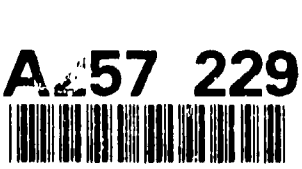

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The prophylactic administration of a drug capable of reacting quickly with cyanide and removing it from the circulation is one approach to the problem of cyanide intoxication. α,β-Unsaturated ketones, esters, nitriles, etc., undergo 1,4-addition with hydrogen cyanide to form stable compounds. We have investigated the reaction of cyanide with 4-phenyl-3-buten-2-one at 37°C and pH 7.4 in aqueous phosphate buffer. Disappearance of 4-phenyl-3-buten-2-one and appearance of the product 4-phenyl-4-cyanobutan-2-one were followed by HPLC analysis on a reverse phase column. The reaction rate was first order in enone and KCN with a rate constant of 0.024 to 0.082 $M^{-1}h^{-1}$. Catalysis of such reactions by an antibody to the transition state for cyanide addition offers a potential for enhancement of the rate to useful levels. The development of such antibodies by use of transition state analogs as haptens is our overall goal. The antibodies would be combined with a drug which is a substrate for cyanide addition. Design and synthesis of the potential drug and its transition state analog are being carried out.

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PI - Signature C. E. Cook DATE 10/10/91

TABLE OF CONTENTS

1.0 Introduction

1.1 Background

The toxicity of cyanide and its ready availability make the development of treatment or prophylactic regimens of importance. Current treatment methods include:

- Administration of drugs that bind cyanide,
- Administration of drugs that induce methemoglobin formation with the resulting methemoglobin binding cyanide, and
- Administration of drugs that augment endogenous enzymatic detoxification.

An extension of the last method would be administration of both a drug that reacts with cyanide and an enzyme that catalyzes the reaction. The enzyme could be replaced with a catalytic antibody. The antibody could be administered prior to anticipated exposure to cyanide, because of the long half-life of antibodies. The drug could be administered prophylactically as long as the threat existed.

The concept of catalytic antibodies is that an antibody to the transition state of a reaction would be able to catalyze the reaction just as an enzyme can--by stabilizing the transition state and thus lowering the energy required for the reaction. Catalytic groups (for example, acid-base catalysts) can also play a role.

To develop an antibody against the transition state of a reaction, one must design an analog that is stable but is close in structure to the transition state. When this compound is conjugated to a protein, the resulting antigen can stimulate the formation of antibodies which stabilize the transition state.

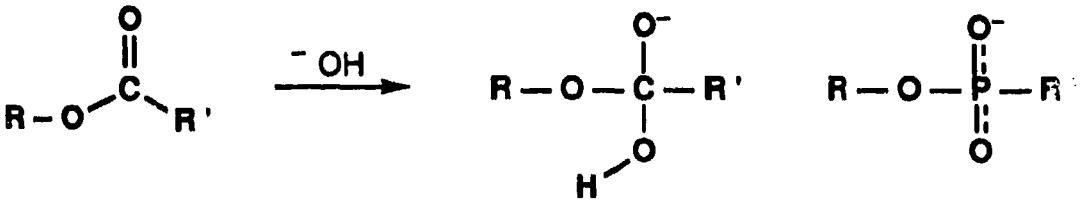
The current status of catalytic antibodies has been recently reviewed by Lerner et al. (1991). The first and simplest example of catalytic antibodies involves ester hydrolysis. The transition state for ester hydrolysis resembles structure A-2 (Chart A). The trigonal carbonyl carbon of ester A-1 becomes tetrahedral before collapsing to yield alcohol and carboxylic acid. Thus tetrahedral phosphonate esters (A-3) are transition-state analogs for ester hydrolysis. Antibodies formed to phosphonate esters have been shown to catalyze ester hydrolysis, with more than 20 such reactions having been reported. Rate accelerations of nearly 10^8 M compared with uncatalyzed reactions have been observed. At least 17 different types of organic reactions have been reported to be catalyzed by antibodies (Lerner et al., 1991). Although the development of antibodies which catalyze bimolecular reactions, such as amide bond formation, has been achieved, this task is more difficult than developing antibodies to catalyze unimolecular or hydrolytic reactions. To date antibodies catalyzing 1,4-additions to conjugated carbonyl systems have not been reported.

1.2 Approach

To apply the catalytic antibody-drug approach to the removal of cyanide requires:

- Selecting a reaction of cyanide;
- Defining the transition state of the reaction;
- Selecting a structural analog that approximates the transition state;
- Designing an appropriate drug;
- Designing the transition-state analog in a form that can be linked covalently to protein;
- Synthesizing the drug, transition-state analog, and protein conjugate;
- Developing monoclonal antibodies to the transition-state analog;

CHART A
ESTER HYDROLYSIS AND TRANSITION STATE ANALOG



A-1 **A-2** **A-3**

- Screening the antibodies to find one which catalyzes the desired reaction; and
- Biological testing.

Cyanide reacts with enones by the pathway shown in Chart B. Study of the kinetics of the process (Agami et al., 1982) indicates that the transition state is product-like (i.e., it resembles structures B-3 and B-4). One approach to a transition-state analog would be to stabilize the enol form B-4 by forming a stable enolate, such as an enol ether, enol silyl ether or perhaps an enol phosphate, phosphonate, or phosphinate (B-6). Alternatively, one could mimic the enolate form B-3 by means of a nitrone.

Once the basic transition state mimic has been defined, the complete structure of the hapten used for conjugation to protein must be designed, together with the structure of the drug which is to react with cyanide under antibody catalysis. The structure of the product of this reaction must also be considered. The structures of a hapten (C-1), drug (C-2), and product (C-3) combination proposed are shown in Chart C, together with an outline of the features considered in their design.

We considered that it would be important to have the relative affinities for catalytic antibody to be drug $<$ transition state $>>$ product. The increased affinity on going from drug to transition state would lower the transition state energy. The product should have lower affinity than the drug to prevent product inhibition of the reaction. Use of a cycloaliphatic ring would provide a semi-rigid platform from which the substituents would shift into different steric relationships in C-1, C-2, and C-3, thus resulting in the desired differential binding to the antibody.

The position of the double bond in C-1 mimics the transition state in which the cyanide would already be almost bonded. The oxygen enolate of C-1

CHART B
REACTION PATHWAY TO HYDROCYANATION OF ENONES

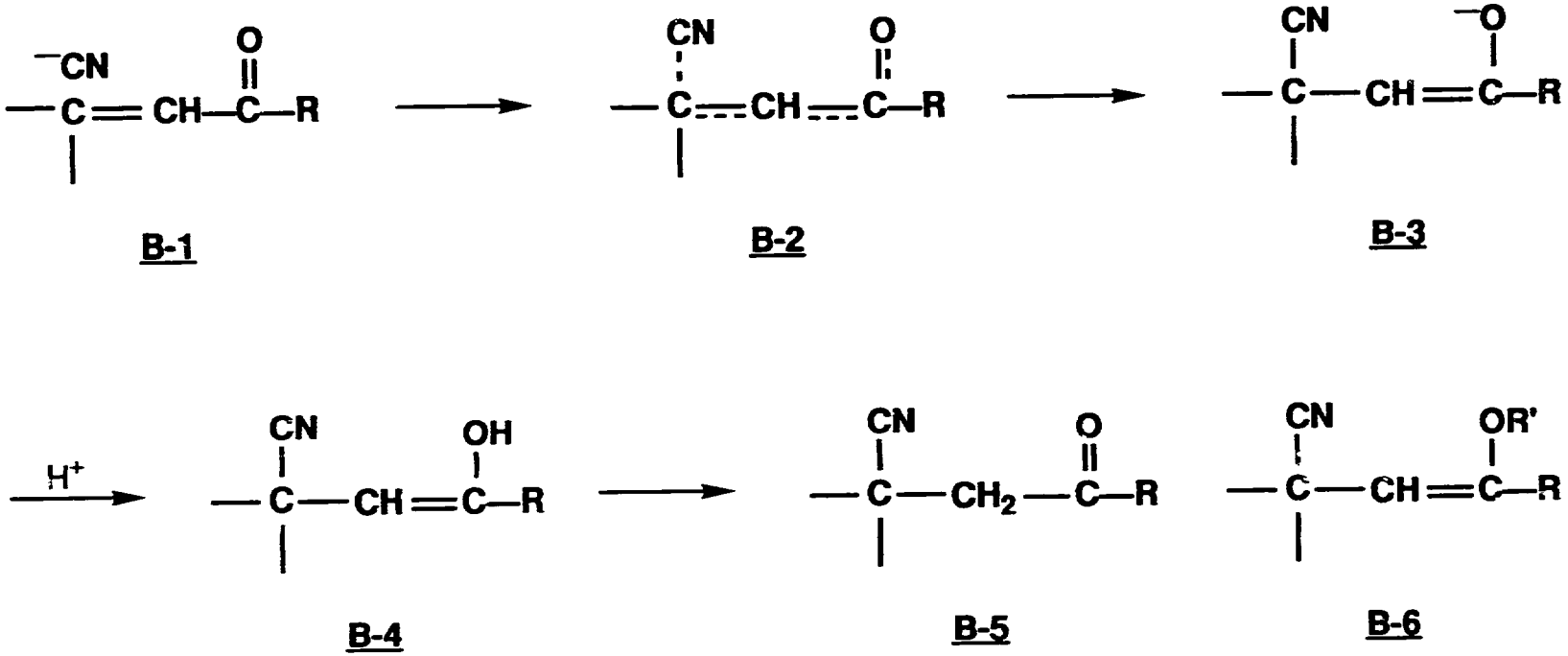
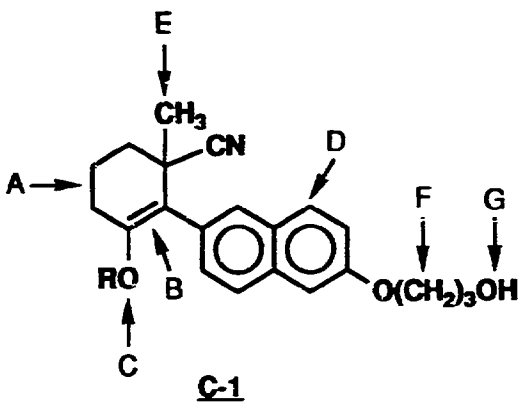
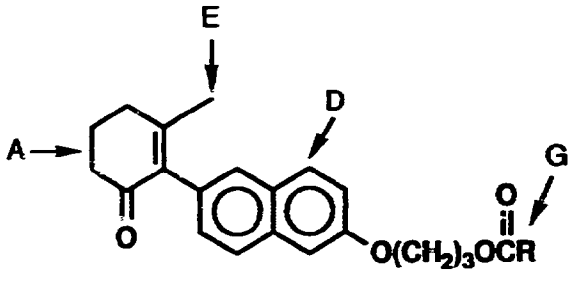


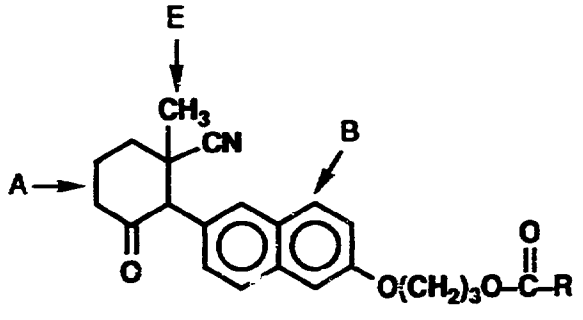
CHART C
FEATURES OF THE TRANSITION STATE ANALOG HAPTEN, DRUG AND REACTION PRODUCT



C-1
Transition State Analog



C-2
Drug



C-3
Product

A. Cycloaliphatic ring provides semiflexible platform for differentiating drug, transition state analog, and product.
B. Enol ether mimics product-like transition state.
C. Oxygen provides hydrogen-bonding site to stabilize transition state.
D. Aromatic moiety provides rigidity, bulk, and immunogenicity. Its shift in position during the reaction helps differentiate binding of drug, transition state, and product.
E. Substituent prevents in vivo α-hydroxylation which could release cyanide.
F. Link separates hapten from protein in conjugate.
G. Hydroxyl allows variation of drug structure by acylation as well a conjugation to protein.

can help to develop a hydrogen-bond donor site in the antibody to further stabilize the transition state.

A large aromatic moiety (e.g., naphthyl) should enhance immunogenicity. Because its steric position would shift during the reaction, differential binding would be enhanced. Since α-aryl enones react more rapidly with cyanide than do β-arylenones (Nagata and Yoshioka, 1977), the α-position is preferable for the aryl group. The nonsteroidal anti-inflammatory drug naproxen has a naphthyl moiety. It is given in fairly large doses and has low toxicity. There is some concern about renal toxicity of naproxen in renally compromised patients (Stewart et al., 1990), but this is likely due to its mode of action (effect on prostaglandin synthesis). Since the proposed drug will not contain the carboxyl moiety of naproxen, it is unlikely to affect prostaglandin synthesis or action. Enone compounds such as the chalcones are not mutagenic. Thus although the toxicity of the selected drugs is subject to experimental findings, a priori low toxicity may be expected.

Placing a substituent α to the cyano substituent would prevent metabolic α-hydroxylation which would yield a cyanohydrin that might dissociate to release cyanide. (Tertiary nitriles are less toxic than primary or secondary nitriles.)

An alkyloxy link to the aryl group was chosen to separate the hapten from the protein in the immunogen. A terminal hydroxyl group provides a means for linking to protein. It also can be acylated or alkylated to provide drug structures of varying degrees of lipophilicity.

2.0 Reaction of Cyanide with α,β-Unsaturated Compounds

2.1 Introduction

As mentioned previously, Agami et al. (1982) have studied the kinetics of hydrocyanation of enones. These studies were carried out at high pH and temperature and in the presence of high concentrations of organic solvent.

Agami et al. (1982) found the rate of 1,4-addition of cyanide to enones to be a second order reaction (first order in both $[CN^-]$ and [enone]). Therefore

$$dS/dt = k\,[CN^-][S] \text{ where } S = \text{substrate (enone)}. \qquad \text{Eq. 1}$$

The reaction has been shown to depend on the $[CN^-]$ rather than the total cyanide (CN^- + HCN) present in the solution. However the ratio $[CN^-]/([CN^-] + HCN])$ will remain constant to a first approximation as long as the pH remains constant.

Under conditions where total cyanide is in large excess throughout the experiments and the pH is constant, the reaction can be treated as a first order kinetic process. The reaction rate is then as follows:

$$dS/dt = k'[S] \qquad \text{Eq. 2}$$

where the constant k' contains the (constant) total cyanide concentration. Half-lives for the reaction can be determined for the total cyanide concentration and pH involved.

In their experiments, Agami et al. (1982) monitored the disappearance of enone by the reduction in ultraviolet absorbance. In particular, the UV of 4-phenyl-3-butene-2-one (λ_{max} ca. 290 nm, ϵ ca. 20,000) decreases on reaction with cyanide ion give 4-phenyl-4-cyanobutan-2-one--a compound with an extinction coefficient much less than 1000.

Since it is the actual cyanide ion concentration rather than the total of CN-plus HCN which is important in determining the rate of the reaction, and

since hydrogen cyanide has a pka of approximately 9.4, one would predict that the rate would decrease markedly with decrease in pH. Thus there was a question whether the reaction would proceed at a measurable rate under physiological conditions (ph 7.4 and 37°C) in the absence of an antibody catalyst. Since this was of considerable concern to the sponsor, a significant amount of effort was invested in exploring it.

2.2 Experimental Results

To compare the effects of ketone (-CO-R), nitrile (-C≡N) and ester [-C(O)-OEt] on the rates of 1,4-addition to a double bond, we chose 3 commercially available compounds--4-phenyl-3-buten-2-one, cinnamonitrile, and ethyl cinnamate and one compound made in-house (3-phenyl-2-cyclohexen-1-one).

ϕ-CH=CH-$COCH_3$ ϕ-CH=CH-CN ϕ-CH=CH-COOEt

These were incubated with KCN at 37°C and the UV absorbance (A) measured at 3-4 times from 0-30 h. Linear regression analysis of ln A as a function of time gave the rate constants in Table 1. These initial data indicated that the ketone reacts with a rate about 2.6 times that of the nitrile and 12 times that of the ester. They also indicated that making the enone part of a cyclohexenone system (e.g., 3-phenyl-2-cyclohexen-1-one as shown in Table 1) reduces the rate by a factor of ca. 4. Although these experiments were preliminary and relatively crude, they did indicate that the order of reactivity was consistent with that given by Nagata and Yoshioka (1977). Therefore further efforts were focused mainly on the reactions of 4-phenyl-3-buten-2-one with cyanide.

Further study of this reaction indicated that when the buffer concentration was increased to 0.15 M phosphate buffer and the KCN concentration to 0.154 M, UV absorbance values were no longer useful for monitoring the reaction. The use of UV absorbance was complicated by changes which resulted in

TABLE 1
RATES OF 1,4-ADDITION OF CYANIDE TO UNSATURATED SUBSTRATES[a]

[KCN] (M)	pH of Added[b] Buffer	k' (h^{-1})[c] 4-Phenyl-3-buten-2-one	Cinnamonitrile	Ethyl Cinnamate	3-Phenyl-2-cyclohexen-1-one
0.0154	7.4	0.0052	-[d]	-	-
0.0154	8.4	0.0137	0.0052	-	0.00326
0.154	7.4	0.127	-	0.011	0.0317

a All substrates were added in acetonitrile solution (4 μL per 2 mL reaction) to give an initial concentration of 20 μM.

b Buffer was 50 mM phosphate buffered saline (containing the KCN). Thus, the actual pH (considering effect of KCN) is probably > nominal pH of buffer.

c k' is the pseudo first order rate constant at given concentration of cyanide and buffer. Values are not based on optimal sampling times.

d - indicates insufficient data to determine k'.

both a bathochromic shift and an increase in absorbance. These changes were shown by control studies to be related to the presence of the KCN alone, whereas the 4-phenyl-3-buten-2-one itself appeared to undergo some reaction with the phosphate buffer solution, which contained a small amount of acetonitrile. Thus it was decided that a more rigorous analytical procedure would have to be adopted. It appeared essential that we be able to analyze both reactant and product. To do this we synthesized the product of the cyanide addition with 4-phenyl-3-buten-2-one and developed an HPLC system by which we could analyze both the starting enone and the final product, 4-cyano-4-phenylbutan-2-one.

4-Phenyl-3-buten-2-one was treated with potassium cyanide by the procedure of Agami et al. (1982), and the resulting product, 4-phenyl-4-cyanobutan-2-one, was isolated. The ^{3}H-NMR spectrum of this compound was completely consistent with the proposed structure: δ ($CDCl_3$, 90 MHz) 2.15 (s, CH_3), 3.1 (m, CH_2-CO), 4.25 (t, CH-CN), 7.45 (s, ArH) ppm.

An HPLC system was developed for the analysis of the enone and the cyano adduct. A C-18 column with a solvent system of 60% methanol and 40% water gave retention times of 11.5 min for the reactant and 6.6 min for the product. UV absorbance was monitored at 220 nm.

Potassium cyanide was added to 0.145 M phosphate buffer which was originally at pH 7.4 and pH 8.4, respectively. The pH of these solutions was then adjusted back to the starting values by addition of a small amount of concentrated hydrochloric acid. Each solution (2.0 mL) was incubated at 37°C for 15 min and 80 μL of an acetonitrile solution of the enone was then added. Final concentrations were 195 mM KCN and 0.376 mM enone. The measured pH was 7.4. The reaction mixture was incubated at 37 ± 1°C. Duplicate 20 μL samples of the reaction mixture were drawn periodically and analyzed directly by HPLC.

Concentrations were measured from peak areas using external standard solutions (substrate 0.98, 0.098, and 0.0098 mM; product 2.88, 0.288, and 0.0288 mM) which were run adjacent to each reaction aliquot. The concentrations reported were the average of the two aliquots. The reaction was followed for 260 h.

The first order equation $C = Ae^{-k't}$, where C is the concentration of the phenylbutenone and t is the time in hours, was fit to the data using the statistics and data handling program RS/1 (BBN). The concentration of the phenylbutenone showed a first order decline (Figure 1).

The pseudo first order rate constant k' based on disappearance of enone in Figure 1 was 0.00478 ± 0.00011 (SE) h^{-1}. The rate of formation of product could also be determined by subtracting the product concentration from the starting concentration of enone and performing a computer fit to a first order equation. This approach assumes that all of the reactant will eventually be converted to product. Although the data points were a little more scattered, the fit was quite reasonable with a k' value of 0.00452 ± 0.00051 (SE) h^{-1}. The close correspondence between the two values of k' indicates that the reaction at pH 7.4 goes relatively cleanly from phenylbutenone to phenylcyanobutanone.

Table 2 summarizes results from experiments of this type. These experiments were deliberately set up utilizing high concentrations of KCN relative to the phenylbutenone. Thus, although the reactions are reported to be first order in both cyanide ion concentration and enone concentration, in effect the KCN concentrations will remain essentially constant throughout the course of the reaction. Cyanide ion concentration should be constant also because the solution is buffered to a given pH. The average k' for these experiments at ph 7.4 was 0.0048 ± 0.0003 (SD) H^{-1}. Assuming $k' = k[KCN]$, $k = \sim 0.025\ M^{-1}h^{-1}$ for the bimolecular reaction.

FIGURE 1

Kinetics of reaction of KCN with 4-phenyl-3-buten-2-one. Initial concentrations were 195 mM in KCN and 0.376 mM in enone, so that reaction was pseudo first order in enone. Lines are computer best fit of $c = Ae^{-kt}$. pH was 7.4, temperature 37°C.

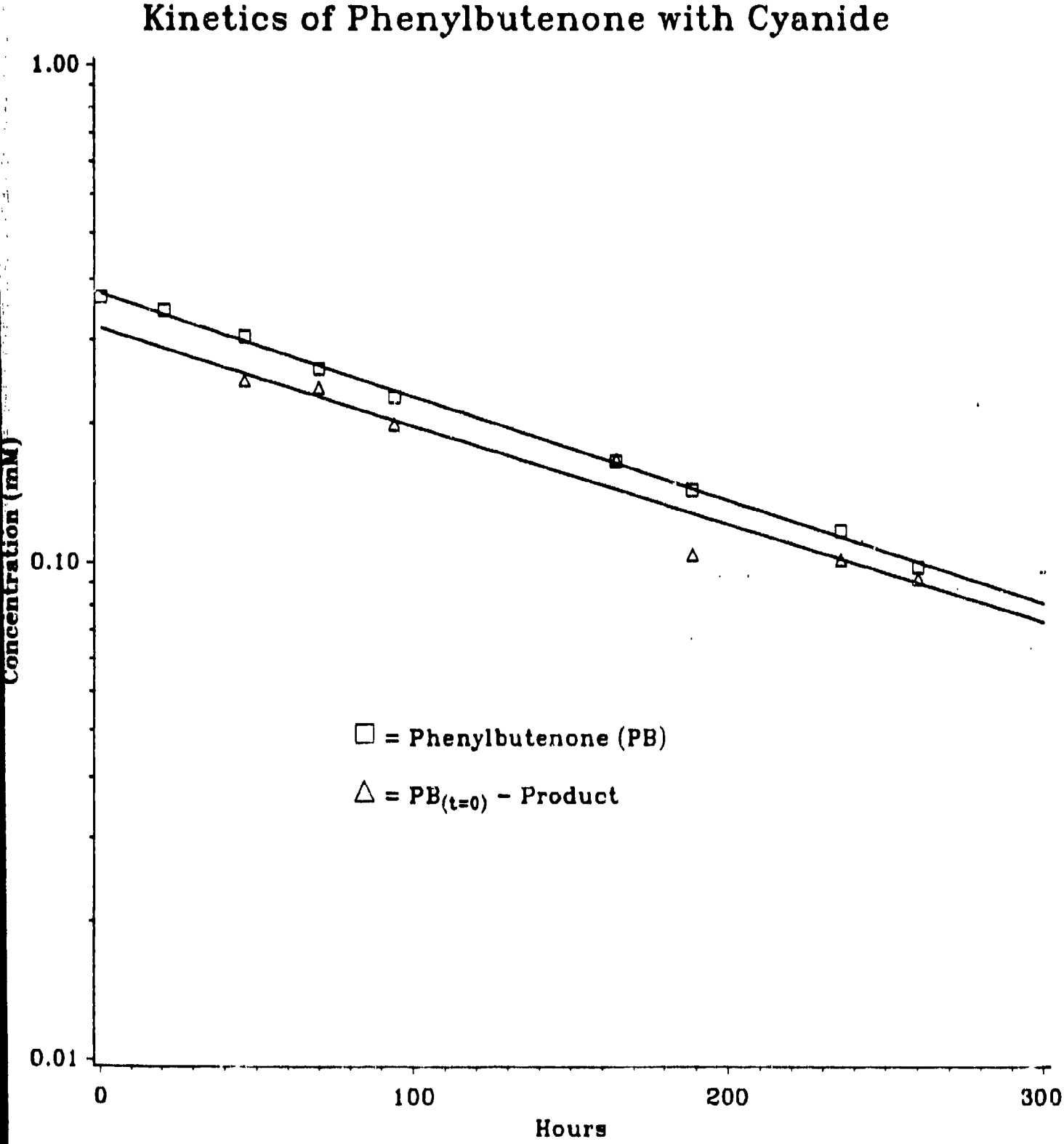


TABLE 2

Rate Constants for the Reaction of KCN With 4-Phenyl-3-buten-2-one at pH 7.4 and 37 °C

Experiment Number[a]	Substrate Concentration	KCN Concentration	Pseudo First Order Rate Constant	Second Order Rate Constant[b]
1	400 μM	204 mM	0.00474 /h[c]	0.0232 L/mole/h[c]
			0.00523 /h[d]	0.0256 L/mole/h[d]
2	200 μM	204 mM	0.00493 /h[c]	0.0242 L/mole/h[c]
3	376 μM	195 mM	0.00478 /h[c]	0.0245 L/mole/h[c]
			0.00452 /h[d]	0.0232 L/mole/h[d]
Mean			0.00482 /h[c]	0.0240 L/mole/h[c]
			0.00488 /h[d]	0.0244 L/mole/h[d]

[a]Each experimental point was analyzed in duplicate.
[b]Calculated from the pseudo first order constant and concentration of KCN.
[c]Determined from disappearance of substrate
[d]Determined from appearance of product

In order to establish pseudo first order kinetic conditions in the experiments above, the concentration of potassium cyanide was in large excess and was also greatly in excess of that which would be achieved under physiological conditions. In order to further prove that the reaction was first order in KCN as well as in the enone, another set of experiments was carried out with KCN as the limiting reagent and 4-phenyl-3-buten-2-one in excess. In this case one would predict that the reaction would be pseudo first order in KCN.

For solubility reasons it was necessary to run these reactions in a 1:1 (v:v) mixture of acetonitrile and 0.145 M phosphate buffer, pH 7.4. The initial concentrations were 0.200 mM KCN and 188.6 mM phenylbutenone. To facilitate analysis, part of the KCN consisted of $K^{14}CN$. Reaction solutions were heated in Teflon capped vials in a heating block. The block was kept at 38°C, but the internal solution temperature was measured to be 34°C. An aliquot of 20 or 25 μL of solution was withdrawn at intervals up to 300-400 h and injected on an HPLC system (C18 column, 1:1 (v:v) methanol:water as solvent, 0.8 mL/min flow rate, detection by UV and radioactivity detectors). The approximate retention times were 3.4 min for $K^{14}CN$ and 11.5 min for ^{14}C-labeled product. (To facilitate collection of product, unlabelled product was added as an internal marker.) Fractions corresponding to these two entities were collected and analyzed for ^{14}C radioactivity by liquid scintillation spectrometry. Cpm were corrected for background and used to calculate rate constants by linear regression analysis of

$$\ln C_t = -k't + \ln Co \qquad \text{Eq. 3}$$

or

$$\ln (Co - P_t) = -k't + \ln Co \qquad \text{Eq. 4}$$

where Co = concentration of cyanide (in cpm) at time 0

C_t = concentration of cyanide (in cpm) at time t

P_t = concentration of product (in cpm) at time t

Figure 2 illustrates the fit to equation 4 for one such experiment. For four separate experiments the value of k' was $1.70 \times 10^{-3} \pm 0.18 \times 10^{-3}$ (SEM) h^{-1} when calculated on disappearance of ^{14}CN and $1.38 \times 10^{-3} \pm 0.19 \times 10^{-3}$ h^{-1} based on appearance of product. The overall mean was 1.54×10^{-3} h^{-1}.

Assuming $k' = k$ [enone], then $k = k'/[\text{enone}] = 8.25 \times 10^{-3}$ M^{-1} h^{-1}. This value of k differs somewhat from that calculated from studies in which the KCN was in excess. The difference may be due to the different solvent composition and (perhaps more important) the 3° difference in temperature in the two studies.

Since HCN is volatile, we were concerned that it might escape from the aqueous solutions sufficiently to have a significant effect on concentration. Therefore vials were tightly capped with Teflon-lined caps. However, they had to be opened and closed during the kinetic studies to remove samples. The possible loss of cyanide was checked during the studies with $K^{14}CN$ by adding the recovered cpm from $K^{14}CN$ and the ^{14}C-cyanoketone product. In two studies run for 169 and 148 h, the decrease in ^{14}CN cpm (4120 and 4624 cpm out of an original 21,300 cpm) and the increase in product ^{14}C (5305 and 4047 cpm) essentially balanced. Thus no significant amount of HCN was lost over this time period in which the vials were sampled 7 to 9 times.

In conclusion it is clear that the reaction between cyanide and a representative enone occurs at a measurable rate under physiological conditions and is first order in each reactant. Catalysis would be required for utility in prophylaxis for cyanide intoxication.

2.3 Computer Simulations

We briefly explored the question of how much overall rate enhancement would be required for a useful prophylactic or therapeutic effect. Assuming $k' = k[KCN]$, $k \approx 0.025$ M^{-1} h^{-1} for the bimolecular reaction. The effect of

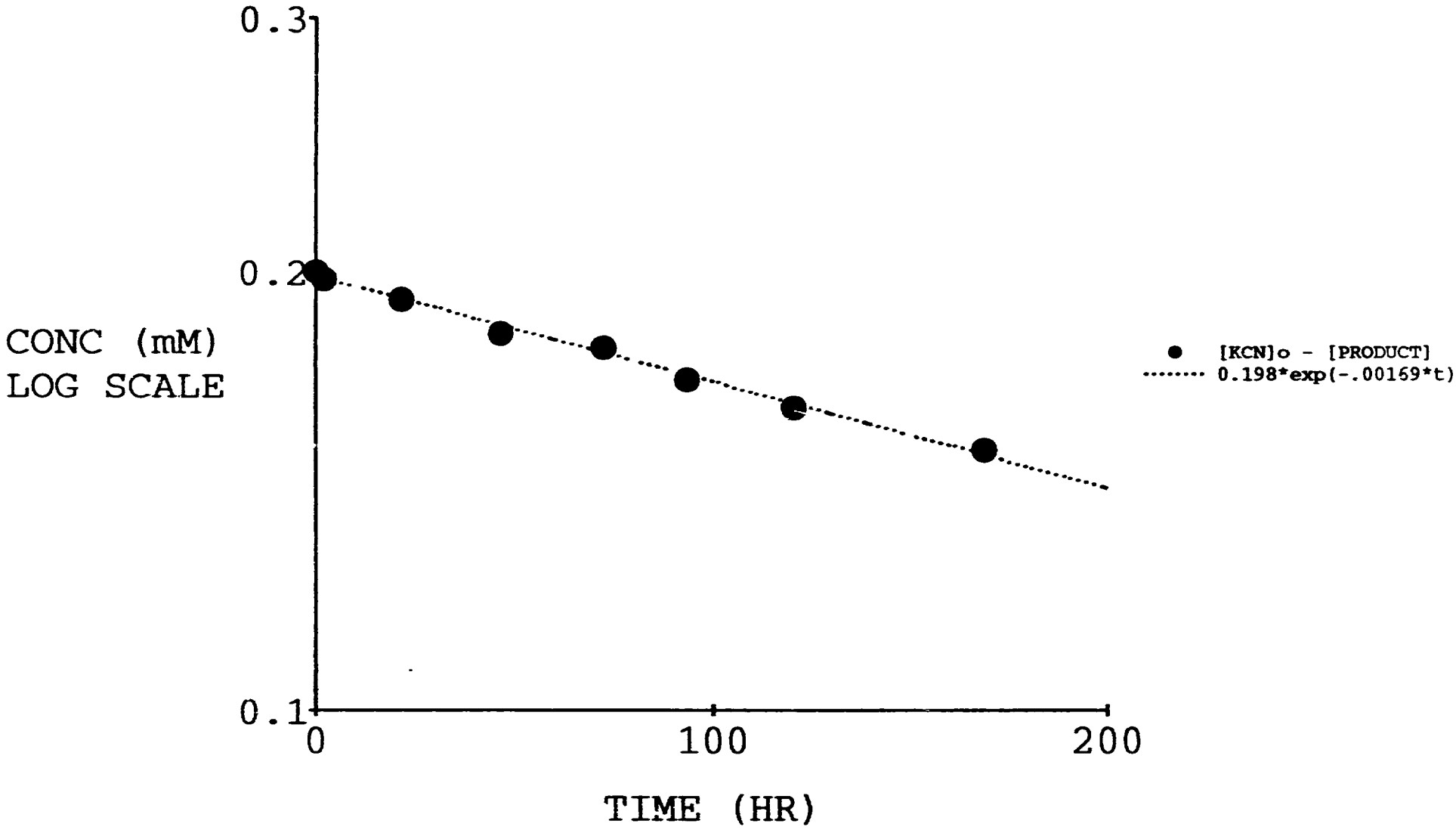


Figure 2. Plot of reaction of C-14 KCN with 4-phenyl-3-buten-2-one. Initial solution was 0.2 mM in KCN, 189 mM in enone, so that reaction was pseudo first order in KCN. Data points are based on conversion of KCN to product, 4-phenyl-4-cyanobutan-2-one, and are calculated from [KCN] at time zero minus [product] at time t as measured by HPLC radiochromatography. pH was 7.4, temperature 34 degrees C.

changing the value of k is shown in Figure 3. A k of 0.25 μM^{-1} h^{-1} would result in reduction of 0.15 mM KCN to about 0.01 mM in 3 min if the initial enone concentration was 0.3 mM. This would be an overall rate enhancement of 10^7. Rate enhancements nearing 10^8 M have been observed in reactions catalyzed by antibodies (Lerner et al., 1991).

To further explore the kinetic aspects of the proposed catalytic reactions, a possible model for antibody catalysis was set up based on enzyme models (Cleland, 1963). This model is illustrated in Figure 4. Kinetic association and dissociation rate constants for antibody binding were selected which were within the ranges usually associated with antibodies (Nisonoff et al., 1975). Simulations of enzyme kinetics were performed using ADAPT II software (Biomedical Simulation Resource, Univ. of Southern California) running on a VAX II work station (DMS 5.4) by Dr. Brian Sadler and Ms. Dorothy Pugh. Figures 5-7 show the effects of various changes in the assumptions made for the rate constants involved in this model. In Figure 5 the effect of changing the association rates for antibody bound compound (k, k_3, and k_8) are modified.

It is assumed that these rate constants vary but remain equal to each other. This simulates changes in antibody affinity. An increase in k_8 alone would oppose cyanide removal, but this could be overcome by increases in k_1 and k_3. An increase in k_5 (the transition from reactants to product, Figure 6) or k_7 (the dissociation rate of product and antibody, Figure 7), enhances the reaction.

Overall these results would indicate that, within the range of affinity constants and catalytic rates associated with known antibodies, pharmacologically relevant concentrations of cyanide and drug could undergo catalytic combination in a time rapid enough to give protection against the toxic

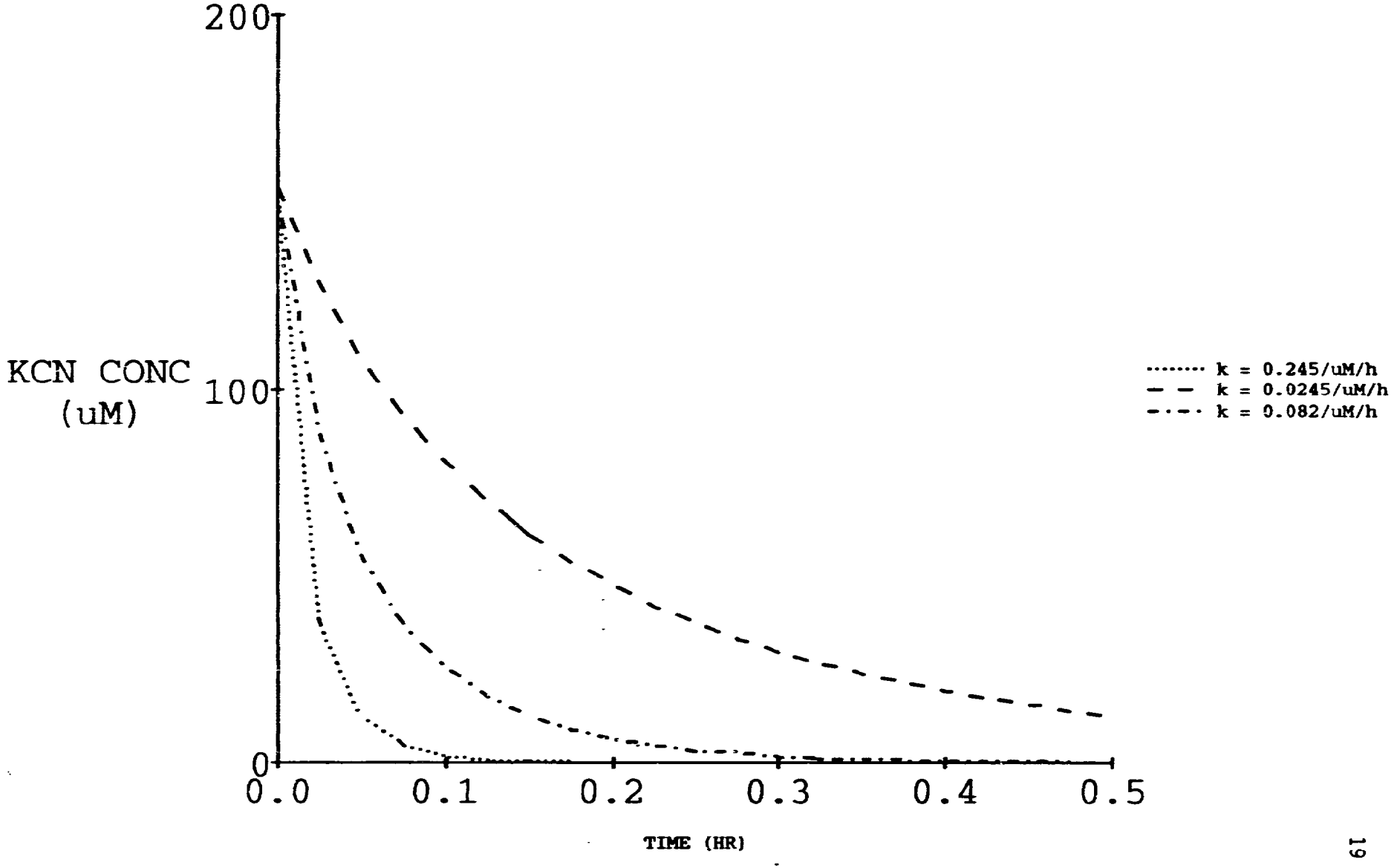
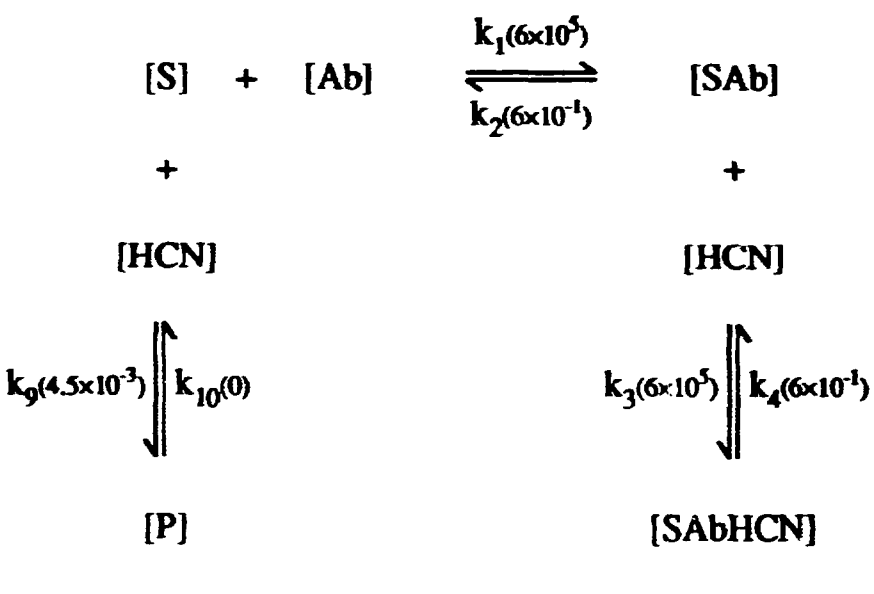


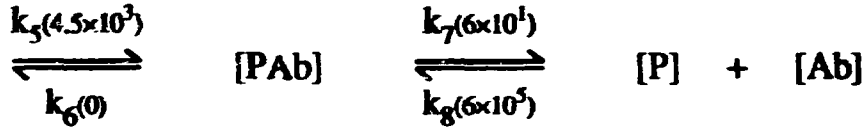
Figure 3. Computer simulations of elimination of KCN by reaction with a drug.
Initial concentrations are 0.154 mM KCN and 0.308 mM drug.
Rate constants (second order) are shown in the legend.

FIGURE 4
Model for removal of cyanide by spontaneous and enzymatic (or catalytic antibody) catalyzed reaction with a drug.

Model



Legend		
[S]	=	Concentration of free substrate (0.3 mM)
[Ab]	=	Concentration of free catalytic antibody (0.03 mM)
[SAb]	=	Concentration of substrate-antibody complex (0 mM)
[HCN]	=	Concentration of free cyanide (0.1 mM)
[SAbHCN]	=	Concentration of substrate-antibody-cyanide complex (0 mM)
[PAb]	=	Concentration of product-antibody complex (0 mM)
[P]	=	Concentration of free product (0 mM)
$k_{1,3,8,9}$	=	Second Order Rate Constants ($L \cdot mMole^{-1} \cdot hours^{-1}$)
$k_{2,4,5,6,7,10}$	=	First Order Rate Constants ($hours^{-1}$)



Differential Equations

$$\frac{d[S]}{dt} = -k_1[S][Ab] + k_2[SAb] - k_9[S][HCN] + k_{10}[P]$$

$$\frac{d[HCN]}{dt} = -k_3[SAb][HCN] + k_4[SAbHCN] - k_9[S][HCN] + k_{10}[P]$$

$$\frac{d[Ab]}{dt} = -k_1[S][Ab] + k_2[SAb] + k_7[PAb] - k_8[P][Ab]$$

$$\frac{d[SAbHCN]}{dt} = k_3[SAb][HCN] - k_4[SAbHCN] - k_5[SAbHCN] + k_6[PAb]$$

$$\frac{d[SAb]}{dt} = k_1[S][Ab] - k_2[SAb] - k_3[SAb][HCN] + k_4[SAbHCN]$$

$$\frac{d[PAb]}{dt} = k_5[SAbHCN] - k_6[PAb] - k_7[PAb] + k_8[P][Ab]$$

$$\frac{d[P]}{dt} = k_7[PAb] - k_8[P][Ab] + k_9[S][HCN] - k_{10}[P]$$

FIGURE 5

Effect of k_1, k_3, and k_8 on Concentration of HCN with Time

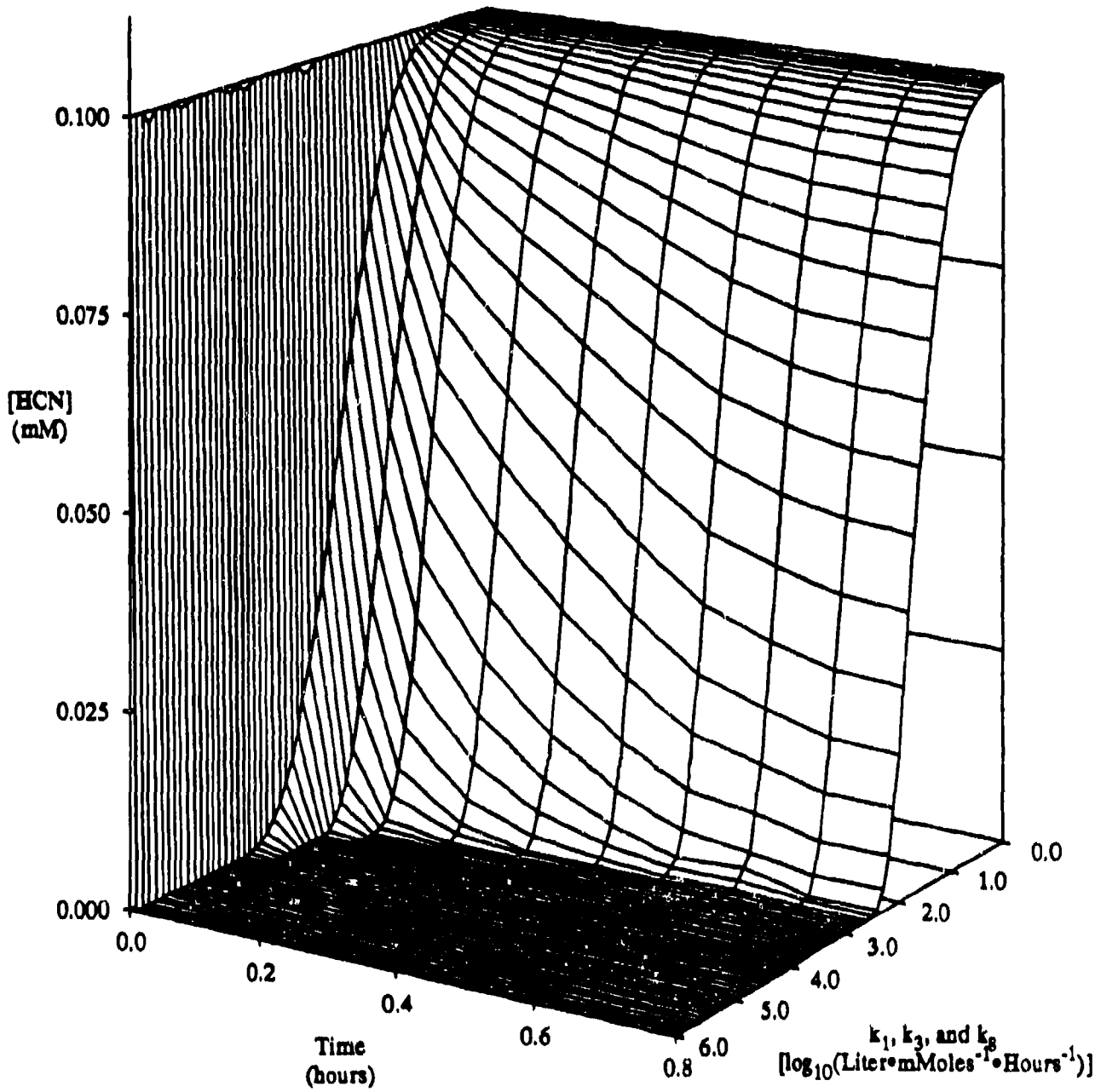


FIGURE 6

Effect of k_5 on Concentration of HCN with Time

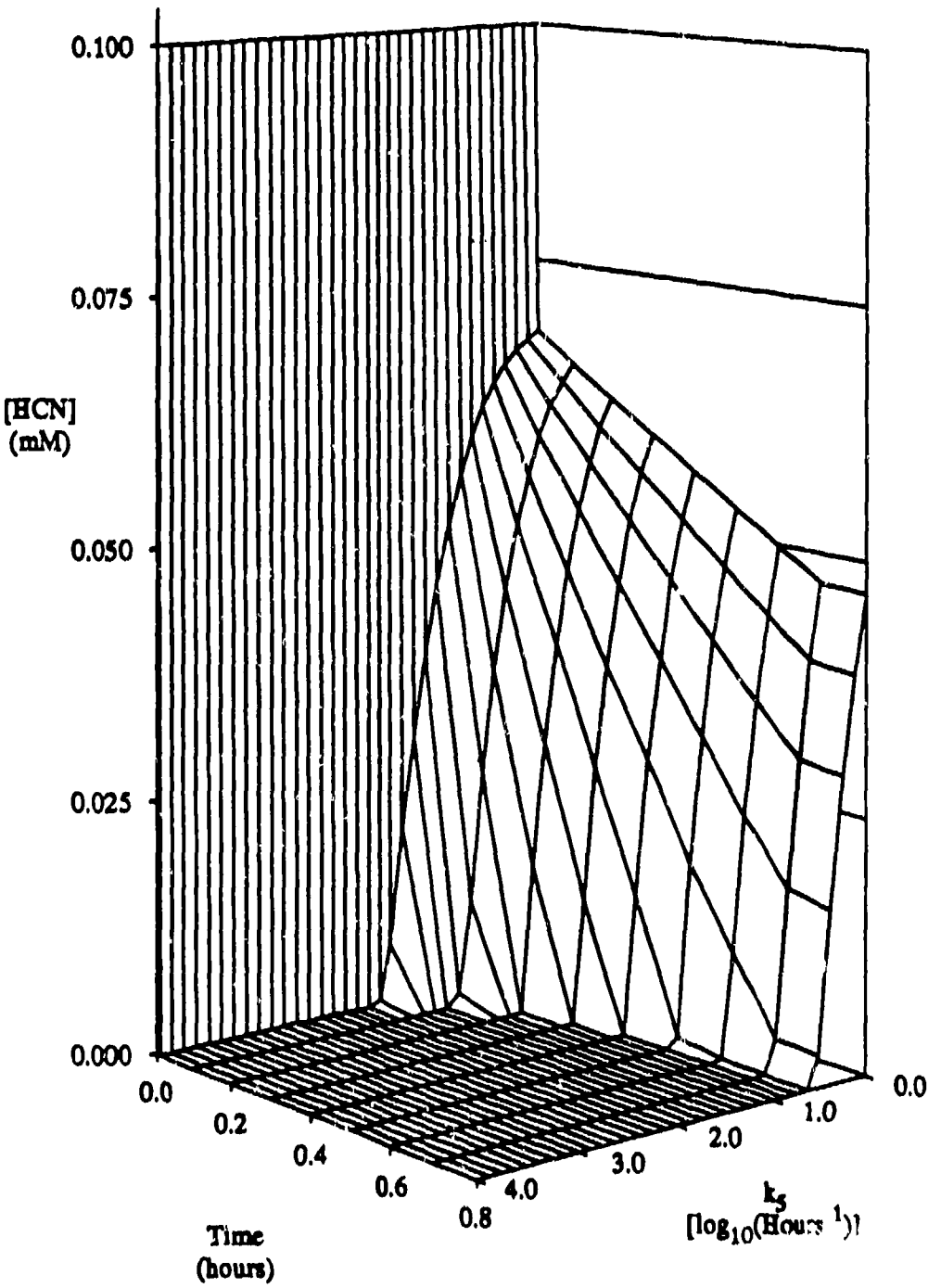
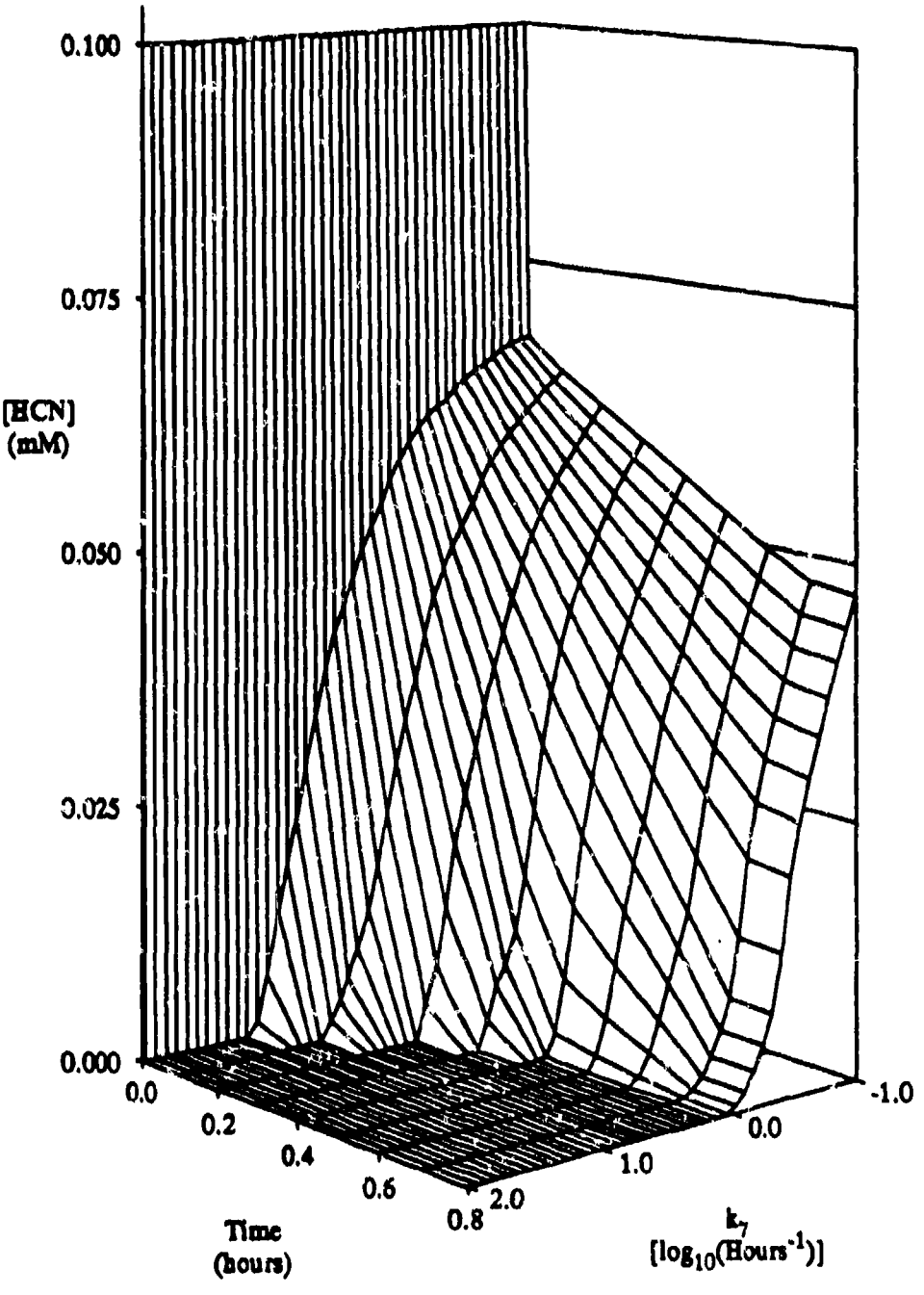


FIGURE 7

Effect of k_7 on Concentration of HCN with Time



effects of cyanide. Note that the affinity constants for antibody binding are relatively modest compared with those which can be achieved for small molecules. These simulations also show that the product should have an affinity constant lower than substrate.

The antibody concentration (0.03 MM in binding sites) would equate to a concentration of ca. 2.25 g/liter or a probable dose of ca. 11 g or 140 mg/kg if we assume an 80 kg person with a volume of distribution of 5 liters for the antibody compartment. Doses of Fab up to 7500 mg/kg given intravenously to rats over a 1 h period were tolerated without apparent toxicity (Pentel et al., 1988), for example.

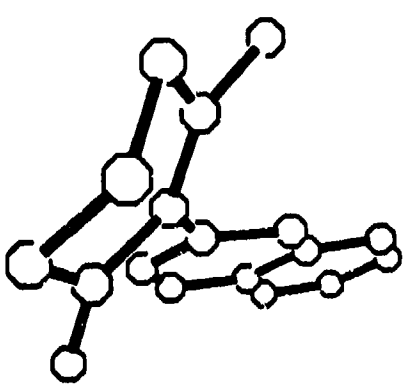
3.0 Computer-Assisted Molecular Modeling Studies of Drug, Hapten and Product

As pointed out above, the preferred order of affinities for the antibody would be transition state > substrate > product. In order to illustrate how this may work with the proposed compounds, we carried out some brief computer assisted molecular modeling studies, the results of which are illustrated in Figures 8-10.

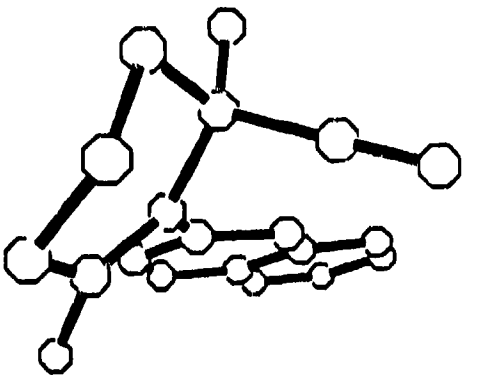
Models of α-naphthyl-β-methylcyclohexenone (drug, A), α-naphthyl-β-cyano-β-methylcyclohexenol (surrogate for transition state, B), α-naphthyl-β-cyano-β-methylcyclohexanone (product, C), methyl enol ether of B (D), and trimethylsilyl enol ether of B (E) were constructed from the fragment library of SYBYL 5.41. By a combination of the Search and Maximin2 features, minimum energy structures were obtained (E is an approximation, as some of the relevant parameters for silicon were only estimates). Figure 8 shows the carbon skeletons of A, B, C, D and E. Figure 9 shows the van der Waals' surfaces for structures A, B, C, and D. Figure 10 shows the superimposition of the carbon skeletons of A, C, D, and E on the transition state surrogate B.

The steric similarity of the molecules was assessed by fitting common nonhydrogen atoms of the minimized structures by a least squares procedure and comparing the root mean square distance (RMSD). If enol structure B was taken as an approximation of the transition state, the proposed methyl enol ether hapten fit it closely (RMSD 0.016 Å). A less good fit was achieved between hapten D and the drug A (RMSD 0.17 Å). The poorest fit was between hapten A and the hydrocyanation product C (RMSD 0.33 Å). Thus the desirable order of affinities should be achieved. Also it appears that either carbon or silyl enol ethers may be suitable transition state analogs.

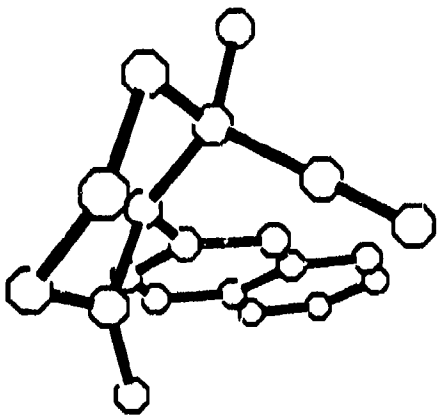
The stereochemistry of these compounds has been given consideration. The enone (A) has no asymmetric carbon atoms. Its mirror image would not be



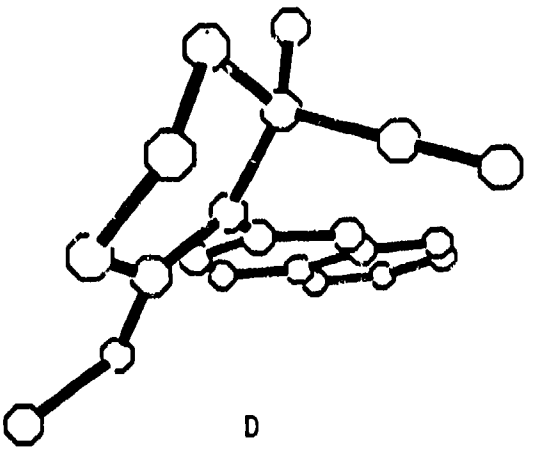
A



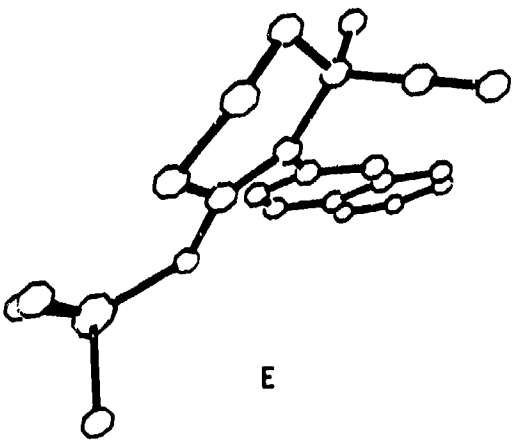
B



C



D



E

FIGURE 8
Carbon skeletons of α-naphthyl-β-methylcyclohexenone (A),
α-naphthyl-β-cyano-β-methylcyclohexenol (B),
α-naphthyl-β-cyano-β-methylcyclohexanone (C), and
α-naphthyl-β-methylcyclohexenol methyl ether (D),
α-naphthyl-β-methylcyclohexenol trimethylsilyl ether (E)

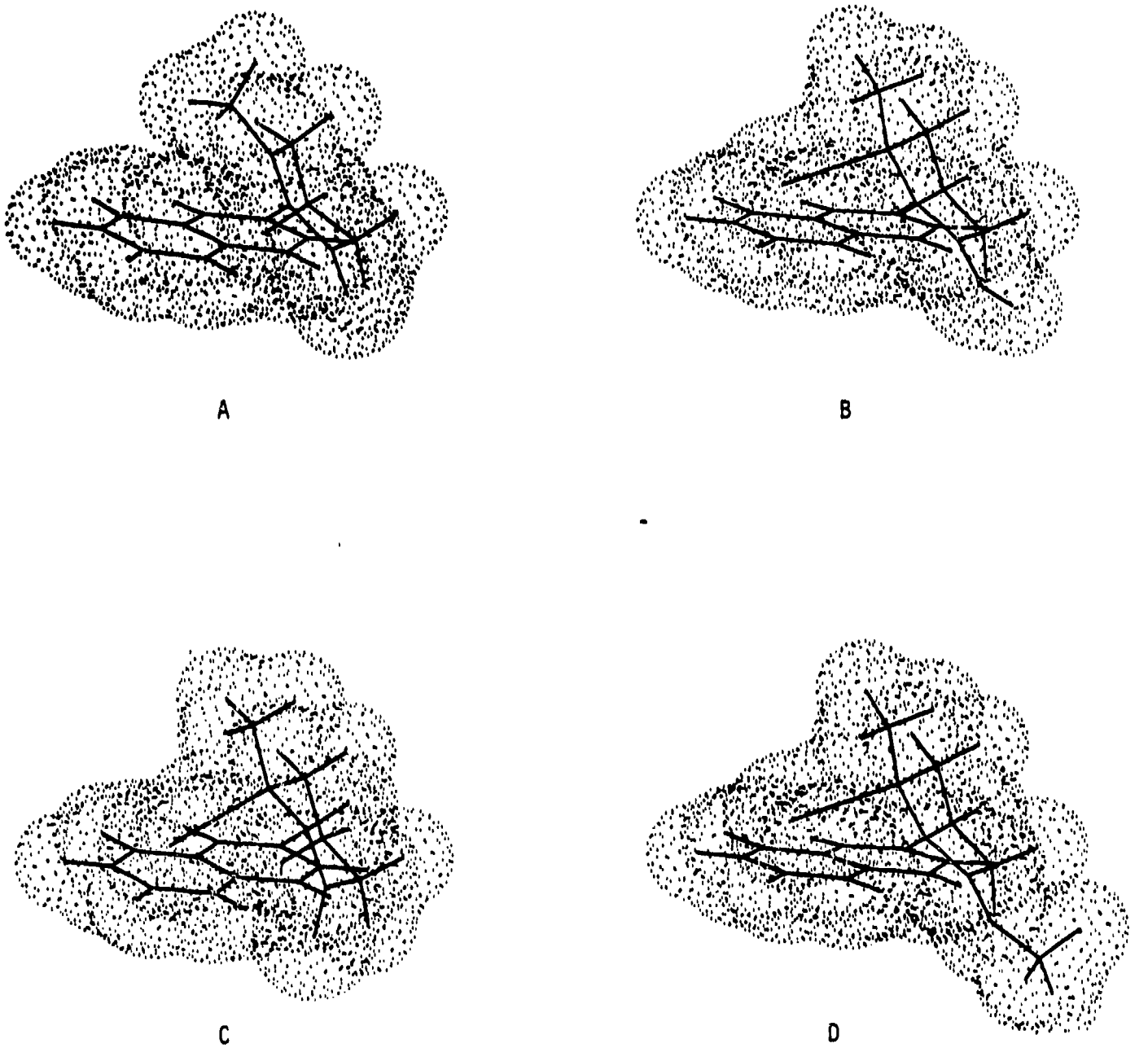
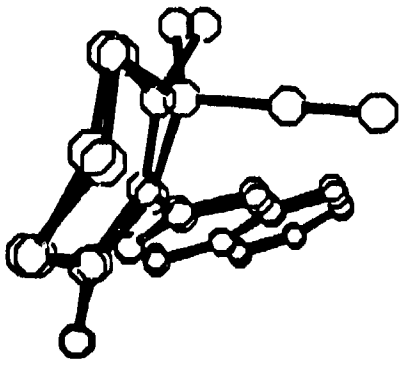
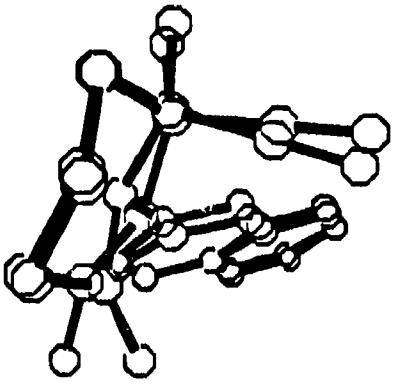


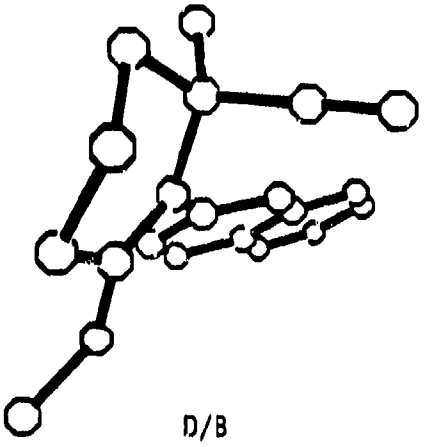
FIGURE 9
van der Waals' surface. For names see Figure 8.



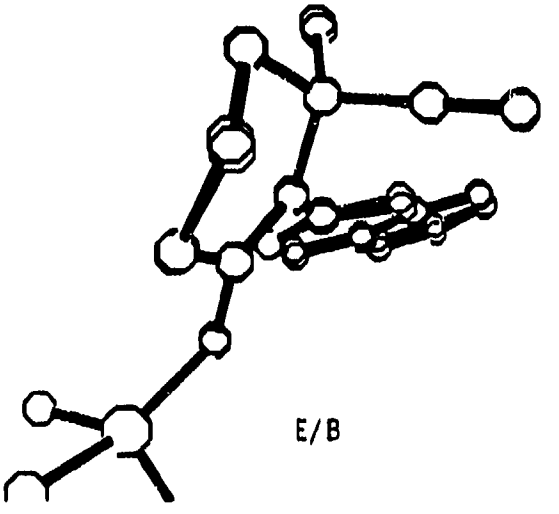
A/B



C/B



D/B



E/B

FIGURE 10
Superimposition of structures A, C, D (Figure 8) and E (α-naphthyl-β-methylcyclohexenol TMS ether) on transition surrogate B (Figure 8).

superimposable without rotation of the C-1 to naphthyl bond, but this is probably energetically allowed. The cyanide atom could approach from either the pro-S or pro-R face of the cyclohexenone. In the final product, there are two asymmetric centers (at C-2 and C-3). We assumed that cyanide ion would approach from the pseudo-axial direction and that the α-naphthyl substituent would assume an equatorial position in the final product. The stereochemistry of the asymmetric centers will be heavily influenced by the chiral antibody molecule, so the overall reaction may be quite enantioselective. However, without an antibody or its structure, further modeling of the stereochemistry does not appear fruitful.

4.0 Synthesis of Hapten and Drug

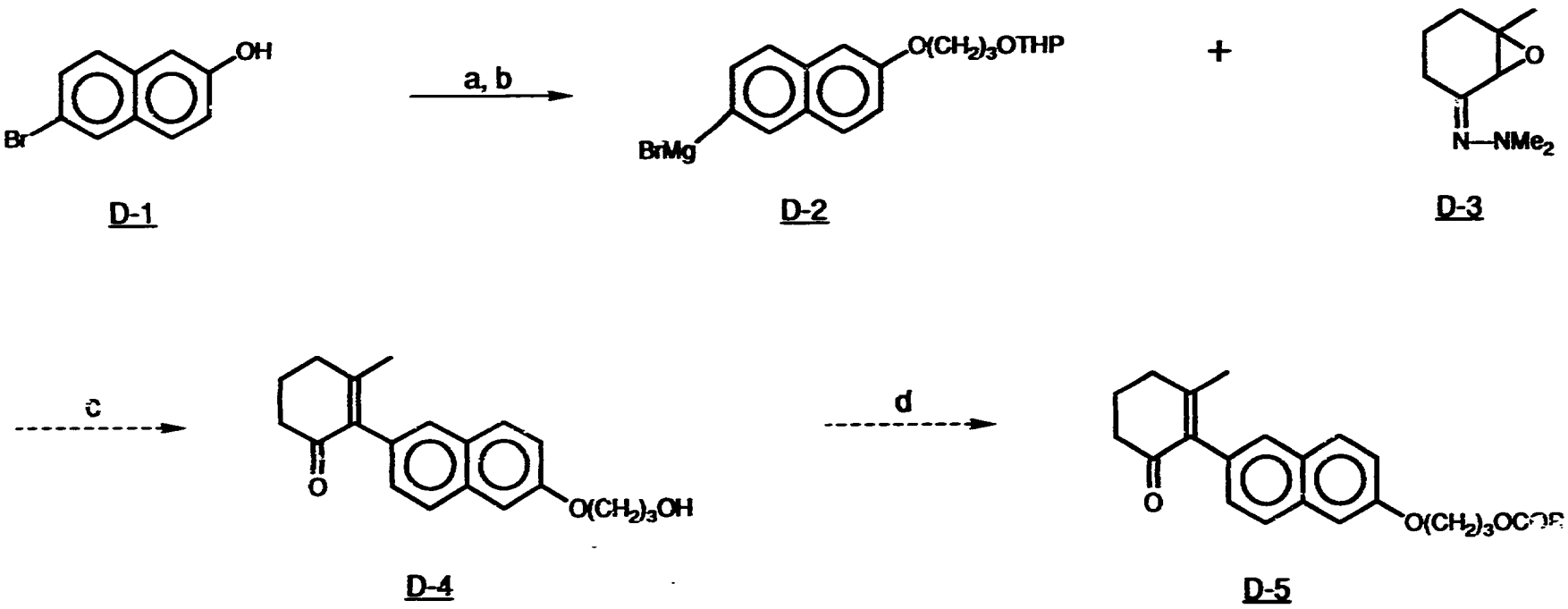
Initial work was directed at the synthesis of 2-[6-(3-hydroxypropyloxy)-2-naphthyl]-3-methylcyclohex-2-en-1-one (D-4).

The originally proposed synthetic route for cyclohexenone D-4 is shown in Chart D. The two intermediates required, compounds E-3 and E-8, were synthesized as shown in Chart E. 3-Methyl-2-cyclohexen-1-one was oxidized with hydrogen peroxide in the presence of monobasic potassium phosphate to the epoxide E-2 in 56% yield. The ketone was then condensed with 1,1-dimethylhydrazine to give epoxyhydrazone E-3. Compound E-3 could not be isolated as a pure material due to decomposition on silica gel and on distillation but was identified on the basis of its NMR spectrum. The reaction of crude epoxyhydrazone E-3 with a Grignard reagent was tested using phenyl magnesium bromide and the resulting mixture hydrolyzed in refluxing 50% aqueous EtOH/1M HCl. 2-Phenyl-3-methylcyclohex-2-en-1-one (E-4) was obtained in 28% yield and its identity established by NMR (cf. Stork and Ponaras, 1976).

The ketal chain of intermediate E-8 was produced in 61% yield from 3-bromo-1-propanol and 3,4-dihydro-2H-pyran. This tetrahydropyranyl (THP) bromide (E-6) was then treated with 6-bromo-2-napthol with potassium carbonate as base in dry acetone to give intermediate E-8 in 71% yield.

Intermediates E-3 and E-8 were to be joined via a Grignard reaction based on the successful formation of 2-phenyl-3-methyl-2-cyclohexen-1-one (above). Formation of the Grignard reagent of E-8 using Mg turnings could not be achieved. Generation of this reagent (Chart F) was accomplished by first forming the lithiate by metal halogen exchange at low temperatures. Treating the lithiate with magnesium bromide etherate, $MgBr_2 \cdot OEt_2$, produced the required Grignard reagent (cf. Patten et al., 1988). In order to test the reactivity of the Grignard reagent, it was treated with benzaldehyde. The

CHART D
ORIGINAL PROPOSED ROUTE TO 2-ARYLCYCLOHEXENONES

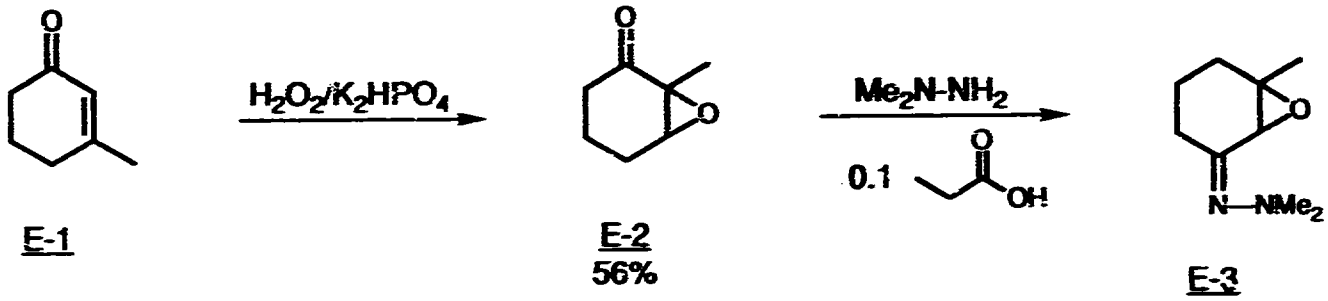


D-1 D-2 D-3

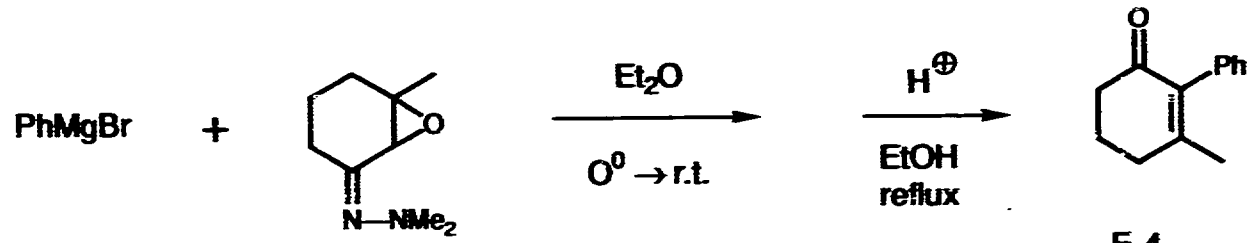
D-4 D-5

a) $THPO(CH_2)_3Br$, K_2CO_3
b) Mg
c) cf. Stork and Ponaras, 1976
d) $(RCO)_2O$, pyr, DMAP

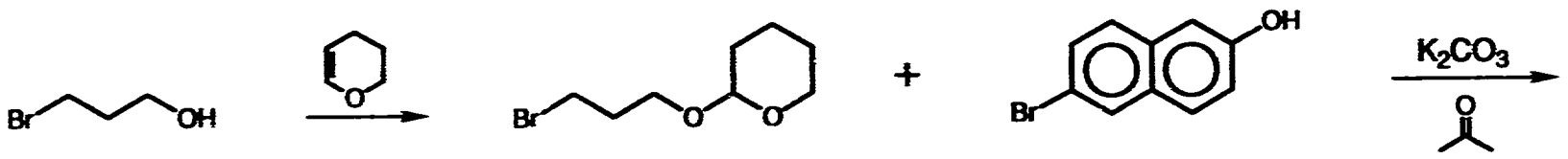
CHART E
SYNTHESIS OF INTERMEDIATES FOR PROPOSED ROUTE



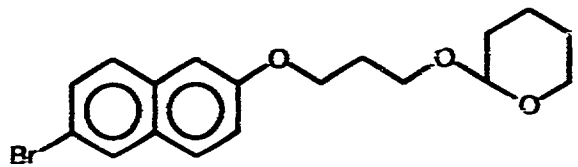
E-1 → E-2 (56%) → E-3



E-3 → E-4 (28%)

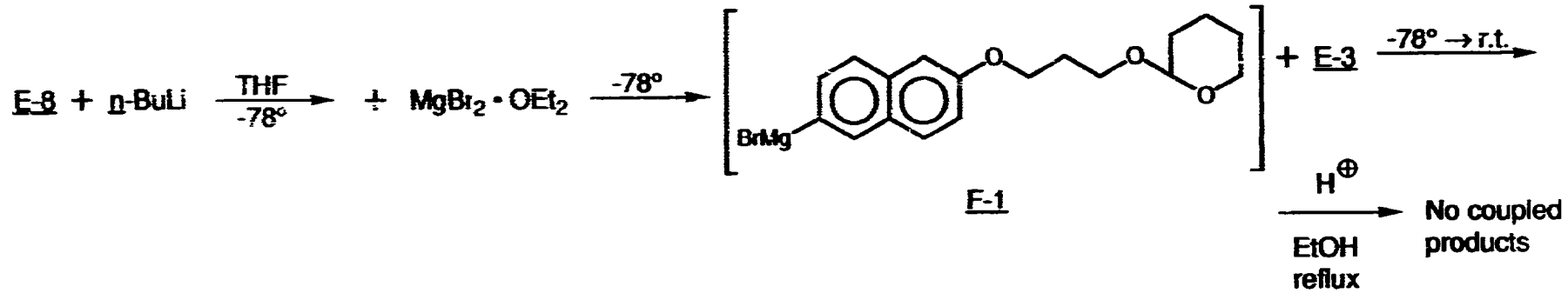


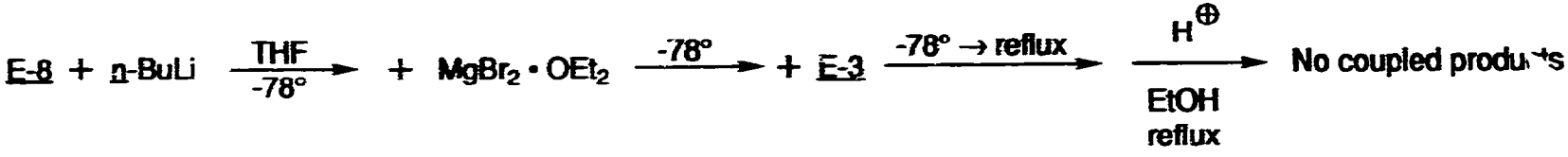
E-5 → E-6 (61%) + E-7

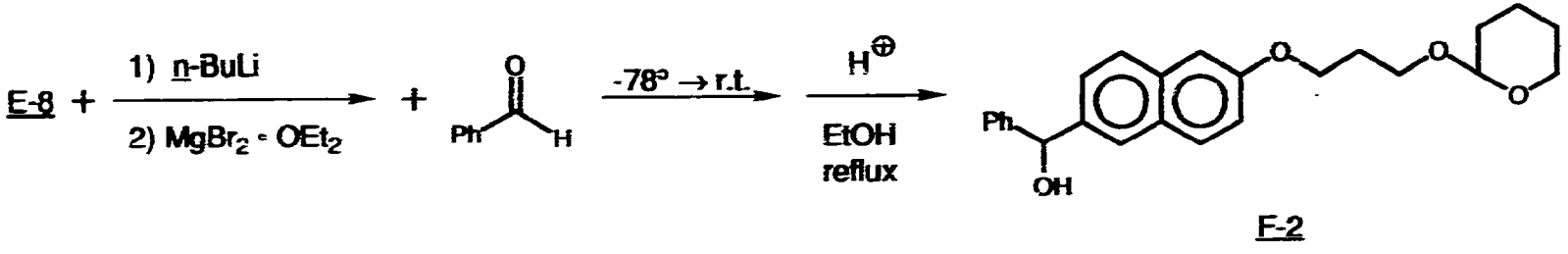


E-8 (71%)

CHART F
GRIGNARD REACTIONS OF NAPHTHALENES







F-2

expected naphthyl-substituted benzyl alcohol (F-2) was observed, showing that the Grignard reagent was generated and can react. However, this reagent failed to give any coupled product on reaction with E-3 either at room temperature or in refluxing tetrahydrofuran (THF). The lithiate of E-8 also failed to yield any coupled product.

Several more attempts were made to react an aryl Grignard or cuprate with the hydrazone epoxide E-3. Table 3 outlines various synthetic reactions studied and their outcomes. For the silylated aryls (entries 2-4), the problem was failure to initiate the Grignard reagent. This problem was resolved by use of ethylene dibromide as an activator (entry 5). When the Grignard reagent was formed (entries 5, 6 and 8), the resulting product mixture was very complex. The same was observed for the cuprate shown in entry 7. Even the reaction of these organometallics with cyclohexene oxide was not straightforward. Thus the reaction product of the silyl protected naphthalenyl lithium cuprocyanide and cyclohexene oxide (entry 9) gave an as yet unidentified product. This material failed to react with either acetyl chloride in the presence of triethyl amine or with acetic anhydride in pyridine and thus does not appear to be the desired 2-substituted cyclohexanol product. However, the reaction of the Grignard reagent from 4-bromoanisole with cyclohexene oxide under copper catalysis was successful (entry 12). The resulting 2-arylcyclohexanol was smoothly oxidized to the corresponding ketone in 75% yield by use of the Dess-Martin reagent (entry 13).

Lee and Oh (1988) have reported that 2-nitrocyclohexene can be readily arylated in the presence of titanium tetrachloride. This reaction, however, in our hands failed to yield the desired products with naphthyl and biphenyl derivatives (entries 14-16). With anisole a product was obtained, but its

TABLE 3
LIST OF REACTIONS ATTEMPTED FOR SYNTHESIS OF 2-ARYLCYCLOHEXENONES

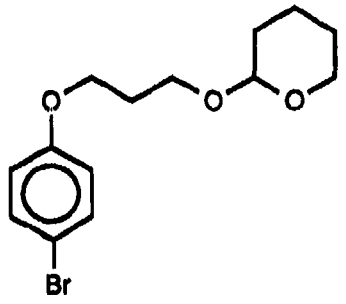
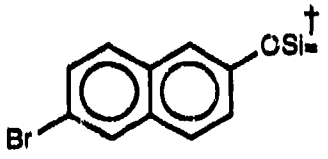
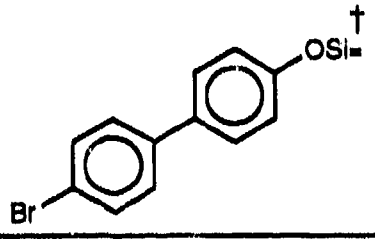
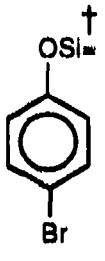
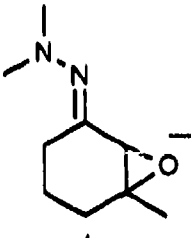
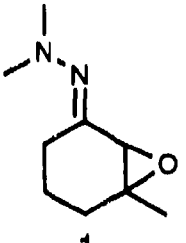
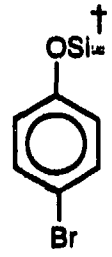
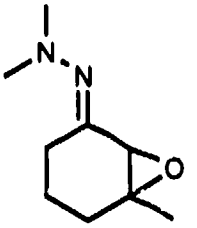
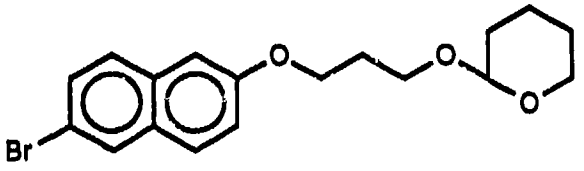
1.		+ Mg (acid washed) → THF, reflux	no initiation of Grignard
2.		+ Mg → THF reflux, I_2 (cat.)	no initiation of Grignard
3.		+ Mg → THF reflux, I_2 (cat)	no initiation of Grignard
4.		+ Mg → THF reflux, I_2 (cat.)	no initiation of Grignard
5.		+ 1.5 Mg + 0.5  → THF, reflux +  (1)	complex mixture
6.		+ Mg → THF reflux, I_2 (cat.) +  (1) → 0° → r.t., 1.5 h	complex mixture

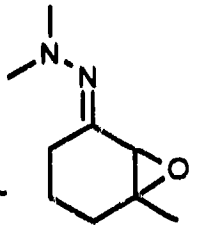
TABLE 3 (continued)
LIST OF REACTIONS ATTEMPTED FOR SYNTHESIS OF 2-ARYLCYCLOHEXENONES

7. 

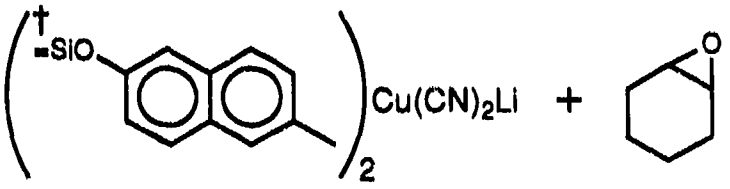
+ n-BuLi/CuCN/THF $\xrightarrow{-78°\ C}$ +  → complex mixture

1

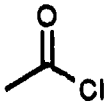
8. 

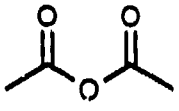
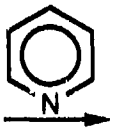
+ Mg* (finely divided) $\xrightarrow[0°]{THF}$ +  → no coupled product

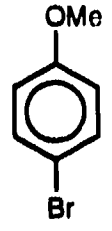
1

9. 

+ $\xrightarrow{-78°\ C}$ unknown product

10. unknown product from 9 +  $\xrightarrow{Et_3N}$ no reaction

11. unknown product from 9 +   no reaction

12. 

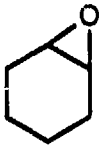
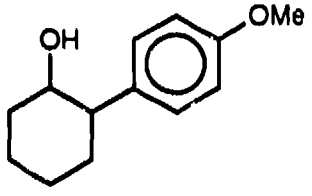
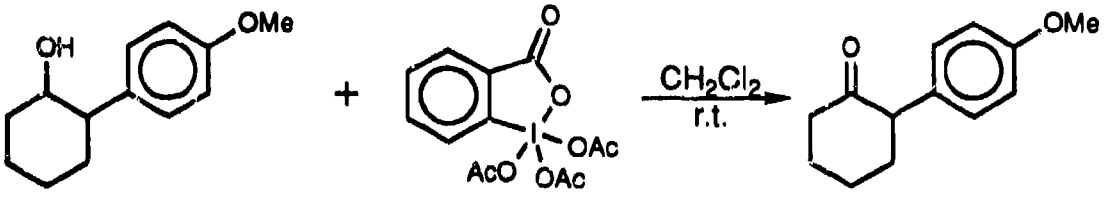
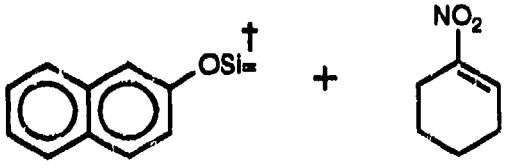
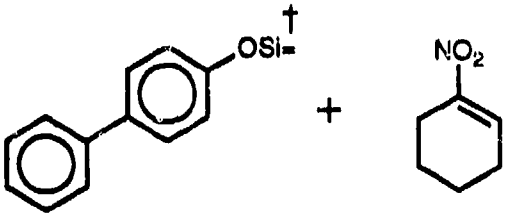
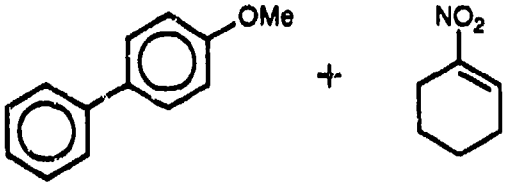
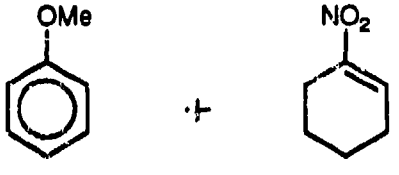
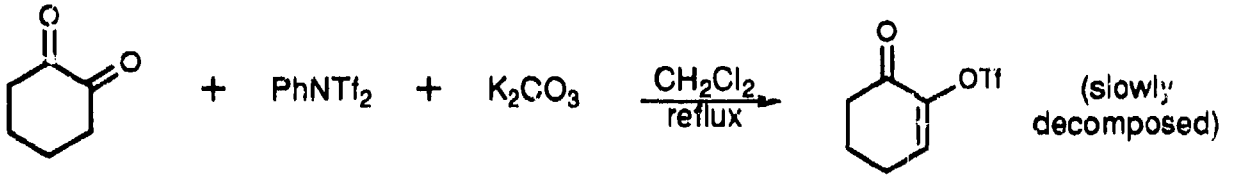
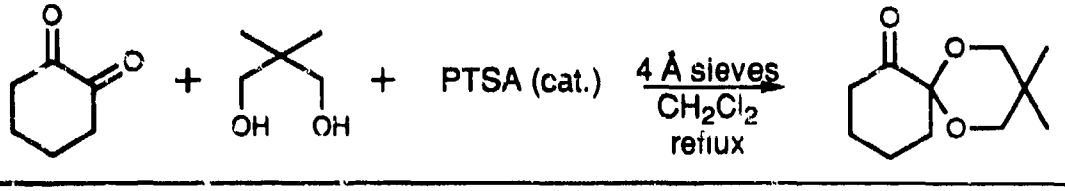
+ Mg $\xrightarrow[I_2\ (cat.)]{THF}$ +  $\xrightarrow[-30°]{CuI}$ 

TABLE 3 (continued)
LIST OF REACTIONS ATTEMPTED FOR SYNTHESIS OF 2-ARYLCYCLOHEXENONES

13.		75% yield
14.		no coupled product
15.		no coupled product
16.		no coupled product
17.		unknown product
18.		(slowly decomposed)
19.		89% yield

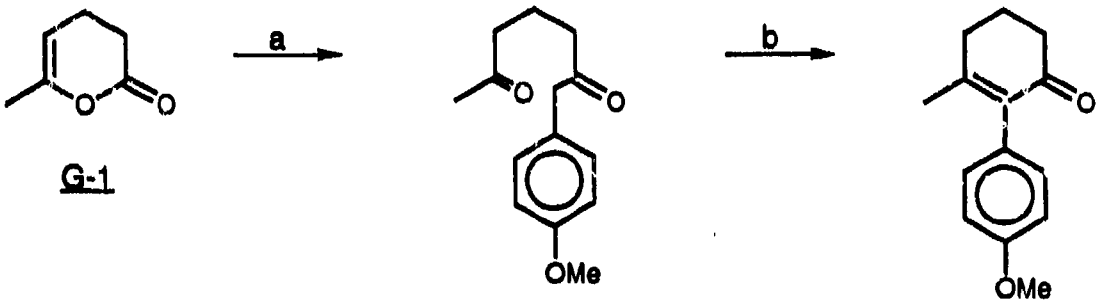
NMR spectrum was not consistent with the desired 2-(p-methoxyphenyl)cyclohexanone. Comparison of this product with that achieved by oxidation of 2-(p-methoxyphenyl)cyclohexanol (entry 13) confirmed that the product from entry 17 was not the desired cyclohexanone. There were no experimental data on this product from the preliminary literature report (Lee and Oh, 1988) of the reaction of anisole with 2-nitrocyclohexene. In view of our ability to make 2-(p-methoxyphenyl)cyclohexanone by the route of entries 12 and 13, we did not further explore this reaction.

The successful synthesis of 2-(p-methoxyphenyl)cyclohexanone opened up the possibility of preparing the desired compound D-4 or its p-methoxyphenyl analog by dehydrogenation reactions involving the use of phenylselenyl chloride followed by hydrogen peroxide. It would then be possible to introduce the methyl group in the 3-position and follow this by subsequent phenylselenyl chloride/H_2O_2 dehydrogenation to give the desired 3-methyl-2-aryl-2-cyclohexen-1-ones. However, the dehydrogenation reaction did not go smoothly, and this approach was abandoned.

Other possible routes to aryl substituted 2-cyclohexanones such as palladium catalyzed tin coupling of vinyl triflates with arylstannanes (Stille, 1986 and Scott and Still, 1986) (eq. 1) and vinyl stannanes with aryl iodides (Stille, 1986; Zimmerman and Stille, 1985; and Bumagin et al., 1984) were briefly considered but not pursued in view of successful synthesis of the desired compound.

The failure of reactions analogous to those reported in the literature when applied to the synthesis of the desired 2-aryl-3-methylcyclohex-2-en-1-ones required us to explore new routes, and two new syntheses of these compounds have now been developed. Initially we prepared 2-(4-methoxyphenyl)-3-methylcyclohex-2-en-1-one (G-3, Chart G). This compound could be used as a

CHART G
ROUTE 1 TO ARYLCYCLOHEXENONES



G-1 G-2 G-3

a) p-Methoxybenzylmagnesium Bromide
b) NaOH

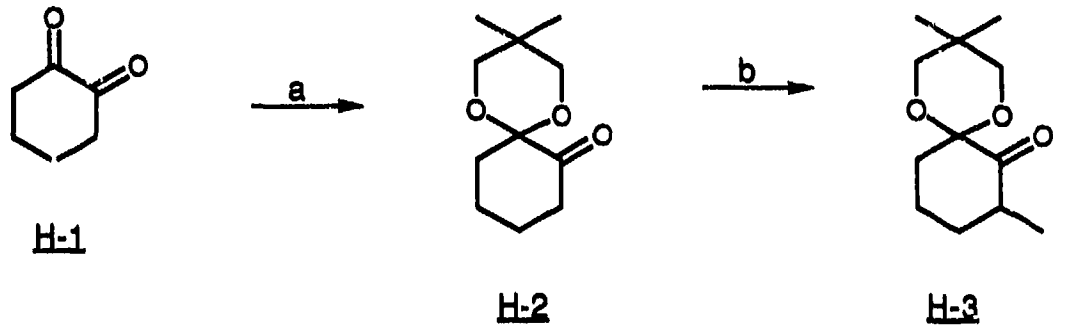
model system for working out the synthetic procedures and could also be converted into a close analog of the proposed hapten C-1 which would serve in its place if the 2-naphthyl series proved unsuitable.

Two new routes, starting with commercially available chemicals, were devised for preparing this compound. In the first route (Chart G-3), p-methoxybenzyl magnesium chloride was allowed to react with 3,4-dihydro-6-methyl-2H-pyran-2-one (G-1). The intermediate from this reaction, 1-p-methoxyphenylheptane-2,6-dione (G-2) was not isolated but treated with sodium hydroxide, whereupon it underwent cyclization to the desired 2-(p-methoxyphenyl)-3-methylcyclohex-2-en-1-one (G-3). This compound was isolated by chromatography. Its NMR spectrum was in complete agreement with the proposed structure.

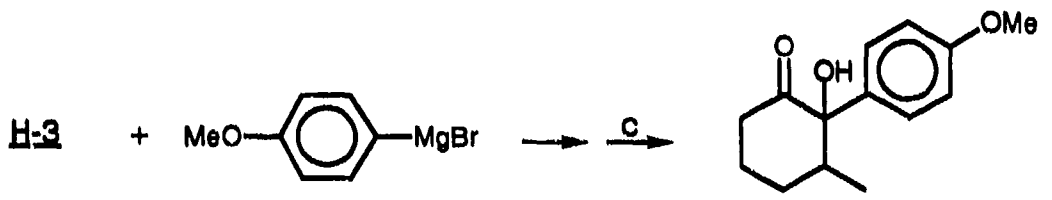
In the second route (Chart H), cyclohexane-1,2-dione (H-1) was smoothly converted to its monoketal H-2 by reaction with 2,2-dimethylpropane-1,3-diol. This compound was monomethylated (lithium diisopropylamide followed by treatment with methyl iodide) α to the remaining carbonyl group. The methylated compound (H-3) underwent ready reaction with p-methoxyphenylmagnesium bromide to yield the tertiary alcohol. This latter compound was deketalized with acidic aqueous ethanol, and the resulting 2-hydroxycyclohexanone (H-4) was dehydrated by treatment with p-toluenesulfonic acid in refluxing benzene. The final product was identical both chromatographically and by NMR with the cyclohexenone G-3 prepared by the first route.

Although the second route has more steps, the starting material is significantly cheaper; and the steps are straightforward. We therefore explored the possibility that this route could be used to introduce a 2-naphthyl group. The tetrahydropyranyl ether from 6-bromo-2-(3-hydroxypropoxy)naphthalene was converted to its lithium derivative (H-5) by exchange with n-butyl lithium.

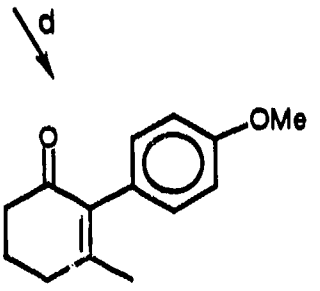
CHART H
ROUTE 2 TO ARYLCYCLOHEXENONES

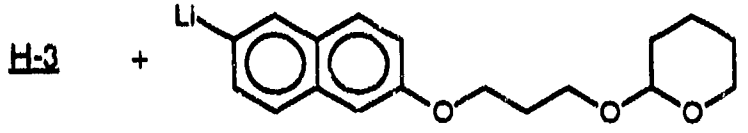


H-1 H-2 H-3



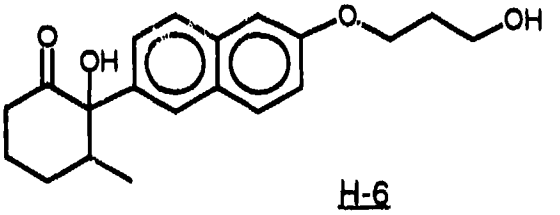
H-4



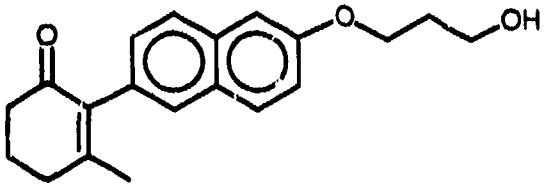


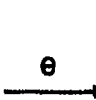
H-5 G-3

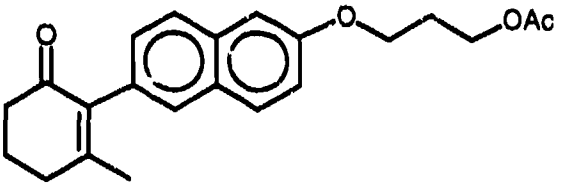
a) 2,2-dimethylpropane-1,3-diol, H^+
b) Li–N(i–Pr)$_2$; CH_3I
c) H^+, EtOH, H_2O
d) p-TsOH, benzene, Δ
e) Ac_2O/Et_3N/DMAP



H-6







H-7 H-8

Reaction of the naphthyl lithium with the protected, methylated derivative of cyclohexane-1,2-dione (H-3) occurred smoothly. When the resulting product was treated with aqueous ethanolic acid, both the ketal and tetrahydropyranyl groups were removed. The crude diol (H-6) was refluxed in benzene with a catalytic amount of tosic acid to produce enone (H-7). Acetylation produced the primary acetate H-8. NMR spectra of the various intermediates and products are consistent with their structures.

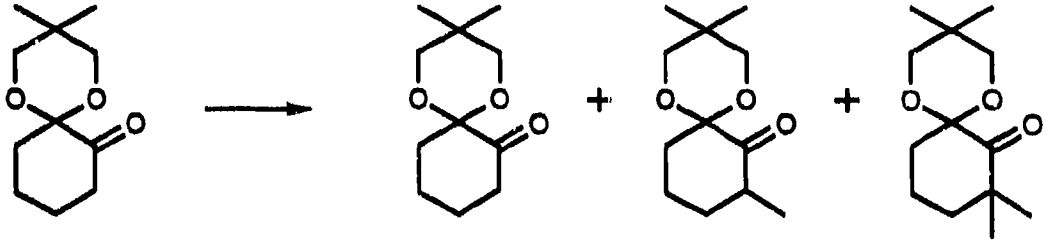
Considerable effort was devoted to trying to improve conditions for making monomethyl ketone H-3, since monomethylation of H-2 yielded a mixture which required tedious chromatography for isolation of H-3.

The alkylation of compound H-2 was studied as shown in Chart I. Use of the strong potassium base, potassium bis-(trimethylsilyl)amide, resulted in excessive dimethylation, and potassium t-butoxide gave only a low yield of H-3. H-2 did not form an imine with cyclohexylamine, but did form an N,N-dimethylhydrazone (I-2) with dimethylhydrazine. However the hydrazone could not be alkylated under conditions (h) or (i) of Chart I.

The next step would be to make the formyl or oxalyl derivative so as to perform monomethylation. However we felt that in the interest of time we should continue to explore the subsequent steps needed to make hapten and conjugate and turned our attention to them.

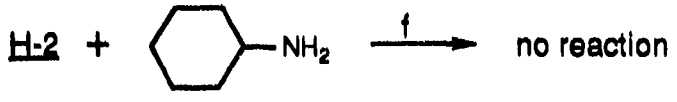
Compound H-7 was readily acetylated to yield acetate H-8 (J-1) in which the primary -OH was protected. Chart J shows several reactions carried out in attempts to convert the enone J-1 to cyano enol ether J-3. Reaction of J-1 with diethylaluminum cyanide followed by aqueous workup yielded the 1,4-hydrocyanation product, presumably via the intermediate aluminum enolate J-2. Attempts to directly trap this intermediate by reaction with Meerwein's reagent to yield the methyl enol ether J-3 were unsuccessful. Although some

CHART I
ALKYLATION EXPERIMENTS



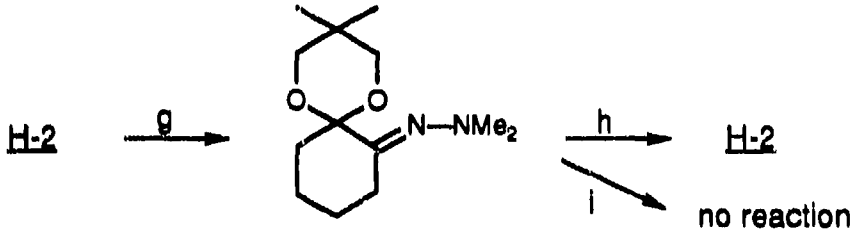
H-2 H-2 H-3 I-1

Conditions	Results
a. LDA; then MeI	H-2 + H-3
b. Repeat addition of LDA followed by MeI twice	H-2 + H-3 + I-1
c. $(TMS)_2NK$; then MeI	H-3 + I-1 (significant amount)
d. $(TMS)_2NK$; then inverse addition to MeI	H-3 + I-1 (significant amount)
e. t-BuOK/t-BuOH, reflux; add MeI	H-3 (very low yield)



I-2

f. $MgSO_4$, CH_2Cl_2, 3 days



I-3

g. $Me_2N–NH_2$, EtOH, reflux

h. $(TMS)_2NK$ (0° to –78°); then MeI (–78° to room temperature); then $NaIO_4$, buffer

i. $(TMS)_2NK$ (0°, THF); then MeI (0° to room temperature overnight)

CHART J
HYDROCYANATION AND ENOL ETHER EXPERIMENTS

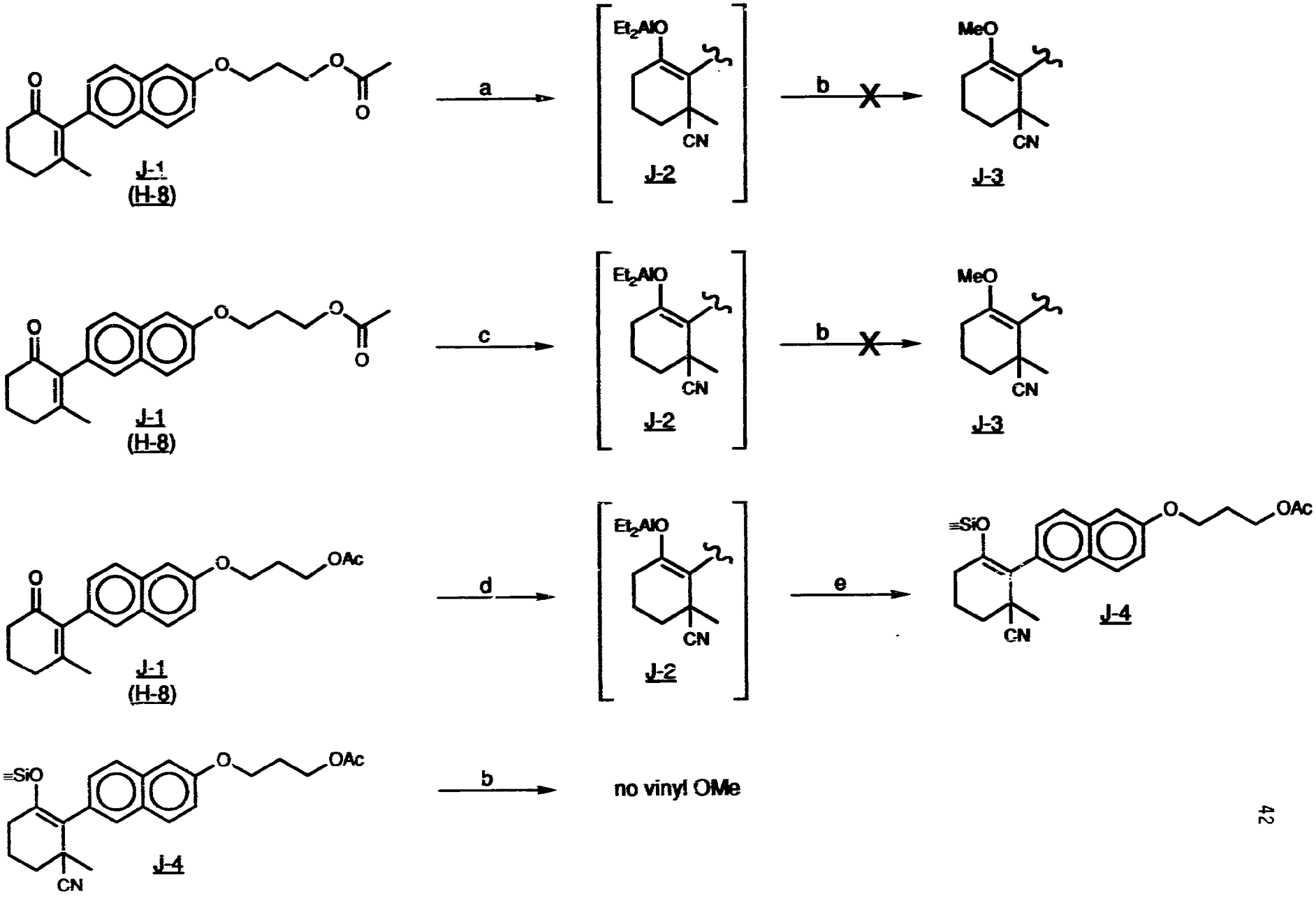
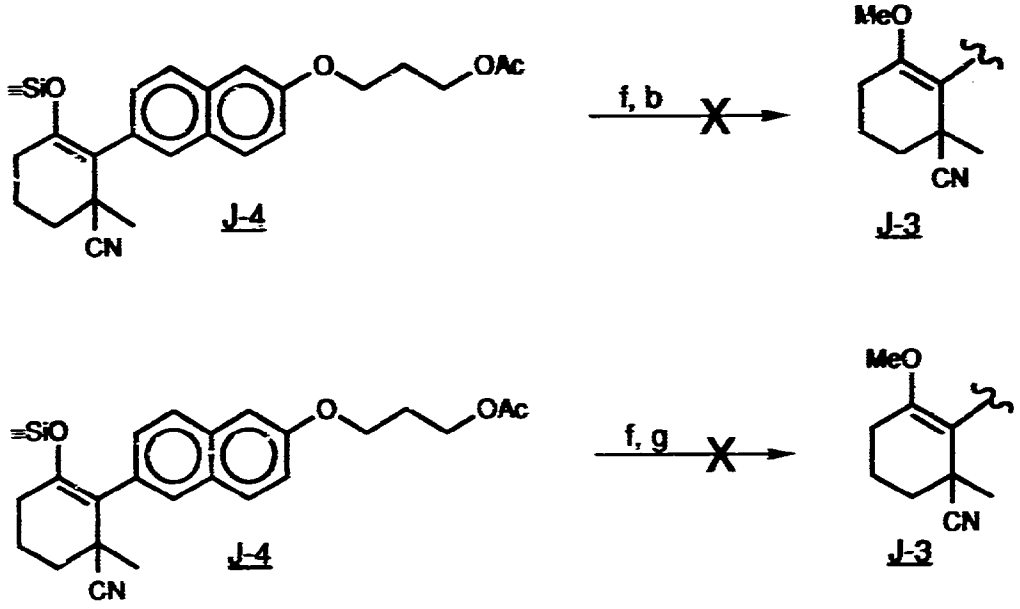
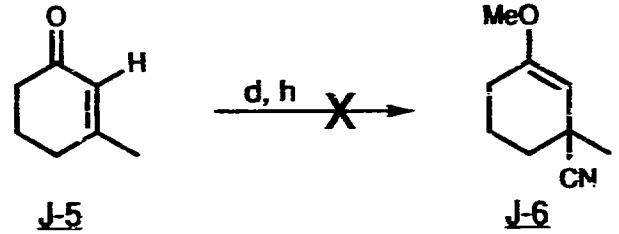


CHART J (Continued)
HYDROCYANATION AND ENOL ETHER EXPERIMENTS



J-4 J-3

J-4 J-3



J-5 J-6

a) 1.2 moles Et_2AlCN in THF/pentane
b) $Me_3O^+BF_4^-$
c) Et_2AlCN in toluene
d) Excess Et_2AlCN
e) Excess trimethylsilyl chloride/pyridine
f) $(n\text{-}Bu)_4NF$
g) CF_3SO_3Me
h) CF_3SO_3Me/HMPA

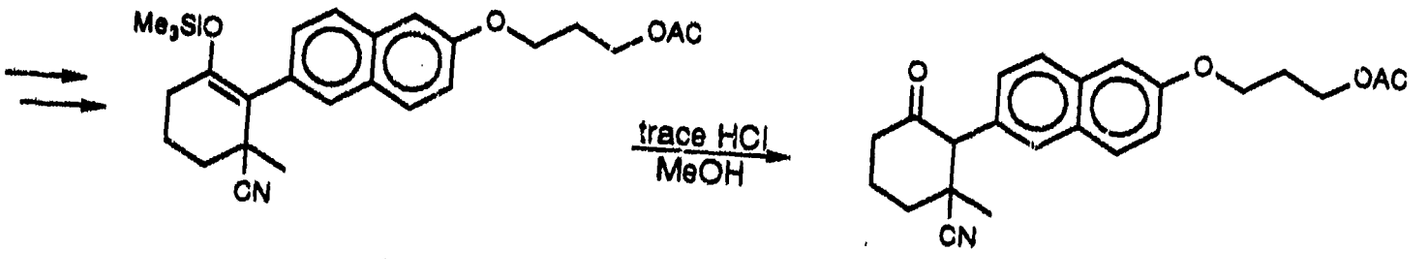
reaction appeared to occur in THF, there was a large amount of apparent by-products from the THF, and no product could be isolated. Reaction of the intermediate aluminum enolate from the model compound 3-methylcyclohex-2-en-1-one (J-5) with another very active O-alkylating agent, methyl triflate, was also unsuccessful.

Samson and Vandewalle (1978) reported that analogous aluminum enolates could be trapped as the trimethylsilyl enol ethers, useful for further synthetic modifications. We found this to be the case and compound J-4 was isolated in good yield. Direct treatment of this compound with Meerwein's reagent gave no evidence of vinyl ether formation. Formation of the anion by reaction with tetra-n-butylammonium fluoride occurred readily, but the resulting enolate could not be trapped with either Meerwein's reagent or methyl triflate.

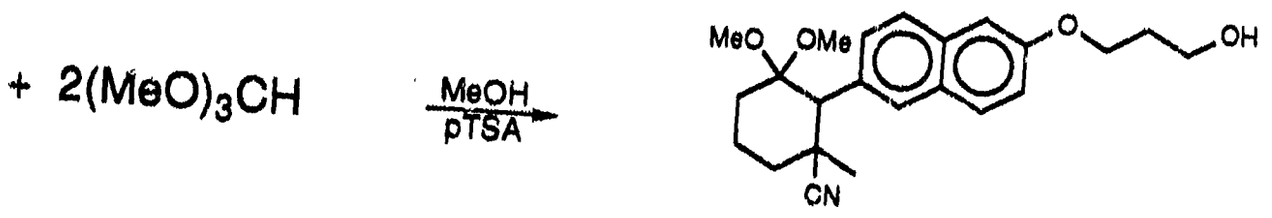
Treatment of trimethylsilyl (TMS) vinyl ether J-4 with trace amounts of HCl in methanol (Samson and Vandewalle, 1978) cleanly produced the β-cyanoketone K-1 in high yield (Chart K). (K-1 would be the product of the drug/cyanide reaction.) This crude material was then gently heated with methyl orthoformate (Napolitano et al., 1986) to yield the dimethyl ketal K-2. It has been reported that dimethyl acetals and ketals can be easily converted to their vinyl methyl ether derivatives simply by treating with trimethylsilyl iodide in the presence of the strong base hexamethyldisilazane (Miller and McKean, 1982). However, no vinyl methyl ether formation could be detected by NMR upon exposure of ketal K-2 to these conditions.

Since molecular modelling indicated that the TMS enol ether could substitute for the methyl enol ether as a transition state analog, we decided to attempt to couple the primary alcohol derived from J-4 to protein. We recognized that silyl ethers are relatively unstable. However, the silyl ether J-4

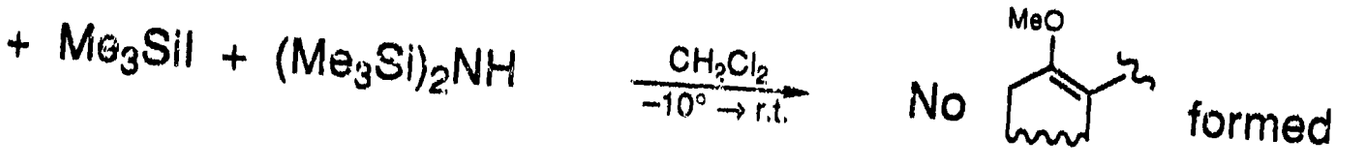
CHART K
ATTEMPTED METHYL VINYL ETHER SYNTHESIS FROM KETAL



J-4 K-1



K-2



had been found stable to aqueous workup conditions in sodium bicarbonate solution. Furthermore we believed it would be possible to confirm the presence of the trimethyl silyl ether in a protein conjugate by NMR, since the Si-CH_3 protons resonate in a portion of the spectrum removed from normal protein resonances.

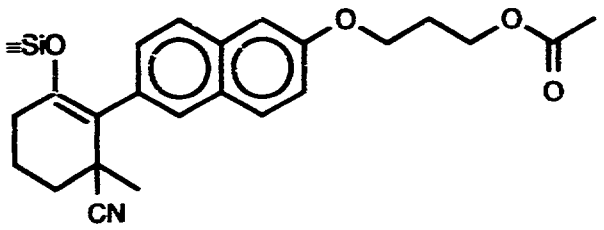
In exploring the coupling of the hapten to protein, we first needed a clean deprotection of the terminal acetate to provide the primary alcohol. The acetate J-4 was first treated with an ethanol/potassium carbonate solution but this only led to decomposition of the TMS vinyl ether (Chart L).

Acetate J-4 was then treated with lithium borohydride (Brown et al., 1982) in dry THF and extracted with ether from saturated sodium bicarbonate to provide the primary alcohol L-1 in high yield (Chart L). The crude product was clean enough to be used without further purification.

As a model for conjugation to protein, the coupling of alcohol L-1 with dimethylamine as the model nucleophile was attempted with 1 equivalent of phosgene (Chart M). The intermediate chloroformate was generated in situ and then added to a solution of dimethylamine in 0.1 M $NaHCO_3$. The reaction mixture was extracted with ether to yield only products no longer containing the TMS vinyl ether moiety. Triphosgene (Eckert and Forester, 1987), a phosgene substitute, was used in the same sequence of reactions when it was feared the phosgene reagent was too harsh. However, the same outcome was observed.

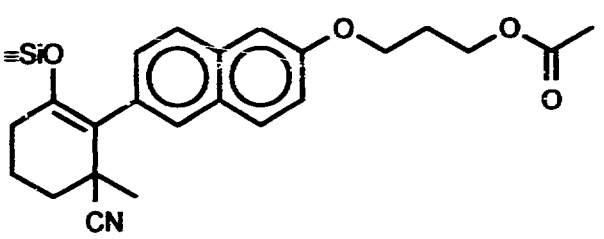
To avoid acidic reagents altogether, the alcohol L-1 was treated with 1,1'-carbonyldiimidazole (Ford and Ley, 1990) (CDI) to produce the imidazolide M-1 in essentially quantitative yield (Chart M) with the silyl ether intact, as shown by NMR. As a model reaction for the conjugation to amino groups of protein, imidazolide M-1 was treated with dimethylamine in 0.1 M $NaHCO_3$, but the coupled product no longer contained the TMS vinyl ether. Thus this approach had to be abandoned.

CHART L
ACETATE CLEAVAGE



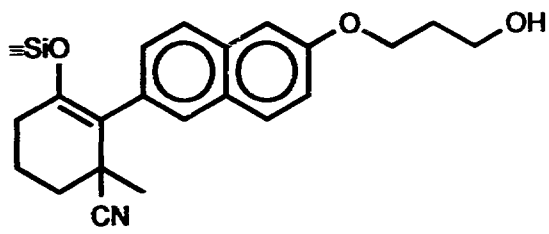
J-4

+ K_2CO_3 / EtOH → TMSO cleavage

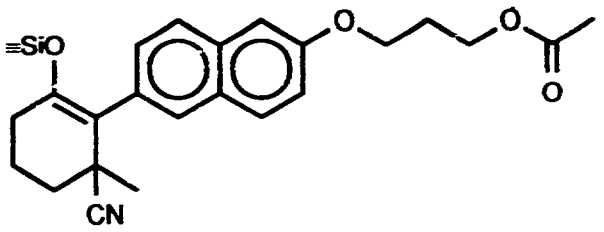


J-4

+ ex. $LiBH_4$ $\xrightarrow[H^{\oplus}\ \text{work-up}]{\text{THF}}$ some

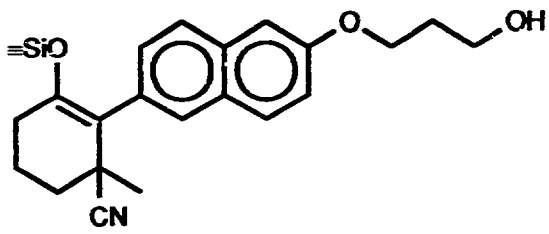


L-1



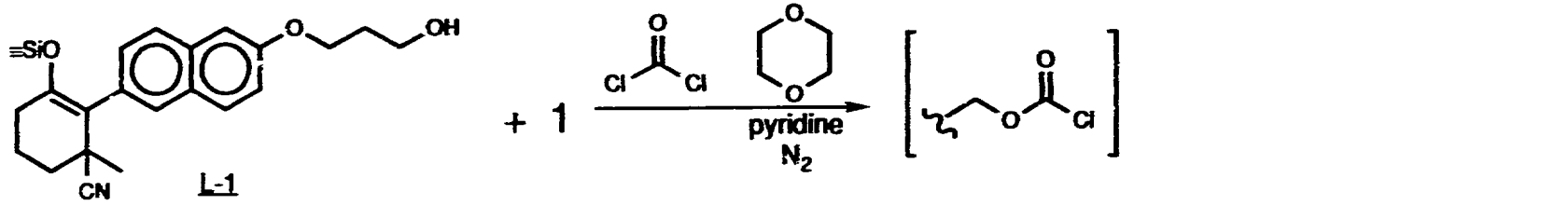
J-4

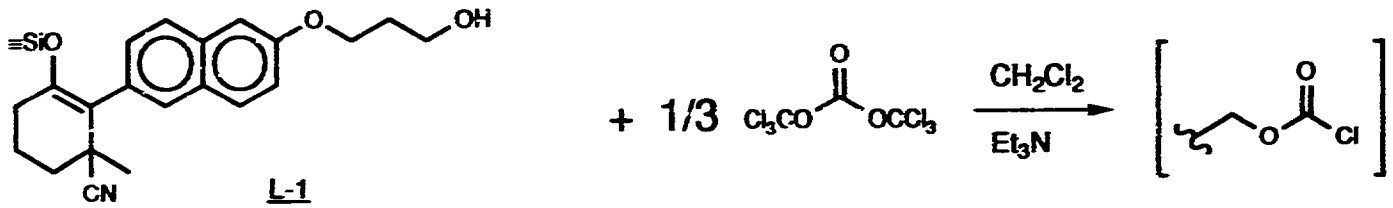
+ ex. $LiBH_4$ $\xrightarrow[NaHCO_3\ \text{work-up}]{\text{THF}}$

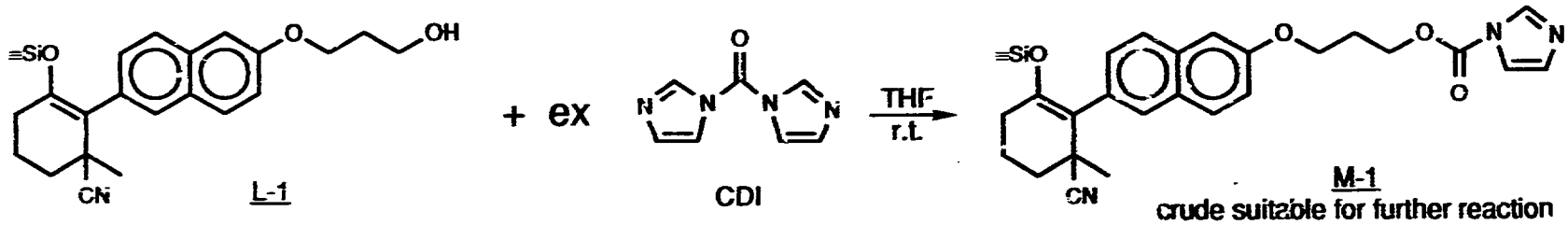


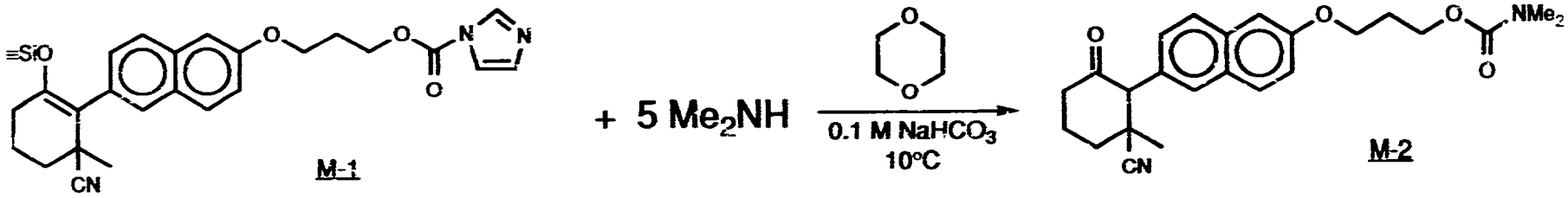
L-1
crude suitable for
further reaction

CHART M
COUPLING MODEL STUDY









In order to improve the stability of the vinyl ether functionality, t-butyldimethylsilyl chloride (TBDMSCl) was substituted for trimethylsilyl chloride in the reaction of the diethylaluminate (J-2) formed from J-1 and diethyl aluminum cyanide. Imidazole and pyridine were used as amine bases to remove aluminum from the enolate but no TBDMS vinyl ether could be detected by NMR.

5.0 Conclusions and Recommendations

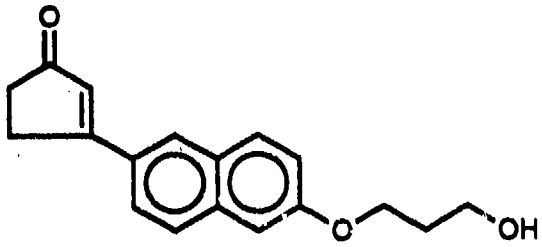
In work to date

- We have clearly established that the reaction of cyanide with α,β-unsaturated ketones, which had previously been studied at high temperature and pH (Agami et al., 1982), will take place under conditions of physiological pH and temperature.
- At physiological pH and temperature, the reaction is second order (first order in both cyanide and the α,β-unsaturated ketone).
- By means of computer simulations we have shown that the uncatalyzed reaction is too slow to be useful as a protection against cyanide intoxication. However, making assumptions of even modest antibody affinity and rate accelerations within those reported to date for reactions catalyzed by antibodies, computer simulations indicate that an antibody which catalyzed the reaction of cyanide with an α,β-unsaturated ketone could be used to protect against cyanide.
- Based on the experimental results of Agami et al. (1982) that show the reaction to have a transition state resembling that of product, two transition state analogs--enolate and nitrone--have been proposed. A drug and transition state analog hapten have been designed based on a cyano-enolate as the transition state analog.
- Other desirable features have been designed into the drug molecule so as to produce pharmacokinetically favorable features.
- Computer assisted molecular modelling experiments support the concept that the order of affinity of the antibody will be transition state analog > drug > product, which is the desired order.
- The drug (H-8) has been synthesized and also converted to the cyanomethyl ketone (K-1) with diethylaluminum cyanide.

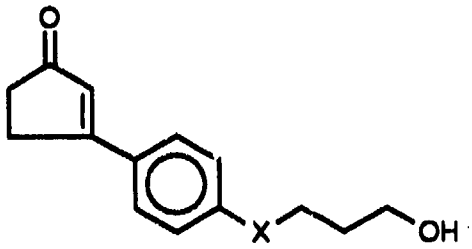
• The drug was converted into an activated transition state analog, M-1, suitable for reaction with amino groups of proteins. However it has been established that the trimethylsilyl enol ether moiety of the transition state analog M-1, although stable to mild aqueous conditions, is not stable enough to permit it to be coupled to protein under standard conditions, and probably is not stable enough to survive in the body.

We therefore are proceeding to modify the structures to take advantage of what we have learned. In particular, it appears that the use of an α-aryl substituent, although advantageous for theoretical reasons, introduces severe synthetic and steric hindrance problems. Each step has required extensive investigation. A β-aryl substituent would be synthetically much easier to obtain and work with. It would retain the advantage of having a tertiary nitrile as the product. It may somewhat reduce the reaction rate with cyanide. However the rate may be increased significantly by the presence of electron-withdrawing substituents on the aryl ring (cf. Agami, et al, 1982). Also, the key features of antibody catalysis should still come into play. Thus in accord with the work scope, we are working on the synthesis of β-arylcycloenones N-1 (Chart N) and subsequent steps. Compound N-1 has already been made by the reaction of commercially available 3-ethoxycyclopent-2-en-1-one with the lithium derivative of E-8. It has the advantage that the chemistry leading up to it is already well worked out. Compound N-2 may have some further advantages in that oxidation of the sulfur to the sulfone should, by means of the inductive effect, significantly accelerate the reaction with cyanide. Both N-1 and N-2 can be made in either the cyclohexenone or cyclopentenone series. Currently we are working in the cyclopentenone series because the corresponding transition state analogs can then be made either as stabilized enolates or as nitrones. The potential of enol phosphates is also under study.

CHART N
β-ARYLCYCLOENONES



N-1



N-2

a. X = S
b. X = SO_2

6.0 Experimental Details of Key Syntheses

3-Methyl-2,3-oxidocyclohexanone (E-2)--A solution of 3-methyl-2-cyclohexen-1-one (10 g, 90.8 mmol) and hydrogen peroxide (30% solution, 30.9 g, 272.4 mmol) in 75 mL of methanol was cooled to 15°C. A 6 M solution of potassium phosphate monobasic (20.7 g, 90.8 mmol) was slowly added over 1 h, and the reaction mixture was stirred at room temperature for 3 h. The mixture was poured into 30 mL of water and extracted (2 x 30 mL) with ether. The organic phase was dried with $MgSO_4$, filtered and concentrated under vacuum to give 21.1 g of crude material. This material (9 g) was purified by column chromatography (SiO_2, 5:1 hexane:ethyl acetate) to yield 2.72 g (56%) of a colorless oil.

^1H NMR (250 MHz, $CDCl_3$) δ 1.46 (s, 3H, CH_3), 1.6-2.6 (m), 3.07 (s, 1H, C(O)CH); IR (neat) 1720 cm^{-1}.

3-Methyl-2,3-oxidocyclohexanone-N,N-dimethylhydrazone (E-3)--The keto-epoxide E-2 (504 mg, 4 mmol) and 1,1-dimethyl hydrazine (608 μL, 8 mmol) were dissolved in 30 mL of ethyl acetate and the mixture cooled to -20°C. Propionic acid (30 μL, 0.4 mmol) was added and the reaction mixture maintained at -20°C for 4 h. The solution was allowed to warm to room temperature and washed with 10% Na_2CO_3 (2 x 20 mL). The organic phase was then dried with Na_2SO_4, filtered, and concentrated to yield 0.64 g of a light yellow oil. Attempts to purify this material only led to decomposition.

^1H NMR (250 MHz, $CDCl_3$) δ 1.20 (s, CH_3), 1.21 (s, CH_3), 2.29 (s, NMe_2), 2.36 (s, NMe_2), 3.08 [s, C(NMe_2)CH]; IR (neat) 1729 cm^{-1}.

2-(3-bromopropyloxy)tetrahydropyran (E-6)--A solution of 3-bromo-1-propanol (2.78 g, 20 mmol) and 3,4-dihydro-2H-pyran (8.4 g, 100 mmol) in 95 mL of dry dichloromethane was cooled to 0°C. A catalytic amount of p-toluene sulfonic acid (38 mg, 0.2 mmol) was added and the mixture stirred for 15 min,

allowed to warm to room temperature and stirred for 2 h, poured into a solution of 40 mL saturated $NaHCO_3$, 40 mL saturated NaCl, 80 mL water and 20 mL ether. The organic phase was separated and washed with 30 mL saturated NaCl solution, dried with $MgSO_4$, filtered, and concentrated under vacuum to yield 3.07 g of a light yellow oil. Purification of 1.038 g of the crude material by silica gel column chromatography (20:1 hexane:ethyl acetate) gave 633 mg (61%) of a clear oil.

^1H NMR (250 MHz, $CDCl_3$) δ 1.4-2.0 (m, 6H, $-CH_2-$), 2.16 (q, 2H, $OCH_2-C\underline{H}_2-CH_2Br$), 3.54 (m, 4H, $O-C\underline{H}_2-$), 3.88 (m, 2H, $Br-C\underline{H}_2-$), 4.60 (s, 1H, O-CH-O).

6-Bromo-2-[2-(3-oxypropyloxy)tetrahydropyran-2-yl]-naphthalene (E-8)--A solution of 6-bromo-2-napthol (2.01 g, 9 mmol), THP-bromide E-6 (2.01 g, 9 mmol) and potassium carbonate (2.5 g, 18 mmol) in 45 mL of dry acetone was refluxed for 48 h. The reaction mixture was filtered and concentrated under vacuum. Purification by silica gel column chromatography (10:1 hexane:ethyl acetate) gave 2.33 g (71%) of a white crystalline solid.

^1H NMR (250 MHz, $CDCl_3$) δ 1.4-2.0 (m, 6H, $-CH_2-$), 2.13 (q, $OCH_2-C\underline{H}_2-CH_2Br$), 3.4-3.7 (m, 2H, $-CH_2-$), 3.8-4.1 (m, 2H, $-CH_2-$), 4.17 (t, 2H, $ArOC\underline{H}_2$), 4.61 (s, 1H, O-CH-O), 7.0-7.9 (m, 6H, Ar-H).

2-(4-Methoxyphenyl)cyclohexanol (Reaction 12, Table 3)--A dry flask was degassed with N_2 and then charged with Mg (1.0 mmol, 24 mg) and 1 mL of THF with a catalytic amount of I_2. A few drops of a 4-bromoanisole solution (1.0 mmol, 125 μL in 5 mL of THF) were added by syringe to the reaction flask. The flask was then heated to induce initiation. The remaining 4-bromoanisole was then added dropwise. Heating was continued at reflux until all the Mg was consumed. After cooling to room temperature, the Grignard reagent was transferred via syringe to a -30°C solution of CuI (0.1 mmol, 19 mg) and cyclohexene oxide (0.66 mmol, 67 μL) in 1 mL of THF. The reaction mixture

temperature was held at -30°C for 5 min and then warmed to 0°C for 2 h. The mixture was then poured into saturated NH_4Cl and extracted with Et_2O. The organic layer was dried with $MgSO_4$, filtered through Celite, and evaporated to dryness. The crude material was identical to authentic sample obtained from Aldrich Chemical Co.

2-(4-Methoxyphenyl)cyclohexanone (Reaction 13, Table 3)--2-(4-Methoxyphenyl)cyclohexanol (2.0 mmol, 412 mg) was dissolved in 5 mL of CH_2Cl_2 at room temperature. Dess-Martin reagent was added in excess until the starting alcohol could not be detected by thin layer chromatography. The reaction mixture was poured into saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic phase was dried with $MgSO_4$, filtered through Celite, and evaporated to dryness. The product was purified by SiO_2 column chromatography (5:1 hexane:ethyl acetate) to yield a white solid (1.50 mmol, 306 mg) in 75% yield.

^1H NMR ($CDCl_3$): δ 1.7-2.6 (m, 8H, ring CH), 3.58 (dd, 1H, C(O)C<u>H</u>C_6H_5OMe), 3.79 (s, 3H, OMe), 6.86 (d, 2H, ArH), 7.06 (d, 2H, ArH).

2,2-(2,2-Dimethylpropane-1,3-dioxy)cyclohexan-1-one (H-2)--1,2-Cyclohexanedione (1.12 g, 10 mmol), 2,2-dimethylpropane-1,3-diol (1.04 g, 10 mmol) and a few mg of p-toluenesulfonic acid were refluxed overnight in 20 mL of methylene chloride. Purification by column chromatography [silica, hexane:ethyl acetate (6:1)] yielded 1.77 g (89%) of H-2.

^1H NMR ($CDCl_3$): δ 3.67 (d, 2H, $(CH_3)_2CC$<u>H</u>-O), 3.46 (d, 2H, $CCH_3)CC$<u>H</u>-O), 2.52 (m, 2H, $C(O)CH_2$), 2.0-1.85 (m, 6H $(CH_2)_3$), 1.18 (s, 3H, CH_3), 0.71 (s, 3H, CH_3).

6-Methyl-2,2-(2,2-dimethyl-1,3-propane dioxy)cyclohexan-1-one (H-3)--A concentrated solution of 2-(2,2-dimethyl-1,3-propylenedioxy)cyclohexene-1-one (H-2) (4.7 mmol, 0.933 g in 1 mL of THF) was added to a -78°C solution of freshly prepared lithium diethylamide (LDA) (5.6 mmol in 5 mL THF) and stirred

for 1 h. Methyl iodide (6.16 mmol, 384 μL) was added via syringe at -78° and the mixture stirred further for 0.5 h. The reaction mixture was then slowly warmed to room temperature, poured into water and extracted with Et_2O. The ether phase was dried with $MgSO_4$, filtered through Celite, and concentrated under vacuum to yield a light yellow oil. The product (H-3) was purified by column chromatography on SiO_2 (50:1 hexane:ethyl acetate) in 13% yield (0.133 mg, 0.63 mmol).

^1H NMR ($CDCl_3$): δ 4.05 (d, 1H, $(CH_3)_2CCH$-O), 3.55-3.40 (m, 2H, $(CH_3)_2CCH$-O), 3.18 (d, 1H, $CCH_3)_2CCH$-O), 3.0-2.80 (m, 1H, C(O)CH), 2.30-2.15 (m, 1H, $-CH_2-$), 2.10-1.80 (m, 1H, $-CH_2-$), 1.70-1.55 (m, 2H, $-CH_2-$), 1.35-1.20 (m, 2H, $-CH_2-$), 1.18 (s, 3H, $C(CH_3)_2$), 1.03 (d, 3H, $CH(CH_3)$, 0.70 (s, 3H, $C(CH_3)_2$).

3-Methyl-2-(4-methoxyphenyl)cyclohex-2-en-1-one (G-3)--4-Methoxyphenyl magnesium bromide was generated as previously described using 0.4 mmol (10 mg) of Mg and 0.4 mmol (50 μL) of 4-bromoanisole in 2 mL of THF. A solution of 6-methyl-2,2-(2,2-dimethylpropane-1,3-dioxy)cyclohexan-1-one in 1 mL of THF was added to the Grignard reagent. When no starting ketone could be detected by thin layer chromatography (TLC), the reaction mixture was poured into sat. NH_4Cl solution and extracted with Et_2O. The organic phase was dried with $MgSO_4$, filtered through Celite, and the solvent removed in vacuo. The crude product was dissolved in acidic $MeOH/H_2O$ (10/2) and refluxed for 4 h. Standard ether workup provided the α-hydroxyketone intermediate. This crude material was then refluxed in benzene with a catalytic amount of p-TsOH for 2 h. Standard ether workup yielded the enone (G-3) which was identical to a sample synthesized from 4-methoxybenzyl bromide and 6-methyl-3,4-dihydro-2H-pyran-2-one (G-1) below.

^1H NMR ($CDCl_3$): δ 7.0-6.85 (m, 4H, ArH), 3.81 (s, 3H, $-OCH_3$), 2.55-2.45 (m, 4H, $-(CH_2)_2-$), 2.10-2.0 (m, 2H, $-CH_2-$), 1.82 (s, 3H, CH_3).

4-Methoxybenzyl Grignard reagent was prepared by dropwise addition of 12.5 g (79 mmol) of 4-methoxybenzyl chloride in 60 mL dry THF over 5 h to a slurry of 4.5 g of magnesium turnings and 4.5 g of magnesium powder in 120 mL dry THF. It was then added to a solution of 7.0 g of 3,4-dihydro-6-methyl-2H-pyran-2-one in 60 mL of dry THF at 0°. Overnight stirring was followed by treatment with saturated aqueous NH_4Cl and extraction with ether/ethyl acetate. The organic extract was evaporated and treated overnight with 6 g of NaOH in 80 mL methanol/20 mL H_2O. Extraction followed by silica chromatography (CH_2Cl_2/acetone) gave 0.65 g of essentially pure (by TLC) G-3 plus another, less pure fraction.

2-[6-(3-hydroxypropyloxy)-2-naphthyl]-3-methylcyclohex-2-en-1-one (H-7)--Compound E-8 (2.54 g, 6.95 mmol) in 10 mL freshly distilled THF was treated with 4.29 mL of n-BuLi solution (6.95 mmol) at -78° for 0.5 h. A solution of ketone H-3 (1.48 g, 6.95 mmol) in 10 mL THF was added. After 0.5 h stirring at -78°, the mixture was allowed to warm to room temperature, added to water and extracted with ether. The crude product was refluxed for 3 h with 20 mL EtOH, 20 mL H_2O and 16 mL of 3N HCl, poured into water and extracted with ether to yield 2.44 g crude H-6. This was dissolved in benzene and refluxed for 5 h with a Dean-Stark trap with a few mg of p-toluenesulfonic acid. Purification of the product by silica chromatography (hexane:ethyl acetate) yielded 0.414 g of H-7.

^1H NMR ($CDCl_3$): δ 7.80-7.65 (m, 2H, ArH), 7.45 (s, 1H, ArH), 7.20-7.10 (m, 3H, ArH), 4.23 (t, 2H, J = 5.9 Hz, $-CH_2-O-$), 3.90 (t, 2H, J = 5.9 Hz, $-CH_2-O-$), 2.55 (m, 4H, $=CH-CH_2-$ and $-C(O)-CH_2-$), 2.10 (m, 4H, $CH_2-CH)_2-CH_2$ and $OCH_2-CH_2-CH_2O$), 1.84 (s, 3H, CH_3).

Synthesis of 2-[6-(3-acetoxypropyloxy-2-naphthyl]3-methylcyclohex-2-en-1-one (H-8)--Alcohol H-7 (0.071 mmol, 22 mg) was dissolved in 4 mL of CH_2Cl_2.

Excess acetic anhydride (1 mL), excess Et_3N (1 mL) and a catalytic amount of N,N-dimethylaminopyridine (DMAP) were added. The reaction was continued for 12 h. The mixture was then poured into water and extracted with CH_2Cl_2. The organic phase was washed with saturated $NaHCO_3$ and dried with $MgOSO_4$. The solution was filtered through Celite and concentrated under vacuum. The product was purified by column chromatography on SiO_2 (2:1 hexane:ethyl acetate) to yield 21 mg (0.60 mmol, 84% yield) of pure acetate H-8.

^1H NMR ($CDCl_3$): δ 7.75-7.65 (m, 2H, ArH), 7.45 (s, 1H, ArH), 7.20-7.10 (m, 3H, ArH), 4.31 (t, 2H, J = 6.3 Hz, $-CH_2-O$), 4.17 (t, 2H, J = 6.2 Hz), 2.55 (m, 4H, $=CH-CH_2-$ and $-C(O)-CH_2-$), 2.25-2.10 (m, 4H, $CH_2-CH_2-CH_2$ and $OCH_2-CH_2-CH_2O$), 2.07 (s, 3H, OAc), 1.84 (s, 3H, CH_3).

3-Cyano-3-methyl-2-[6-(3-acetoxypropyloxy)naphth-2-yl]-1-trimethylsilyloxycyclohex-1-ene (J-4)--To a room temperature solution of the starting enone H-8 (0.057 mmol, 20 mg) in 300 μL of dry THF, diethylaluminum cyanide (0.114 mmol, 114 μL of 1M toluene solution) was added dropwise and allowed to react for 1.5 h. Excess TMSCl (0.171 mmol, 22 μL) and excess pyridine (0.278 mmol, 22 μL) were then added dropwise and the solution stirred for 1.5 h. The mixture was diluted with ether and washed with ice cold saturated NH_4Cl, 5% HCl, and then saturated $NaHCO_3$. The ether was dried with Na_2SO_4, filtered through Celite, and concentrated under vacuum to yield the almost pure TMS vinyl ether J-4. The product was purified by column chromatography on silica gel (6:1 hexane:ethyl acetate) to yield 5 mg of pure material.

^1H NMR ($CDCl_3$): δ 7.75-7.10 (m, 6H, ArH), 4.31 (t, 2H, $-CH_2O-$), 4.17 (t, 2H, $-CH_2O-$), 2.35-2.05 (m, 4H, ring CH), 2.07 (s, 3H, OAc), 1.70 (m, 2H, ring CH), 1.23 (s, 3H, CH_3), -0.15 (s, 9H, $OSi(-CH_3)_3$).

3-Cyano-3-methyl-2-[6-(3-acetoxypropyloxy)naphth-2-yl]cyclohexanone (K-1)--TMS vinyl ether J-4 (0.057 mmol) was dissolved in ~5 mL MeOH with 3-4

drops of 5% HCl, stirred at room temperature for 0.5 h and extracted with ether from sat. $NaHCO_3$. The organic phase was dried with Na_2SO_4, filtered through Celite, and solvent removed to yield a light yellow oil.

^1H NMR ($CDCl_3$): δ 1.29 (s, 3H, CH_3), 2.07 (s, 3H, OAc), 2.18 (q, 2H, $-CH_2-$), 2.7-2.2 (m, ring CH), 4.17 (t, 2H, $-CH_2O-$), 4.31 (t, 2H, $-CH_2O-$), 7.8-7.1 (m, 6H, ArH).

3-Cyano-1,1-dimethoxy-3-methyl-2-[6-(3-acetoxypropyloxy)naphth-2-yl]-cyclohexane (K-2)--β-Cyanoketone K-1 was dissolved in ~5 mL MeOH, to which was added a large excess of methyl orthoformate with a catalytic amount of p-TsOH. The mixture was heated gently for 3 h, diluted with Et_2O, and extracted from sat. $NaHCO_3$ solution. The extract was dried with Na_2SO_4, filtered through Celite, and solvent removed to yield crude ketal K-2, purified by column chromatography (2:1 hexane/EtOAc) to yield pure ketal K-2 (5 mg, 0.013 mmol).

^1H NMR ($CDCl_3$): δ 1.08 (s, 3H, CH_3), 2.12 (q, 2H, $-CH_2-$), 1.4-2.3 (m, ring CH), 2.63 (s, 3H, OMe), 3.33 (s, 3H, OMe), 3.91 (t, 2H, $-CH_2OH$), 4.25 (t, 2H, $-CH_2O-$), 7.1-7.7 (m, 6H, ArH).

3-Cyano-3-methyl-2-[6-(3-hydroxypropyloxy)-naphth-2-yl]-1-trimethylsilyloxycyclohexene (L-4)--TMS vinyl ether J-4 (0.028 mmol crude) was dissolved in ~1 mL of freshly distilled THF and excess $LiBH_4$ was added. After 2.5 h, no starting material could be detected by TLC. The reaction mixture poured into ice cold $NaHCO_3$ solution and extracted with Et_2O. When bubbling from destruction of excess $LiBH_4$ had stopped, the organic phase was separated, dried with Na_2SO_4, filtered through Celite, and solvent removed, providing a light yellow oil (9 mg, 0.022 mmol), 79% from enone).

^1H NMR ($CDCl_3$): δ -0.15 (s, 9H, $OSiMe_3$), 1.23 (s, 3H, CH_3), 2.15 (m, 2H, $-CH_2-$), 1.8-2.5 (m, ring CH), 3.92 (s, 2H, $-CH_2OH$), 4.26 (s, 2H, $-CH_2O-$), 7.2-7.9 (m, 6H, ArH).

Formation of Imidazolocarbonyloxy Derivative of L-1 (M-1)--The primary alcohol L-1 was dissolved in 100 μL of freshly distilled THF. A THF solution (200 μL) containing excess carbonyldiimidazole was added, and the mixture was stirred at room temperature for 3 h, filtered through a pad of silica and solvent removed. Essentially pure compound was isolated.

^{1}H NMR ($CDCl_3$): δ -0.15 (s, 9H, $OSiMe_3$), 1.21 (s, 3H, CH_3), 2.36 (m, 2H, $-CH_2-$), 2.36-1.70 (m, ring CH), 4.24 (t, 2H, $-CH_2O$), 4.69 (t, 2H, $-CH_2O-$), 7.05-7.75 (m, 8H, ArH and -CH=CH-), 8.16 (s, 1H, N-CH=N).

7.0 References

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