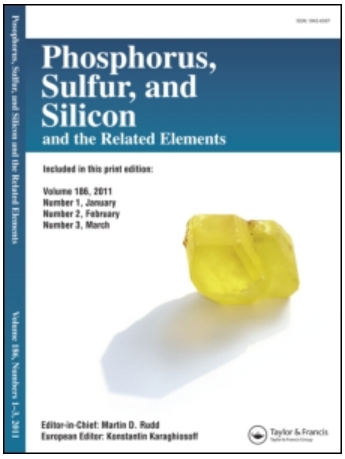

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From Phosphonates to Catalytic Antibodies. A Novel Route to Phosphonoester Transition State Analogs and Haptens

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FROM PHOSPHONATES TO CATALYTIC ANTIBODIES. A NOVEL ROUTE TO PHOSPHONOESTER TRANSITION STATE ANALOGS AND HAPTENS

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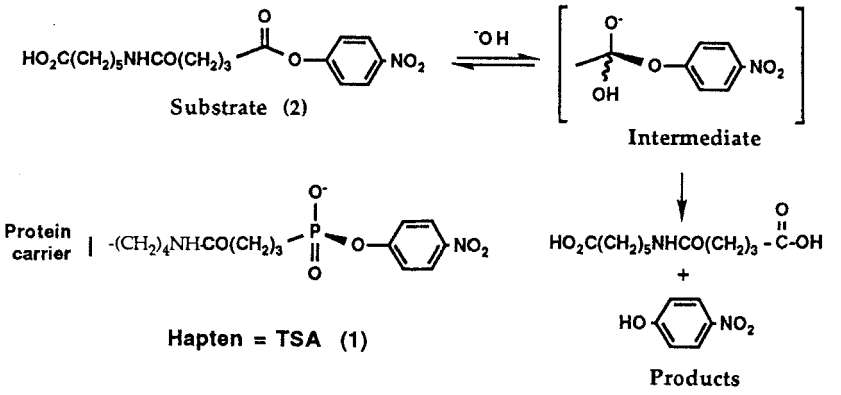
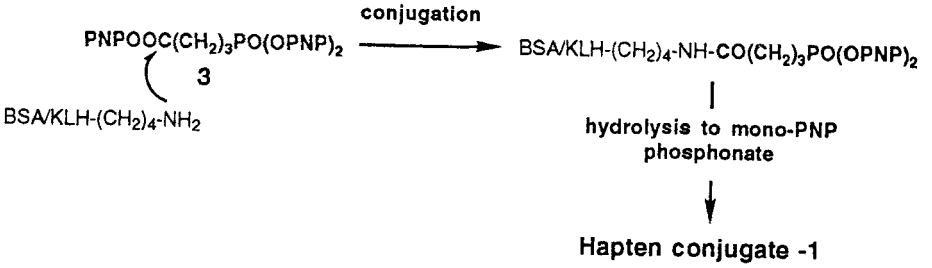
Abstract We present here a newly developed approach based on DBU catalyzed transesterification of bis-PNP-phosphonates, used for the preparation of phosphonoesters and their protein conjugates. These are being used, as transition state analogs, to elicit catalytic antibodies having enzyme-like properties.

Phosphono and phosphoro esters and amidates mimic the tetrahedral negativly charged intermidiate, and transition state, involved in ester and amide hydrolysis (Scheme 1). These transition state analogs (TSA) were shown to be potent inhibitors of many hydrolytic enzyme, and have also been used to elicit catalytic antibodies for a variety of acyl transfer reactions (1). Antibodies raised against phosphonates selectivly bind to the TS for a specific reaction with high affinity reduce the activation energy barrier and thereby catalyze that reaction.

We have described the production of esterolytic antibodies elicited against a mono-p-nitrophenyl (PNP) phosphonate hapten, **1** (Scheme 1). Several of the resulting hapten-binding monoclonal antibodies catalyze the hydrolysis of the corresponding PNP ester (**2**) and carbonate with enzyme-like properties, i.e., rate enhancement ($k_{cat}/k_{uncat} = 10^4$), turnover and high substrate specificity (2). Recently, we have found that the catalytic activity of these antibodies can be assigned to their differential affinity for the phosphonate TSA vs. the substrate, as predicted by transition state theory. A newly developed approach was used for the preparation of the phosphonate hapten, **1** (Scheme 2). p-Nitrophenyl 4-bis-p-nitrophenylphosphonobutyrate **3** was linked to the carrier protein via amidation of the carboxy PNP ester by the ε-amino groups of lysine side chains. One of the PNP phosphonate bonds was then hydrolyzed on the protein to yield the desired mono-PNP-phosphonate hapten-protein conjugate **1**.

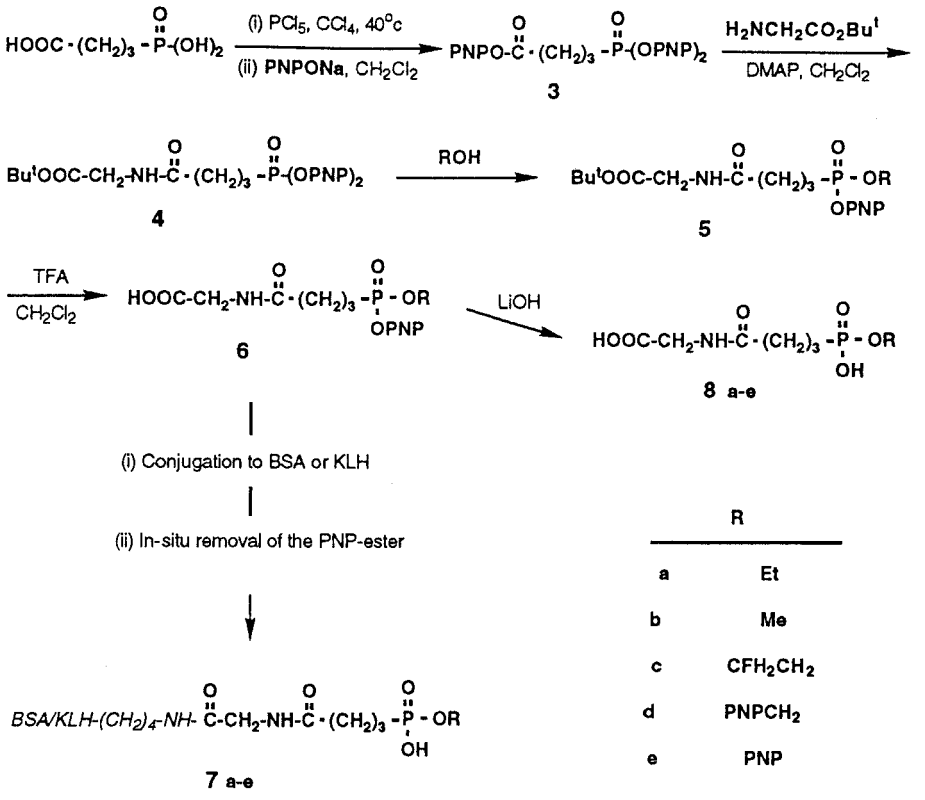


SCHEME 1 Hydrolysis of a p-nitrophenyl ester (**2**) via a tetrahedral intermidiate and the structure of the phosphonate hapten **1**.



SCHEME 2 Preparation of the protein conjugates of hapen **1**.

We have shown that the conjugation, i.e., amidation, takes place solely at the carboxy-PNP ester and that the bis-PNP-phosphonate is cleanly hydrolyzed to the mono-PNP-phosphonic acid. This novel approach is simple and reliable; it avoids the free carboxy-phosphonic acid, which is commonly used as a hapten (1), but which is hard to synthesize, isolate, and may also conjugate to proteins via the phosphonic acid.
The scope of this approach was expanded to other phosphonate haptens (Scheme 3). We found that the preparation of phosphonoesters using DBU (1,8-diazabicyclo [5.4.0]-undecene) catalyzed ester exchange of bis-PNP-phosphonates is significantly shorter than other routes used to obtain such compounds (3).



SCHEME 3 Preparation of a series of phosphonoester haptens via DBU catalyzed transesterfication of PNP-phosphonate **4**.

The resulting monoalkyl-mono-PNP-phosphonates are then easily hydrolyzed to the corresponding phosphonoacids. The transesterification of bis-PNP-phosphonates proceeds rapidly: in the presence of one equivalent of DBU, a period of 10 minutes-3 hours (depending on the excess of alcohol) is required to complete the exchange. Unlike the esterification of dichlorophosphonates by alcohols, where monosubstitution is not easily achieved, the rate of exchange of the second PNP-ester is significantly slower so that the monoalkyl phosphonates **5** were obtained in high yield and, as judged by P^{31} NMR, bis-alkyl phosphonates or any other side products are not formed. Isolation and handling of the intermediates and products is convenient since PNP-phosphonates are very often crystalline and easy to monitor and purify by TLC/PLC due to their intense UV absorption.

The carboxy group of **6** (as NHS or other activated ester) was covalently linked to a protein carrier. *In situ* hydrolysis of the PNP ester afforded the immunogen or protein conjugate. It was shown, by P^{31} and H^1 NMR, that base-catalyzed hydrolysis of the monoalkyl-monoPNPphosphonates, **6**, proceeds cleanly to give the corresponding phosphonoacids, **8**.The released p-nitrophenol (OD at 405 nm) allows the convinient determination of the hapten density.

All these TSA haptens were shown to elicit hapten-specific monoclonal and polyclonal antibodies. Several monoclonal antibodies out of those obtained against the p-nitrobenzyl phosphonate (as KLH conjugate, **7d**) were found to efficiently catalyze the hydrolysis of the corresponding p-nitrobenzyl ester.

The DBU catalyzed transesterification of PNP phosphonates was developed to allow the rapid and convenient synthesis of phosphonoester haptens. In view of the wide-ranging importance of phosphonates in chemistry and as biologically active compounds, and the difficulties frequently encountered in their synthesis, we intend to further investigate the mechanism of the DBU-catalyzed transesterification reaction and its scope as a general route to phosphonoesters and amidates.

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1. For reviews see: B.S. Green and D.S. Tawfik Trends Biotech., 7, 304 (1989); R.A. Lerner, S.J. Benkovic and P.G. Schultz Science, 252, 659 (1991).
2. D.S. Tawfik, R.R. Zemel, R. Arad-Yellin, B.S. Green and Z. Eshhar Biochemistry, 29, 9916 (1990).
3. The preparation of mono and disubstituted phosphonates usualy involves selective hydrolysis of bis-methylphosphonates to yield the corresponding monophosphonic acid which is then chlorinated and reacted with the appropriate alcohol; hydrolysis of the remaining methyl ester yields the monoalkylphosphonic acid (for example see, N.E.Jacobsen and P.A. Bartlett J.Am. Chem. Soc., 103, 654 (1981). Potent enzyme inhibitors were prepared by this method which was also adopted for the synthesis of most of the phosphonate haptens used to raise catalytic antibodies (1).