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CONTRACT NO: DAMD17-93-C-3081

TITLE: Synthesis of Phosphorus Based Haptens for
Monoclonal Catalytic Antibody Production

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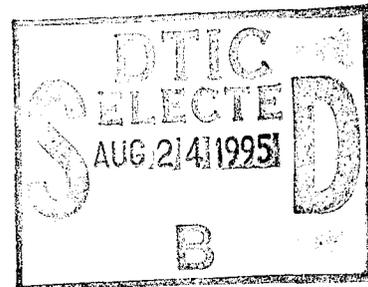
REPORT DATE: March 22, 1995

TYPE OF REPORT: Midterm Report, Phase II

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE March 22, 1995	3. REPORT TYPE AND DATES COVERED Midterm Report: Feb. 22,1994 to Feb. 21,1995	
4. TITLE AND SUBTITLE Synthesis of Phosphorus Based Haptens for Monoclonal Catalytic Antibody Production			5. FUNDING NUMBERS Contract No. DAMD17-93-C-3081	
6. AUTHOR(S) Kaiming Liu, Ph.D. Sudersan M. Tuladhar, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) STEROIDS, LTD. Chicago Technology Park 2201 West Campbell Park Drive Chicago, IL 60612			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U. S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Covers Phase II research.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to DOD Components only, Specific Authority. Other requests shall be referred to the Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, MD 21702-5012 <i>22 Mar 95</i>			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Our objective in this Phase II project is to synthesize haptens for production of monoclonal antibodies which will catalyse the hydrolysis of phosphorus-based nerve agents. Our syntheses followed three strategies. The first is synthesis of stable pentacoordinate unsymmetrical oxyphosphoranes as haptens for monoclonal catalytic antibodies based on a transition state for phosphonate hydrolysis: 2-(2'-pyridyldithio)propionylamino-2-methyl-3-(S)-[(1(-)-menthoxy) (1",3"-dioxo-4",6"-di-tert-butylbenzo]-methylphosphorane (29) is typical of this approach. In the second approach, the "bait and switch" concept is pursued and methyl [2-(2'-pyridyldithio)propionylaminomethyl-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane]-methylphosphonate (51) and its enantiomer (60), and [2-(3',5'-dinitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane]-aminophosphonate (64) are typical of suitable haptens. The third approach is based upon the potential addition of water to the phosphorus atom of a tetracoordinate phosphate to yield an intermediary pentacoordinate system. The typical examples are methyl 2-(p-nitrobenzoyl)aminomethyl-2-methyl-3-(S)-(methoxy)(trifluoromethyl)-hydroxybutane phosphonate (69), (2'-pyridyldithio)ethyl diethyl-3,3-difluoro-3-phosphonopropionic acid ester (70) and diethyl-4,4-difluoro-4-phosphonobutyl (2'-pyridyldithio)propionic acid ester (72). The syntheses and structures of various representatives as well as conjugation of 29 with BSA, KLH and PTG are presented in this report.				
14. SUBJECT TERMS Catalytic antibodies, pentacoordinated phosphorus, bifunctional "bait and switch" haptens, fluorinated haptens, protein conjugates.			15. NUMBER OF PAGES 60	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

FOREWORD

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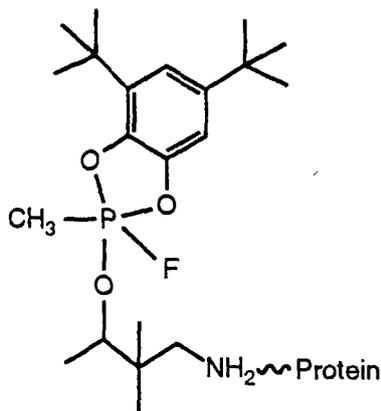
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INTRODUCTION

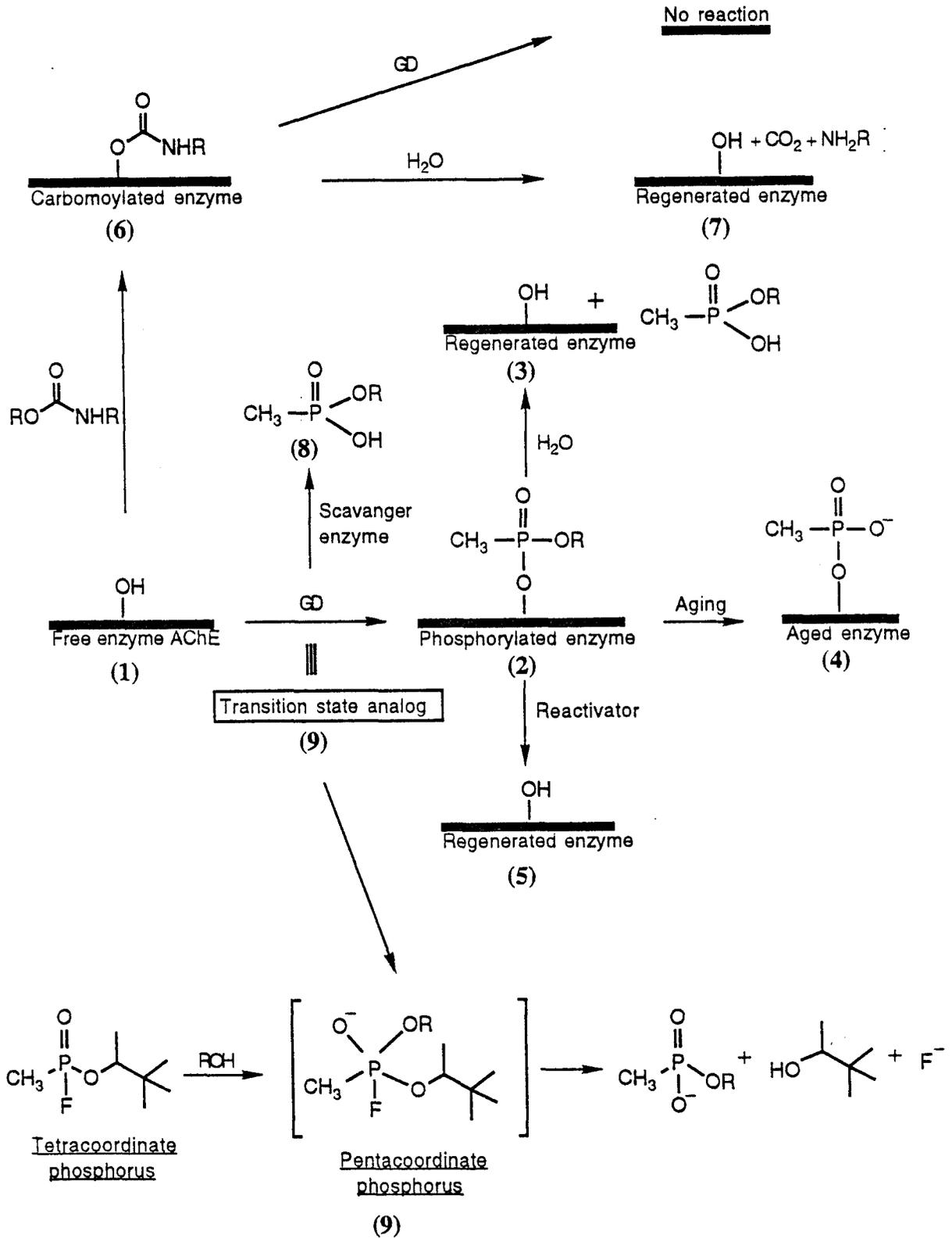
New initiatives in combating organophosphorus nerve agents are very important both in military applications as well as in the public domain, especially with respect to terrorist attack as occurred recently in Japan. Advances over pretreatment drugs which reversibly inhibit acetylcholinesterase as well as reactivators of inhibited AChE are active areas of research and development¹. A novel approach is prophylactic pretreatment using scavengers. Thus a purified recombinant organophosphorus acid anhydase is reported to protect mice against soman². Other scavengers administered in stoichiometric amounts such as acetylcholinesterase, butyrylcholinesterase and carboxylesterase have been investigated^{3,4}. A radically different approach originates in the area of catalytic antibodies⁵. This concept as applied to the catalytic hydrolysis of soman depends upon synthesis of a transition state analog for this process and then upon conjugation of this transition state analog as a hapten to carrier proteins and immunological generation of monoclonal antibodies. The various processes which can occur in the phosphorylation of AChE and subsequent reactions are shown in Figure 1. These include slow spontaneous hydrolysis (1→2→3), aging (1→2→4), reactivation as with PAM (1→2→5), reversible inhibition and hydrolysis (1→6→7) and scavenging (1→8). The basic concept of the application of catalytic antibodies to phosphonate hydrolysis is indicated in the lower part of Figure 1. The tetracoordinate phosphorus center of GD is converted upon addition of a nucleophilic to a pentacoordinate center in **9**. This is the transition state analog (GD→**9**)⁶. As a hapten transition state analog, **9** is used to generate catalytic antibodies for the hydrolytic cleavage.

In our approach to this problem we successfully synthesized a stable pentacoordinate phosphoranes (**10**) which served as a transition state analog for the phosphonate hydrolysis and in collaboration with USAMRICD⁷ immunochemical studies had been carried out.

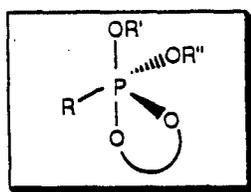
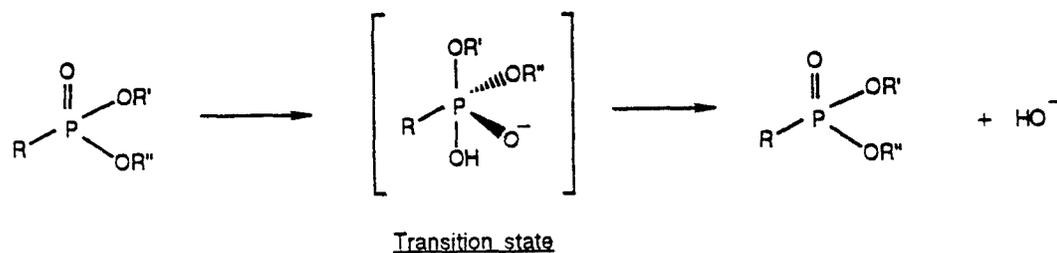


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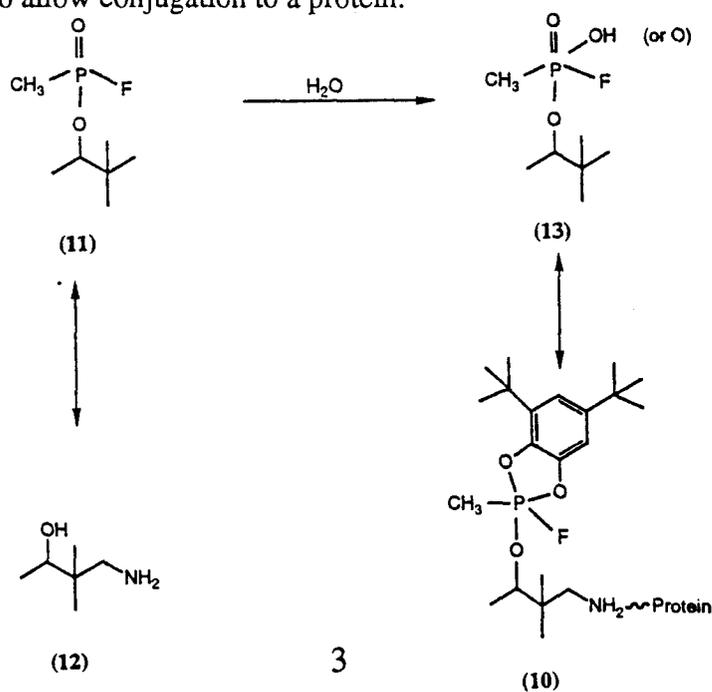
Figure 1. Phosphonylation of AChE



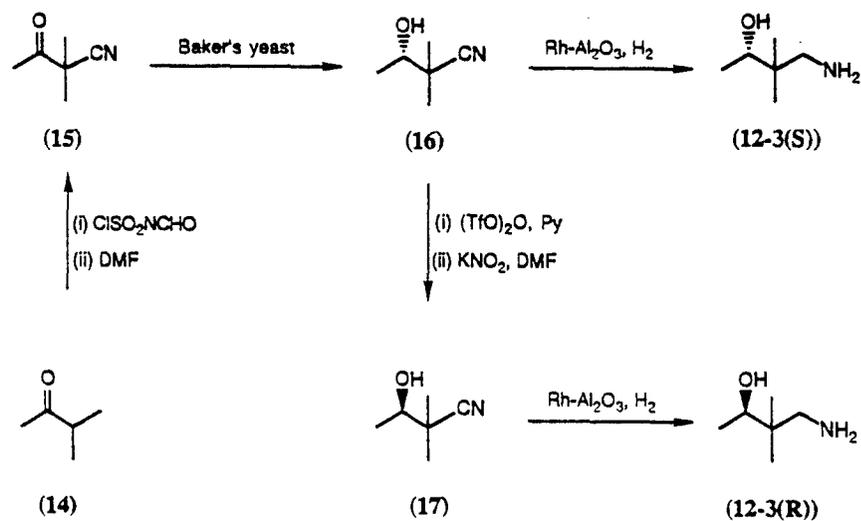
The model we selected for the pentacoordinate intermediate possessed a chelate-like bridge because this structural feature was required for stability. The additional requirements were hydrolytic stability and the ability to be conjugated *via* a suitable linker arm to a protein for immunization.



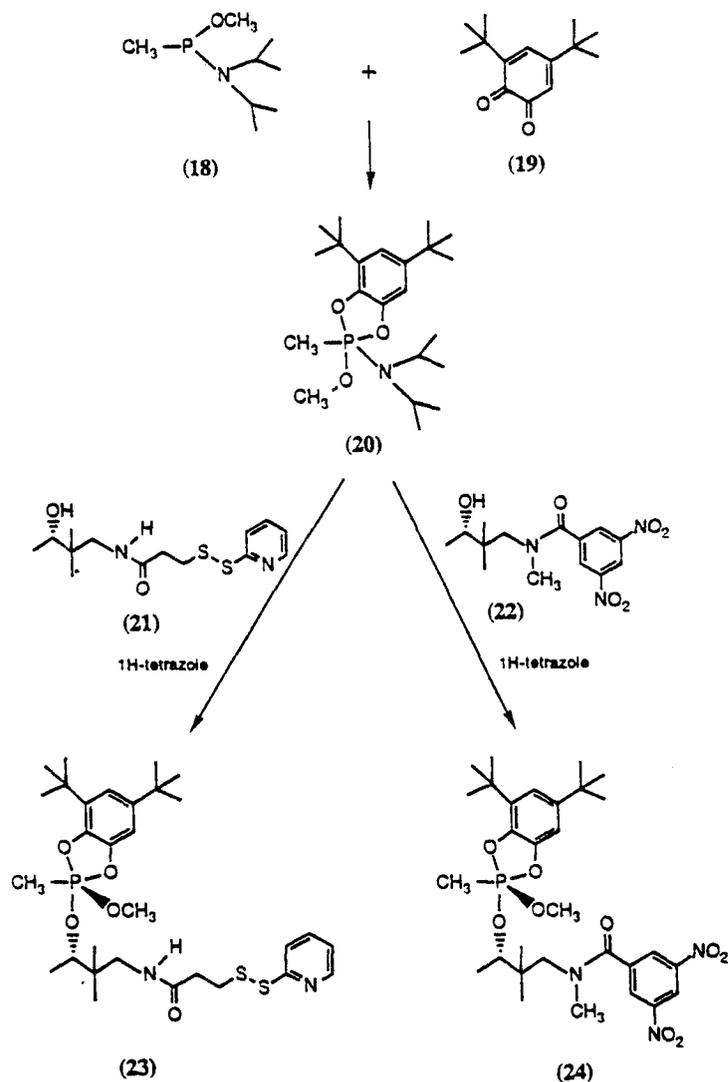
Furthermore, in the model the asymmetry at the secondary carbon atom of the pinacol group in soman (11) was mimicked. All of these requirements were met in hapten molecule 10 in that the stereochemical center at carbon atom in 2,2-dimethyl-3-hydroxybutylamine (12) was controlled and an amino group was positioned at the terminal carbon atom in 12 to allow conjugation to a protein:



The two enantiomers of 12-(3S) and 12-(3R) were synthesized as follows⁷:



The synthesis of the pentacoordinated system was accomplished as follows⁸:



The X-ray structure of the 3,5-dinitrobenzoyl derivative (**24**) revealed the trigonal bipyramidal structure at phosphorus as well as the absolute configuration at the chiral carbon and phosphorus atoms as shown in Figure 2:

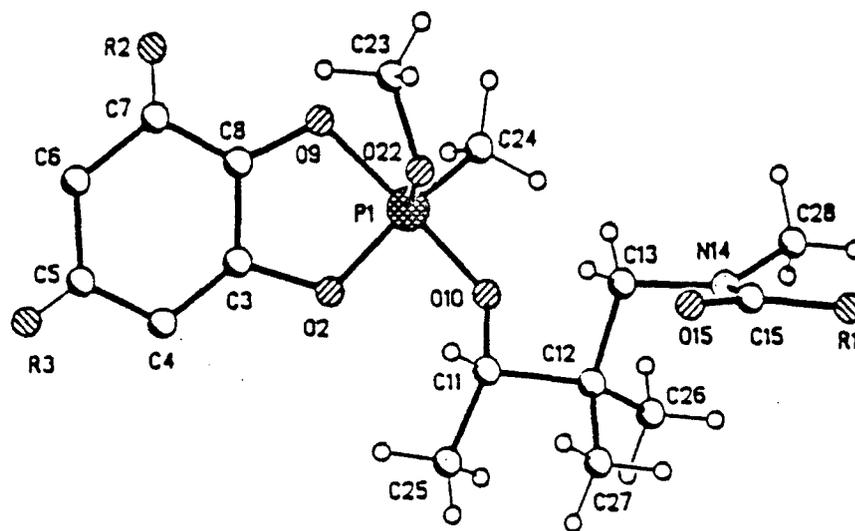
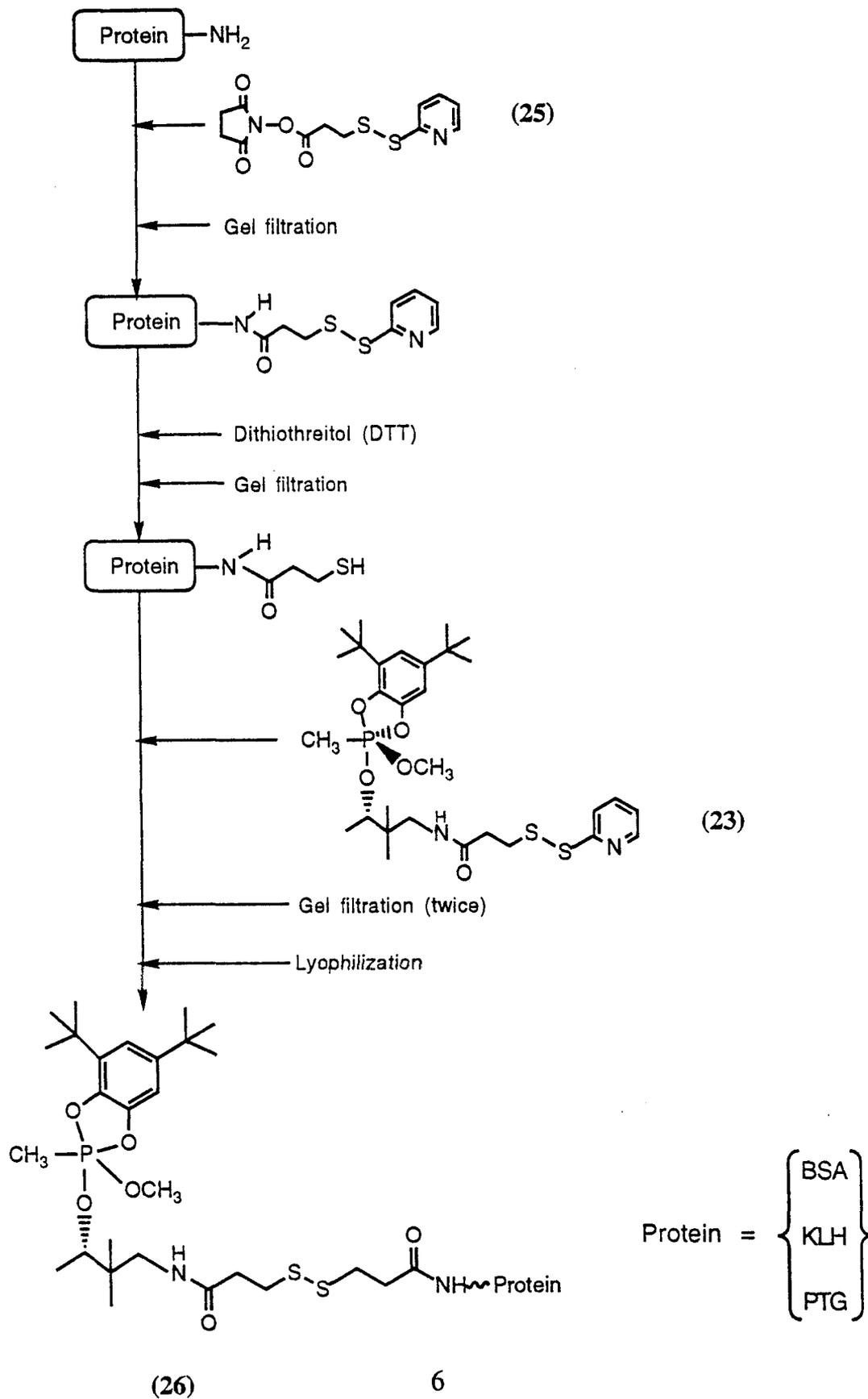


Figure 2.

The molecular structure and numbering scheme for the chiral phosphorane (**24**); R1 is the 3,5-dinitrophenyl group, and R2 and R3 are tert-butyl group.

The disulfide (**23**) was used for conjugation to BSA (**26-BSA**), KLH (**26-KLH**) and PTG (**26-PTG**) as shown in Scheme 1, and the BSA analog was used for immunization and antibody production using female Balb/c mice. A monoclonal antibody was obtained which catalyzed the hydrolysis of soman⁷.

Scheme 1. Protein Conjugation of Disulfide (23)

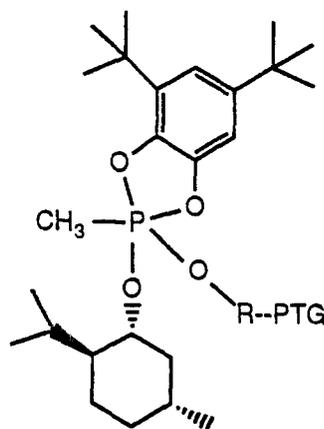


BODY:

In this midterm report of phase II project, we have synthesized three types of haptens for the generation of catalytic monoclonal antibodies: (i) an unsymmetrical monocyclic oxyphosphoranes, namely **29**; (ii) haptens for bifunctional catalytic antibodies: 'bait and switch' strategy, namely **51**, **60** and **64**; and (iii) fluorinated haptens, namely **69**, **70** and **72**. The goal of this project is to supply these complex haptens and their protein (BSA, KLH and PTG) conjugates to scientists at USAMRICD who will produce antibodies of varying catalytic activity.

Unsymmetrical Monocyclic Oxyphosphoranes.

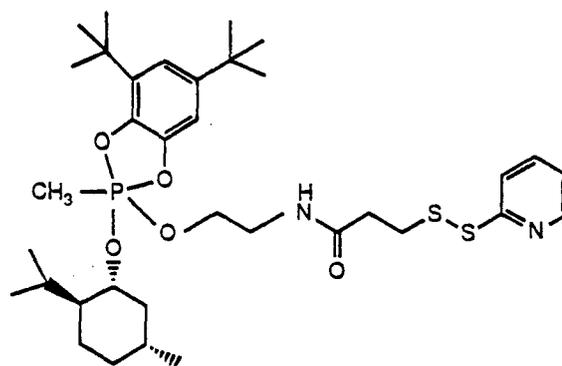
Unsymmetrical monocyclic oxyphosphoranes are complex organic structures. We now can produce this type hapten reasonably efficiently. We represent a sole source for these phosphoranes and we are capable of synthesis, protein conjugation and quantitation. The synthesis of stable protein conjugates of unsymmetrical monocyclic oxyphosphoranes have been carried out. These compounds have already proven useful in immunochemical work. Schlager, *et al.*⁹ used the porcine thyroglobulin (PTG) conjugate of **27** to obtain catalytic antibodies for the hydrolysis of 4-nitrophenyl (1,2,2-trimethyl)propylmethylphosphonate.



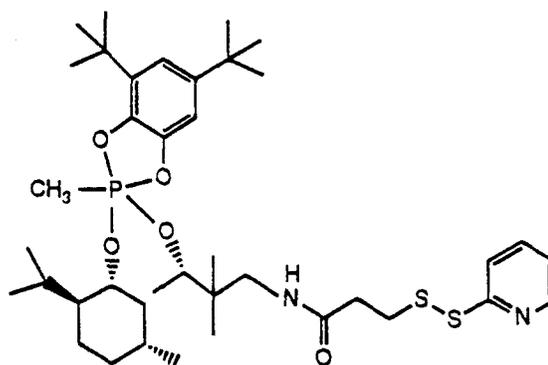
(27)

This is an important result and further validates the original concept that a pentacoordinate phosphorus atom can function as an appropriate transition-state analog and hapten for tetracoordinate phosphate and phosphonate ester hydrolysis.

We have found that the methyl group imparts greater stability compared with simple methoxyl. Therefore we plan to introduce the methyl group into candidate phosphoranes in order to achieve greater stability and survivability in the immunochemical procedure. In order to broaden and amplify this genetic engineering aspect of phosphate-active monoclonal antibodies, two important synthetic goals must be accomplished. The first is the synthesis of the phosphorane **28** and protein conjugates, and the second is the synthesis of the soman-like phosphorane **29** and its protein conjugates.



(28)



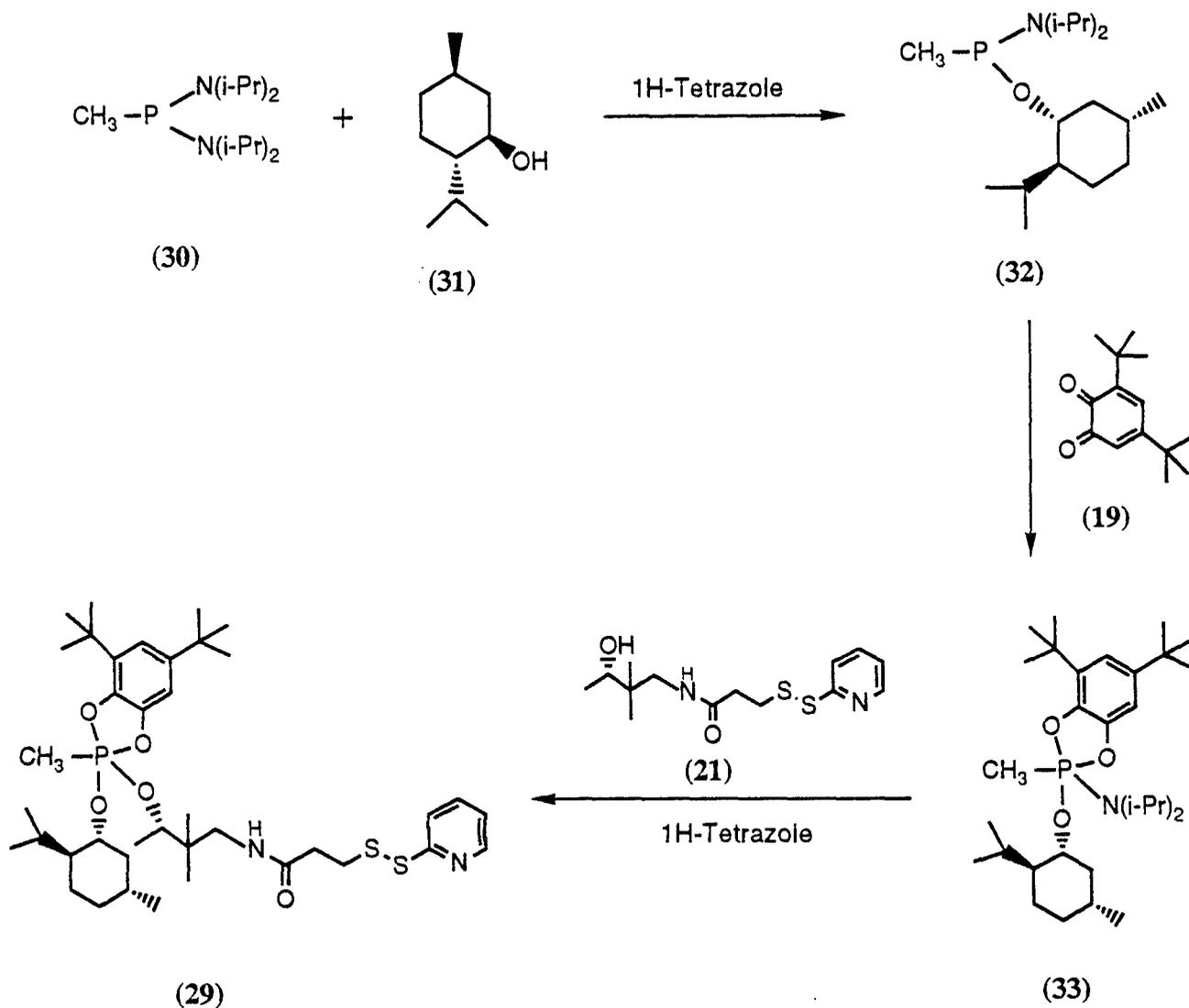
(29)

The rationale for phosphorane (**28**) is that the *l*(-)-menthyl group that imparts stability upon the phosphorane and substantially facilitates the immunochemical procedures of obtaining ultimate antibodies. This phosphorane focuses on the pentacoordinate phosphorus.

Phosphorane (**29**) incorporates two features, namely, the stability-enhancing effect of the *l*(-)-menthyl group as well as soman mimic we have used. Catalytic antibodies elicited in response to **29** will have specific activity towards soman.

Synthesis of 29:

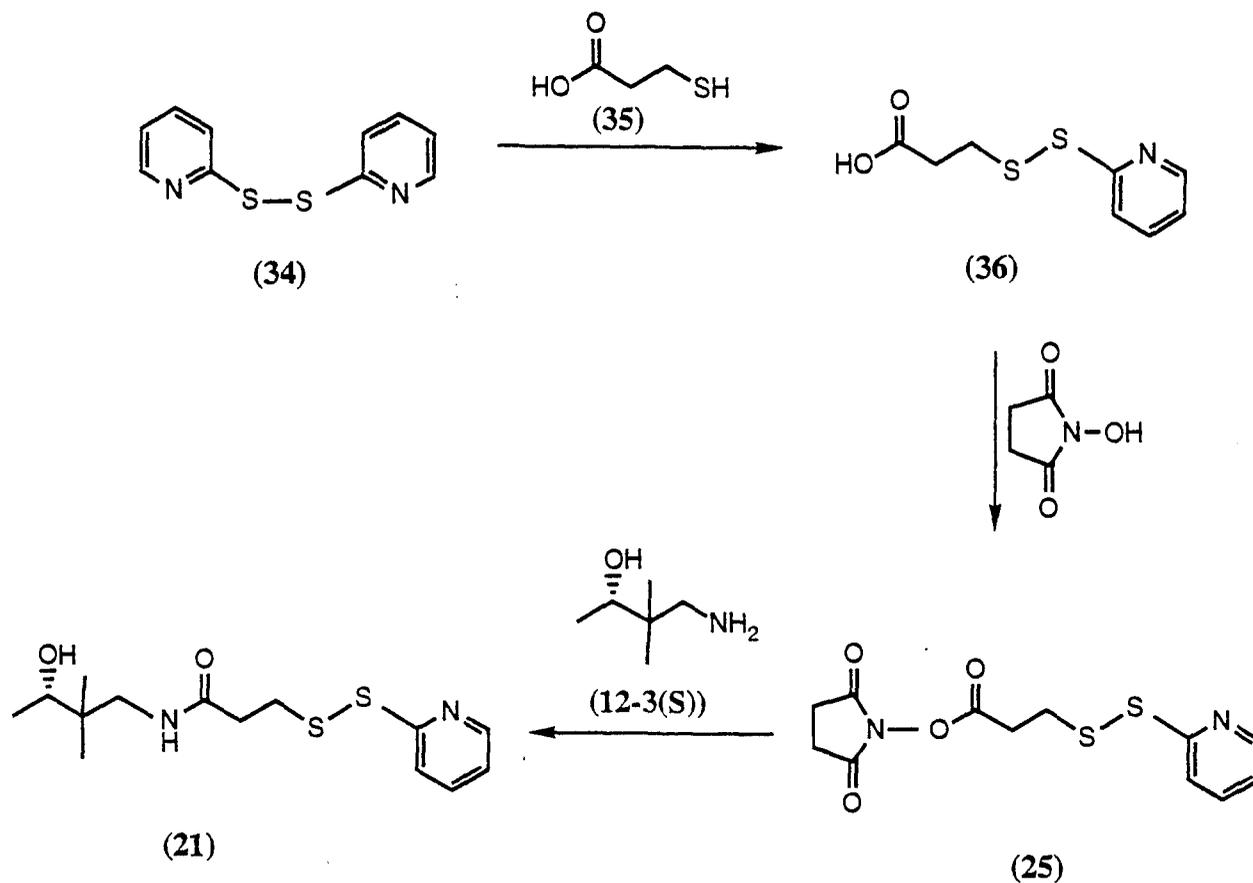
Synthesis of phosphorane **29** was accomplished as shown in Scheme 2. (N,N-Diisopropylamine)(*l*-(-)-menthoxy)methylphosphine (**32**) was prepared by displacement of one of the N,N-diisopropylamine groups from *bis*(N,N-diisopropylamine) (menthyl)phosphine (**30**) by *l*-(-)-menthol (**31**) in the presence of 1*H*-tetrazole. Treatment



Scheme 2

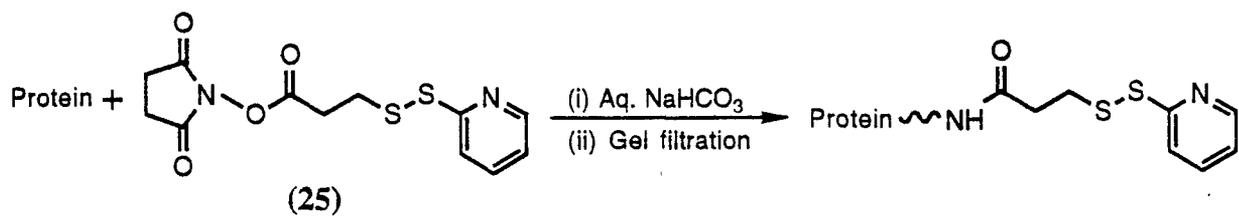
of phosphine **32** with 3,5-di-*tert*-butyl-1,2-benzoquinone (**19**) gave monocyclic oxyphosphorane (**33**) subsequent reaction with linker arm alcohol (**21**) produced the

desired oxyphosphorane hapten (**29**) in 39% overall yield. The linker alcohol **21** was synthesized in four steps as shown in Scheme 3.

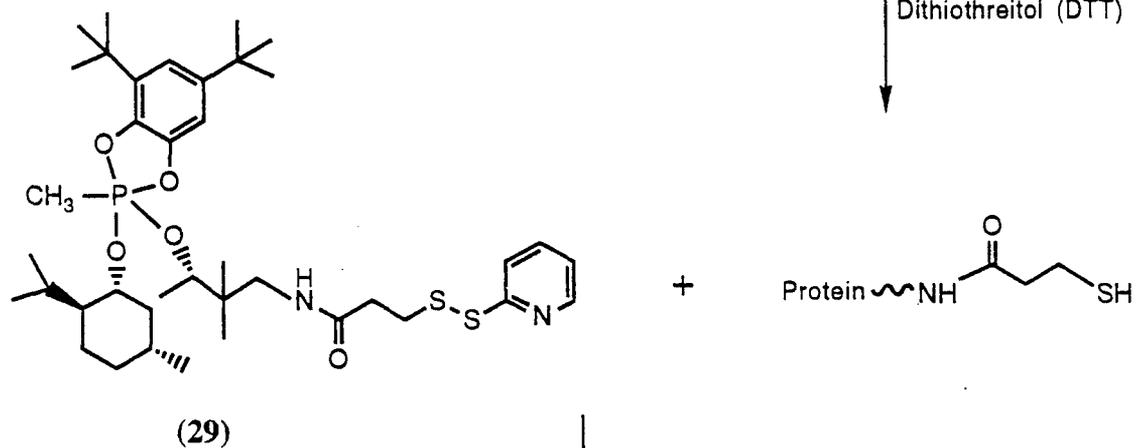


Scheme 3

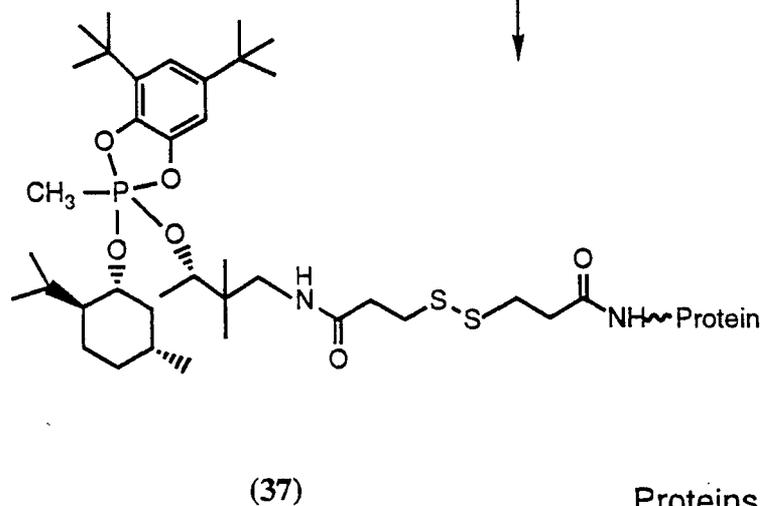
The hapten **29** was conjugated with proteins (BSA, KLH and PTG) as shown in Scheme 4 to produce **37-BSA**, **37-KLH** and **37-PTG**.



Dithiothreitol (DTT)



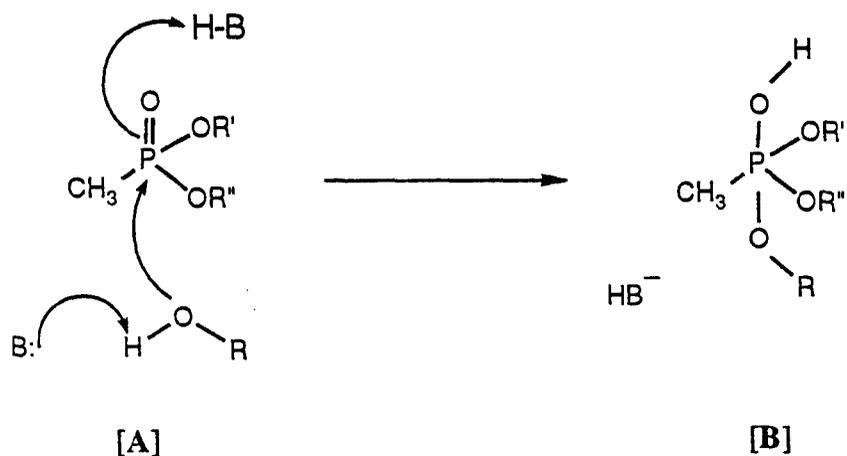
(i) THF
(ii) Gel filtration
(iii) Lyophilization



Proteins = $\left\{ \begin{array}{l} \text{BSA} \\ \text{KLH} \\ \text{PTG} \end{array} \right\}$

Scheme 4

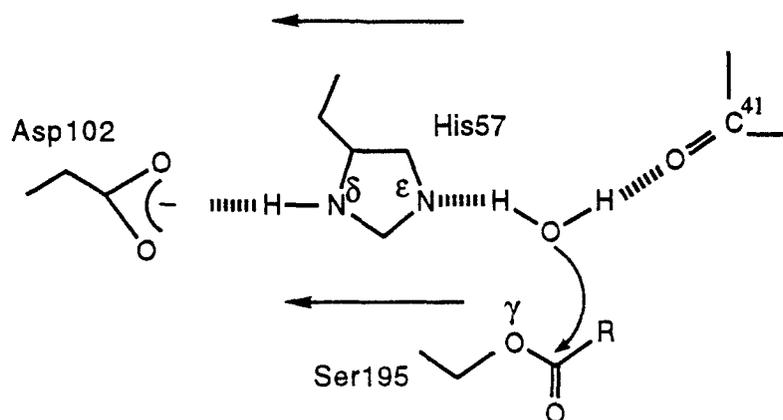
Extrapolating either from physical organic chemical mechanistic information or studies on the actual enzyme involved, one formulates a model for the interactions of various groups. In the case of hydrolysis of a phosphonate the following steps may intervene (**A**→**B**→**products**):



In the phosphorane approach we focused on the pentacoordinate transition-state analog.

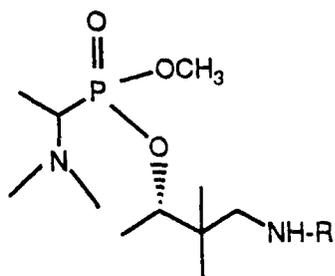
This was an induced fit strategy. In the case of bifunctional catalysis we focus upon inducing a binding pocket that provides general acid, general base or electrostatic stabilization. We propose a series of increasing complexity which should prove progressively more catalogenic.

At this point it should be emphasized that a good deal of empiricism underlies these experiments. We are designing a hapten for a catalytic process for which exact details are known. Certainly in the case of serine proteases, even though the structure of AChE is known, details of the role of catalytic groups at the active site are not known. For example, the details of the position of the water molecule in the serine protease is still a matter of debate. As recently as July 1993 this question has been vigorously discussed¹⁰. Perone *et al.*¹⁰ offer the following depiction for the role of the catalytic water molecule in deacylation:



According to these workers the His57 is shown in a shifted position relative to the ground state, which could be adopted during donation of the proton from N ϵ to the leaving group. In this position the hydrogen bond with Asp102 may be weakened or broken. The catalytic water occupies a position just vacated by the P1' residue of the leaving group. Large arrows indicate possible movement of His57 after acceptance of the proton from the water. After this movement, the hydrogen bond between N ϵ of His57 and Asp102 is reformed as the N ϵ proton to O γ of Ser195. For an alternative description see Singer *et al.* ¹¹

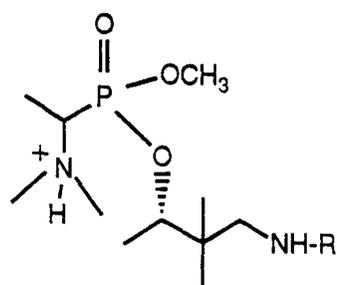
Catalytic antibodies have been successful in accomplishing the binding aspect of enzymatic catalysis. The next level is construction of a hapten which might induce the complementary groups present at the active site in an enzymatic process in a monoclonal antibody. We have succeeded in synthesizing such a hapten, named **39**, for nucleophilic-assisted phosphonate hydrolysis¹².



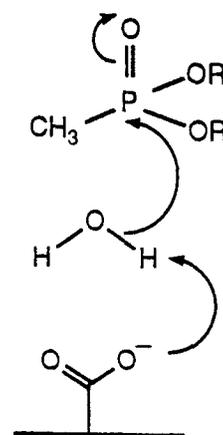
R = linker arm

(39)

The rationale for this compound is that the protonated secondary amino group would induce a complementary negatively charged group in the binding pocket of the catalytic antibody derived from this structure [A]. In the case that the negatively charged group were carboxylate, this could act as a catalytic component in the hydrolysis of a phosphonate [B].

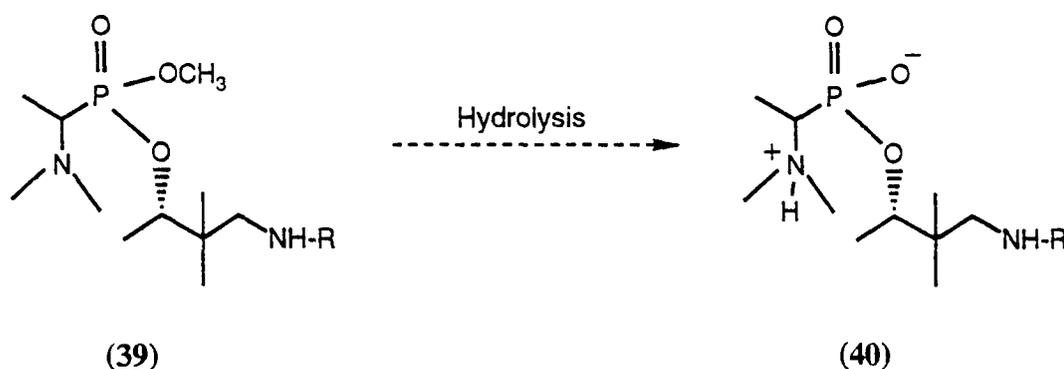


[A]



[B]

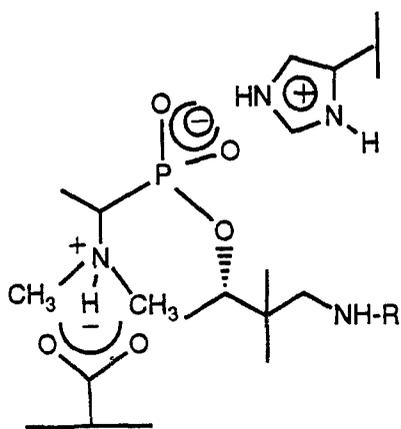
In this Phase II project, we took this hapten a step further. Hydrolysis of the methylphosphonate group in **39** expected to give a zwitterionic compound (**40**).



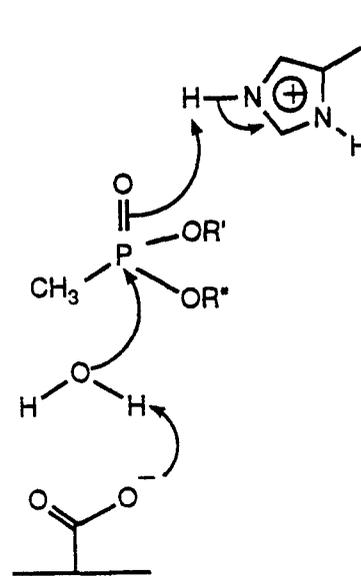
(39)

(40)

This hapten has the potential to induce both a negative and positive center in the vicinity of the binding site [C]. For example, a histidinium cationic group could be induced complementary to the phosphonate anion. The catalytic system derived [D] is now bifunctional.

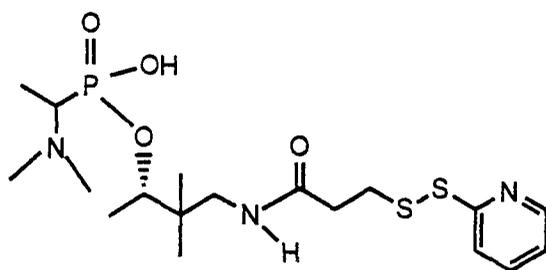


[C]

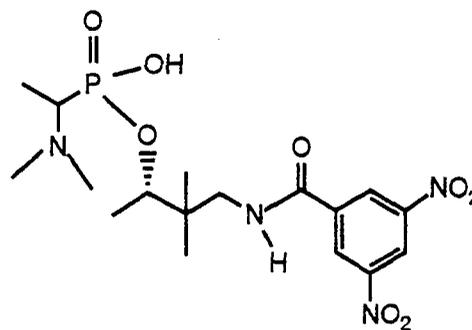


[D]

Synthesis of the diastereomeric mixtures **41** and **42** is in progress, these haptens will be conjugated to BSA, KLH and PTG.

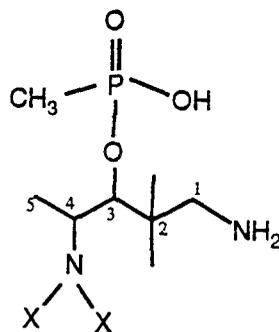


(41)



(42)

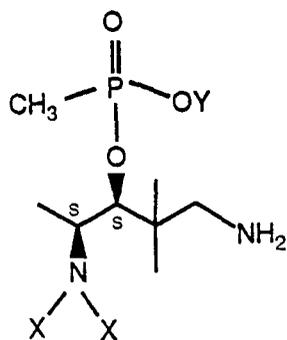
An alternative substrate which likewise incorporates zwitterionic groups at stereogenic center as embodied in (43) ($X = \text{CH}_3$ or $\text{C}_6\text{H}_5\text{CH}_2$).



$X = \text{CH}_3$ or $\text{C}_6\text{H}_5\text{CH}_2$

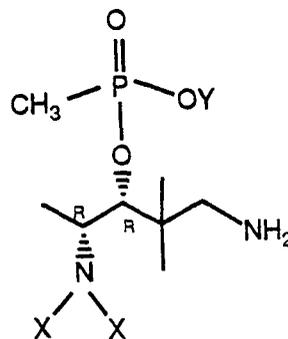
(43)

The structure **43** has two chiral centers at C-3 and C-4, and therefore four separate enantiomers **43-3(S),4(S)**, **43-3(R),(R)**, **43-3(S),4(R)** and **43-3(R),4(S)** (X = C₆H₅CH₂, Y = CH₃) are expected as shown below:



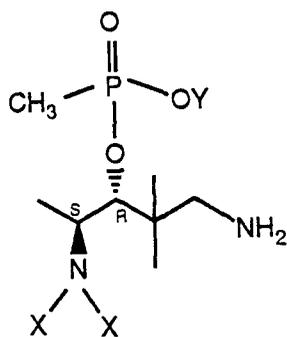
X = C₆H₅CH₂, Y = CH₃

(43)-3(S),4(S)



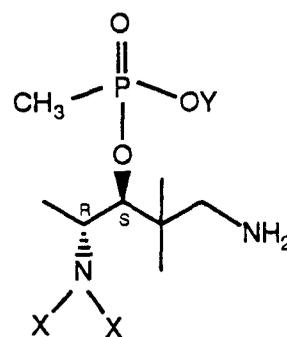
X = C₆H₅CH₂, Y = CH₃

(43)-3(R),4(R)



X = C₆H₅CH₂, Y = CH₃

(43)-3(R),4(S)

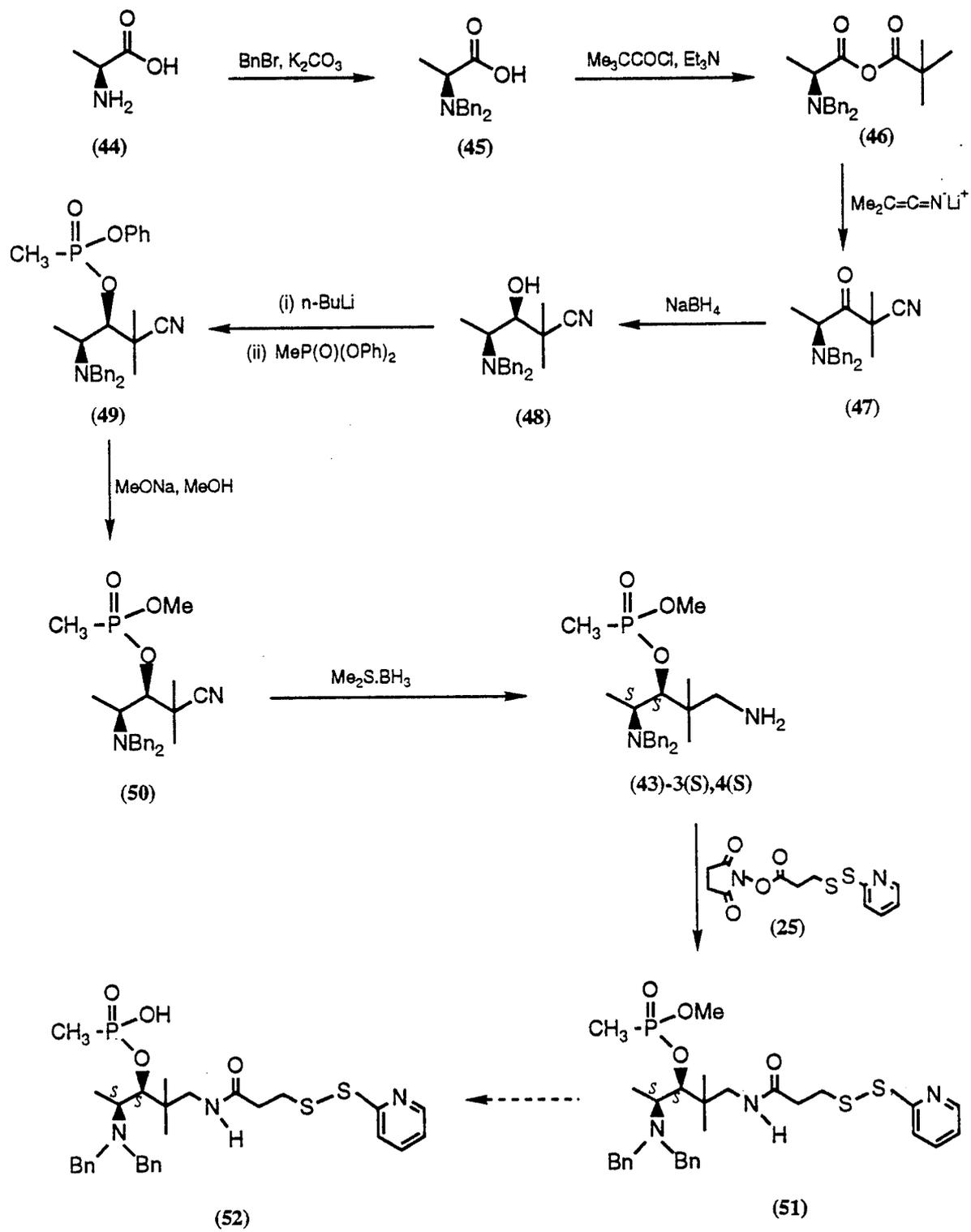


X = C₆H₅CH₂, Y = CH₃

(43)-3(S),4(R)

Synthesis of 51 and 60:

We have synthesized two enantiomers of methyl aminophosphonates namely **43-3(S),4(S)** and **43-3(R),4(R)** starting from 2-(S)-alanine (**44**) and 2-(R)-alanine (**53**) respectively as shown in Scheme 5 and Scheme 6. N,N-Dibenylation of (S)-alanine (**44**)

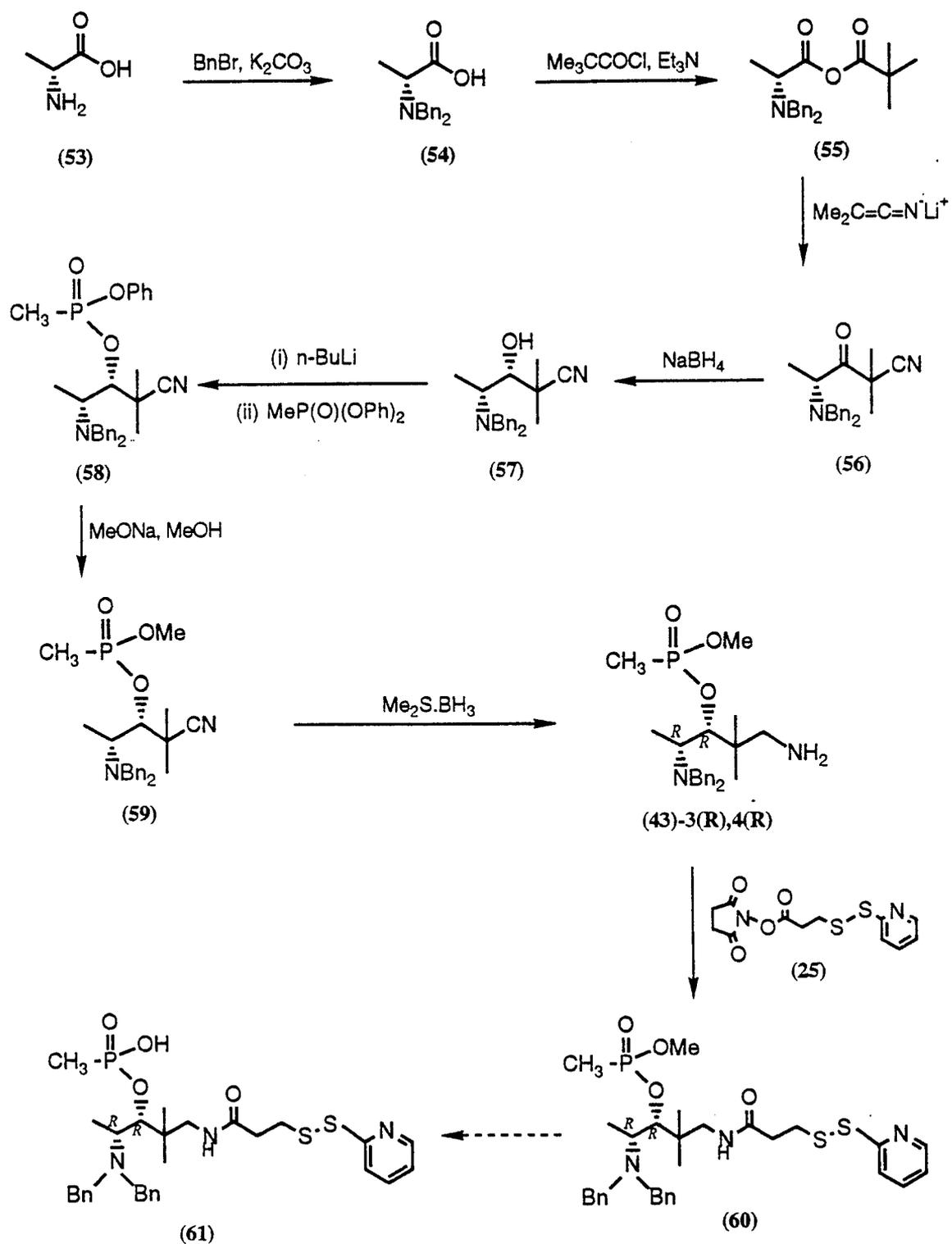


Scheme 5

with benzyl bromide in the presence of potassium carbonate gave N,N-dibenzyl-(S)-alanine (**45**) which was converted to mixed anhydride (**46**) using trimethylacetyl chloride in tetrahydrofuran in 80% yield. The mixed anhydride **46** was treated with lithium salt of isobutyronitrile to produce 4-(S)-(N,N-dibenzylamino)-2,2-dimethyl-3-oxopentanenitrile (**47**) in 92% yield. Low temperature stereoselective reduction of **47** with sodium borohydride in tetrahydrofuran gave exclusively 4-(S)-(N,N-dibenzylamino)-2,2-dimethyl-3-(S)-hydroxypentanenitrile (**48**) in 81% yield. The stereochemistry of this reaction was controlled by bulky groups at nitrogen atom, that is, N,N-dibenzyl groups. This gives *trans* addition product, no chelation control, resulting only *threo* isomer¹³. Phosphonylation of **48** with diphenyl methylphosphonate (prepared from methyl dichlorophosphonate and phenol in the presence of triethylamine) in the presence of n-butyllithium in tetrahydrofuran gave phenyl 4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentanenitrile (**49**). Transesterification of **49** with methanol in the presence of sodium methoxide gave methyl 4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentanenitrile (**50**). Reduction of nitrile **50** with borane-dimethylsulfide complex afforded the corresponding methylamine derivative (**43-3(S),4(S)**) which was coupled with N-succinimidyl-3-(2'-pyridyldithio)propionate (**25**) in the presence of triethylamine produced methyl [2-(2'-pyridyldithio)propionylaminomethyl-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane)]-methylphosphonate (**51**) in 27% yield based on nitrile **48**.

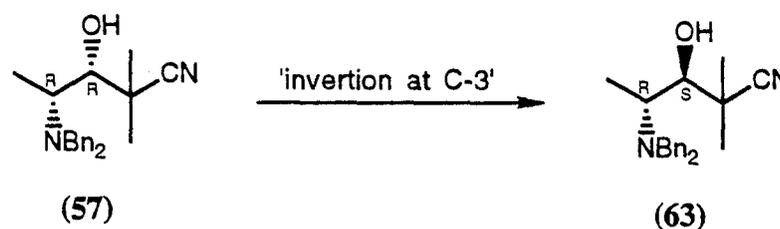
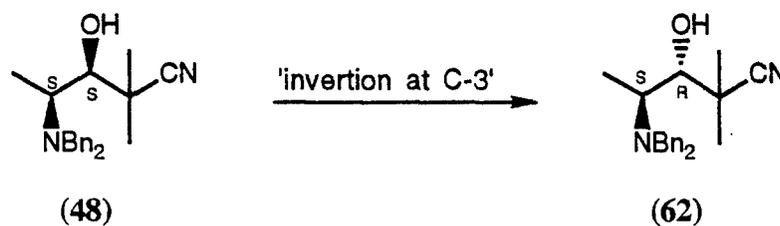
Similarly, we have also synthesized the enantiomer of **51**, that is, methyl [2-(2'-pyridyldithio)propionylaminomethyl-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)]-methylphosphonate (**60**) in the same pathway starting from 2-(R)-alanine (**53**) as shown in Scheme 6.

Hydrolysis of methyl phosphonates **51** and **60** to phosphonate derivatives (**52**) and (**61**) respectively are in progress. Each of these pure enantiomers will be conjugated to BSA, KLH and PTG. These stereoisomers will provide an ultimate test of the stereoselection quality of catalytic monoclonals and indicate the influence of the attacked ligand on activity as opposed to the tetrahedral phosphorus.



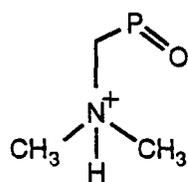
Scheme 6

The *threo* stereoisomers **48-3(S),4(S)** and **57-3(R),4(R)** can be inverted at the secondary alcohol to yield *erythro* stereoisomers (**62-3(R),4(S)**) and (**63-3(S),4(R)**) respectively as shown below:

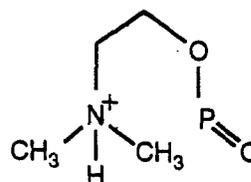


Attempted inversion at C-3 alcohol by a well-established method (i, Tf_2O ; ii, KNO_2/DMF) used for a neopentyl type secondary alcohol system¹⁴ has not been successful in this type of aminoalcohol. Further investigation on this type of reaction or to explore a new synthetic pathway for other *erythro* stereoisomers is in progress.

Finally, the distance between the quaternary nitrogen center in **40** is shorter compared to **43**:

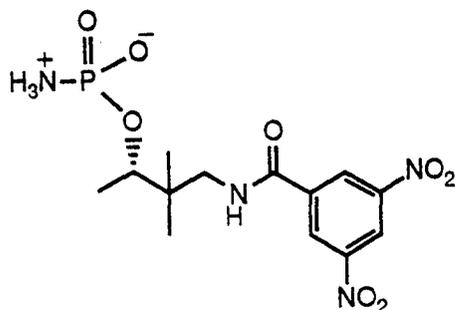


as in **40**

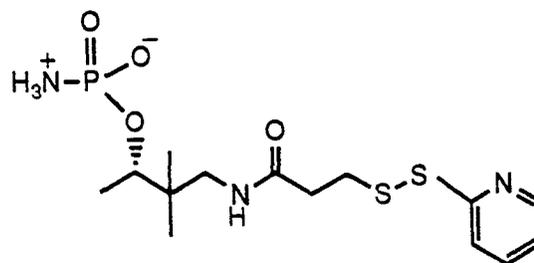


as in **43**

A structurally simpler zwitterionic phosphonate (aminophosphonate or phosphoramidate), in which ammonium center and phosphorus atom are adjacent, may act in the same way to induce complementary general acid and base groups as in examples - (64) and (65):



(64)

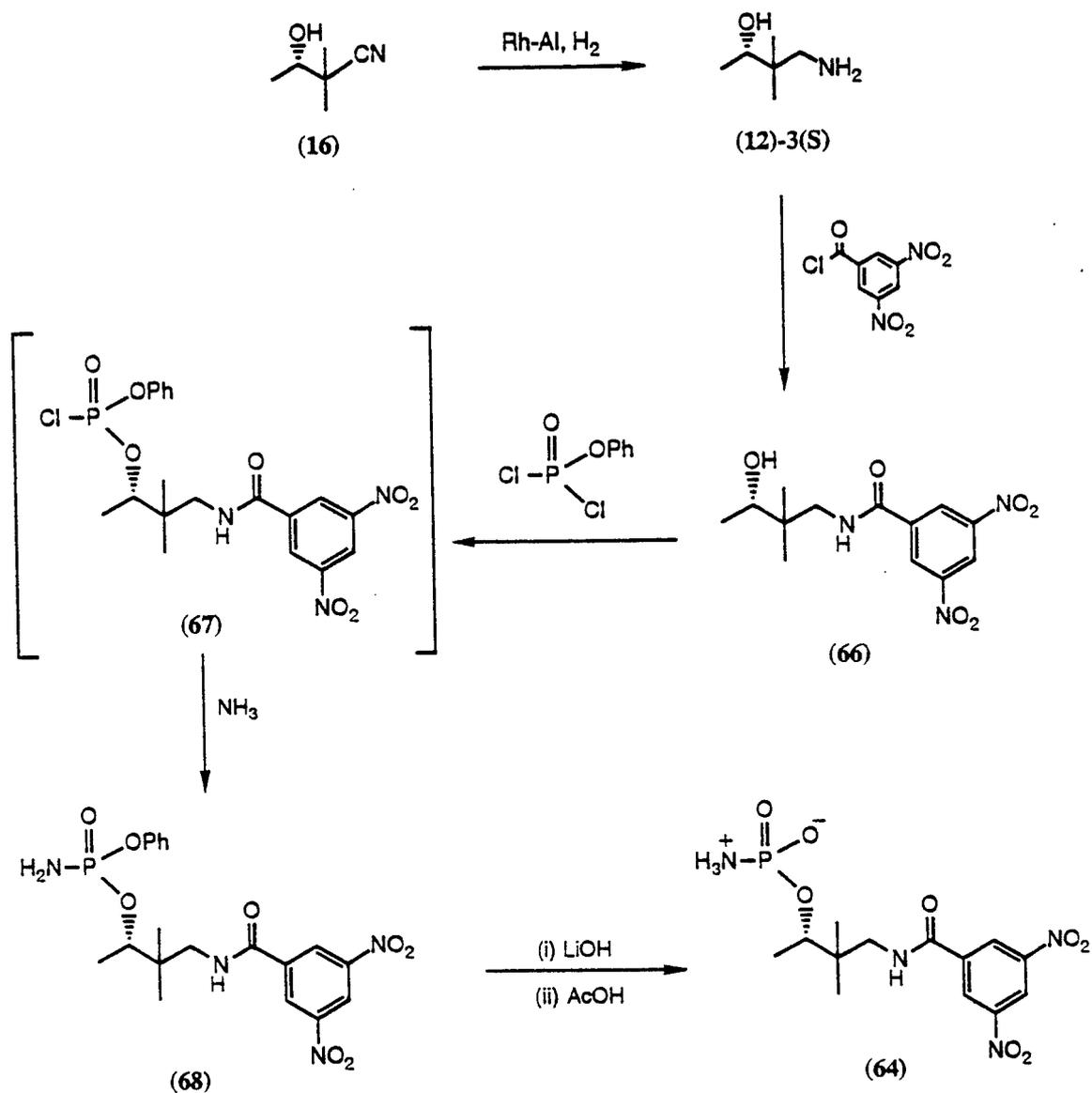


(65)

In the above structures the positive and negative centers are obviously closer than 40 and 43 and this hapten will assist in elucidating the distance factor.

Synthesis of 64:

We have recently synthesized one of the aminophosphonates as a model (64) as shown in Scheme 7. Catalytic hydrogenation of 2-methyl-2-cyano-3-(S)-hydroxybutane (16) in the presence of 5% rhodium on alumina at atmospheric pressure gave corresponding 2-aminomethyl-2-methyl-3-(S)-hydroxybutane (12-3(S)) in 94% yield. The coupling of this aminoalcohol 12 with 3,5-dinitrobenzoyl chloride in the triethylamine gave 2-(3',5'-dinitrobenzoyl)aminomethyl-2-methyl-3(S)-hydroxybutane (66) in 96% yield. Phosphonylation of hydroxyamide 66 with phenyldichlorophosphite in the presence of quinoline gave phosphonylated product (67) which was, without isolation, treated with ammonia gas produced phenyl [2-(3',5'-dinitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane]-aminophosphonate (68) in 51% yield. Hydrolysis of 68 with 1N lithium hydroxide followed by flash chromatography in silica gel using chloroform:methanol:water system afforded [2-(3',5'-dinitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane]-aminophosphonate (64) as a light yellow solid which was characterized by ^1H NMR spectrum and mass spectra (CI and FAB). The synthesis of other aminophosphonate hapten (65) is in progress and will be reported.

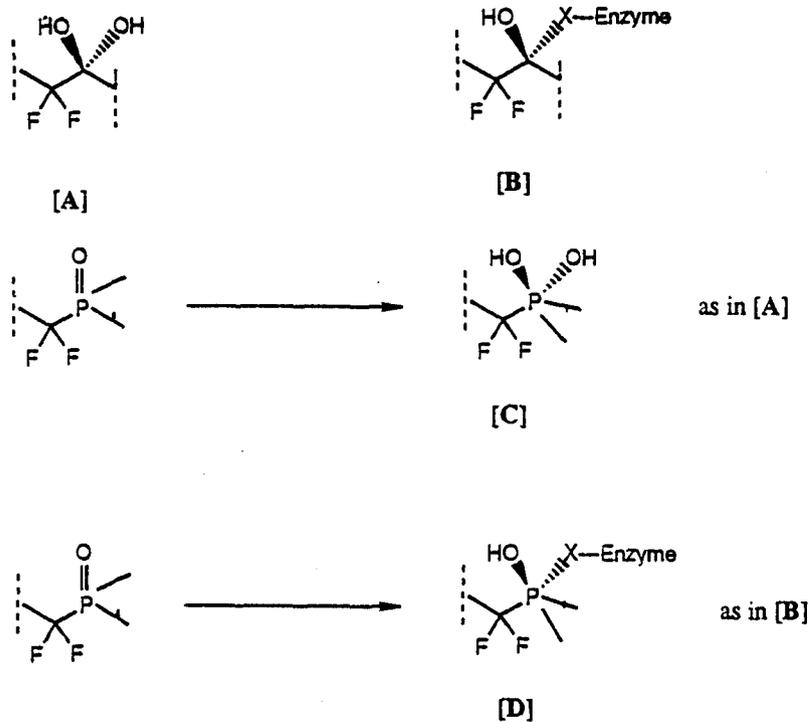


Scheme 7

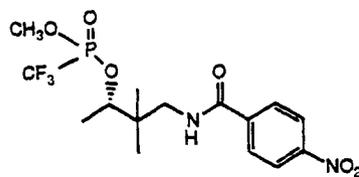
Fluorinated Haptens.

Difluoromethylene ketones are predominantly hydrated in aqueous solution. This property accounts for the fact that substrate analogs incorporating the difluoro ketone group act as hydrolytic enzyme inhibitors¹⁵. Linderman *et al.*¹⁶ using ¹⁹FNMR showed that the fluoroketone inhibitor binds to the active site of AChE in a tetrahedral form.

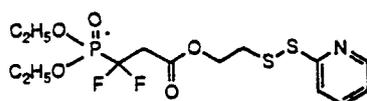
The difluoromethylene ketone group may act as a transition-state analog in the hydrated form [A] or a covalently bound hemiketal [B] if one extrapolates this concept to the phosphonate system the analogous structures are [C] and [D]¹⁷.



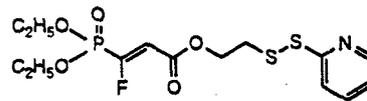
In order to elucidate the above concept, we have synthesized some typical fluorinated haptens which have $P(O)CF_3$ and $P(O)(CF_2)R$ systems in the molecules. These fluorinated haptens are:



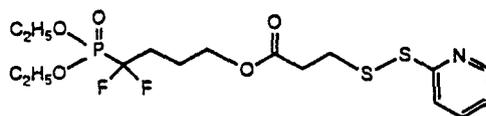
(69)



(70)



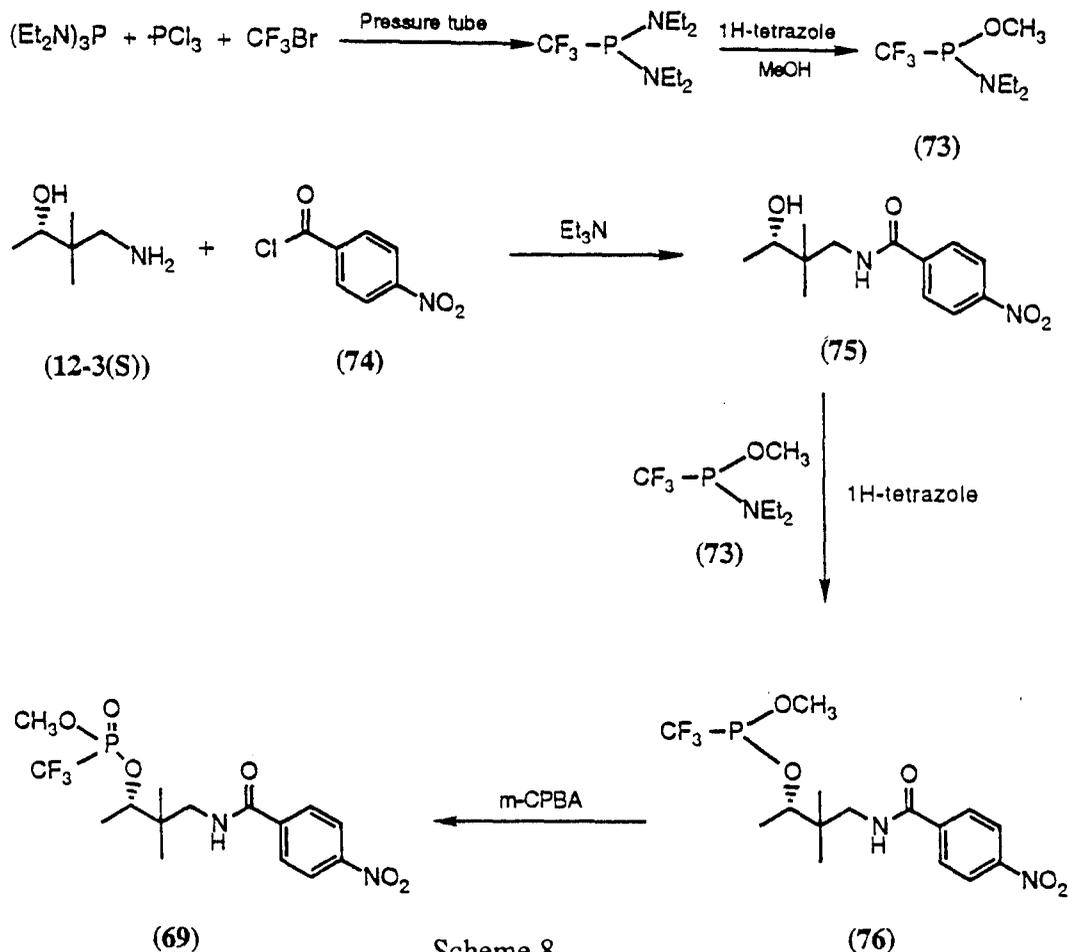
(71)



(72)

Synthesis of 69:

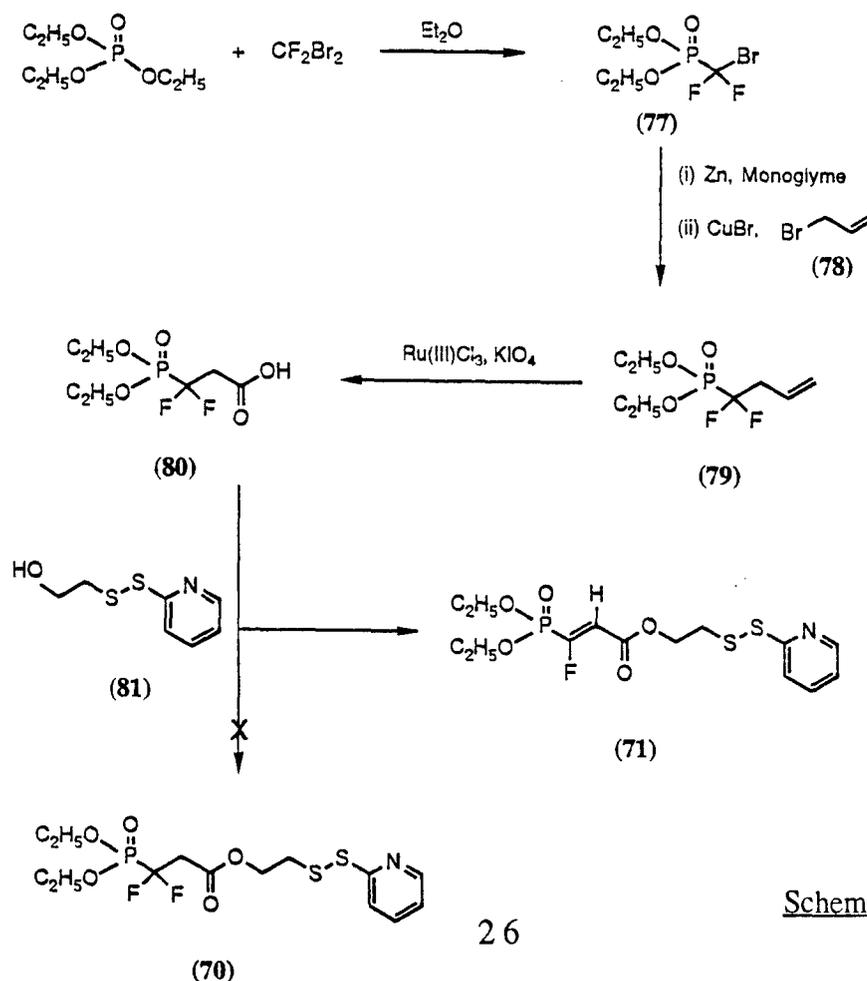
The fluorinated hapten (**69**) was synthesized as shown in Scheme 8. The coupling of 2-aminomethyl-3-(S)-hydroxybutane (**12-3(S)**) with p-nitrobenzoyl chloride (**74**) in the presence of triethylamine gave 2-(p-nitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane (**75**) in 27% yield. Phosphinylation of secondary alcohol **75** with (diethylamino) (methoxy)trifluoromethylphosphine (**73**) (prepared from bis(diethylamino)trifluoromethyl phosphine which in turn was obtained by reaction of hexaethylphosphorus triamide, phosphorus trichloride and bromotrifluoromethane in a pressure tube) in the presence of 1H-tetrazole produced 2-(p-nitrobenzoyl)aminomethyl-2-methyl-3(S)-(methoxy)(trifluoromethyl)hydroxybutane phosphine (**76**) in 60% yield. The final compound, 2-(p-nitrobenzoyl)aminomethyl-2-methyl-3(S)-(methoxy)(trifluoromethyl)hydroxybutane phosphonate **69** was obtained by mild oxidation of phosphine **75** with *meta*-chloroperbenzoic acid in 60% yield. The trifluoronated hapten **69** was fully characterized by ¹H, ¹³C, ¹⁹F and ³¹P NMR and mass spectra.



Scheme 8

Synthesis of 70:

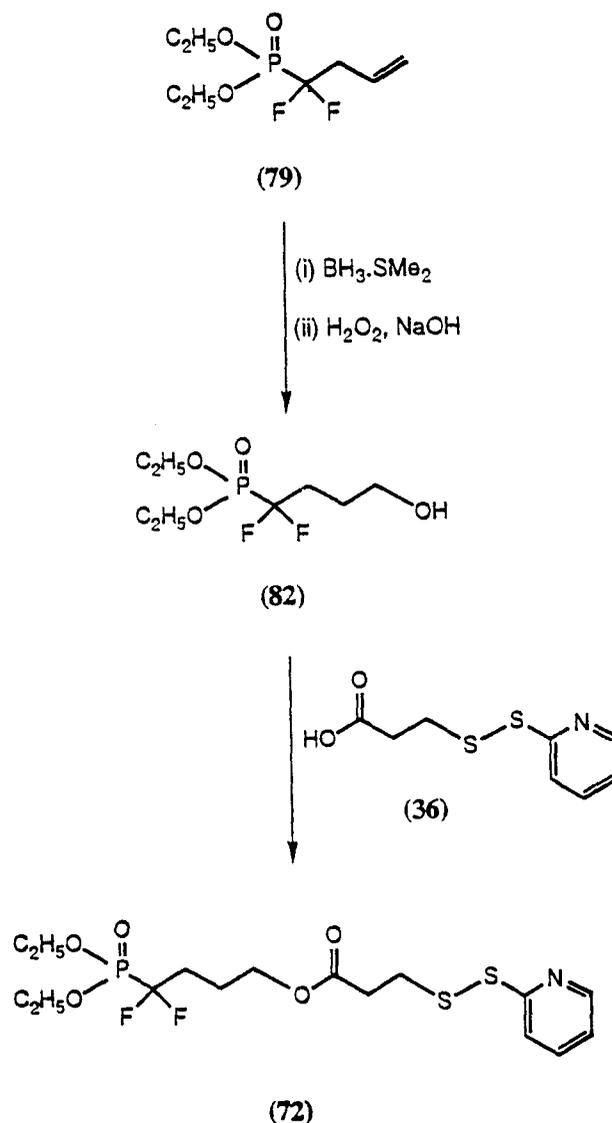
Synthesis of (2'-pyridyldithio)ethyl diethyl-3,3-difluoro-3-phosphonopropionic acid ester (70) was attempted as shown in Scheme 9. Reformatsky reaction of diethyl-bromodifluoromethylphosphonate (77)¹⁸ with 3-bromopropene (78) in the presence of zinc powder along with a catalytic amount of copper (I) bromide gave diethyl (1,1-difluoro-3-butenyl)phosphonate (79)¹⁹ in 58% yield. Treatment of 79 with ruthenium (III) chloride and potassium periodate produced diethyl 3,3-difluoro-3-phosphonopropionic acid (80)²⁰ in 58% yield. Attempted coupling of acid 80 with 2-pyridyldithioethanol (81) in the presence of dicyclohexyldicarbodiimide (DCC) or 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and a catalytic amount of additive, 1-hydroxybenzotriazole (HOBT) did not produce a desired compound, (2'-pyridyldithio)ethyl diethyl-3,3-difluoro-3-phosphonopropionic acid ester (70), instead a hydrogen fluoride eliminated product, (2'-pyridyldithio)ethyl diethyl-3-fluoro-3-phosphono-2-propenic acid ester (71) was obtained. Similar result was obtained when acid chloride of 80 was attempted to be coupled with 81 in the presence of triethylamine. An eliminated product, 71 was fully confirmed by IR, ¹H, ³¹P NMR and mass spectra. Attempted synthesis of 70 is in progress.



Scheme 9

Synthesis of 72:

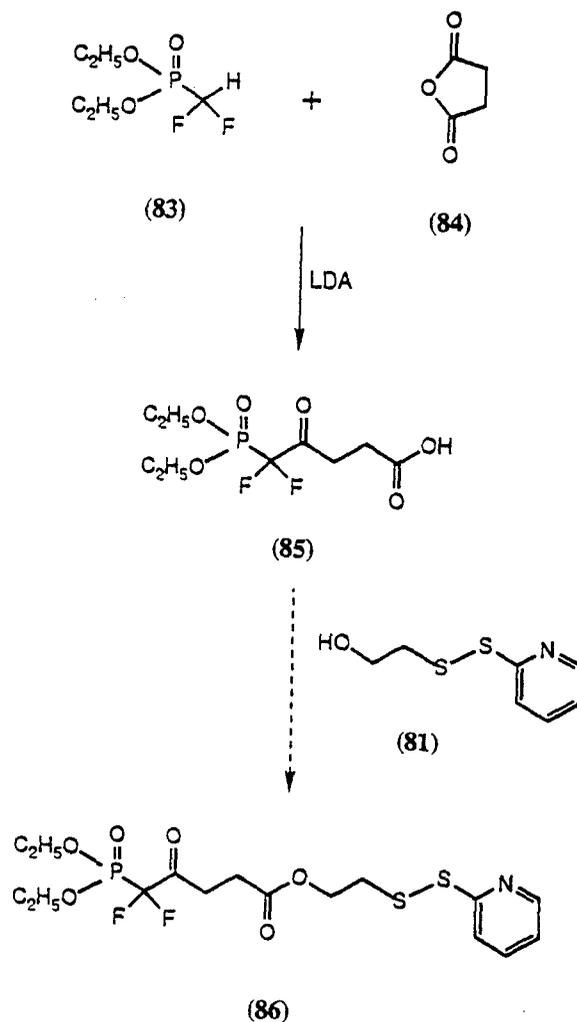
We have also designed an alternative fluorinated hapten compound (72) which was synthesized as shown in Scheme 10.



Scheme 10

Hydroboration²¹ of diethyl (1,1-difluoro-3-butenyl)phosphonate (79) with borane-dimethylsulfide complex followed by oxidation with hydrogen peroxide in the presence of sodium hydroxide afforded diethyl (4,4-difluoro-1-hydroxybutyl)phosphonate (82) in 68% yield. The coupling of alcohol 82 with 3-(2'-pyridyldithio)propionic acid (36) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBT) produced an expected product, diethyl-4,4-difluoro-4-phosphonobutyl (2'-pyridyldithio)propionic acid ester (72) in 34% yield. This hapten was fully characterized by IR, ^1H , ^{31}P and mass spectra.

Finally, the synthesis of (2'-pyridyldithio)ethyl diethyl-4,4-difluoro-3-oxo-phosphonobutyric acid ester (**86**) has been in progress as shown in Scheme 11. We have already synthesized diethyl-4,4-difluoro-3-oxo-phosphonobutyric acid (**85**) in 52% yield by reacting the lithium derivative of diethyl (α,α -difluoromethyl)phosphonate (**83**) with succinic anhydride (**84**) in tetrahydrofuran. The coupling of **85** with (2'-pyridyldithio)ethanol (**81**) is expected to give **87**. The result will be reported.



Scheme 11

Conjugated of diethyl-4,4-difluoro-4-phosphonobutyl (2'-pyridyldithio)propionic acid ester (**72**) and (2'-pyridyldithio)ethyl diethyl-4,4-difluoro-3-oxo-phosphonobutyric acid ester (**86**) with BSA, KLH and PTG will be carried out and these haptens will be submitted for the generation of catalytic monoclonal antibodies.

CONCLUSIONS

A catalytic antibody towards organophosphates would not only have military application but would also be tremendously useful in protecting other workers who are in contact with these compounds. This includes the agricultural sector, those engaged in industrial production of insecticides, and the general public who might be accidentally exposed to toxic organophosphorus compounds. Such a catalytic monoclonal antibody would have commercial value.

Furthermore, the technology developed in this Phase II project could be extended to biosensors for organophosphates, fluorescent-based diagnostic devices for organophosphates and also in construction of restriction abzymes for nucleic acid cleavage.

EXPERIMENTAL

Melting points were determined using a Thomas-Hoover Unimelt capillary tube melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on Bruker AM-400 at 400MHz and Varian VXR-300 at 300MHz spectrometers. ^{13}C NMR spectra were recorded on a Bruker AM-400 at 100MHz and Varian VXR-300 at 75MHz spectrometers. Tetramethylsilane (TMS) was used as an internal standard for ^1H NMR. ^{31}P NMR spectra were recorded on Varian VXR-300 at 121.4MHz and Bruker WP200SY at 81.0MHz spectrometers, and the chemical shifts downfield from the reference (85% phosphoric acid, H_3PO_4) are indicated as positive. ^{19}F NMR spectra were recorded on Bruker WP-200-SY spectrometer at 188.3MHz with trichlorofluoromethane (CFCl_3) as an external reference and are indicated as negative upfield from the reference. All chemical shifts were reported in part per million (ppm), and the coupling constant (J) values were calculated in hertz (Hz) based on the chemical shifts which were given by the spectrometer, hence a slight differences in the coupling constants may be noticed. The deuteriochloroform (CDCl_3) was used as solvent for all NMR experiments with residual chloroform as an internal standard for ^{13}C NMR. IR spectra were recorded on IBM IR/30-32 and ATI Mattson ICON FTIR spectrometers. Mass spectra were obtained by positive chemical ionization (CI) or by fast atomic bombardment (FAB) technique with a Finnigan MAT-90 mass spectrometer. Unless otherwise noted, all reactions were carried out in oven-dried glasswares. Reagent and solvent transfers were made with oven-dried syringes and needles. Dichloromethane was distilled from calcium hydride. Tetrahydrofuran (THF) was freshly distilled from sodium metal/benzophenone prior to use. All reagents were purchased from Aldrich Chemical Co. and used as received. All solvents for chromatography were obtained commercially and used as received. Reactions were monitored by analytical thin layer chromatographic (tlc) methods with the use of E. Merck silica gel, 230-400 mesh (60A) glass plates (0.25mm) and all spots on tlc plates were visualized either by ultra-violet (UV) light or detected by dipping the plate into 5% solution of phosphomolybdic acid in ethanol and heat. The pure products were isolated by short-path distillation under an argon atmosphere for low boiling compounds or vacuum distillation for high boiling compounds, or by flash column chromatography with the use of E. Merck grade silica gel, 230-400 mesh (60A). Basic alumina was purchased from Aldrich (No. 199443, basic Brokmann I). Bovine serum albumin (BSA), keyhole limpets hemocyanin (KLH) and porcine thyroglobulin (PTG) were purchased from Sigma Chemical Co. Gel column (Sephadex G-25, PD-10) was purchased from Pharmacia.

Bis(N,N-diisopropylamine)(methyl)phosphine (30): To a stirred suspension of *bis*(N,N-diisopropylamino)chlorophosphine (35.5gm, 130mmol) in dry ether (600ml) was added a solution of methyllithium (1.4M in ether, 100ml, 140mmol) at -78°C under dry argon. After complete addition, the reaction mixture was stirred at room temperature for 16hrs. The mixture was filtered, and the white residue of lithium chloride was washed with dry ether. The combined filtrate and washings were concentrated *in vacuo* to give a crude product as a pale yellow oil. Distillation of the crude oil under high vacuum afforded a pure colorless oil (28.4gm, 87%), bp. 70-74°C at 0.07mmHg; ¹HNMR (400MHz, CDCl₃) δ: 1.09 & 1.18 (24H, two d, J = 6.65Hz & 6.72Hz, 4 x (CH₃)₂C), 1.21 (3H, d, J_{H-P} = 10.2Hz, CH₃P), 3.40 (4H, m, 4 x CH(CH₃)₂), ³¹PNMR (121MHz, CDCl₃) δ: 40.8; MS(Cl): 246 (M⁺+1, 100%), 231 (231%) and 146 (87%).

(N,N-Diisopropylamino)(l-(-)-menthoxy)methylphosphine (32): To a solution of *bis*(N,N-diisopropylamine)(methyl)phosphine (30) (1.71gm, 6.904mmol) in dry dichloromethane (50ml) were added *l*-(-)-menthol (31) (1.1gm, 7.04mmol) and 1*H*-tetrazole (59.1mg, 0.345mmol) successively at 0°C (ice-bath). The reaction mixture was stirred at 0°C for 15min, then at room temperature for 40hrs. The reaction mixture was concentrated *in vacuo* to give a colorless oil with a small amount of solid (tetrazole salt). Dry hexane (50ml) was added to the residue and filtered. The filtrate was concentrated *in vacuo* to give oil free from salt. The crude oil was distilled under high vacuum to afford a pure colorless oil (1.89gm, 91%); bp. 73-90°C at 0.005mmHg; ¹HNMR (400MHz, CDCl₃) δ: 0.74 & 0.81 (6H, two d, J = 6.92Hz & 6.93Hz, CH₃CH), 0.89 (6H, m, (CH₃)₂CH) 0.8-1.0 (4H, m, (CH₂)₂), 1.10 (6H, d, J = 6.73Hz, (CH₃)₂CH₂N), 1.18 & 1.20 (6H, two d, J = 6.64Hz, (CH₃)₂CH₂N), 1.21 & 1.22 (3H, two d, J_{H-P} = 6.95Hz & 6.51Hz, CH₃P), 1.4 (1H, m), 1.6 (2H, m, CH₂), 2.0-2.3 (2H, m), 3.39 & 3.51 (1H, two m, OCH), 3.53 (2H, m, N[CH(CH₃)₂]₂); ³¹PNMR (121MHz, CDCl₃) δ: +112.5 (58%) and +121.7 (42%) (diastereomeric mixture).

2-(2'-Pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-hydroxybutane (21): (a) Preparation of 3-(2-pyridyldithio)propionic acid²² (36) To a solution of 2,2'-dipyridyl disulfide (34) (7.8gm, 35.6mmole) in absolute ethanol (100ml) and glacial acetic acid (1.6ml, 17.8mmole) was added a solution of 3-mercaptopropionic acid (35) (1.6ml, 17.8mmole) in absolute ethanol (25ml) dropwise at room temperature. The reaction mixture was stirred at room temperature overnight. After 18hrs, the reaction mixture was concentrated *in vacuo* and the last traces of acetic acid were removed by azeotropeing with

carbon tetrachloride to get a residual oil. The residual oil was dissolved in saturated sodium bicarbonate solution (30ml). The aqueous bicarbonate solution was washed with dichloromethane (3 x 40ml), and then carefully acidified with 3NHCl until no more oil separated, and extracted the oil with dichloromethane (3 x 50ml), dried over magnesium sulfate, filtered and concentrated *in vacuo* to give oil. The crude product was column chromatographed on basic alumina using ethanol:dichloromethane (2:3) to remove unwanted impurities, followed by ethanol:dichloromethane:acetic acid (2:3:5) to get a pure compound as pale yellow needles (2.3gm, 56%); single spot in tlc on silica gel, ethyl acetate:hexane = 5:1; mp 57-59°C; IR (KBr): 2976 (br, OH), 1713 (COOH), 1606, 1535, 1512, 1471, 1419 (S-S), 765 and 717 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 2.69 (2H, t, J = 6.91Hz, CH_2COOH), 2.94 (2H, t, J = 6.91Hz, CH_2S), 7.06 (1H, m, 5'-H-pyridine), 7.57 - 7.67 (2H, m, 3'-H & 4'-H-pyridine), 8.38 (1H, m, 6'-H-pyridine); ^{13}C NMR (100MHz, CDCl_3) δ : 33.76, 33.99, 120.51, 121.46, 138.22, 149.21, 159.75 and 175.28.

(b) Preparation of N-succinimidyl-3-(2-pyridyldithio)propionate (25): To a solution of 3-(2-pyridyldithio)propionic acid (36) (0.5gm, 2.3mmole) in dry dichloromethane (10ml) was added thionyl chloride (186 μl , 2.6mmole) dropwise at 0°C (ice-bath) under argon, and stirred at 0°C for 30min. A small amount of white precipitate was formed. The reaction mixture was stirred at room temperature for 30min and the solution became clear; then to the resulting mixture was added a solution of N-hydroxysuccinimide (267mg, 2.3mole) and triethylamine (405 μl , 2.76mmole) in dry dichloromethane (10ml) at 0°C. The mixture was stirred at 0°C for 30min and at room temperature for additional 2hrs. The reaction mixture was diluted and extracted with dichloromethane (20ml), followed by washing with saturated sodium bicarbonate (2 x 20ml), saturated sodium chloride (2 x 20ml), dried over sodium sulfate, filtered and concentrated *in vacuo* to give a pale yellow gummy solid residue. The crude product was recrystallized from ethyl acetate:hexane (1:5) to afford colorless crystals (0.614gm, 47%), single spot on silica gel tlc, ethyl acetate:hexane (5:1); mp 78-80°C (mp 79.5-80.5°C); IR (KBr): 1786, 1740 (C=O), 1574, 1446, 1417, 1246, 1205, 1116, 1078, 763 and 648 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 2.84 (4H, s, succinimidyl-H), 3.06 - 3.15 (4H, m, SCH_2CH_2), 7.10 - 7.14 (1H, m, 5'-H-pyridine), 7.64 - 7.67 (2H, m, 3'-H & 4'-H-pyridine), 8.49 - 8.50 (1H, m, 6'-H-pyridine); ^{13}C NMR (100MHz, CDCl_3) δ : 25.82, 31.25, 33.13, 120.26, 121.29, 137.47, 150.13, 166.83 and 169.08.

(c) Synthesis of 2-(2'-pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-hydroxybutane (21): To a solution of 2-aminomethyl-2-methyl-3-(S)-hydroxybutane (12-3(S)) (0.533gm, 4.55mmol) and dry triethylamine (0.69gm, 6.82mmol) in dry dichloromethane (20ml) was

added a solution of N-succinimidyl-3-(2-pyridyldithio)propionate (25) (1.49gm, 4.77mmol) in dry dichloromethane (15ml) dropwise at 0°C (ice-bath). The reaction mixture was stirred at 0°C for 30min, and then at room temperature for 1hr. The reaction mixture was diluted with dichloromethane (40ml) and washed with saturated sodium bicarbonate (2 x 15ml), saturated sodium chloride (1 x 15ml), dried over sodium sulfate, filtered and concentrated *in vacuo* to give a light yellow oil. The crude product was flash chromatographed in silica gel using hexane:ethyl acetate (1:3) to afford a colorless oil (1.5gm, 99%). IR (neat): 3500-3300 (br OH and amide-NH), 2971, 1647 (amide-CO), 1563 (NH), 1574, 1447, 1418, 1117 and 762 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 0.87 (6H, two s, $(\text{CH}_3)_2\text{C}$), 1.10 (3H, d, $J = 6.45\text{Hz}$, CH_3CH), 2.64-2.68 (4H, m, CH_2CO & CH_2NH), 3.09 (2H, t, $J = 6.55\text{Hz}$, CH_2S), 3.50 (1H, m, CHOH), 3.76 (1H, dd, $J = 13.96\text{Hz}$ & 8.16Hz , CH_2NH), 4.07 (1H, d, $J = 3.97\text{Hz}$, OH), 7.13 (1H, m, 5'-H), 7.22 (1H, bs s, NH), 7.60-7.69 (2H, m, 3'-H & 4'-H) and 8.45 (1H, m, 6'-H); ^{13}C NMR (100MHz, CDCl_3) δ : 16.70 & 18.80 ($(\text{CH}_3)_2\text{C}$), 23.44 (CH_3CH), 35.16 & 35.78 (CH_2CO & CH_2S), 38.89 ($(\text{CH}_3)_2\text{C}$), 48.78 (CH_2N), 70.30 (CHOH), 120.50 & 121.10 (3'-C & 5'-C), 137.10 (4'-C), 149.5 (6'-C), 159.30 (2'-C) and 172.4 (C=O); MS(CI): 315 (M^++1 , 100%).

2-(2'-Pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-[(l-(-)menthoxy)(1'',3''-dioxo-4'',6''-di-tert-butylbenzo]-methylphosphorane (29):

(a) Synthesis of (N,N-diisopropylamine)(1',3'-dioxo-4',6'-di-tert-butylbenzo)(l-(-)-menthoxy)-methylphosphorane (33): To a solution of 3,5-di-tert-butyl-1,2-benzoquinone (19) (0.34gm, 1.54mmol) in dry dichloromethane (15ml) was added a solution of (N,N-diisopropylamino)(l-(-)-menthoxy)methylphosphine (32) (0.465gm, 1.54mmol) in dry dichloromethane (15ml) dropwise at room temperature. The reaction mixture was stirred at room temperature for 2hrs. The dark-purple color of the benzoquinone gradually faded in approximately 2hrs. Thus formed (N,N-diisopropylamine)(1',3'-dioxo-4',6'-di-tert-butylbenzo)(l-(-)-menthoxy)-methylphosphorane (33) in dichloromethane was used in the next step without any purification as the compound was found unstable.

(b) Synthesis of 2-(2'-pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-[(l-(-)menthoxy)(1'',3''-dioxo-4'',6''-di-tert-butylbenzo]-methylphosphorane (29): To an *in situ* generated (N,N-diisopropylamine)(1',3'-dioxo-4',6'-di-tert-butylbenzo)(l-(-)-menthoxy)-methylphosphorane (33) in dichloromethane (30ml) (from above) was added a solution of 2-(2'-pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-hydroxybutane (21) (0.485gm, 1.54mmol) and 1H-tetrazole (10.8mg, 0.154mmol) in dry dichloromethane (10ml)

dropwise at room temperature under argon. The resulting reaction mixture was stirred at room temperature overnight. After 24hrs, the reaction mixture was concentrated *in vacuo* to give an oil. The crude product was flash chromatographed in silica gel (which was pretreated with a mixture of hexane:triethylamine (9:1) for 10min) using a mixture of hexane:ethyl acetate:triethylamine (6:1:0.7) to afford a pure colorless oil (0.442gm, 39%), $R_f = 0.29$ (hexane:ethyl acetate:triethylamine = 6:1:0.7). IR (neat): 3317 (NH), 2956, 2869, 1660 (CONH), 1574 (NH-bend), 1519, 1426, 1362, 1300 (P=O), 1253, 1202, 1145, 1113, 1033, 993 (P-O-C), 911, 851, 758, 720 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 0.05 & 0.79 (3H, two d, $J = 6.8\text{Hz}$ & 7.07Hz , CH_3), 0.69, 0.79 0.86, 0.93 (6H, four d, $J = 6.48, 6.33, 7.60$ and 6.22Hz , $(\text{CH}_3)_2\text{C}$), 0.75-1.0 (2H, m), 1.26, 1.27, 1.37 and 1.38 (18H, four s, 2 x t-Bu), 1.78, 1.86 (3H, two d, $J_{\text{HP}} = 17.5$ and 17.36Hz , $\text{CH}_3\text{-P}$), 4.02, 4.14 (1H, two m, OCH), 2.50-2.64 (3H, m, CH_2CO & CH_2NH), 3.10 (2H, t, $J = 7.08\text{Hz}$, CH_2CO), 3.37 (1H, two m, OCH), 6.59, 6.65 (1H, two m, NH), 6.79-6.83 (2H, m, aromatic-H), 7.04 (1H, m, 5'-H), 7.59-7.71 (2H, m, 3'-H & 4'-H), 8.43 (1H, m, 6'-H); ^{13}C NMR (100MHz, CDCl_3) δ : 14.31, 16.00, 20.79, 21.01, 21.27, 21.85, 21.92, 22.04, 25.26, 26.00, 29.48, 29.65, 31.36, 31.63, 33.90, 34.01, 34.07, 34.23, 34.55, 38.38, 46.14, 47.99, 48.55, 78.05, 104.9, 105.3, 114.70, 119.65, 120.6, 131.63, 136.9, 142.6, 144.0, 149.5, 159.88 and 170.1; ^{31}P NMR (121MHz, CDCl_3) δ : -21.158 and -21.657 (two singlets due to two diastereomers); MS(CI): 735 (M^++1 , 10%), 297 (100%).

Preparation of BSA conjugate of 2-(2'-pyridyldithio)propionylamino-methyl-2-methyl-3-(S)-[(L-(-)menthoxy)(1'',3''-dioxo-4'',6''-di-tert-butylbenzo]-methylphosphorane (37-BSA): To a previously deaired solution of BSA (150mg, $2.27 \times 10^{-3}\text{mmol}$) in 0.1mol aqueous solution of sodium bicarbonate (9ml) was added a solution of N-succinimidyl-3-(2-pyridyldithio)propionate (25) (22mg, 0.0704mmol) in ethanol (1ml) at 0°C (ice-bath) under argon. After stirring the mixture for 10min at 0°C and at room temperature for 30min, the reaction mixture was passed through gel-filtration columns (Sephadex G-25 PD-10, pre-equilibrated with water), and the eluent at the void volumn was collected. To the combined solutions (15ml) was added dithiothreitol (DTT) (11mg, 0.0713mmol) at 0°C (ice-bath) under argon, and the reaction mixture was stirred at 0°C for 10min, and then at room temperarture for 30min. The reaction mixture was passed through gel-filtration columns using aqueous 100mmol sodium bicarbonate solution (de-aired) as eluent. The eluent at void volume was collected (18ml) and diluted with equal volume of tetrahydrofuran (THF) (peroxide free). To this

protein solution was added a solution of 2-(2'-pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-[(*l*-(-)menthoxy)(1'',3''-dioxo-4'',6''-di-*tert*-butylbenzo)]-methylphosphorane (29) (50mg, 0.068mmol) in tetrahydrofuran (1ml) at 0°C and stirred for 30min and then at room temperature for 15min. The reaction mixture became slightly yellow and turbid, and was subjected to gel-filtration with water. The collected eluent was lyophilized to afford a white fluffy powder. ³¹P NMR (121MHz, DMSO-d₆, 85% H₃PO₄ as external standard) δ: -20.754 and -20.025 (singlets corresponding to a mixture of diastereomers in almost equal amounts).

Preparation of KLH conjugate of 2-(2'-pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-[(*l*-(-)menthoxy)(1'',3''-dioxo-4'',6''-di-*tert*-butylbenzo)]-methylphosphorane (37-KLH): To a previously deaired solution of KLH (100mg, 1.3x10⁻⁴mmol) in 0.1mol aqueous solution of sodium bicarbonate (5ml) was added a solution of N-succinimidyl-3-(2-pyridyldithio)propionate (25) (5.0mg, 0.016mmol) in ethanol (1ml) at 0°C (ice-bath) under argon. After stirring the mixture for 20min at 0°C and at room temperature for 20min, the reaction mixture was passed through gel-filtration columns (Sephadex G-25 PD-10, pre-equilibrated with water), and the eluent at the void volumn was collected. To the combined solutions (12ml) was added dithiothreitol (DTT) (2.4mg, 0.0156mmol) at 0°C (ice-bath) under argon, and the reaction mixture was stirred at 0°C for 5min, and then at room temperarture for 20min. The reaction mixture was passed through gel-filtration columns using aqueous 100mmol sodium bicarbonate solution (de-aired) as eluent. The eluent at void volume was collected (18ml) and diluted with equal volume of tetrahydrofuran (THF) (peroxide free). To this protein solution was added a solution of 2-(2'-pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-[(*l*-(-)menthoxy)(1'',3''-dioxo-4'',6''-di-*tert*-butylbenzo)]-methylphosphorane (29) (10mg, 120eq) in tetrahydrofuran (1ml) at 0°C and stirred for 30min and then at room temperature for 15min. The reaction mixture became slightly yellow and turbid, and was subjected to gel-filtration with water. The collected eluent was lyophilized to afford a white fluffy powder. ³¹P NMR (121MHz, DMSO-d₆, 85% H₃PO₄ as external standard) δ: -20.57 and -20.02 (singlets corresponding to a mixture of diastereomers in almost equal amounts).

Preparation of PTG conjugate of 2-(2'-pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-[(*l*-(-)menthoxy)(1'',3''-dioxo-4'',6''-di-*tert*-butylbenzo)]-methylphosphorane (37-PTG): To a previously deaired solution of PTG

(150mg, 0.0002mmol) in 0.1mol aqueous solution of sodium bicarbonate (9ml) was added a solution of N-succinimidyl-3-(2-pyridyldithio)propionate (25) (8.6mg, 0.0277mmol) in ethanol (1ml) at 0°C (ice-bath) under argon. After stirring the mixture for 20min at 0°C and at room temperature for 20min, the reaction mixture was passed through gel-filtration columns (Sephadex G-25 PD-10, pre-equilibrated with water), and the eluent at the void volume was collected. To the combined solutions (14ml) was added dithiothreitol (DTT) (5mg, 120eq) at 0°C (ice-bath) under argon, and stirred the reaction mixture for at 0°C for 5min, and then at room temperature for 20min. The reaction mixture was passed through gel-filtration columns using aqueous 100mmol sodium bicarbonate solution (deaired) as eluent. The eluent at void volume was collected (18ml) and diluted with equal volume of tetrahydrofuran (THF) (peroxide free). To this protein solution was added a solution of 2-(2'-pyridyldithio)propionylaminomethyl-2-methyl-3-(S)-[(1(-)-menthoxy)(1",3"-dioxo-4",6"-di-*tert*-butylbenzo]-methylphosphorane (29) (20mg, 120eq) in tetrahydrofuran (1ml) at 0°C and stirred for 30min and then at room temperature for 15min. The reaction mixture became slightly yellow and turbid, and was subjected to gel-filtration with water. The collected eluent was lyophilized to afford a white fluffy powder. ³¹P NMR (121MHz, DMSO-d₆, 85% H₃PO₄ as external standard) δ: -20.729 and -20.180 (singlets corresponding to a mixture of diastereomers in almost equal amounts).

N,N-Dibenzyl-2(S)-alanine²³ (45): To a stirred mixture of 2(S)-alanine (6.0gm, 67.4mmole), and potassium carbonate (40.93gm, 296.63mmole) in dry methanol (120ml) was added dropwise benzyl bromide (23.06gm, 134.83mmole) at room temperature, and the reaction mixture was stirred at room temperature overnight. After 18hrs, the reaction mixture was filtered, and the filtrate was concentrated *in vacuo*. The residue was treated with water (60ml), and the resulting solution was acidified with 2NHCl to pH = 6.5, then extracted with ethyl acetate (3 x 80ml), washed with saturated sodium chloride (1 x 80ml), dried over magnesium sulfate, filtered, concentrated *in vacuo* to afford a colorless sticky oil which on standing became solid (15.0gm). The crude product was ground to a fine powder and then washed twice with hexanes to get a pure white solid (13.8 gm, 76%) (one spot on tlc, ethyl acetate). IR (neat): 3061, 3028, 3000 (br, OH), 2980, 2933, 2849, 1716 (C=O), 1608, 1494, 1454, 1373, 1211, 1148, 748, 689 cm⁻¹; ¹H NMR (300MHz, CDCl₃): δ 1.39 (3H, d, J = 7.20Hz), 3.49 (2H, d, J = 12.9Hz), 3.53 (1H, q, J = 7.20), 3.83 (2H, d, J = 13.8Hz), 7.26-7.39 (10H, m).

4-(S)-(N,N-Dibenzylamino)-2,2-dimethyl-3-oxopentanenitrile²⁴ (47):

(a) Preparation of lithium salt of isobutyronitrile: To a solution of diisopropylamine (12.48ml, 89.22mmole) in dry tetrahydrofuran (100ml) was added a solution of n-butyllithium (1.6M in hexane, 55.76ml, 89.22mmole) at -78°C, and stirred at that temperature for 1hr, and to the resulting lithium diisopropylamide (LDA) was added a solution of isobutyronitrile (6.75ml, 74.35mmole) in tetrahydrofuran (50ml) dropwise, and stirring was continued for another hour. The resulting lithium salt of isobutyronitrile was kept at -78°C for the use in the next step of the reaction (see below).

(b) Preparation of mixed anhydride of N,N-dibenzyl-2-(S)-alanine (46): To a stirred solution of N,N-benzyl-2-(S)-alanine (5.0gm, 18.59mmole) in dry tetrahydrofuran (200ml) at -78°C was added triethylamine (3.88ml, 27.88mmole) dropwise, and the mixture was stirred for 30min. To the resulting mixture was added a solution of butyryl chloride (2.37ml, 19.14mmole) in dry tetrahydrofuran (50ml) dropwise at the same temperature, and stirred for 1hr, and thus formed a mixed anhydride solution that was used in next step (see below).

(c) Synthesis of 4-(S)-(N,N-dibenzylamino)-2,2-dimethyl-3-oxopentanenitrile (47): To the above solution of mixed anhydride (46) in dry tetrahydrofuran (250ml) was added a solution of lithium salt of isobutyronitrile in dry tetrahydrofuran (see above) by using double-ended needle at -78°C. The reaction mixture was stirred at -78°C for 40min. Then the mixture was warmed slowly to -30°C, and then quenched with a solution of saturated sodium chloride. The volatile organic solvent was removed *in vacuo*, and to the resulting aqueous solution was added another portion of saturated sodium chloride, followed by extraction with chloroform (3 x 70ml), dried over magnesium sulfate, filtered and concentrated *in vacuo* to give a light brown liquid (10.0gm). The crude product was purified by flash chromatography on silica gel using hexanes:ethyl acetate (14:1) to a pure colorless liquid (5.45gm, 92%). IR (neat) : 3062, 3029, 2985, 2938, 2844, 2812, 2235 (CN), 1722 (CO), 1602, 1494, 1454, 1380, 1203, 1148, 1124, 1074, 1028, 745 and 701 cm^{-1} ; ¹HNMR (300MHz, CDCl₃): δ 1.27 (3H, d, J = 7.2Hz, CH₃CN), 1.39 (3H, s, CH₃), 1.43 (3H, s, CH₃), 3.71 (2H, d, J = 14.1Hz, CH₂Ph), 3.83 (2H, d, J = 14.1Hz, CH₂Ph), 4.16 (1H, q, CHCH₃) and 7.28-7.36 (10H, m, aromatic-H); ¹³CNMR (75 MHz, CDCl₃): 11.32, 24.62, 24.73, 26.47, 42.46, 53.98, 59.40, 122.05, 127.07, 128.24, 128.87, 138.76 and 205.26; MS(CI): 321 (100%), 320, 243, 224.

4-(S)-(N,N-Dibenzylamino)-2,2-dimethyl-3-(S)-hydroxypentanenitrile(48):

To a stirred solution of 4-(S)-(N,N-dibenzylamino)-2,2-dimethyl-3-oxopentanenitrile (47)

(3.6gm, 11.25mmole) in methanol (36ml) and tetrahydrofuran (36ml) was added sodium borohydride (0.86gm, 22.50mmole) in added in portions at -20°C . The reaction mixture was stirred at that temperature for 40min, and then neutralized with 2NHCl to pH = 6-7. The volatile organic solvents were removed *in vacuo* from the reaction mixture, and to the residue was added water (100ml), extracted with ethyl acetate (3 x 30ml). The combined ethyl acetate extracts were washed with saturated sodium chloride, dried with magnesium sulfate, filtered and concentrated *in vacuo* to give a semisolid compound (3.28gm). The flash chromatography of the crude product on silica gel afforded a colorless oil which on standing became fine needle-like crystals (2.89, 81%), (one spot on tlc, hexane:ethyl acetate = 4:1). IR (neat): 3247 (OH, br), 3086, 3063, 3028, 2978, 2940, 2844, 2234 (CN), 1603, 1495, 1453, 1416, 1380, 1358, 1200, 1140, 1055, 1029, 753 and 701 cm^{-1} ; $^1\text{HNMR}$ (300MHz, CDCl_3): δ 0.93 (3H, s, CH_3), 1.32 (3H, d, $J = 6.6\text{Hz}$, CH_3CH), 1.38 (3H, s, CH_3), 2.84 (1H, m, CHNBn_2), 3.35 (2H, d, $J = 13.20\text{Hz}$, CH_2Ph), 3.46 (2H, d, $J = 8.70\text{Hz}$, CH_2Ph), 5.43 (1H, s, OH) and 7.26-7.35 (10H, m, aromatic-H); $^{13}\text{CNMR}$ (75MHz, CDCl_3) δ : 10.53, 19.67, 25.71, 34.80, 53.21, 54.39, 74.58, 124.58, 127.47, 128.55, 129.07 and 137.97; MS(CI): 323 (100%), 254, 224.

Phenyl (2-cyano-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane)-methylphosphonate²⁵ (49): Preparation of diphenyl methylphosphonate: To a stirred solution of methyl dichlorophosphonate (3.82gm, 28.74mmole) in dry dichloromethane (25ml) was added a solution of phenol (6.22gm, 66.11mmole) and triethylamine (9.2ml, 66.11mmole) in dry dichloromethane (15ml) dropwise under nitrogen at room temperature. The reaction mixture was stirred for 1hr at room temperature. The solid precipitate from the reaction mixture was filtered off, and the filtrate was washed with saturated sodium chloride (2 x 10ml), dried (MgSO_4), filtered, and concentrated *in vacuo* to give a light yellow oil (7.0g). The flash chromatography of the crude product on silica gel using hexane:ethyl acetate (2:1) afforded a colorless oil (6.12gm, 86%), (single spot on tlc, hexane:ethyl acetate = 2:1). IR (neat): 3068, 3043, 2928, 1592, 1490, 1456, 1415, 1268 (P=O), 1216, 1192, 1164, 1072, 1028, 995, 934 (P-O-C), 796, 762, 690 cm^{-1} ; $^1\text{HNMR}$ (300MHz, CDCl_3) δ : 1.79 (3H, d, $J_{\text{PH}} = 17.70\text{Hz}$, $\text{CH}_3\text{-P}$), 7.25 (10H, m, aromatic-H); $^{31}\text{PNMR}$ (121MHz, CDCl_3) δ : 24.524.

Synthesis of phenyl (2-cyano-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane)-methylphosphonate (49): To a solution of 4-(S)-(N,N-dibenzylamino)-2,2-dimethyl-3-(S)-hydroxypentanenitrile (48) (1.7gm, 5.27mmole) in dry tetrahydrofuran (30ml) was added a solution of n-butyllithium (1.6M in hexane, 3.3ml, 5.27mmole) dropwise under nitrogen at

0°C (ice-bath). The mixture was stirred at 0°C for 30min and then at room temperature for another 30min, then a solution of diphenyl methylphosphonate (1.31gm, 5.27mmole) in dry tetrahydrofuran (30ml) was added dropwise. The resulting reaction mixture was stirred for 1hr, then a saturated solution of sodium chloride (60ml) was added to the mixture. The organic layer was separated from the aqueous; the aqueous layer was extracted further with ether (3 x 30ml). The combined organic layers were dried over magnesium sulfate, filtered, concentrated *in vacuo* to give a light brown oil (2.87gm). The flash chromatography of the crude product on silica gel using ethyl acetate:hexane (1:1) afforded a colorless oil (2.08gm, 83%), (single spot on tlc, ethyl acetate:hexane = 1:1). ¹HNMR (300MHz, CDCl₃) δ: 0.94 (3H, s, CH₃), 1.30 (3H, s, CH₃), 1.31 (3H, d, J = 6.9Hz, CHCH₃), 1.68 (3H, d, J_{P-H} = 17.1Hz), 3.21 (1H, m, CHNBn₂), 3.27 (2H, d, J = 13.2Hz, CH₂Ph), 4.12 (2H, d, J_{P-H} = 13.5Hz, CH₂Ph), 4.32 (1H, dd, J₁ = 1.80Hz, J₂ = 11.70Hz, CH-O-P) and 7.16-7.51 (15H, m, aromatic-H); ³¹PNMR (121MHz, CDCl₃) δ: 27.67 (2%), 28.49 (1%), 28.69 (96%), 28.82 (1%); MS(CI): 477 (100), 475 (3.4), 385 (7.0), 305 (12.3), 243 (3.7), 224 (18.0).

Methyl (2-cyano-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane)-methylphosphonate (50): To a stirred solution of pheny (2-cyano-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane)-methylphosphonate (49) (1.0gm, 2.10 mmole) in methanol (10ml) was added a solution of sodium methoxide (25% in methanol, 0.53ml, 2.32mmole) in anhydrous methanol (40ml) dropwise at 0°C (ice-bath). The reaction mixture was allowed to warm to room temperature and stirred overnight. After 18hrs, the methanol was removed from the reaction mixture *in vacuo*, and the residue was extracted with chloroform (30ml). The chloroform extract was washed with saturated sodium chloride (2 x 20ml), dried (MgSO₄), filtered and concentrated *in vacuo* to give a light yellow oil (1.0gm). The flash chromatography of the crude product on silica gel using ethyl acetate afforded a colorless oil which on standing at room temperature became leaf-like crystals (0.7gm, 81%), (single spot on tlc, ethyl acetate). IR (neat): 3071, 3027, 2982, 2952, 2826, 2807, 2237 (CN), 1603, 1495, 1454, 1384, 1313, 1247 (P=O), 1190, 1008, 916, 812, 448 and 702 cm⁻¹; ¹HNMR (300MHz, CDCl₃) δ: 0.88 (3H, s, CH₃), 1.30 (3H, d, J = 7.50Hz, CHCH₃), 1.35 (3H, s, CH₃), 1.56 (3H, d, J = 17.40Hz, CH₃P), 3.18 (1H, m, CH-OP), 3.31 (2H, d, J = 13.2Hz, CH₂Ph), 3.75 (3H, d, J = 11.1Hz, CH₃OP), 4.03 (2H, d, J = 13.2Hz, CH₂Ph), 4.15 (1H, dd, J₁ = 2.4Hz, J₂ = 11.4Hz, CHOP) and 7.21-7.50 (10H, m, aromatic-H); ³¹PNMR (121MHz, CDCl₃) δ: 31.48 (1%), 31.70 (95%), 31.9 (1%), 33.13 (2%); MS(CI): 415 (100), 337 (4.2), 323 (8.6), 305 (10.7), 243 (3.4), 215 (9.3).

Methyl (2-aminomethyl-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane)-methylphosphonate (43-3(S),4(S)): To a stirred solution of methyl (2-cyano-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)-methylphosphonate (50) (50mg, 0.121mmole) in dry tetrahydrofuran (2ml) was added a solution of borane dimethylsulfide complex (2M in ether, 0.18ml, 0.36mmole) dropwise at room temperature. After complete addition, the reaction mixture was refluxed for 1hr. and then cooled to room temperature, followed by acidification with 6NHCl (5 drops) to pH 3. The mixture was refluxed for 30min, and then the mixture was cooled to room temperature followed by addition of solid sodium hydroxide (40mg) and stirred for 10min. The mixture was extracted with ether (10ml), washed with saturated sodium chloride solution (2 x 2ml), dried (MgSO₄), filtered and concentrated *in vacuo* to sticky oil (50mg). IR (neat): 3379 (NH₂), 3027, 2934, 2850, 1602, 1495, 1231, 1130, 997 and 727 cm⁻¹; ¹HNMR (300MHz, CDCl₃) δ: 0.57 (3H, s, CH₃), 0.99 (3H, s, CH₃), 1.27 (3H, d, J = 6.6Hz, CH₃), 1.51 (3H, d, J = 17.10Hz, CH₃P), 2.28 (1H, d, J = 13.20Hz, CH-NH₂), 2.64 (1H, d, J = 13.20Hz, CH-NH₂), 3.14 (1H, m, CHBn₂), 3.27 (2H, d, J = 13.20Hz, CH₂Ph), 3.70 (3H, d, J = 11.40Hz, CH₃OP), 3.99 (2H, d, J = 12.90Hz, CH₂Ph), 4.25 (1H, d, J = 11.1Hz, CHOP) and 7.21-7.41 (10H, m, aromatic-H). The crude product was used in the next step without further purification.

Methyl [2-(2'-pyridyldithio)propionylaminomethyl-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane]-methylphosphonate (51):

(a) Preparation of 3-(2-pyridyldithio)propionic acid (36) To a solution of 2,2'-dipyridyl disulfide (34) (7.8gm, 35.6mmole) in absolute ethanol (100ml) and glacial acetic acid (1.6ml, 17.8mmole) was added a solution of 3-mercaptopropionic acid (35) (1.6ml, 17.8mmole) in absolute ethanol (25ml) dropwise at room temperature. The reaction mixture was stirred at room temperature overnight. After 18hrs, the reaction mixture was concentrated *in vacuo* and the last traces of acetic acid were removed by azeotroping with carbon tetrachloride to get the residual oil. The residual oil was dissolved in saturated sodium bicarbonate solution (30ml). The aqueous bicarbonate solution was washed with dichloromethane (3 x 40ml), and then carefully acidified with 3NHCl until no more oil separated, and extracted the oil with dichloromethane (3 x 50ml), dried over magnesium sulfate, filtered and concentrated *in vacuo* to give oil. The crude product was column chromatographed on basic alumina using ethanol:dichloromethane (2:3) to remove unwanted impurities, followed by ethanol:dichloromethane:acetic acid (2:3:5) to get a pure compound as a pale yellow needles (2.3gm, 56%); single spot in tlc on silica gel, ethyl acetate:hexane

= 5:1; mp 57-59°C; IR (KBr): 2976 (br, OH), 1713 (COOH), 1606, 1535, 1512, 1471, 1419 (S-S), 765 and 717 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 2.69 (2H, t, $J = 6.91\text{Hz}$, CH_2COOH), 2.94 (2H, t, $J = 6.91\text{Hz}$, CH_2S), 7.06 (1H, m, 5'-H-pyridine), 7.57 - 7.67 (2H, m, 3'-H & 4'-H-pyridine), 8.38 (1H, m, 6'-H-pyridine); ^{13}C NMR (100MHz, CDCl_3) δ : 33.76, 33.99, 120.51, 121.46, 138.22, 149.21, 159.75 and 175.28.

(b) Preparation of N-succinimidyl-3-(2-pyridyldithio)propionate (25): To a solution of 3-(2-pyridyldithio)propionic acid (36) (0.5gm, 2.3mmole) in dry dichloromethane (10ml) was added thionyl chloride (186 μl , 2.6mmole) dropwise at 0°C (ice-bath) under argon, and stirred at 0°C for 30min. A small amount of white precipitate was formed. The reaction mixture was stirred at room temperature for 30min and the solution became clear, then to the resulting mixture was added a solution of N-hydroxysuccinimide (267mg, 2.3mole) and triethylamine (405 μl , 2.76mmole) in dry dichloromethane (10ml) at 0°C. The mixture was stirred at 0°C for 30min and at room temperature for additional 2hrs. The reaction mixture was diluted and extracted with dichloromethane (20ml), followed by washing with saturated sodium bicarbonate (2 x 20ml), saturated sodium chloride (2 x 20ml), dried over sodium sulfate, filtered and concentrated *in vacuo* to give a pale yellow gummy solid residue. The crude product was recrystallized from ethyl acetate:hexane (1:5) to afford a colorless crystals (0.614gm, 47%), single spot on silica gel tlc, ethyl acetate:hexane (5:1); mp 78-80°C (lit mp 79.5-80.5°C); IR (KBr): 1786, 1740 (C=O), 1574, 1446, 1417, 1246, 1205, 1116, 1078, 763 and 648 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 2.84 (4H, s, succinimidyl-H), 3.06 - 3.15 (4H, m, SCH_2CH_2), 7.10 - 7.14 (1H, m, 5'-H-pyridine), 7.64 - 7.67 (2H, m, 3'-H & 4'-H-pyridine), 8.49 - 8.50 (1H, m, 6'-H-pyridine); ^{13}C NMR (100MHz, CDCl_3) δ : 25.82, 31.25, 33.13, 120.26, 121.29, 137.47, 150.13, 166.83, and 169.08.

(c) Synthesis of methyl [2-(2'-pyridyldithio)propionylaminomethyl-4-(S)-N,N-dibenzyl-amino-2-methyl-3-(S)-hydroxypentane]-methylphosphonate (51) To a solution of methyl (2-aminomethyl-4-(S)-N,N-dibenzylamino-2-methyl-3-(S)-hydroxypentane)-methylphosphonate (43-3(S),4(S)) (50mg, 0.12mmole) and dry triethylamine (34 μl) in dry dichloromethane (2ml) was added a solution of N-succinimidyl-3-(2-pyridyldithio)propionate (25) (45mg, 0.14mmole) in dry dichloromethane (1ml) dropwise at room temperature and stirred overnight. After 18hr the reaction mixture was concentrated *in vacuo* to get a sticky oil. The crude product was flash chromatographed on silica gel using ethyl acetate:triethylamine (12:1) to afford a pure light yellow oil (20mg, 27% based on nitrile (50)), (single spot on tlc, ethyl acetate:hexane = 5:1). IR (neat): 3306 (NH), 3029, 2958, 1672 (C=O), 1560, 1447, 1417, 1310, 1244 (P=O) and 1120 cm^{-1} ;

^1H NMR (300MHz, CDCl_3) δ : 0.42 (3H, s, CH_3), 1.08 (3H, s, CH_3), 1.23 (3H, d, $J = 6.79\text{Hz}$, CH_3), 1.58 (3H, d, $J = 17.09\text{Hz}$, PCH_3), 2.57 (2H, t, $J = 8.0\text{Hz}$, CH_2CO), 2.86 (1H, dd, $J_1 = 14.06\text{Hz}$, $J_2 = 4.30\text{Hz}$, CH-NH), 3.05 (2H, t, $J = 7.60\text{Hz}$, CH_2S), 3.14 (1H, m, $J = 6.98\text{Hz}$, CHNBn_2), 3.27 (2H, d, $J = 13.15\text{Hz}$, CH_2Ph), 3.41 (1H, dd, $J_1 = 14.01$, $J_2 = 8.94\text{Hz}$, CHNH), 3.70 (3H, d, $J = 11.14\text{Hz}$, POCH_3), 3.97 (2H, d, $J = 13.09\text{Hz}$, CH_2Ph), 4.12 (1H, dd, $J_1 = 10.92$, $J_2 = 1.44\text{Hz}$, POCH), 7.03 - 7.09 (1H, m, 5'-H-pyridine), 7.23 - 7.33 (10H, m, aromatic-H), 7.56 - 7.70 (2H, m, 3'-H & 4'-H-pyridine) and 8.43 (1H, m, 6'-H-pyridine).

N,N-Dibenzyl-2(R)-alanine (54): To a stirred mixture of 2-(R)-alanine (6.0gm, 67.4mmole), and potassium carbonate (40.93gm, 296.63mmole) in dry methanol (120ml) was added dropwise benzyl bromide (23.06gm, 134.83mmole) at room temperature, and the reaction mixture was stirred at room temperature overnight. After 18hrs, the reaction mixture was filtered, and the filtrate was concentrated *in vacuo*. The residue was treated with water (60ml), and the resulting solution was acidified with 2N HCl to pH = 6.5, then extracted with ethyl acetate (3 x 80ml), washed with saturated sodium chloride (1 x 80ml), dried over magnesium sulfate, filtered, concentrated *in vacuo* to afford a colorless sticky oil which on standing became solid (15.0gm). The crude product was ground to the fine powder and then washed twice with hexanes to get a pure white solid (14.5gm, 80%) (one spot on tlc, ethyl acetate). IR (KBr): 3062, 3018, 3000 (br, OH), 2959, 2939, 2852, 1725 (C=O), 1609, 1494, 1454, 1376, 1216, 1139, 748, 698 cm^{-1} ; ^1H NMR (300MHz, CDCl_3): δ 1.39 (3H, d, $J = 7.20\text{Hz}$), 3.49 (2H, d, $J = 12.9\text{Hz}$), 3.53 (1H, q, $J = 7.20$), 3.83 (2H, d, $J = 13.8\text{Hz}$), 7.26 - 7.39 (10H, m).

4-(R)-(N,N-Dibenzylamino)-2,2-dimethyl-3-oxopentanenitrile (56):

(a) Preparation of lithium salt of isobutyronitrile: To a solution of diisopropylamine (12.48ml, 89.22mmole) in dry tetrahydrofuran (100ml) was added a solution of n-butyllithium (1.6M in hexane, 55.76ml, 89.22mmole) at -78°C , and stirred at that temperature for 1hr, and to the resulting lithium diisopropylamide (LDA) was added a solution of isobutyronitrile (6.75ml, 74.35mmole) in tetrahydrofuran (50ml) dropwise, and stirring was continued for another hour. The resulting lithium salt of isobutyronitrile was kept at -78°C for use in the next step of the reaction (see below).

(b) Preparation of mixed anhydride of N,N-dibenzyl-2-(R)-alanine (55): To a stirred solution of N,N-benzyl-2-(R)-alanine (5.0gm, 18.59mmole) in dry tetrahydrofuran (200ml) at -78°C was added triethylamine (3.88ml, 27.88mmole) dropwise, and the

mixture was stirred for 30min. To the resulting mixture was added a solution of butyryl chloride (2.37ml, 19.14mmole) in dry tetrahydrofuran (50ml) dropwise at the same temperature, and stirred for 1hr, and thus formed a mixed anhydride solution that was used in next step (see below).

(c) Synthesis of 4-(R)-(N,N-dibenzylamino)-2,2-dimethyl-3-oxopentanenitrile (56): To the above solution of mixed anhydride (55) in dry tetrahydrofuran (250ml) was added a solution of lithium salt of isobutyronitrile in dry tetrahydrofuran (see above) by using double-ended needle at -78°C. The reaction mixture was stirred at -78°C for 40min. Then the mixture was warmed slowly to -30°C, and then quenched with a solution of saturated sodium chloride. The volatile organic solvent was removed *in vacuo*, and to the resulting aqueous solution was added another portion of saturated sodium chloride and followed by extraction with chloroform (3 x 70ml), dried over magnesium sulfate, filtered and concentrated *in vacuo* to give a light brown liquid (10.0gm). The crude product was purified by flash chromatography on silica gel using hexanes:ethyl acetate (14:1) to a pure colorless liquid (5.45gm, 92%). IR (neat) : 3062, 3029, 2985, 2938, 2844, 2812, 2235 (CN), 1722 (CO), 1602, 1494, 1454, 1380, 1203, 1148, 1124, 1074, 1028, 745 and 701 cm^{-1} ; ^1H NMR (300MHz, CDCl_3): δ 1.27 (3H, d, $J = 7.2\text{Hz}$, CH_3CN), 1.39 (3H, s, CH_3), 1.43 (3H, s, CH_3), 3.71 (2H, d, $J = 14.1\text{Hz}$, CH_2Ph), 3.83 (2H, d, $J = 14.1\text{Hz}$, CH_2Ph), 4.16 (1H, q, CHCH_3) and 7.28-7.36 (10H, m, aromatic-H); ^{13}C NMR (75MHz, CDCl_3): 11.32, 24.62, 24.73, 26.47, 42.46, 53.98, 59.40, 122.05, 127.07, 128.24, 128.87, 138.76 and 205.26; MS(CI): 321 (100%), 320, 243, 224.

4-(R)-(N,N-Dibenzylamino)-2,2-dimethyl-3-(R)-hydroxypentanenitrile(57):

To a stirred solution of 4-(R)-(N,N-dibenzylamino)-2,2-dimethyl-3-oxopentanenitrile (56) (3.6gm, 11.25mmole) in methanol (36ml) and tetrahydrofuran (36ml) was added sodium borohydride (0.86gm, 22.50mmole) in portions at -20°C. The reaction mixture was stirred at that temperature for 40min, and then neutralized with 2NHCl to pH = 6-7. The volatile organic solvents were removed *in vacuo* from the reaction mixture, and to the residue was added water (100ml), extracted with ethyl acetate (3 x 30ml). The combined ethyl acetate extracts were washed with saturated sodium chloride, dried with magnesium sulfate, filtered and concentrated *in vacuo* to give a semisolid compound (3.28gm). The flash chromatography of the crude product on silica gel afforded a colorless oil which on standing became fine needle-like crystals (2.89, 81%), (one spot on tlc, hexane:ethyl acetate = 4:1). IR (KBr): 3247 (OH, br), 3086, 3063, 3028, 2978, 2940, 2844, 2234 (CN), 1603, 1495, 1453, 1416, 1380, 1358, 1200, 1140, 1055, 1029, 753 and 701 cm^{-1} ;

^1H NMR (300MHz, CDCl_3): δ 0.93 (3H, s, CH_3), 1.32 (3H, d, $J = 6.6\text{Hz}$, CH_3CH), 1.38 (3H, s, CH_3), 2.84 (1H, m, CHNBn_2), 3.35 (2H, d, $J = 13.20\text{Hz}$, CH_2Ph), 3.46 (2H, d, $J = 8.70\text{Hz}$, CH_2Ph), 5.43 (1H, s, OH) and 7.26-7.35 (10H, m, aromatic-H); ^{13}C NMR (75MHz, CDCl_3) δ : 10.53, 19.67, 25.71, 34.80, 53.21, 54.39, 74.58, 124.58, 127.47, 128.55, 129.07 and 137.97; MS(CI): 323 (100%), 254, 224.

Phenyl (2-cyano-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)-methylphosphonate (58): Preparation of diphenyl methylphosphonate: To a stirred solution of methyl dichlorophosphonate (3.82gm, 28.74mmole) in dry dichloromethane (25ml) was added a solution of phenol (6.22gm, 66.11mmole) and triethylamine (9.2ml, 66.11mmole) in dry dichloromethane (15ml) dropwise under nitrogen at room temperature. The reaction mixture was stirred for 1hr at room temperature. The solid precipitate from the reaction mixture was filtered off, and the filtrate was washed with saturated sodium chloride (2 x 10ml), dried (MgSO_4), filtered, and concentrated *in vacuo* to give a light yellow oil (7.0gm). The flash chromatography of the crude product on silica gel using hexane:ethyl acetate (2:1) afforded a colorless oil (6.12gm, 86%), (single spot on tlc, hexane:ethyl acetate = 2:1). IR (neat): 3068, 3043, 2928, 1592, 1490, 1456, 1415, 1268 (P=O), 1216, 1192, 1164, 1072, 1028, 995, 934 (P-O-C), 796, 762, 690 cm^{-1} ; ^1H NMR (300MHz, CDCl_3) δ : 1.79 (3H, d, $J_{\text{PH}} = 17.70\text{Hz}$, $\text{CH}_3\text{-P}$), 7.25 (10H, m, aromatic-H); ^{31}P NMR (121MHz, CDCl_3) δ : 24.524.

Synthesis of phenyl (2-cyano-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)-methylphosphonate (58): To a solution of 4-(R)-(N,N-dibenzylamino)-2,2-dimethyl-3-(R)-hydroxypentanenitrile (57) (1.7gm, 5.27mmole) in dry tetrahydrofuran (30ml) was added a solution of n-butyllithium (1.6M in hexane, 3.3ml, 5.27mmole) dropwise under nitrogen at 0°C (ice-bath). The mixture was stirred at 0°C for 30min and then at room temperature for another 30min, then a solution of diphenyl methylphosphonate (1.31gm, 5.27mmole) in dry tetrahydrofuran (30ml) was added dropwise. The resulting reaction mixture was stirred for 1hr, then a saturated solution of sodium chloride (60ml) was added to the mixture. The organic layer was separated from the aqueous; the aqueous layer was extracted further with ether (3 x 30ml). The combined organic layers were dried over magnesium sulfate, filtered, concentrated *in vacuo* to give a light brown oil (2.87gm). The flash chromatography of the crude product on silica gel using ethyl acetate: hexane (1:1) afforded a colorless oil (2.08gm, 83%), (single spot on tlc, ethyl acetate: hexane = 1:1). ^1H NMR (300MHz, CDCl_3) δ : 0.94 (3H, s, CH_3), 1.30 (3H, s, CH_3), 1.31 (3H, d, $J = 6.9\text{Hz}$, CHCH_3), 1.68 (3H, d, $J_{\text{P-H}} = 17.1\text{Hz}$), 3.21 (1H, m, CHNBn_2), 3.27 (2H, d, J

= 13.2Hz, CH₂Ph), 4.12 (2H, d, J_{P-H} = 13.5Hz, CH₂Ph), 4.32 (1H, dd, J₁ = 1.80Hz, J₂ = 11.70Hz, CH-O-P) and 7.16-7.51 (15H, m, aromatic-H); ³¹P NMR (121MHz, CDCl₃) δ: 27.67 (2%), 28.49 (1%), 28.69 (96%), 28.82 (1%); MS(CI): 477 (100), 475 (3.4), 385 (7.0), 305 (12.3), 243 (3.7), 224 (18.0).

Methyl (2-cyano-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)-methylphosphonate (59): To a stirred solution of phenyl (2-cyano-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)-methylphosphonate (58) (1.0gm, 2.10 mmole) in methanol (10ml) was added a solution of sodium methoxide (25% in methanol, 0.53ml, 2.32mmole) in anhydrous methanol (40ml) dropwise at 0°C (ice-bath). The reaction mixture was allowed to warm to room temperature and stirred overnight. After 18hrs, the methanol was removed from the reaction mixture *in vacuo*, and the residue was extracted with chloroform (30ml). The chloroform extract was washed with saturated sodium chloride (2 x 20m), dried over magnesium sulfate, filtered and concentrated *in vacuo* to give a light yellow oil (1.0gm). The flash chromatography of the crude product on silica gel using ethyl acetate afforded a colorless oil which on standing at room temperature became leaf-like crystals (0.7gm, 81%), (single spot on tlc, ethyl acetate). IR (neat): 3071, 3027, 2982, 2952, 2826, 2807, 2237 (CN), 1603, 1495, 1454, 1384, 1313, 1247 (P=O), 1190, 1008, 916, 812, 448 and 702 cm⁻¹; ¹H NMR (300MHz, CDCl₃) δ: 0.88 (3H, s, CH₃), 1.30 (3H, d, J = 7.50Hz, CHCH₃), 1.35 (3H, s, CH₃), 1.56 (3H, d, J = 17.40Hz, CH₃P), 3.18 (1H, m, CH-OP), 3.31 (2H, d, J = 13.2Hz, CH₂Ph), 3.75 (3H, d, J = 11.1Hz, CH₃OP), 4.03 (2H, d, J = 13.2Hz, CH₂Ph), 4.15 (1H, dd, J₁ = 2.4Hz, J₂ = 11.4Hz, CHOP) and 7.21-7.50 (10H, m, aromatic-H); ³¹P NMR (121MHz, CDCl₃) δ: 31.48 (1%), 31.70 (95%), 31.9 (1%), 33.13 (2%); MS(CI): 415 (100), 337 (4.2), 323 (8.6), 305 (10.7), 243 (3.4), 215 (9.3).

Methyl (2-aminomethyl-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)-methylphosphonate (43-3(R),4(R)): To a stirred solution of methyl (2-cyano-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)-methylphosphonate (59) (50mg, 0.121mmole) in dry tetrahydrofuran (2ml) was added a solution of borane dimethylsulfide complex (2M in ether, 0.18ml, 0.36mmole) dropwise at room temperature. After complete addition, the reaction mixture was refluxed for 1hr. and then cooled to room temperature, followed by acidification with 6NHCl (5 drops) to pH 3. The mixture was refluxed for 30min, and then the mixture was cooled to room temperature followed by addition of solid sodium hydroxide (40mg) and stirred for 10min. The mixture was

extracted with ether (10ml), washed with saturated sodium chloride solution (2 x 2ml), dried over magnesium sulfate, filtered and concentrated *in vacuo* to sticky oil (50mg). IR (neat): 3379 (NH₂), 3027, 2934, 2850, 1602, 1495, 1231, 1130, 997 and 727 cm⁻¹; ¹HNMR (300MHz, CDCl₃) δ: 0.57 (3H, s, CH₃), 0.99 (3H, s, CH₃), 1.27 (3H, d, J = 6.6Hz, CH₃), 1.51 (3H, d, J = 17.10Hz, CH₃P), 2.28 (1H, d, J = 13.20Hz, CH-NH₂), 2.64 (1H, d, J = 13.20Hz, CH-NH₂), 3.14 (1H, m, CHBn₂), 3.27 (2H, d, J = 13.20Hz, CH₂Ph), 3.70 (3H, d, J = 11.40Hz, CH₃OP), 3.99 (2H, d, J = 12.90Hz, CH₂Ph), 4.25 (1H, d, J = 11.1Hz, CHOP) and 7.21-7.41 (10H, m, aromatic-H). The crude product was used in the next step without further purification.

Methyl [2-(2'-pyridyldithio)propionylaminomethyl-4-(R)-N,N-dibenzyl-amino-2-methyl-3-(R)-hydroxypentane]-methylphosphonate (60) To a solution of methyl (2-aminomethyl-4-(R)-N,N-dibenzylamino-2-methyl-3-(R)-hydroxypentane)-methylphosphonate (43-3(R),4(R)) (50mg, 0.12mmole) and dry triethylamine (34 μl) in dry dichloromethane (2ml) was added a solution of N-succinimidyl-3-(2-pyridyldithio)propionate (25) (45mg, 0.14mmole) in dry dichloromethane (1ml) dropwise at room temperature and stirred overnight. After 18hr, the reaction mixture was concentrated *in vacuo* to get a sticky oil. The crude product was flash chromatographed on silica gel using ethyl acetate:triethylamine (12:1) to afford a pure light yellow oil (20mg, 27% based on nitrile (57)), (single spot on tlc, ethyl acetate: hexane = 5:1). IR (neat): 3306 (NH), 3029, 2958, 1672 (C=O), 1560, 1447, 1417, 1310, 1244 (P=O) and 1120 cm⁻¹; ¹HNMR (300MHz, CDCl₃) δ: 0.42 (3H, s, CH₃), 1.08 (3H, s, CH₃), 1.23 (3H, d, J = 6.79Hz, CH₃), 1.58 (3H, d, J = 17.09Hz, PCH₃), 2.57 (2H, t, J = 8.0Hz, CH₂CO), 2.86 (1H, dd, J₁ = 14.06Hz, J₂ = 4.30Hz, CH-NH), 3.05 (2H, t, J = 7.60Hz, CH₂S), 3.14 (1H, m, J = 6.98Hz, CHNBn₂), 3.27 (2H, d, J = 13.15Hz, CH₂Ph), 3.41 (1H, dd, J₁ = 14.01, J₂ = 8.94Hz, CHNH), 3.70 (3H, d, J = 11.14Hz, POCH₃), 3.97 (2H, d, J = 13.09Hz, CH₂Ph), 4.12 (1H, dd, J₁ = 10.92, J₂ = 1.44Hz, POCH), 7.03 - 7.09 (1H, m, 5'-H-pyridine), 7.23 - 7.33 (10H, m, aromatic-H), 7.56 - 7.70 (2H, m, 3'-H & 4'-H-pyridine) and 8.43 (1H, m, 6'-H-pyridine).

2-Aminomethyl-2-methyl-3-(S)-hydroxybutane (12-3(S)): To a solution of 2-methyl-2-cyano-3-(S)-hydroxybutane (16) (0.5gm, 4.46mmole) in absolute ethanol presaturated with ammonia gas (40ml) was added 5% rhodium on alumina (0.2gm) in one portion at room temperature. The mixture was installed in a hydrogenation apparatus under 50psi at room temperature, and shaken for overnight. After 18hrs, the reaction mixture

was filtered through a pad of Celite, and the filtrate was concentrated *in vacuo* to give a pure colorless oil (0.49gm, 94%); ^1H NMR (300MHz, CDCl_3) δ : 0.79 & 0.86 (6H, two d, $J = 1.8\text{Hz}$, $(\text{CH}_3)_2\text{C}$), 1.07 (3H, dd, $J = 1.8, 6.3\text{Hz}$, CH_3CH), 2.63 & 2.80 (2H, two dd, $J = 12.3, 1.8\text{Hz}$, CH_2NH_2) and 3.69 (1H, m, CH_3CH).

2-(3',5'-Dinitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane (66):

To a solution of 2-aminomethyl-2-methyl-3-(S)-hydroxybutane (12-3(S)) (0.512gm, 4.37mmole) and triethylamine (0.92ml, 6.56mmole) in dry dichloromethane (30ml) was added dropwise a solution of 3,5-dinitrobenzoyl chloride²⁶ (1.09gm, 4.72mmole) in dry dichloromethane (5ml) at 0°C (ice-bath), and stirred for 3hrs. During the course of reaction, a white solid was formed. The reaction mixture was then filtered to remove the solid, and the filtrate was washed with saturated sodium bicarbonate (2 x 20ml), water (2 x 30ml), saturated sodium chloride (2 x 20ml), dried over magnesium sulfate, filtered and concentrated *in vacuo* to give solid residue. The crude product was recrystallised from ethyl acetate:hexane (1:2) to afford colorless crystals (1.31gm, 96%). ^1H NMR (300MHz, CD_3COCD_3) δ : 0.91 & 0.94 (6H, two s, $(\text{CH}_3)_2\text{C}$), 1.08 (3H, d, $J = 6.6\text{Hz}$, CH_3CH), 3.16 (2H, m, CH_2NH_2), 3.65 (1H, m, CHCH_3) and 8.65 & 9.07 (3H, aromatic-H).

Phenyl [2-(3',5'-dinitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane]-aminophosphonate (68): To a stirred solution of phenyldichlorophosphite (0.112gm, 0.53mmole) in dry tetrahydrofuran (3ml) was added dropwise a solution of 2-(3',5'-dinitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane (66) (0.15gm, 0.484 mmole) and quinoline (0.062gm, 0.482mmole) in dry tetrahydrofuran (3ml) over a period of 30min at room temperature. After complete addition, there was some precipitation which dissolved into a clear solution in about 2hrs. The reaction mixture was then kept at room temperature for 2 days; during that period the solution became cloudy. The reaction mixture was cooled to 0°C (ice-bath), and into that cold solution was passed dry ammonia gas for 5min, and the reaction mixture was allowed to stand for about 30min at room temperature. During that time a fine white precipitate of ammonium chloride salt was formed. The mixture was filtered and the solid residue was washed several times with tetrahydrofuran. The combined filtrates were concentrated *in vacuo* to give a solid. The crude product was flash chromatographed on silica gel using ethyl acetate:hexane (1:1) to get colorless solid which was recrystallised from ethyl acetate:hexane to afford a pure compound (115mg, 51%); mp $203\text{-}204^\circ\text{C}$. ^1H NMR (300MHz, CD_3COCH_3) δ : 0.94 & 0.99 (6H, two s, $(\text{CH}_3)_2\text{C}$), 1.31 (3H, d, $J = 6.3\text{Hz}$, CH_3CH), 3.10 & 3.80 (2H, two

dd, CH₂NH₂), 4.66 (1H, m, CH₃CH), 4.70 (1H, br s, NH), 7.38 (5H, m, aromatic-H), 9.06 (1H, t, J = 2.40Hz, aromatic-H), 9.24 (2H, d, J = 2.40Hz, aromatic-H), 9.47 (2H, br s, NH₂). MS(CI): 467 (M⁺+1, 9.4%), 373 (M⁺-OPh, 12.3%), 294 (100%). Found: C, 48.89; H, 4.95; N, 11.93; P, 6.65. C₁₉H₂₃O₈N₄P requires C, 48.93; H, 4.97; N, 12.01; P, 6.64%

[2-(3',5'-Dinitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane]

aminophosphonate (64): To a solution of phenyl [2-(3',5'-dinitrobenzoyl)-aminomethyl-2-methyl-3-(S)-hydroxybutane]-aminophosphonate (68) (100mg, 0.214 mmole) was added dropwise a solution of 1N lithium hydroxide²⁷ (3ml) at room temperature, and the mixture was stirred for 1hr. The pH of the reaction mixture was adjusted to 4.2 with acetic acid, and then the mixture was concentrated *in vacuo* to get the residue. The residue was treated with water (2ml) and the pH of the solution was maintained to 4.2 with acetic acid. The aqueous layer was concentrated *in vacuo*, and the last traces of water were removed by azeotroping with absolute ethanol to give a sticky residual oil. The crude product was flash chromatographed in silica gel using chloroform and followed by a mixture solution of chloroform:methanol:water = 16:8:1 to afford a light yellow solid (64mg, 76%), single spot on tlc, chloroform:methanol:water = 12:7:1.

¹HNMR (300MHz, D₂O) δ: 0.87 & 0.89 (6H, two dd, (CH₃)₂C), 1.20 (3H, d, J = 6.3Hz, CH₃CH), 3.26 (1H, d, J = 13.8Hz, CH₂NH), 3.46 (1H, d, J = 13.2Hz, CH₂NH), 4.07 (1H, m, CHCH₃), 8.91 & 9.15 (3H, two s, aromatic-H); MS(CI) : 391 (M⁺+1, 0.51%), 373 (M⁺-OH, 0.98%), 356 (M⁺-OH & -NH₂, 0.62%), 312 (2.35%), 294 (100%); MS(FAB): 391 (M⁺+1, 4.5%), 355 (M⁺ -OH & -NH₂, 0.26%), 299 (M⁺ -2xNO₂, 2.8%), 207 (M⁺-C₆H₃N₂O₅, 21.1%).

Bis(diethylamino)trifluoromethylphosphine (73): To a stirred hexaethylphosphorus triamide (25gm, 101.2mmol) in a 150-ml Pyrex pressure tube was added phosphorus trichloride (2.77gm, 20.1mmol) dropwise at 0°C under argon. The mixture turned yellow, then brown, while stirring continued for 20min at 0°C and 30min at room temperature. The reaction tube was cooled down to liquid nitrogen temperature (liquid nitrogen-bath), then after 5min an equal volume of bromotrifluoromethane gas (9.8gm, 65.8mmol) was collected in the same pressure tube (uncapped). The pressure tube was sealed with a Teflon screw stopper and placed behind a safety shield, then reaction mixture was stirred at room temperature overnight. After 20hrs, an excess bromotrifluoromethane was removed under reduced pressure, and the remaining liquid was

distilled under argon to afford a pure colorless oil (15.3gm, 62%), bp 40-45°C at 1.6mmHg; $^1\text{H NMR}$ (400MHz, CDCl_3): 3.12 (4H, m, 2 x CH_2CH_3), 1.07 (6H, t, $J = 7.10\text{Hz}$, 2 x CH_2CH_3); $^{31}\text{P NMR}$ (121MHz, CDCl_3) δ : 74.94 (quartet, $\text{JPCF} = 93.98\text{Hz}$).

2-(p-Nitrobenzoyl)aminomethyl-2-methyl-3-(S)-hydroxybutane (75): To a solution of 2-aminomethyl-2-methyl-3-(S)-hydroxybutane (12-3(S)) (2.4gm, 20.48mmol) and dry triethylamine (2.487gm, 24.58mmol) in dry dichloromethane (25ml) was added a solution of p-nitrobenzoyl chloride (74) (3.61gm, 19.46mmol) in dry dichloromethane (150ml) dropwise at 0°C under argon. The reaction mixture was stirred at 0°C for 30min and at room temperature for 12hrs. The reaction was monitored by tlc (hexane:ethyl acetate, 1:1). After completion of the reaction, the mixture was washed with saturated sodium bicarbonate solution (2 x 100ml), saturated sodium chloride (2 x 100ml), dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to give a crude product as a yellow oil. The flash chromatography of the crude product in silica gel using hexane:ethyl acetate, 1:1) afforded a pure compound as pale yellow needles (1.48gm, 27%), mp. 111-112°C, single spot in tlc (hexane:ethyl acetate, 1:1), $R_f = 0.38$. IR (neat): 3307, 3086, 2976, 2876, 1639, 1598, 1557, 1524, 1448, 1391, 1346, 1310, 1175, 1099, 913, 869 and 703 cm^{-1} ; $^1\text{H NMR}$ (400MHz, CDCl_3) δ : 0.94 & 0.99 (6H, two s, $(\text{CH}_3)_2\text{C}$), 1.19 (3H, d, $J = 6.42\text{Hz}$, CH_3CH), 2.85 (1H, d, $J = 3.87\text{Hz}$, OH), 3.10 (1H, dd, $J = 13.77\text{Hz}$ & 4.93Hz , CHNH), 3.71 (2H, m, CHOH & CHNH), 7.35 (1H, br s, NH), 7.93 (2H, m, aromatic-H) and 8.28 (2H, m, aromatic-H).

Methyl 2-(p-nitrobenzoyl)aminomethyl-2-methyl-3-(S)-(methoxy)(trifluoromethyl)-hydroxybutane phosphonate (69): (a) Preparation of (diethylamino)(methoxy)trifluoromethylphosphine (73): To a stirred solution of bis(diethylamino)trifluoromethylphosphine (0.864gm, 3.54mmol) was added dry methanol (0.143ml, 3.54mmol) dropwise at 0°C (ice-bath) under argon and to that mixture was added 1H-tetrazole (0.248gm, 3.54mmol) in one portion. The reaction mixture was stirred at 0°C for 15min and then at room temperature for 30min, and this reaction gave (diethylamino)(methoxy)trifluoromethylphosphine which was unstable to isolate and therefore used in the next step without purification.

(b) Synthesis of 2-(p-nitrobenzoyl)aminomethyl-2-methyl-3-(S)-(methoxy)(trifluoromethyl)-hydroxybutane phosphine (76): To the above (diethylamino)(methoxy)trifluoromethylphosphine (73) was added a solution of 2-(p-nitrobenzoyl)aminomethyl-2-methyl-3-

(S)-hydroxybutane (75) (0.941gm, 3.54mmol) in dry dichloromethane (10ml) and dry tetrahydrofuran (5ml) at room temperature, and to mixture was added 1*H*-tetrazole (0.248gm, 3.54mmol) in one portion. The reaction mixture was stirred at room temperature for 2hrs under argon. ³¹P NMR of the reaction mixture showed the completion of the reaction. The resulting product was 2-(*p*-nitrobenzoyl)aminomethyl-2-methyl-3-(*S*)-(methoxy)(trifluoromethyl)hydroxybutane phosphine; ³¹P NMR (121MHz, CDCl₃) δ: 142.19 and 137.75 (two quartets, *J* = 85.0Hz & 81.96Hz respectively), corresponding to 50:50 diastereomeric mixture. The product was used in the next step without further purification.

(c) Synthesis of methyl 2-(*p*-nitrobenzoyl)aminomethyl-2-methyl-3-(*S*)-(methoxy)-(trifluoromethyl)hydroxybutane phosphonate (69): To the above solution of methyl 2-(*p*-nitrobenzoyl)aminomethyl-2-methyl-3-(*S*)-(methoxy)(trifluoromethyl)hydroxybutane phosphine (76) in dichloromethane was added *meta*-chloroperbenzoic acid (1.0gm, 5.8mmol) in one portion. The reaction mixture was stirred at room temperature for 20min. The reaction was complete as indicated by ³¹P NMR spectrum which showed two quartets had moved upfield compared to phosphine. The reaction mixture was concentrated *in vacuo* to give the residue which was diluted with dichloromethane (200ml), washed with saturated sodium bicarbonate (2 x 50ml), 10% sodium sulfite solution (3 x 50ml), finally with saturated sodium chloride (1 x 100ml), dried over magnesium sulfate, filtered and evaporated *in vacuo* to give a yellow oil (1.15gm). The flash chromatography of the crude product in silica gel using hexane:ethyl acetate (2:1) afforded a colorless oil (0.87gm, 60%). IR (neat): 2971 (NH), 1724, 1651, 1601, 1527, 1347, 1276, 1107, 1049, 1013, 870 and 717 cm⁻¹; ¹H NMR (400MHz, CDCl₃) δ: 0.96 & 0.99 (6H, two s, (CH₃)₂C), 1.22 (3H, d, *J* = 7.07Hz, CH₃C), 3.08 (1H, dd, *J* = 13.87 Hz & 5.07Hz, CHNH), 3.68 (1H, m, CHNH), 3.86 (1H, m, CHOH), 4.01 (3H, d, *J*_{P-H} = 10.87Hz, CH₃), 7.82 (1H, br s, NH), 8.05 (2H, m, aromatic-H) and 8.25 (2H, m, aromatic-H); ¹³C NMR (100MHz, CDCl₃) δ: 14.40, 17.88, 19.59, 20.07, 21.19, 23.73, 37.06, 49.23, 73.74, 123.58 (two signals), 128.1 (two signals), 130.60, 149.5 and 151.3; ¹⁹F NMR (188MHz, CDCl₃) δ: -73.81 and -73.19 (two doublets, *J*_{FH} = 105.6 & 125.6Hz, ratio 2.6:1); ³¹P NMR (121MHz, CDCl₃) δ: 2.17 (quartet) and 0.564 (quartet) (*J*_{P-F} = 127Hz) corresponding to two diastereomers in a ratio of 2.76:1; MS(CI): 413 (M⁺+1, 1.84%).

Diethyl bromodifluoromethylphosphonate (77): To a stirred solution of triethylphosphite (103ml, 600mmol) in dry diethyl ether (300ml) was added dibromodifluoromethane (58.3ml, 640mmol) dropwise at 0°C. The colorless reaction

mixture was allowed to reach the room temperature and refluxed overnight. After 24 hrs, ether was removed from the reaction mixture *in vacuo* to give oil. Distillation of the crude oil under high vacuum afforded colorless oil (142.1gm, 89%), bp. 90°C at 3mmHg. ^{31}P NMR (121MHz, CDCl_3) δ : 0.425 (triplet, $J_{\text{PF}} = 93.98\text{Hz}$; MS(CI): 269 (100%) and 267 (100%).

Diethyl (1,1-difluoro-3-butenyl)phosphonate (79): To a well-stirred suspension of zinc powder (100mesh) (108.4gm, 1.66gm-atom) in dry monoglyme (835ml) was added diethyl bromodifluoromethylphosphonate (77) (422.11gm, 1.581mol) dropwise at room temperature under argon. Cooling bath was used in order to control the exothermic reaction during addition of the reagent. The reaction mixture was stirred at room temperature overnight. After 20hrs, the reaction mixture was filtered through a medium fritted Schlenk funnel under argon to remove excess unreacted zinc. The filtrate solution containing [(diethoxyphosphinyl)difluoromethyl]zinc bromide in monoglyme was used immediately in the next step without isolation or purification. To this solution was added a catalytic amount of copper(I) bromide (0.886gm, 6.17mmol) followed by 3-bromopropene (78) (139ml, 1.62mol) at room temperature. The reaction mixture was stirred at room temperature for 12hrs, then approximately 300ml of monoglyme was removed *in vacuo* from the reaction mixture, and to the remaining liquid was added water (600ml). The aqueous layer was extracted with dichloromethane (3 x 600ml). The combined organic extracts were washed with saturated sodium chloride, dried over sodium sulfate, filtered and concentrated *in vacuo* to give crude oil. Distillation of the crude oil under high vacuum afforded a pure colorless oil (210gm, 58%), bp. 92°C at 4.5mmHg. ^1H NMR (400MHz, CDCl_3) δ : 1.36 (3H, t, $J = 7.0\text{Hz}$, CH_3C), 2.81 (2H, m, CH_2CF_2), 4.25 (2H, dq, CH_2O), 5.24-5.29 (2H, m, $\text{CH}_2=\text{CH}$), 5.75-5.85 (1H, m, $\text{CH}=\text{CH}_2$); IR (neat): 3010, 1650, 1460, 1420, 1395, 1290 (P=O), 1180, 1050 (C-O-P), 915, 820 and 770 cm^{-1} ; ^{19}F NMR (188MHz, CDCl_3) δ : 111.7 (dt, $J_{\text{FP}} = 107\text{Hz}$ & $J_{\text{FH}} = 20\text{Hz}$); ^{31}P NMR (121MHz, CDCl_3) δ : 6.62 (triplet).

Diethyl 3,3-difluoro-3-phosphonopropionic acid (80): To a vigorously stirred biphasic solution of diethyl (1,1-difluoro-3-butenyl)phosphonate (79) (10gm, 43.86mmol) in carbon tetrachloride (62ml), acetonitrile (62ml) and water (130ml) was added potassium periodate (40gm, 174mmol) in one portion followed by ruthenium (III) chloride trihydrate (0.14gm, 0.67mmol) at room temperature. The reaction mixture was stirred at room temperature for 12hrs. Then dichloromethane (60ml) was added to the reaction mixture,

the solution was filtered through Celite. The organic layer was separated from the aqueous, the aqueous layer was extracted further with dichloromethane (3 x 40ml). The combined organic extracts were dried over magnesium sulfate, filtered and concentrated *in vacuo* to give clear oil. The crude product was flash chromatographed in silica gel using ethyl acetate:dichloromethane (4:1) to afford a colorless oil (6.2gm, 58%). In a separate experiment, an extractive work-up was modified as follows: dichloromethane layer was extracted with 50% saturated sodium carbonate solution (3 x 75ml), careful neutralization of the aqueous extract with concentrated hydrochloric acid to pH = 1-2, followed by saturation with solid sodium chloride, and then extracted with dichloromethane (3 x 50ml). The combined dichloromethane extracts were dried over magnesium sulfate, filtered and concentrated *in vacuo* afforded almost pure colorless oil. The alkaline extractive procedure was used in the scale-up experiment. IR (neat) : 3350, 1745, 1275 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 1.36 (6H, t, $J = 7.06\text{Hz}$, 2 x CH_3CH_2), 3.12 (2H, dt, $J_{\text{FP}} = 18.3\text{Hz}$, $J_{\text{FH}} = 6.04\text{Hz}$, CH_2CF_2), 4.28 (4H, q, 2 x $\text{CH}_3\text{CH}_2\text{O}$), 9.69 (1H, br s, COOH); ^{19}F NMR (188MHz, CDCl_3) δ : 111.16 (dt, $J = 105.19\text{Hz}$); ^{31}P NMR (121MHz, CDCl_3) δ : 5.98 (triplet, $J = 103.2\text{Hz}$); MS(FAB): 246 (M^+ , 100%).

Attempted synthesis of (2'-pyridyldithio)ethyl diethyl-3,3-difluoro-3-phosphonopropionic acid ester (70): To a stirred diethyl 3,3-difluoro-3-phosphonopropionic acid (80) (0.46gm, 1.87mmol) was added thionyl chloride (0.56gm, 4.67mmol) dropwise at room temperature under argon. The reaction mixture was stirred at 60°C for 12hrs. Excess thionyl chloride was removed by distillation at reduced pressure, and the yellow oil of diethyl 3,3-difluoro-3-phosphonopropionyl chloride was used as such in the next step without purification. The resulting acid chloride was diluted with dry dichloromethane (10ml), and to that solution was added a solution of 2-pyridyldithioethanol (81) (0.333gm, 1.78mmol) in dry dichloromethane (5ml) dropwise at room temperature followed by dry triethylamine (0.4ml, 2.81mmol). The reaction mixture was stirred at room temperature for 12hrs. The reaction mixture was diluted with dichloromethane (25ml), washed with water (2 x 50ml), saturated sodium chloride (1 x 25ml), dried over magnesium sulfate, filtered and concentrated *in vacuo* to give brown oil (0.74gm). This was found to be (2'-pyridyldithio)propionyl diethyl-3-fluoro-3-phosphono-2-propenic acid ester (71), a hydrogen fluoride elimination product of (2'-pyridyldithio)propionyl diethyl-3,3-difluoro-3-phosphonopropionic acid ester (70). The compound 71 was fully characterized. IR (neat): 2985, 1732, 1653, 1574, 1447, 1418, 1314, 1103, 1018, 1270, 993 and 818 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 1.35 (6H, t,

$J = 7.05\text{Hz}$, $2 \times \text{CH}_3\text{CH}_2$), 3.05 (2H, t, $J = 6.5\text{Hz}$, CH_2S), 4.14-4.23 (4H, t, $2 \times \text{CH}_2\text{O}$), 6.16 (1H, dd, $J_{\text{FP}} = 35\text{Hz}$, $J_{\text{FH}} = 8.6\text{Hz}$, CHCFP), 7.06 (1H, m, 5'-H), 7.60-7.64 (2H, m, 3'-H & 4'-H) and 8.45 (1H, m, 6'-H); $^{31}\text{PNMR}$ (121MHz, CDCl_3) δ : 2.72 (d, $J_{\text{PF}} = 97\text{Hz}$); $\text{MS}(\text{CI})$: 396 (M^++1 , 100%).

Diethyl (4,4-difluoro-1-hydroxybutyl)phosphonate (82): To a solution of diethyl (4,4-difluoro-1-butenyl)phosphonate (79) (13.68gm, 60mmol) in dry hexane (20ml) was added a solution of borane-dimethylsulfide complex (2M in tetrahydrofuran, 11.0ml, 22.0mmol) dropwise at 0°C (ice-bath) under argon, the mixture solution became slightly brown color. The reaction mixture was stirred at room temperature 3hrs and to that mixture was added ethanol (20ml) followed by 3N sodium hydroxide solution (6.6ml). The mixture was cooled again to 0°C , then hydrogen peroxide (30% aqueous solution, 7.4ml) was added dropwise in such a rate that reaction temperature did not go above 35°C . After complete addition of hydrogen peroxide, the cooling bath was removed and the mixture was heated to gentle reflux for 4hrs. The reaction mixture was then poured into ice-water (240ml). The volatile organic solvents from the reaction mixture were removed *in vacuo*, to that aqueous residue was added water (100ml), and then extracted with ether (3 x 40ml), washed with water (1 x 25ml), saturated sodium chloride (1 x 50ml), dried over anhydrous potassium carbonate, filtered and concentrated *in vacuo* to give an oil (10gm, 68%). Distillation of the crude oil under high vacuum afforded pure colorless oil (7.0gm), bp. 115°C at 0.10mmHg; $R_f = 0.46$ (ethyl acetate:hexane, 1:1); IR (neat): 3445, 2986, 2938, 1447, 1371, 1263, 1165, 1023, 817, 756 and 600 cm^{-1} ; $^1\text{HNMR}$ (400MHz, CDCl_3) δ : 1.35 (6H, t, $J = 7.0\text{Hz}$, $2 \times \text{CH}_3\text{CH}_2$), 1.81 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 2.13 (3H, m, CH_2CF_2 and OH), 3.66 (2H, t, $J = 6.25\text{Hz}$, CH_2OH) and 4.24 (4H, q, $J = 6.87\text{Hz}$, $2 \times \text{CH}_3\text{CH}_2\text{O}$); $^{31}\text{PNMR}$ (121MHz, CDCl_3) δ : 10.375 (t, $J = 109\text{Hz}$); $^{19}\text{FNMR}$ (188MHz, CDCl_3) δ : 113.25 (dt, $J_{\text{FP}} = 109.6\text{Hz}$ & $J_{\text{FH}} = 19.5\text{Hz}$).

Diethyl-4,4-difluoro-4-phosphonobutyl (2'-pyridyldithio)propionic acid ester (72): To a stirred solution of 3-(2'-pyridyldithio)propionic acid (36) (215mg, 1.0mmol) in dry dichloromethane (5ml) was added 1-hydroxybenzotriazole (HOBT) (148.5mg, 1.1mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (288mg, 1.5mmol) at room temperature. After stirring for 5min, to the resulting yellow mixture was added a solution of diethyl (4,4-difluoro-1-hydroxybutyl)phosphonate (82) (492mg, 2.0mmol) in dry dichloromethane (2ml) dropwise at room temperature, and

stirred overnight. After 20hrs, the reaction mixture was diluted with dichloromethane (15ml) and then washed with 0.1NHCl (2 x 10ml), saturated sodium chloride (2 x 10ml), dried over sodium sulfate, filtered and concentrated *in vacuo* to give yellow oil (480mg). The flash chromatography of the crude oil in silica gel using ethyl acetate:hexane, 1:1 then 3:1, finally ethyl acetate to afford pure compound (150mg, 34%), single spot in tlc, $R_f = 0.24$ (ethyl acetate:hexane, 1:1). IR (neat): 2988, 2915, 1715, 1555, 1434, 1390, 1255, 1164, 1095, 1034, 799 and 766 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 1.33 (6H, t, $J = 7.06\text{Hz}$, 2 x $\text{CH}_3\text{CH}_2\text{O}$), 1.90 (2H, quintet, $J = 6.91\text{Hz}$, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CF}_2$), 2.09 (2H, m, $\text{CH}_2\text{CH}_2\text{CF}_2$), 2.74 (2H, t, $J = 7.06\text{Hz}$, $\text{C}(\text{O})\text{CH}_2\text{CH}_2\text{S}$), 3.01 (2H, t, $J = 7.06\text{Hz}$, $\text{C}(\text{O})\text{CH}_2\text{CH}_2\text{S}$), 4.11 (2H, t, $J = 6.35\text{Hz}$, $\text{CH}_2\text{CH}_2\text{O}$), 4.24 (4H, q, $\text{CH}_3\text{CH}_2\text{O}$), 7.08 (1H, m, 3'-H-pyridine), 7.63 (2H, m, 4'-H & 5'-H-pyridine), 8.44 (1H, d, 6'-H-pyridine); ^{31}P NMR (121MHz, CDCl_3) δ : 8.22 (triplet, $J_{\text{P-F}} = 109.15\text{Hz}$, PCF_2); ^{19}F NMR (188MHz, CDCl_3) δ : 30.48 & 31.06 (dt, $J_{\text{F-P}} = 19.33\text{Hz}$ & $J_{\text{F-P}} = 108.5\text{Hz}$, PCE_2CH_2); MS(CI): 444 (M^++1 , 100%).

Diethyl phosphono-5,5-difluoro-4-oxopentanoic acid (85): To a solution of lithium diisopropylamide (LDA) (1.5M in cyclohexane, 3.63ml, 3.25mmol) in dry tetrahydrofuran (5ml) was added a solution of diethyl (α,α -difluoromethyl)phosphonate (83) (611mg, 3.25mmol) in dry tetrahydrofuran (5ml) at -78°C under argon. The mixture was stirred at -78°C for 30min, and then to the resulting lithium derivative of diethyl (α,α -difluoromethyl)phosphonate was added a solution of succinic anhydride (84) (325mg, 3.25mmol) in dry tetrahydrofuran (10ml). The reaction mixture was stirred at -78°C for 2hrs and then at room temperature overnight. After 18hrs, the volatile solvents from the reaction mixture were removed by blowing argon through the flask to get a yellowish-white salt which was dissolved in partially saturated sodium chloride (15ml), and then extracted with ethyl acetate (3 x 50ml), washed with saturated sodium chloride, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to give yellow oil (260gm). The flash chromatography of the crude oil in silica gel using ethyl acetate:hexane (1:1) afforded an oil (170mg, 52%). IR (neat): 2986, 2935, 1737, 1604, 1397, 1273, 1030 and 798 cm^{-1} ; ^1H NMR (400MHz, CDCl_3) δ : 1.21-1.38 (6H, m, 2 x CH_3CH_2), 2.65 (4H, br m, $\text{CH}_3\text{CH}_2\text{COOH}$), 4.04-4.16 (4H, m, 2 x OCH_2CH_3). ^{31}P NMR (121MHz, CDCl_3) δ : 3.489 (triplet, $J_{\text{FP}} = 97.0\text{Hz}$); ^{19}F NMR (188MHz, CDCl_3) δ : -116.3 (doublet, $J_{\text{FP}} = 96.8\text{Hz}$).

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