a TSA hapten to elicit antibodies for the hydrolysis of bisaryl carbonate **2** and aryl esters **9a–b**

This report describes the utilization of a phosphorodithioate hapten (**1**), as a member of a new class of TSAs, for the elicitation of catalytic antibodies for the hydrolysis of the bisaryl carbonate (**2**). Hapten **1** was synthesized as outlined in Scheme 3. Incorporation of the phosphorodithioate core of **1** was achieved using a modification of Martin's[11] procedure which involves the base-induced reaction of Boc-protected 4-aminophenol **3** with 2-thio-1,3,2-dithiaphospholane **4**.

Phosphorodithioate **5**, which is unstable under both basic and acidic conditions, was protected as its 2,4-dinitrobenzyl phosphorodithioate ester (**6**).[12] Removal of the Boc-group of **6** then occurred smoothly under acidic conditions and was followed by subsequent reaction of aniline **7** with glutaric anhydride to give the linker-appended precursor **8** in excellent yield (91% from **6**). Removal of the 2,4-dinitrobenzyl group, with 4-thiocresole and triethylamine, gave hapten **1** in good yield (88%).

Following conjugation of hapten **1** to the carrier proteins bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), Balb/c mice were immunized with the KLH-**1** conjugate. Monoclonal antibodies were generated and purified by standard hybridoma protocols[13] and 25 clones were obtained which bound to the BSA-**1** conjugate. In a preliminary screening assay,[14] six monoclonal antibodies were



Scheme 3. *Reagents and conditions*: (i) DBU, 74%; (ii) 2,4-dinitrobenzyl chloride, lutidine, 89%; (iii) TFA, CH_2Cl_2, 99%; (iv) glutaric anhydride, 92%; (v) 4-thiocresole, NEt_3, 85%

found to catalyze the hydrolysis of carbonate **2**. The kinetic parameters of the most proficient antibody, 48F10, were studied in detail.

48F10 exhibits classical Michaelis–Menten kinetics and performs multiple turnovers without any reduction in activity. The turnover number, k_{cat}(**2**)=2.7 min^{-1}, is equivalent to a rate enhancement, k_{cat}(**2**)/k_{uncat}(**2**), of 3×10^4 and is amongst the highest noted for any carbonate hydrolyzing antibody.[15] The catalytic activity of 48F10 is competitively inhibited[16] by phosphorodithioate **1** (K_i=1.2 μM). Preliminary substrate specificity studies with antibody 48F10 have revealed limited tolerance for any changes in the core structure of bisaryl carbonate **2**. Aryl ester **9a**, where a bridging oxygen atom has ben replaced with a methylene group is still a substrate [K_m(**9a**)=599 μM, k_{cat}(**9a**)=0.27 min^{-1}, k_{cat}(**9a**)/k_{uncat}(**9a**)=5×10^3], but ester **9b** where methylene replacement is coupled with 4-acetamido-substitution for a hydrogen atom, is a very poor substrate. Furthermore, alkyl esters where the 4-acetamidophenyl group of **9a** has been replaced with ethyl or cyclohexyl groups are not substrates.

Despite the considerable body of knowledge that has been generated, both structural and kinetic, regarding the link between hapten design and antibody catalysis, there is still considerable uncertainty regarding the haptenic structural requirements necessary to elicit proficient catalysts.[3] This study has shown that phosphorodithioates are excellent haptens for the elicitation of antibodies with acyl transferase activity. The most efficient catalyst, 48F10, has an enhancement ratio for the catalysis of hydrolysis of bisaryl carbonate **2** superior to those achieved by utilizing more classical phosphate haptens. Based on this one study it is preliminary yet to conclude as to whether the phosphorodithioate hapten system will be a better transition-state mimic than the other phosphorus(V) systems vide supra. However, we are pursuing an ongoing study to substitute the atoms around the tetrahedral anionic phosphorus(V) core with moieties that serve to increase the bond lengths (relative to oxygen) and thus help to resolve this question more fully.

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14. Measured at pH 7.0 (50 mM HEPES, with 1% Tween-80 and 4% DMSO as co-solvent) at room temperature. Kinetic assays were followed using reversed-phase HPLC [CH_3CN/H_2O (1% TFA) 18:82 mobile phase, with 4′-fluoroacetanilide as an internal standard (IS)] by observing the formation of 4-acetamidophenyl acetic acid at λ=254 nm. Reactions were performed at 200–800 μM substrate (**2**) concentrations and 2 μM antibody (purified by protein-G affinity and mono-Q ion-exchange column chromatography) concentration. At appropriate times during the assay an aliquot (30 μL) of the reaction mixture was removed and added to an equal volume of IS solution (100 μM in water). This mixture was then analyzed by HPLC vide supra. The assay was followed for no more than 5% of the reaction progress, during which time the progress curves were linear (r^2>0.985). The non-catalyzed rates, k_{uncat}, of bisarylcarbonate **2** and ester **9a** are 9.0×10^{-5} min^{-1} and 5.3×10^{-5} min^{-1}, respectively, and were determined by the method of initial rates using the buffer system vide supra.
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16. Phosphorodithioate **1** is a tight-binding inhibitor of both 48F10, therefore K_i determinations was not made using the classical Lineweaver–Burke analysis over a range of inhibitor concentrations which is known to be subject to error. Rather the method of Copeland et al. (Copeland, R. A.; Lombardo, D.; Glannaras, J.; Decicco, C. P. *Bioorg. Med. Chem. Lett.* **1995**, 1947) was employed, which allows for the accurate determination of the K_i values of tight-binding inhibitors.