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SYNTHESIS OF CONJUGATES OF OLIGOPEPTIDES AND ANTIMETABOLITES

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SYNTHESIS OF CONJUGATES OF OLIGOPEPTIDES AND ANTIMETABOLITES

BY

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ABSTRACT

Advisor: Professor Robert Engel

The present thesis program has had as its purpose the synthesis of phosphonic acid analogues of natural phosphates bound to oligopeptides. The purpose of oligopeptide is to enable transport of potential agents that might serve as drugs for metabolic regulation via the oligopeptide transport system. This was to be accomplished by the attachement of the isosteric phosphonic acid analogue, i.e., 3,4-dihydroxybutyl-1-phosphonic acid (DHBP) and 4-hydroxy-3-oxobutyl-1-phosphonic acid ($DHAP_a$) to the β-carboxyl group of the aspartyl unit in the tripeptide aspartylalanylalanine and alanylaspartylalanine. In addition, the conjugation of the isosteric phosphonic acid analogue of phosphomevalonate, 5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonic acid and nalidixic acid with alanylserylalanine and the conjugation of sulfanilamide and other drugs to aspartylalanylalanine were performed.

The studies on peptide transport in barley have shown that peptides may be of considerable importance in certain plant systems and considering these studies, we have tried to use aspartylalanylalanine in the transport of certain antimetabolites into fruit plants.

Initially, the potential for *in vivo* antimetabolic activity for dihydroxyacetone phosphate was not promising

due to the lack of transport mechanism for the natural material. However, *in vivo* activity has been accomplished by the coupling of 3,4-dihydroxybutyl-1-phosphonic acid and 4-hydroxy-3-oxobutyl-1-phosphonic acid with the aspartylalanylalanine.

ABBREVIATIONS

AcOH	Acetic Acid
Ac_2O	Acetic Anhydride
Ala	Alanine
Asc	Aminosuccinyl
Asp	Aspartic Acid
BOC	tert-Butyloxycarbonyl
t-Bu	tert-Butyl
DCC	N,N'-Dicyclohexylcarbodiimide
DCU	N,N'-Dicyclohexylurea
DMF	Dimethylformamide
DMAP	4-Dimethylaminopyridine
Gly	Glycine
IBC	Isobutylchloroformate
IR	Infrared
Leu	Leucine
MA	Mixed Anhydride
NMR	Nuclear Magnetic Resonance
PCP	Pentachlorophenyl
Ser	Serine
Sar	Sarcosyl
sn	Stereospecific numbering
TEA	Triethylamine
TFA	Trifluoroacetic Acid
Z	Carbobenzoxy Group

LIST OF FIGURES & SCHEMES

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my advisor, Dr. Robert R. Engel, for his support and guidance throughout this project. His patience, understanding and unwavering confidence in me made this thesis a reality.

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Special thanks to Professor David C. Locke who has been a great help throughout this program and who was never too busy to sit down and help with my difficulties.

I would also like to thank to Dr. Daniel Swerdlik for his help in computer language for typing this thesis.

To my best friend, my wife Shirin,
for her support, encouragement and love,

To Amina and Haris,
for the times I didn't play,

To my wonderful parents, Tasleem and Aziza,
for their love and belief,
who always said that I could ...

This thesis is dedicated.

TABLE OF CONTENTS

INTRODUCTION

Phosphonic acids and their derivatives, which are structurally related to the naturally occurring phosphates have been given considerable attention during the last twenty years. This attention has been generated by the recognition that phosphonic acids and their derivatives have the potential to function as analogues of naturally occurring phosphates. In particular, they possess a potential to serve as metabolic regulators and drugs. Phosphonic acid analogues are important probes for studying intermediary metabolism; unfortunately, as many are charged organic compounds, cannot enter cells. We therefore decided to search for vectors that would permit these analogues to go into cells.

In consideration of the use of phosphonic acids as analogues of natural phosphates, it is presumed that the carbon-phosphorus bond is incapable of being hydrolysed by the 'ordinary' enzymes involved in phosphate cleavage. With this presumption, several mechanistic possibilities may be envisioned for metabolic regulation by compounds bearing such a linkage. This is of particular interest in light of the fact that the presence of a carbon-phosphorus linkage does not preclude enzymatic cleavage of other phosphate ester linkages which might also be present (1).

In the design of an analogue of a natural metabolite, it is desirable that the analogue bear only one structural variation from the parent compound. The structural variation considered here is the presence of a carbon-phosphorus linkage in place of the normal phosphate ester linkage. There is a good reason to believe that such phosphonic acid analogues of natural phosphates might serve as very useful probes for studying metabolic regulation.

As a substitute for a natural phosphate metabolite, a phosphonic acid or phosphonate may be capable of inhibiting or perturbing the regular metabolism of a biological system simply by non-participation in a normal phosphate ester cleavage process. Also, the phosphonic acid, substituting for a natural metabolite in its entrance to an organism might be capable of specific or nonspecific inhibition of one or more enzymatic processes. In this way, the use of phosphonic acids as analogues of natural phosphate would seem to be a reasonable approach to metabolic problems (2).

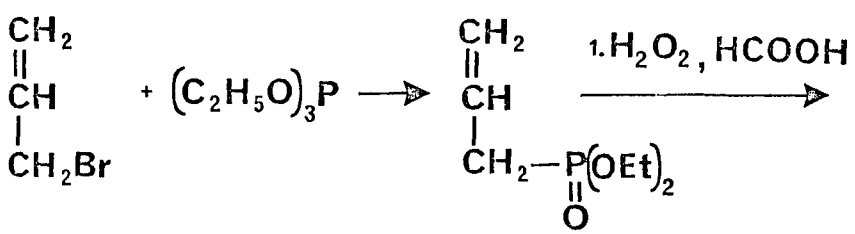
Analogues, both isosteric and nonisosteric for almost all simple phosphorus-containing products and by-products of carbohydrate degradation have been synthesized and investigated. The applicability of the term 'isosteric' to analogues and natural compounds discussed herein is reasonable as they are almost identical in size and shape. This has been demonstrated by the use of available crystallographic data illustrating that the distances between the phosphoryl oxygen and other positions for the natural

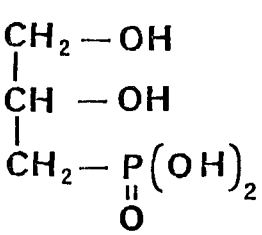
compounds in comparison to the nominally isosteric phosphonic acid analogue vary by less than 1 percent. Suitable data of this type has been obtained for the related compounds, 2-aminoethyl phosphate (3,4) and 2-aminoethyl phosphonic acid (5) as well as for other simple phosphate esters (6-8).

Glycerol-3-phosphate provides one of the most interesting species for investigation of intermediary metabolism. Scheme I illustrates that sn-glycerol-3-phosphate is on the branch point between phosphoglyceride and carbohydrate metabolism.

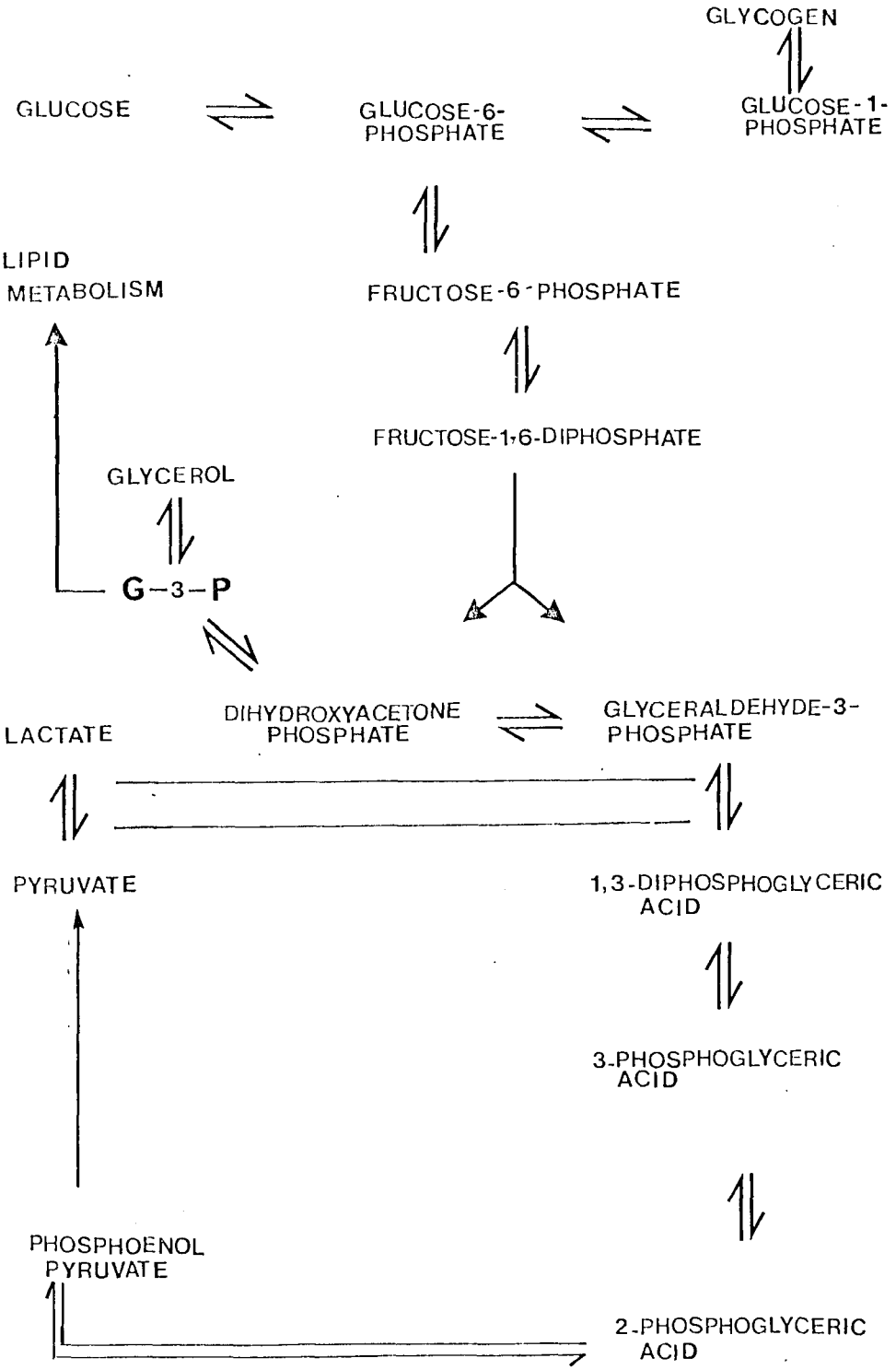
If one were to apply analogues of glycerol-3-phosphate or related compounds in an attempt to regulate the metabolism of an organism, one might anticipate either pathway to be influenced.

Rosenthal and Geyer (9) first synthesized 2,3-dihydroxypropyl-1-phosphonic acid (I) as a nonisosteric analogue of glycerol-3-phosphate by an Arbuzov reaction on allyl bromide





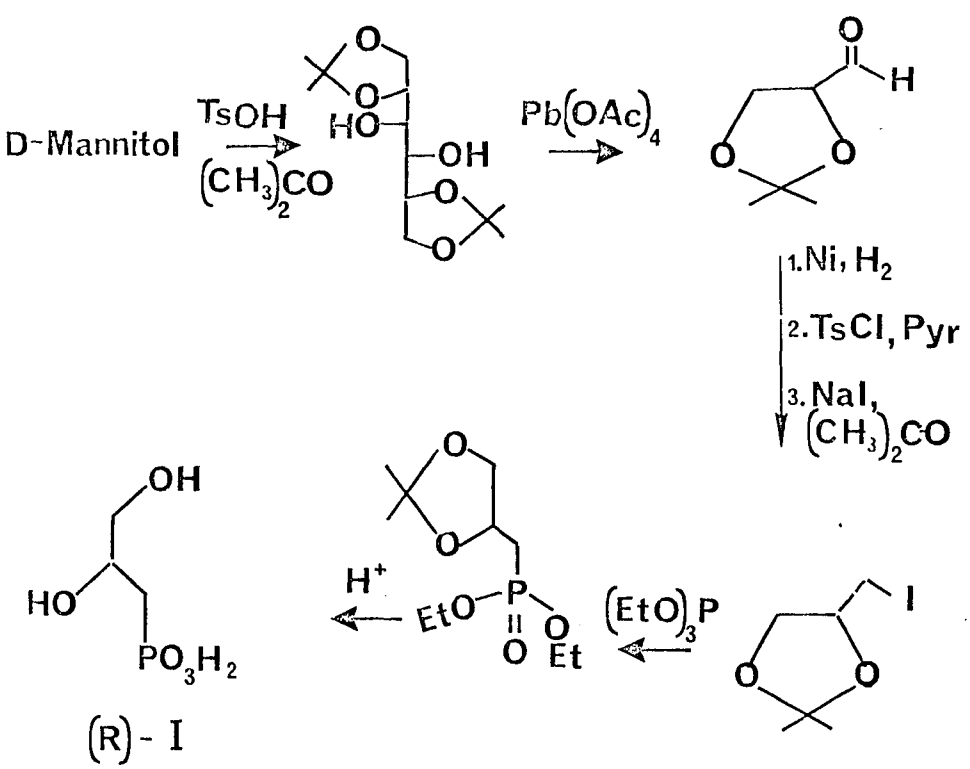
(I)



SCHEME I

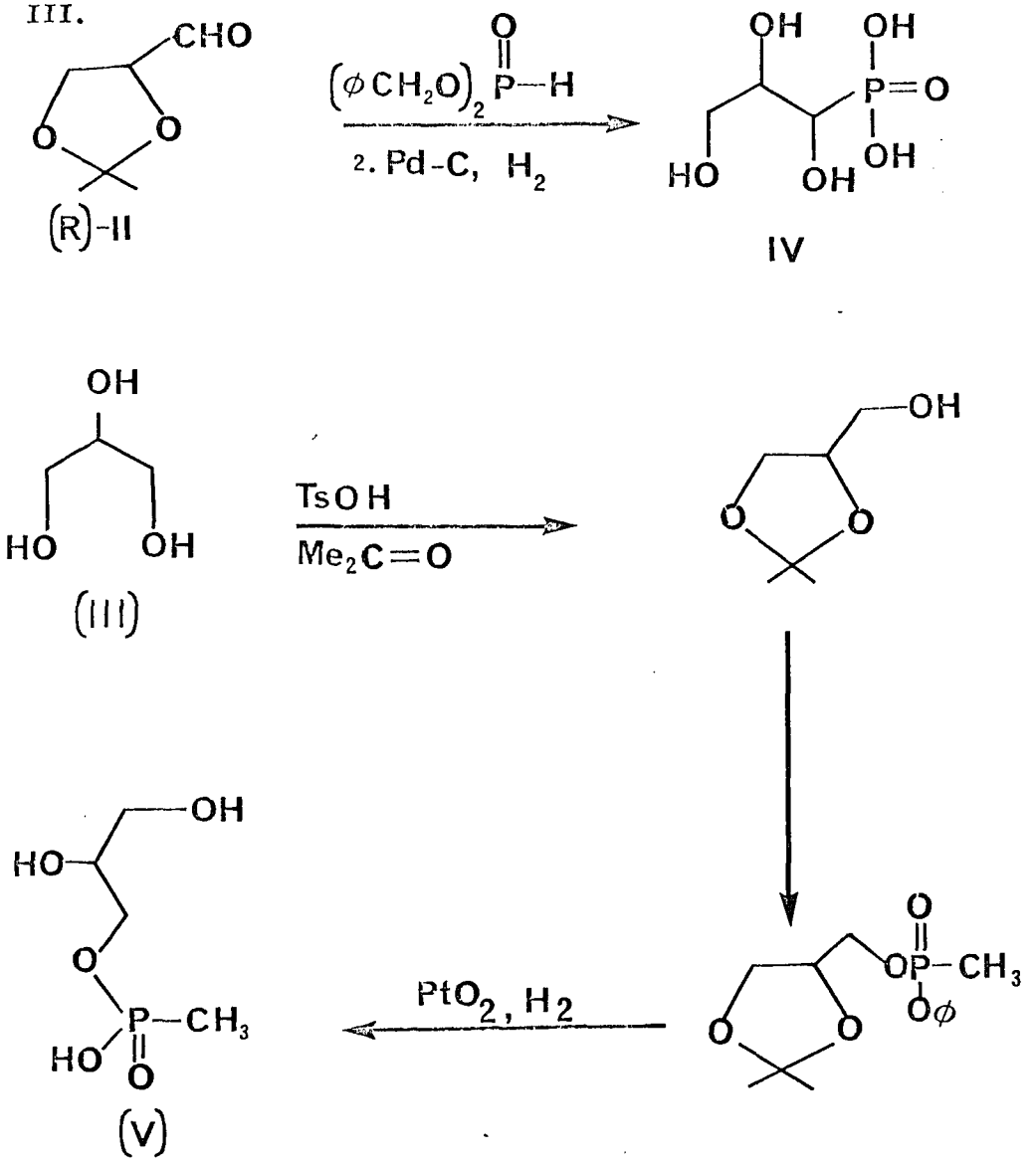
followed by hydoxylation and ester hydrolysis. It should be noted that the dilithium salts of this and subsequently synthesized phosphonic acids are convenient forms for purification due to their particular solubility properties, although this particular hydrolytic technique often presents difficulties.

The optically active form, R-(-)-2,3-dihydroxypropyl-1-phosphonic acid (R-(I)), bearing the same absolute configuration as natural sn-glycerol-3-phosphate about the internal hydroxyl was later reported by Baer and Basu (10). This route began with D-mannitol diacetonide and involved an Arbuzov reaction on the iodide generated after lead tetraacetate cleavage (Scheme II).



Scheme II

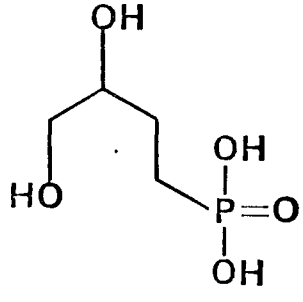
Two other nonisosteric analogues of glycerol-3-phosphate have also been reported (11). These are 1,2,3-trihydroxypropyl-1-phosphonic acid (IV) and the phosphonate (V), derived from compounds (II) and (III) respectively, Scheme III.



SCHEME III

Because of the relatively major structural changes (relative to the natural material) in a small molecule, it might be expected that these might exhibit significant deviation from the biochemical nature of glycerol-3-phosphate. Using I, Baer et al., (12) concluded that the ester oxygen was necessary for the activity. The racemic form of the isosteric analogue, 3,4-dihydroxybutyl-1-phosphonic acid (VI) was then reported by Kabak et al., (13), utilizing a route paralleling that of Rosenthal and Geyer (9) begining with 4-bromo-1-butene. This material was used by Shopsis et. al., (14-16) who demonstrated that it was capable of inhibiting the growth of mutant strains of Escherichia coli at rather low concentration.

The bacteriostatic (growth inhibited without cell death) effect appeared to be associated with perturbation of normal phospholipid metabolism (17-19). This effect derived from an inability of the organism to cleave the phosphonate linkage now present in place of

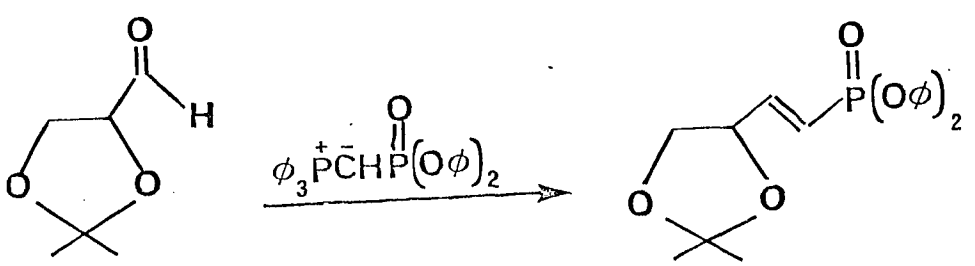


(VI)

an ordinary phosphate. This isosteric analogue substitutes for glycerol-3-phosphate in one stage of the normal metabolic process but a point of inhibition is reached as a result of the inability to cleave phosphate.

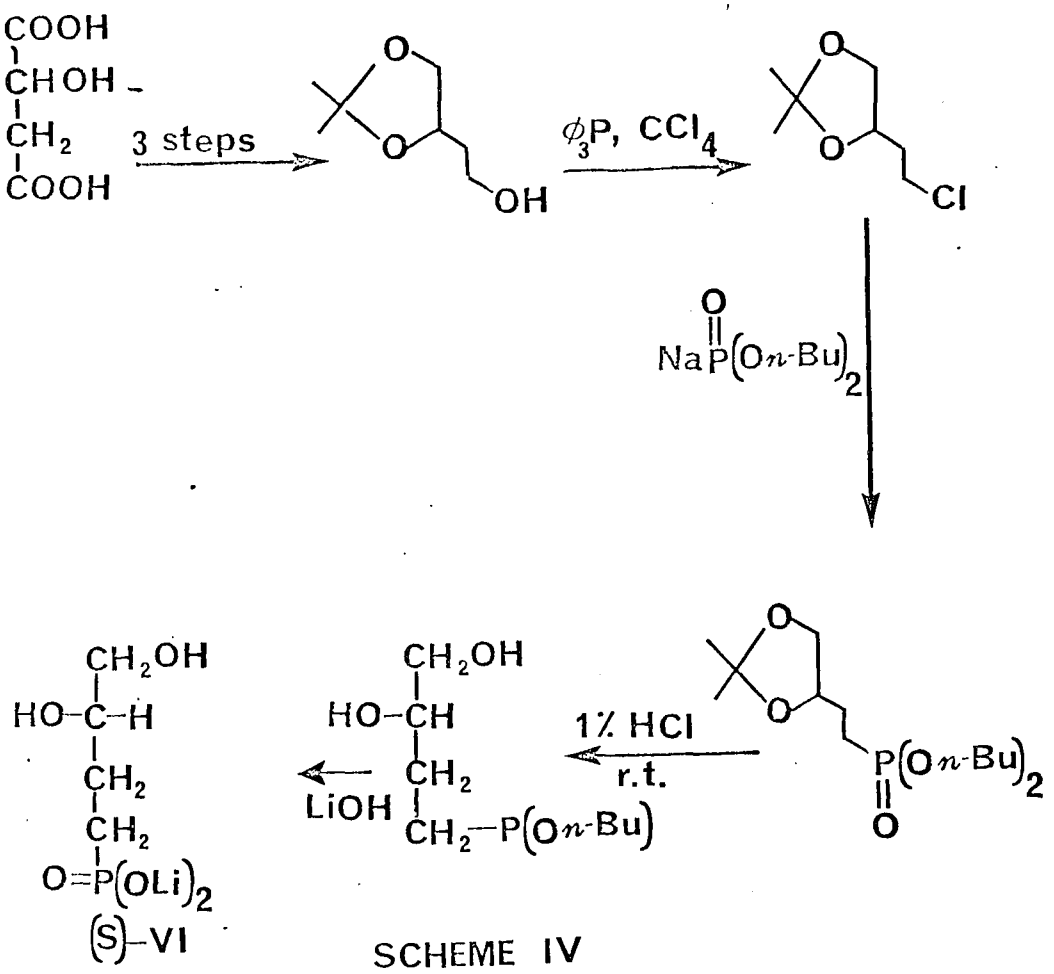
An important conclusion of this study is that for biological activity, there should be correspondence of size between the analogue and the natural substrate. Contrary to Baer's conclusion, the ester oxygen is not necessary for activity, but correspondence of the size with the natural substrate is. This result was also confirmed by Adams et al., (11) with the synthesis of optically active form of (VI) by a route begining with phosphonylation by a Wittig reaction on the aldehyde followed by hydrogenation.

Paulsen and Bartsch (20) reported a similar sequence for the synthesis of the racemate of (VI) using a Horner reaction of tetraethyl methylene diphosphonate on racemic (II). Phosphonate ester cleavage was accomplished using trimethylchlorosilane.



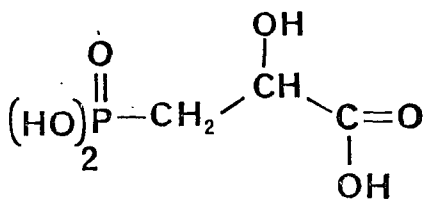
(R)-II (S)-VI

K.C. Tang et al., (21) reported the synthesis of the optically active form of (S)-(VI) starting with the commercially available optically active malic acid as illustrated in Scheme IV.

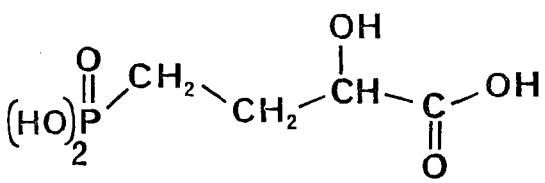


SCHEME IV

There are four synthesis of analogues of 3-phosphoglyceric acid which have been reported. Pfeiffer et al. (22) described both the nonisosteric and isosteric analogues (VII) and (VIII).

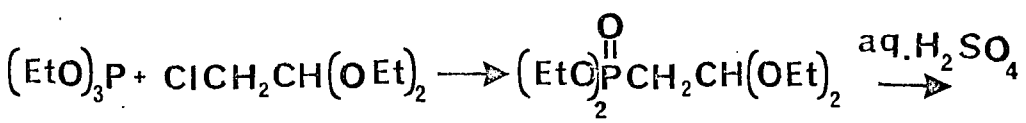


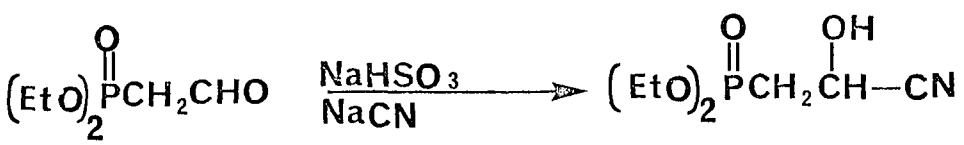
(VII)

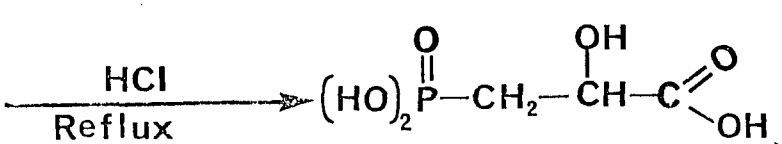


(VIII)

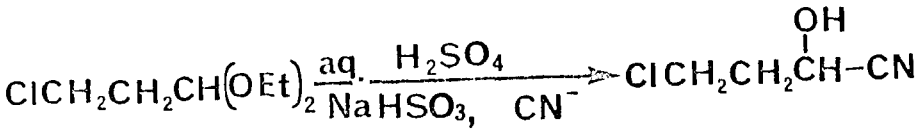
The synthesis involves C-P bond formation by an Arbuzov reaction followed by the hydrolysis of the cyanohydrin as follows:

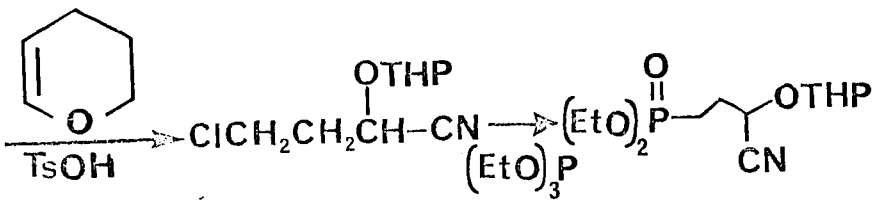


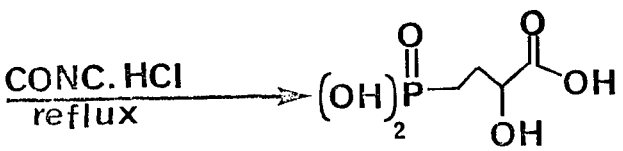




VII

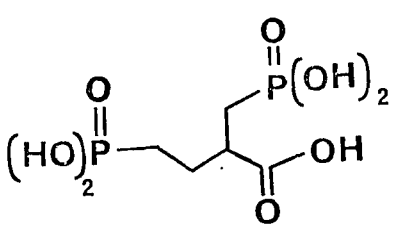






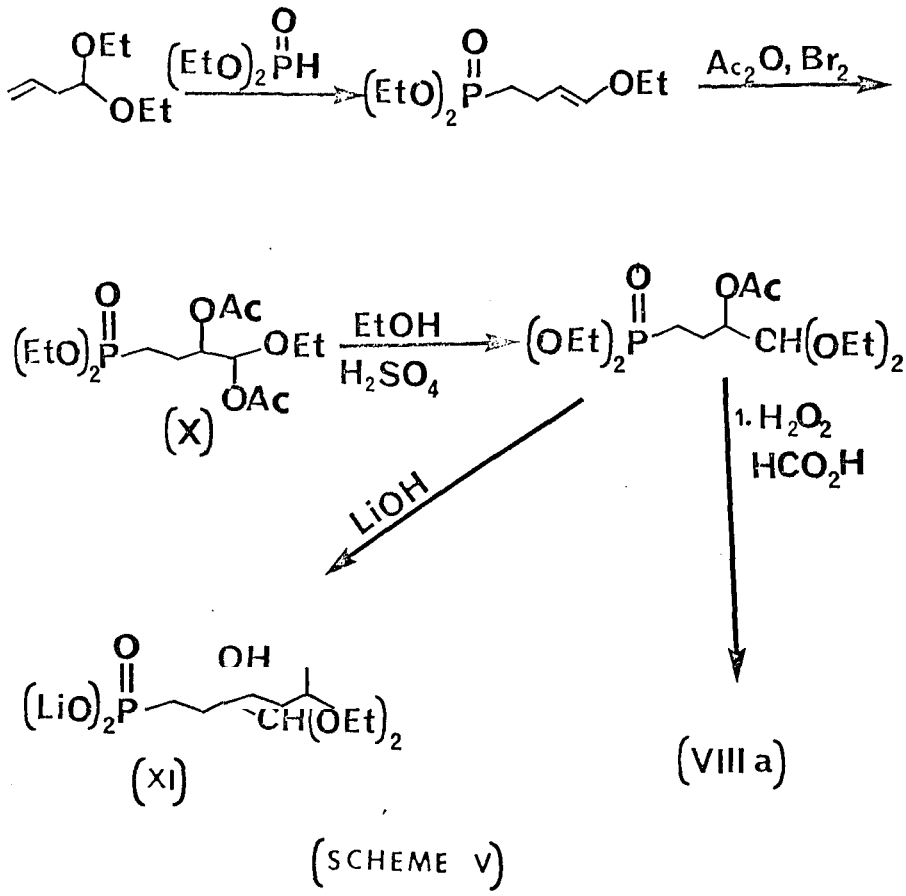
(VIII)

Also reported was the synthesis of the isosteric analogue (IX) of 2,3-diphosphoglyceric acid (22).



(IX)

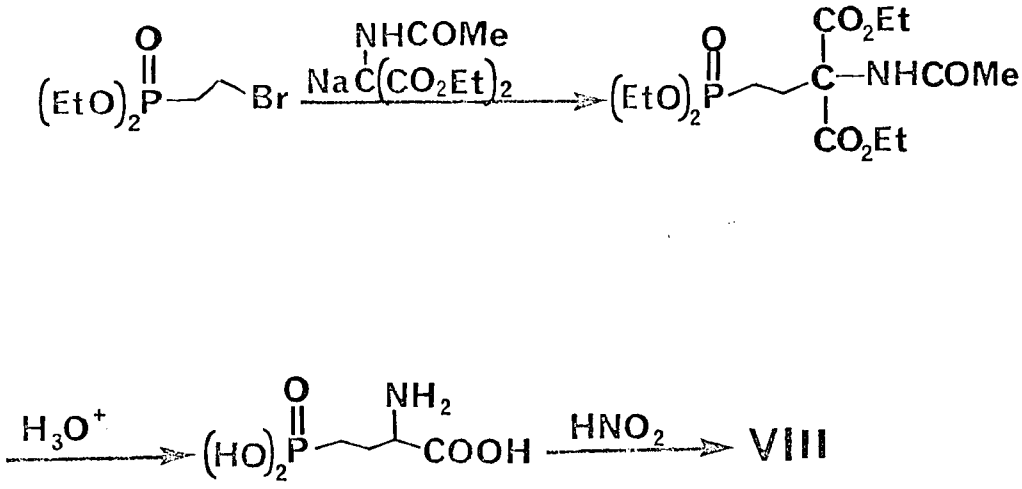
Goldstein et al. (23) reported the preparation of the trilithium salt of (VIII) by the oxidation of an intermediate (X) in the preparation of the analogue (XI) of glceraldehyde-3-phosphate (Scheme V).



(SCHEME V)

Dixon and Sparkes (24) reported a multistep route for the preparation of (VIII) from diethyl 2-bromoethyl phosphonate (Scheme VI).

Initial biochemical investigation (22) of (V) and (VI) indicated neither to have any effect on the binding capabilities of human red cells. However, it was later found (24) that (VI) was capable of replacing the natural material in oxidation of NADH. Orr and Knowles (25) observed it to be a substrate for phosphoglycerate kinase with a similar kinetic value (Km) to that of the natural material.

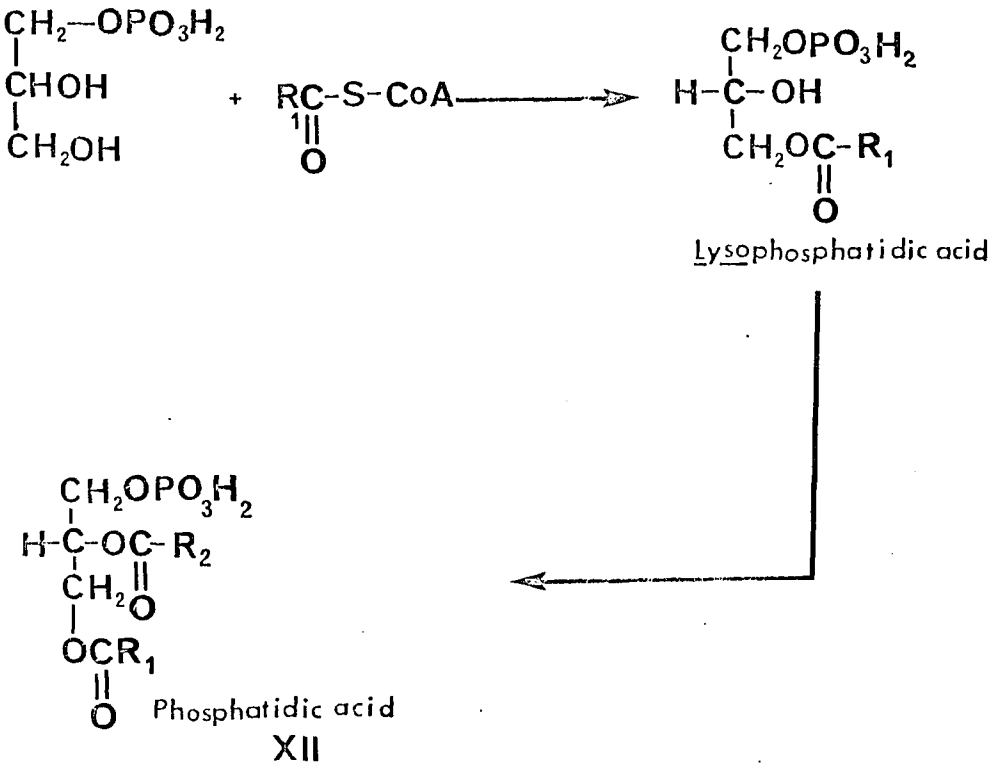


SCHEME VI

The phosphonic acid analogue of dihydroxyacetone phosphate, 4-hydroxy-3-oxybutyl-1-phosphonic acid is reduced by the anabolic glycerol-3-phosphate dehydrogenase of E. coli; unfortunately, this material does not have any effect on intact cells presumably as it cannot be transported into the intact cells.

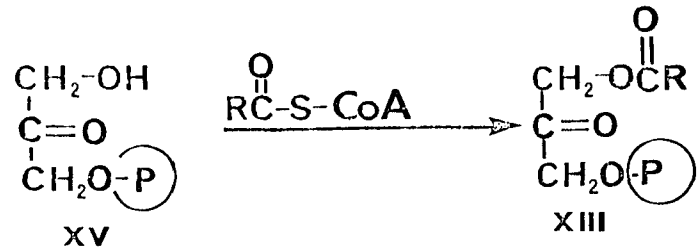
As described in Scheme I, dihydroxyacetone phosphate is converted to glycerol-3-phosphate which, in turn, leads in another direction, to lipid. Phosphatidic acid (XII) serves as an

intermediate in the synthesis of both triglycerides and phospholipids. This is formed from sn-glycerol-3-phosphate and the coenzyme A derivatives of fatty acids.

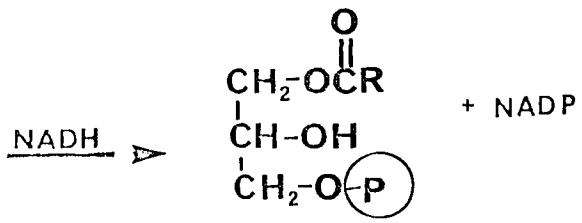


XII

In addition to the above pathway for the formation of (XII), acylation of dihydroxyacetone phosphate (XV) occurs in liver and the product is subsequently reduced in the presence of NADH: to give lysophosphatidic acid (XVI).

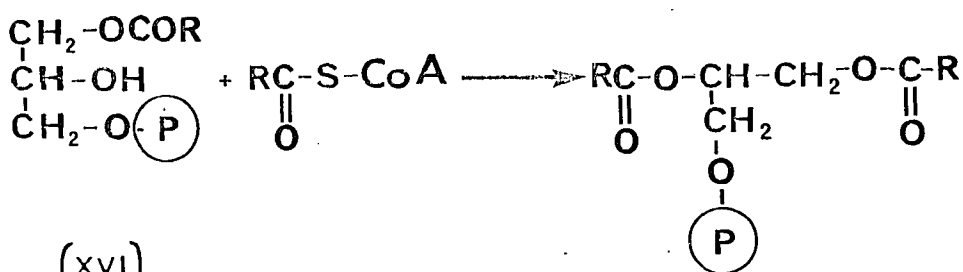


XV XIII



XVI

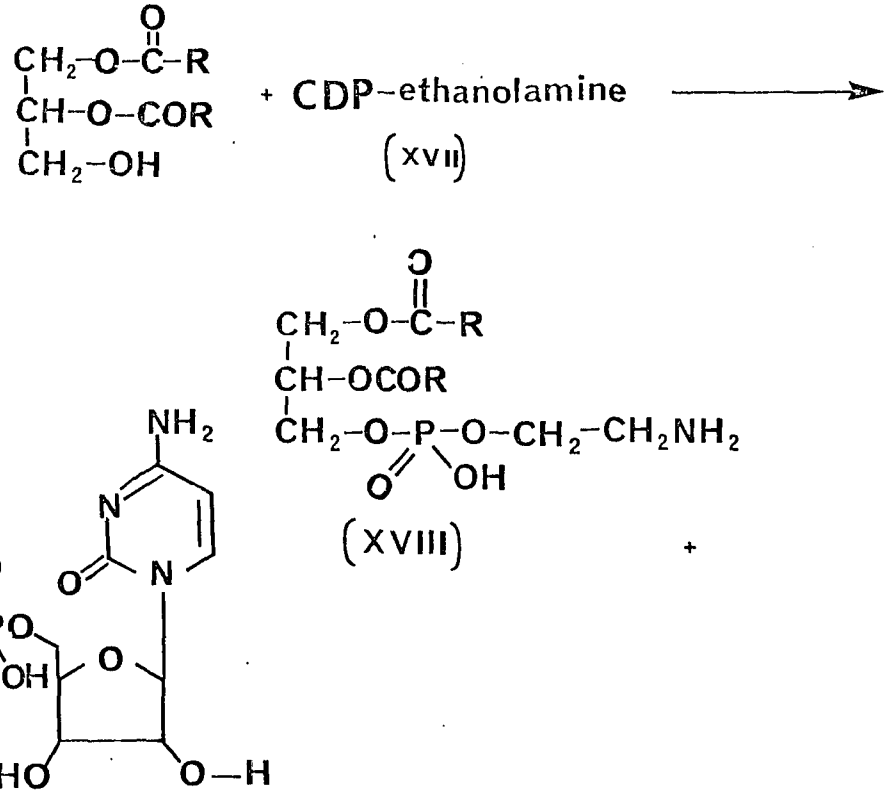
The transacylase is found in both mitochondria and microsomes and is specific for unsaturated fatty acids. Reduction of acyl dihydroxyacetone phosphate (XIII) is accomplished by a microsomal enzyme which uses an unsaturated fatty acyl CoA yielding (XIV).



(XVI) (XIV)

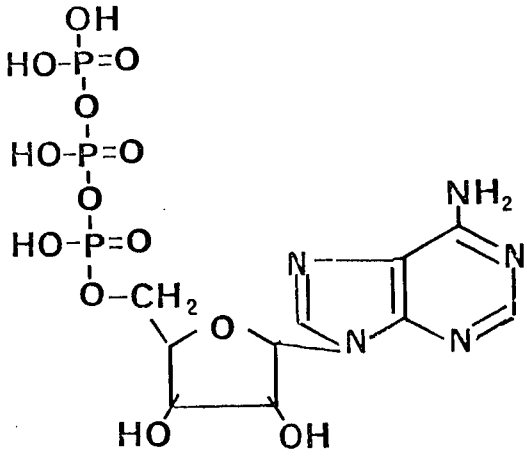
The formation of other phospholipids is subsequent to that of phosphatidic acid. There are two pathways for the synthesis of phospholipids from phosphatidic acid, one of which occurs almost exclusively in plants and micro-organisms. Both of them involve activation by cytidine triphosphate (CTP).

In the first pathway, which occurs chiefly in animals, the base ethanolamine or choline that is to be inserted into the phospholipid is phosphorylated. The phosphoryl derivative then reacts with CTP to give the corresponding CDP derivative. The CDP-ethanolamine (XVII) or CDP-choline (XXI) can now react with a diglyceride to form the corresponding phosphatidyl derivative such as (XVIII) and cytidine monophosphate (CMP, XXII).

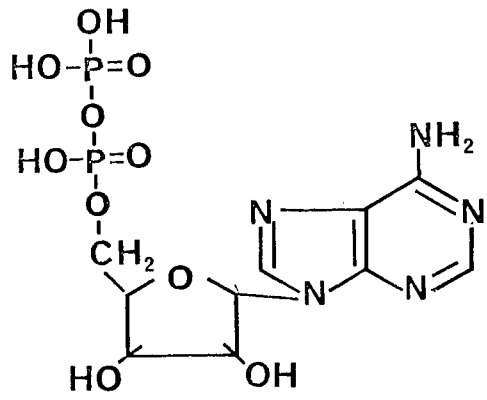


CMP

(XXII)



ATP + $NH_2CH_2CH_2OH$ ⟶

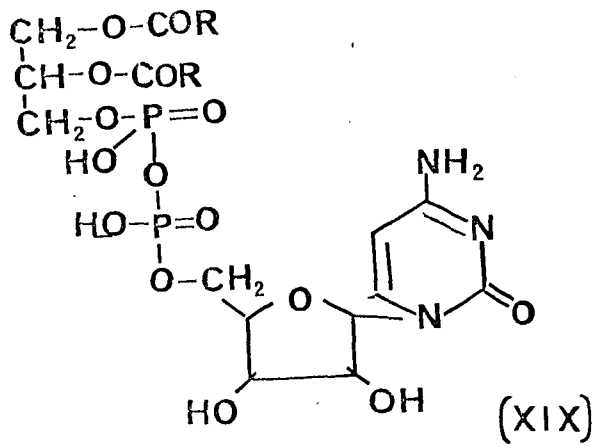


ADP

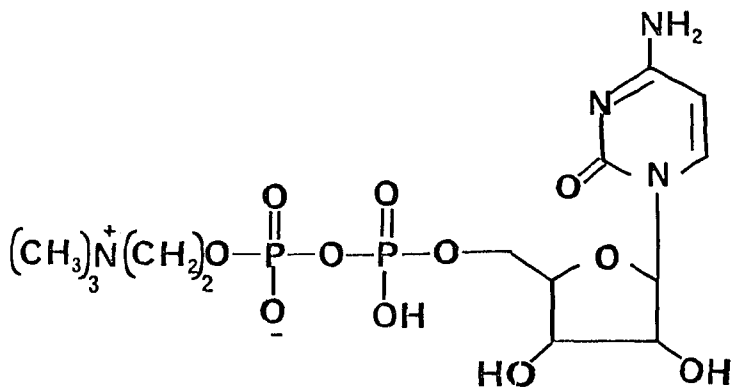
$NH_2CH_2CH_2O$-(P) +

CTP + $NH_2CH_2CH_2O$-(P) ⟶ CDP + PP_i

In the alternative pathway of synthesis of phospholipids, CTP is involved in activation of the phosphatidic acid (XII). The compound thus formed is CDP-diglyceride (XIX), a compound believed to be involved in the inhibitory route of 3,4-dihydroxbutyl-1-phosphonic acid in the studies (15-18) of E. coli in vivo and in vitro.

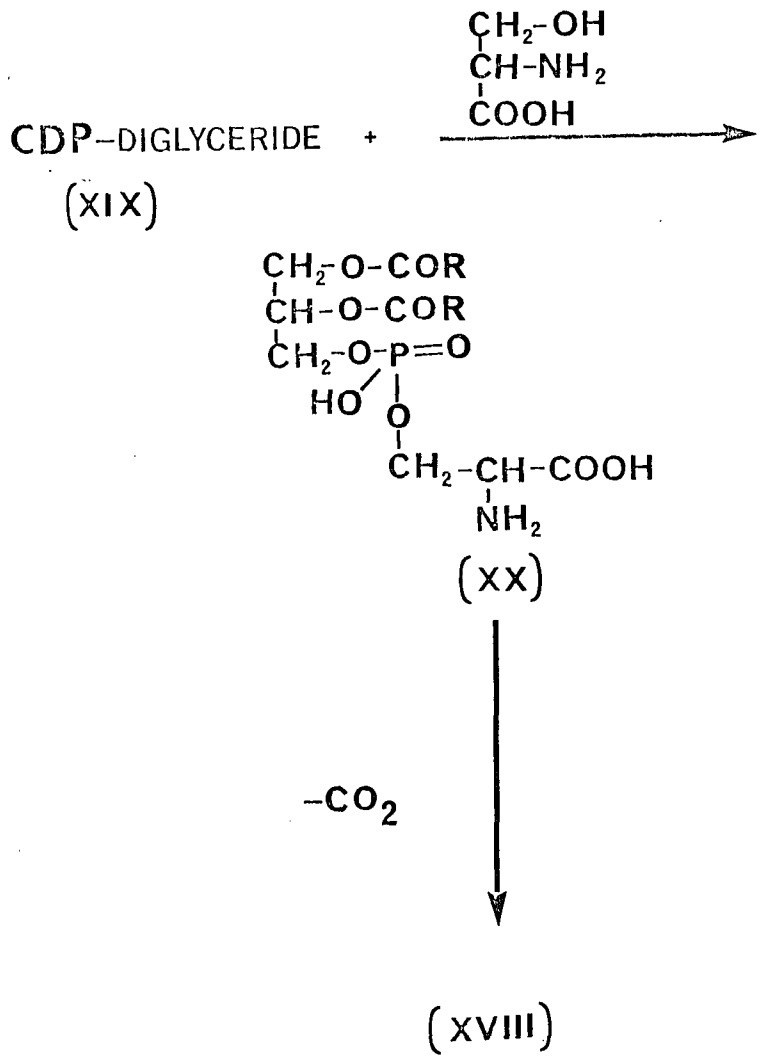


(XIX)



(XXI)

CDP-diglyceride can now react with serine in an enzymatic process to give phosphatidyl serine (XX), which generates phosphatidyl ethanolamine (XVIII) by decarboxylation.



Baer introduced the general term <u>phosphonolipids</u> for analogues of phospholipids. One may consider here two main categories of phosphonolipids; one with structural changes in the glycerol portion (a C-P bond being present instead of the glycerol ester

oxygen) and the other related to aminoethyl phosphonic acid (a C-P bond being present instead of the esteric oxygen of the head group). It should be noted that this latter category constitutes a 'natural' system found in numerous organisms (26-33). As a substitute for sn-glycerol-3-phosphate, (S)-3,4-dihydroxybutyl-1-phosphonic acid (VI) is one of the primary isosteric species in reaction involving L-glycerol-3-phosphate:NAD oxidoreductase. The syntheses of this material have been developed, generating it in racemic, chiral and specifically labelled forms.

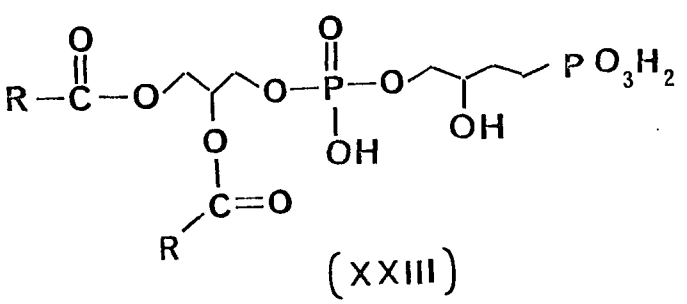
The compound, 3,4-dihydroxybutyl-1-phosphonic acid (VI) was of particular interest as bacterial species were readily available which actively transported and used the natural phosphate. Thus there was a significant possibility that (VI) might be transported and would be of use in vivo. In fact the enantiomer of (VI) corresponding in absolute configuaration to sn-glycerol-3-phosphate is transported into a variety of bacterial species and its transport properties have now been thoroughly studied in E. coli (34,35). It is also noteworthy that its antipode does not appear to be transported (36). Thus it would appear that the transport of (VI) correlates well with that of the natural material.

For those species which transport (VI), growth inhibition is observed, a characteristic not enjoyed by the non-isosteric species. At low external concentration of (VI) suitable strains of

E. coli undergo significant perturbation of phospholipid synthesis and growth stasis (13,15,16,37). The mode of antimetabolic activity of (VI) in E. coli has been noted by both in vivo and in vitro efforts to involve perturbation of phosphatidylglycerol synthesis (38,39): (VI) replaces sn-glycerol-3-phosphate in reaction mediated by CDP-diglyceride; sn-glycerol-3-phosphate phosphatidyltransferase and thus generates a polar lipid material (XXIII) which is incapable of undergoing phosphate cleavage and thus precludes phosphatidylglycerol synthesis. As a result it seriously perturbs the normal lipid composition.

Moreover, at low concentration the synthesis of lipoprotein is strongly inhibited (40).

The material (VI) has also been noted to be active in the inhibition of growth of strains of B. subtilis. A major difficulty in working with phosphonic acid analogues of natural compounds is the inability of many of these charged compounds to enter the cell. In fact, the impermeability of intact cells is a frequent problem in biochemistry. When there is no transport system for a compound,



(XXIII)

the cell membrane is a formidable barrier. For charged compounds, positive diffusion seldom produces the desired internal concentration and the metabolism of these compounds must be studied in a cell-free extract.

Prominent among techniques used to circumvent the cell barrier is chemical modification of the compound under study in the hope that it will then be soluble in the cell membrane. One successful example is the use of N(6)-2'-O-dibutyryl cyclic AMP in place of cyclic AMP in both prokaryotic and eukaryotic systems (41). Another approach is to link the non-penetrating substances covalently to a molecule that is actually transported by a pre-existing transport system; an obvious advantage of using an active transport system is that high internal concentrations of the material can be reached very quickly and secondly, such a method maintains to the greatest degree possible the structural integrity of the substance. In addition, the extreme nature of masking the charges of anionic or cationic compounds need not be used, as molecules for which there exists a transport system need not be soluble in the cell membrane to reach the cytoplasm. It is known that due to special transport systems, bacteria are capable of taking up nutrients even from very dilute solutions, resulting in an accumulation of solutes inside the cell. Likewise, the bactericidal effect of various antibiotics can be obtained at extremely low concentration of the drug. In some cases the minimal

inhibitory concentration is found to be lower *in vivo* than *in vitro* (42). It is believed that certain antibiotics are capable of 'misusing' existing transport system(s) which the bacterium needs to take up various nutrients. This raises the question of which transport system(s) can be used by which drug(s).

For several years, Gilvarg has been concerned with the utilization of oligopeptides by *Escherichia coli* and has delineated the structural features of a peptide substrate which govern its transportability. It is a requirement of the transport system that the N-terminal α-amino group of a peptide be unacylated. This was shown by performing comparative studies on the growth response of *E. coli* lysine auxotroph to lysine (43,44), oligolysine peptides and α-N-acetyl-derivatives of oligolysine. It was observed that the lysine auxotroph could use di-, tri-, and tetralysine as source of lysine, while α-N-acetylated derivatives were inactive. This conclusion was further substantiated by the results with acetylated arginine oligopeptides. The importance of the C-terminal carboxyl group in peptide transport was also studied by Gilvarg and Payne (45,46). They concluded that the C-terminal carboxyl group is not necessary for the uptake of oligopeptide. To this end, a series of peptides without the free carboxyl group were synthesized. The compounds prepared were lysylcadaverine peptides. Cadaverine, which is a diamine obtained on decarboxylation of lysine, comprised only the C-terminal residue in these peptides: all other residues

were lysine. All these analogues were able to enter E. coli as shown by their capacity to support the growth of lysine auxotroph. The oligopeptide system, while showing preferences for all L-forms, seems able to tolerate a degree of 'steric wobble' with respect to the third and presumably later amino acid residues relative to the N-terminus. Studies with E. coli indicate that this organism possesses an accessory permeability barrier that excludes peptides with a diffusion radius greater than a certain critical value. This feature is reflected in the inability of the higher member of homologous peptides series to enter the organism. Some of the structural specifications for oligopeptides transport in E. coli are shown in diagramatic form in Fig.1 (47).

A striking finding was that a permease is capable of transporting peptides of widely varying amino-acid components and size (47). The non-stringent nature of oligopeptide permease suggests that it would be an ideal candidate for carrying out the transport of normally impermeant compounds.

A particularly suitable test substance should be an amino acid which alone could not reach the cytoplasm, but which could be linked covalently to a potentially transporting peptide. To prove that the peptide carried such an amino-acid into the cell it would be highly desirable that the amino-acid be involved in a normal metabolic scheme so that a mutant unable to synthesize the amino-acid could be isolated and tested for its growth response to

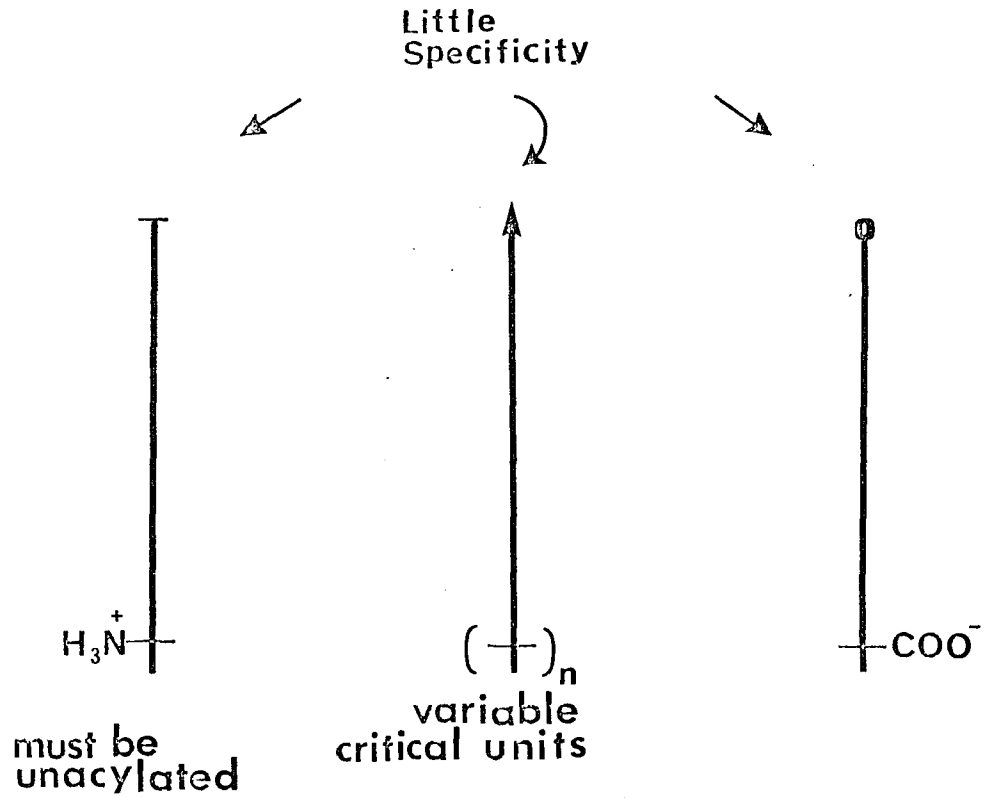


FIG. 1

the peptide. Gilvarg and Fickel (48) showed transport of a threonine precursor, homoserine phosphate into E. coli using the oligopeptide permease as lysyl-lysyl-homoserine phosphate.

Ames et al., (49) have shown the existence of oligopeptide permease in Salmonella typhimurium. They used this transport system for smuggling a histidine biosynthetic intermediate, histidinol phosphate ester into the bacterium as its glycylglycyl derivative, gly-gly-histidinol phosphate. Free histidinol phosphate ester is not transported into Salmonella.

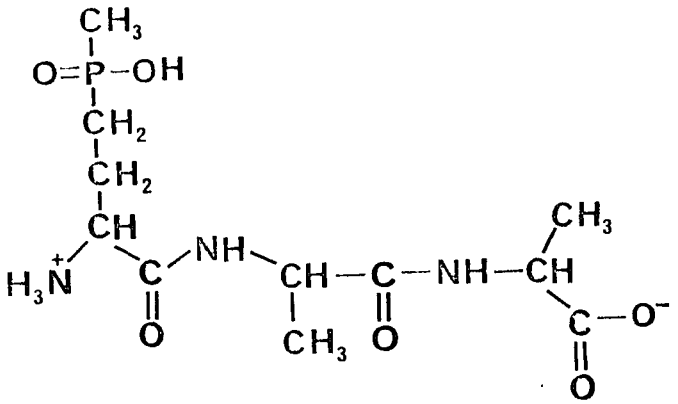
The transport of small peptides is found in mammalian systems as well. The first reports of peptide transport in mammals were those of Newey and Smyth (50-53) who demonstrated the uptake of peptides by, and hydrolysis to amino-acid within, the mucosal cells of the small intestine. Subsequent studies by Craft (54), Sadikali (55) and Matthews (56,57) confirmed and extended this observation. They investigated the absorption of glycine and glycylglycine in gastrointestinal disease in man. Their results indicated that a given dose of glycine was absorbed more rapidly from glycylglycine than from the free amino-acid, and most rapidly when given in the form of triglycine. Similar results were obtained in their kinetic studies (58,59). These workers indicated a resemblance between peptide transport in mammalian gut and in bacteria (60).

Extension of the work to the series L-methionine, L-methionyl-L-methionine and L-methionyl-L-methionyl-L-methionine gave essentially the same results as those obtained from the glycine series. Further examples of more rapid transport of neutral amino-acids from peptides than from the equivalent free amino acids in mammalian gut were reported from the laboratories of Adibi and Philips (61,62) who extended the finding to glycyl-L-leucine and L-leucylglycine. Edwards (63) reported the phenomenon with L-leucyl-L-alanine, L-leucyl-L-tyrosine, L-alanylglycine, and L-alanine and tryptophyl-glycine in the rat *in vivo* and Rubino *et al*. (64) found the flux of glycine residue of glycyl-L-proline from intestinal lumen into mucosal cell in rabbit ileum.

Bayer et. al., (65) have pointed out that the tripeptide antibiotic 1-phosphinothricyl-alanyl-alanine (XXIV, Fig.II) exhibits much greater antibacterial activity against intact cells than does the constituent amino acid, phosphinothricin. In contrast, in the cell free system only the amino-acid shows effective inhibition of the E. coli glutamine synthetase. It is suggested that the uptake of the antibiotic into the cell is strongly favored by the tripeptide form which is then hydrolysed to the free phosphinothricin and alanine. This shows that the tripeptide form, but not the free inhibitor, is capable of invading the cell via the oligopeptide system (66,67).

A similar transport effect has been used to explain the greater antibacterial activity of the tripeptide antibiotic 1-(N(5)-phosphono)- methionine-5-sulfoximinyl-alanyl-alanine (XXV, Fig.II), (68) compared to the amino acid 1-(N(5)-phosphino)-methionine-5-sulfoximine, which also inhibits the glutamine synthetase of E. coli. Zahner and Jung (69) investigated the uptake of L-methionine-S-oxide. They found that L-methionine-S-oxide-alanyl-alanine (XXVI, Fig.II) is taken up via the oligopeptide transport system. Inside the cell, it specifically inhibits the glutamine synthetase.

Thus the oligopeptide transport system provides a basic and within certain limits, widely applicable carrier system.



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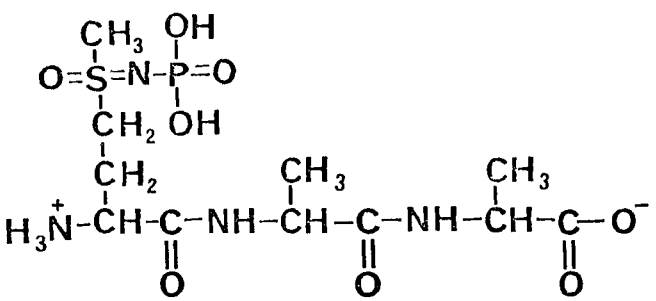

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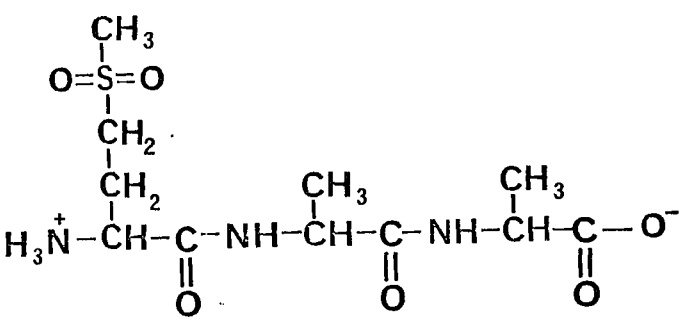
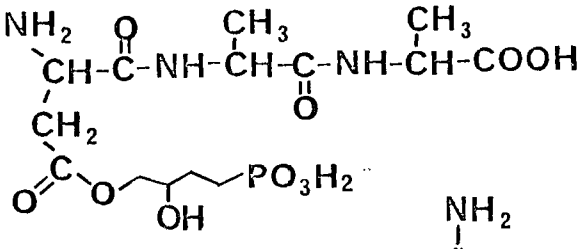


(XXVI)

FIGURE II

The absence of a transport system for simple phosphonic acid analogues of natural phosphate has already been found to be a problem. The phosphonic acid analogue of dihydroxyacetone phosphate, 4-hydroxy- 3-oxobutyl-1-phosphonate (DHAP) is reduced by the anabolic glycerol-3-phosphate dehydrogenase of E. coli (39): unfortunately the analogue does not have any effect on intact cells because it cannot be transported. It would appear reasonable from the examples cited before that if a phosphonic acid analogue species were coupled to a tripeptide, it would have a good probability of being transported into the cell. Thus, studies were initiated with 3,4-dihydroxybutyl-1-phosphonic acid (DHBP) and 4-hydroxy-3- oxobutyl-1-phosphonic acid as metabolic regulators and L-aspartyl-L-leucyl-L-alanine , L-aspartyl-L-alanyl-L-alanine and L-alanyl-L-aspartyl-L-alanine as peptide carriers. A tripeptide linked to an analogue should have the proper hydrodynamic volume. Initial thoughts as to the design of the tripeptide resulted in the choice of aspartate as the 'linking' portion, with alanine or leucine for the 'bulk' portion of the tripeptide. Investigations subsequent to the syntheses indicate this to be a non-optimal choice (vide infra). Having three types of the tripeptide systems and two sets of phosphonate analogues, the following target molecules (XXVII, XXVIII, XXIX, XXX and XXXI) might be synthesized and investigated.



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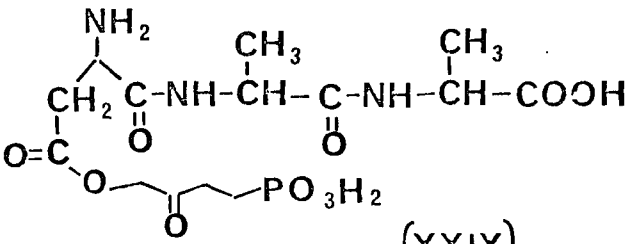

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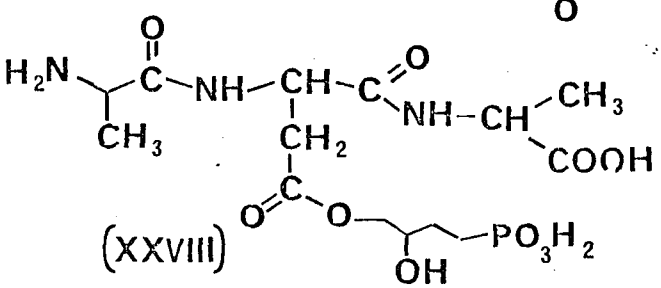

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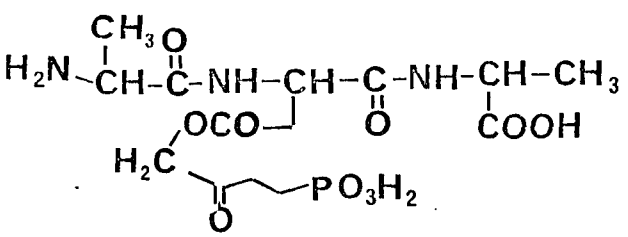

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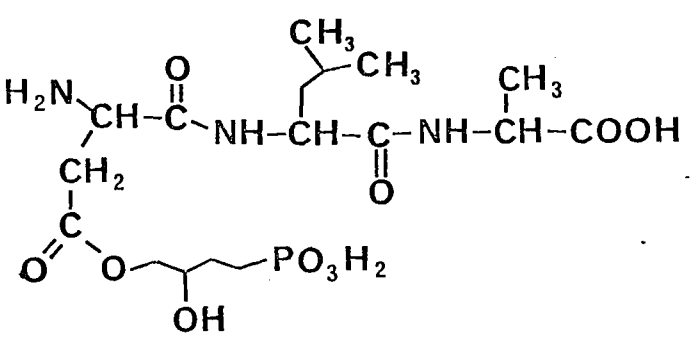


(XXXI)

In addition to the phosphonates which are analogues of naturally-occurring phosphates, there may be considered other drugs that can be modified by linkage to the tripeptides and transported.

Unlike the microorganism, higher plants are generally autotrophic; thus, the utilization of external amino acids, peptides and proteins is normally unimportant. However, several interesting examples may be envisaged, although these are of minor importance considering the number of species involved. One might expect some parasitic species to utilize organic nitrogenous compounds present in their hosts; the uptake of amino acids and/or peptides from symbiotic microorganisms in root nodules may also be considered in this category. Similarly, certain aquatic plants may be able to utilize organic compounds present in highly eutropic waters. A third, and better established example is the utilization of captured insects as a nitrogen source by carnivorous plants. Finally, the uptake and utilization of the endosperm storage reserves by monocot embryos can also be considered as a system in which growth is dependent upon an external (although in this case controlled) supply of organic nitrogen.

Fortunately, this paucity of examples is adequately compensated for by two situations which rarely apply to microorganisms. First, being eukaryotic, the cells of higher plants contain a number of organelles, each of which may be capable of specific transport and utilization of amino acids. Second, and more importantly, higher

plants are multicellular. Thus, we have a situation, almost unique amongst the organisms, in which amino acids are produced by some cells and utilized by others, thus necessitating their transport within the plant body; this transport may be over a short distance (cell to cell transfer), or a long distance (translocation).

It is a fact that our knowledge of amino acid transport processes in higher plants is limited, especially when compared with our understanding of similar systems in microorganisms and mammals. This seems to be due not simply to the difficulties in handling and complexities of the tissue, but also to a feeling amongst many biochemists, that plants are generally of lesser significance than other organisms.

About 400 plants species, out of a world total of over a quater million, are now known to be carnivorous, each being specially adapted to trap and digest insects, and to utilize nutrients derived from them. The traps and digestive structures are very varied, ranging from simple, sticky leaves (Pinguicula) and the cup-like leaves of pitcher plants (Sarracenia and Nepenthes) to the complex structure of the well known Venus flytrap (70-73).

The ability of such carnivorous plants to utilize organic compounds as a source of nitrogen has been known for over a hundred years, yet surprisingly little information is available concerning the nature of the absorption process. In his classic work Insectivorous Plants, Darwin (70) showed that members of the

genera Utricularia (bladderworts) and Drosera (sundews) exhibit enhanced growth and reproductive capacity when supplied with insects or with animal proteins. Furthermore, he concluded that members of two other genera, Pinguicula (butterworts) and Dionaea (Venus's flytrap), were also capable of specifically absorbing certain organic nitrogenous compounds, to which they responded by secreting digestive enzymes. Subsequent studies, on a wide variety of species, have confirmed that growth and reproductive capacity are promoted by a supply of insects, proteins, peptones,or other forms of organic nitrogen, especially under conditions of nitrogen limitation (74-83). This, added to the fact that these species are commonly found in soil deficient in nitrogen, has led to the hypothesis that the carnivorous habit is an important means of obtaining nitrogen (74). However, digested insects may also supply phosphorus, sulpher, and other elements to the growing plant (83).

It is well known that nutrients can pass from a trapped insect into the tissues of an insectivorous plant. Plummer and Kethley in 1964 (84) raised ants on a $^{32}P/^{35}S$-labelled diet. The isotopes became incorporated into insects protein and when fed to a pitcher plant (Sarracenia). The label was found to be absorbed into the plant tissue. Similarly, ^{14}C-labelled nutrients are absorbed from Daphnia by Drosera (85) and from Chlorella by Pinguicula (86). No reports have appeared indicating the uptake of intact protein by pinocytosis. On the contrary, the secretion of

proteases into the traps of carnivorous plants [(Nepenthes, 87-91). Sarrcenia: Hepburn et al., (92). Drosera:Amagase (90,93). Dionaea: (96)], often as a specific response to insects or other forms of organic nitrogen (70,95), indicates that absorption may well be in the form of amino acids and/or small peptides. However, the degree to which protein is digested is unknown. Total degradation to amino acids has been claimed in the pitcher plants, Nepenthes (93) and Sarracenia (84), while in the Venus flytrap there is apparently no peptidase activity secreted into the traps (94), indicating that peptide uptake may be important.

Only a few reports of specific amino acid uptake have appeared. Hepburn et al. (92) showed that asparginine and peptones are absorbed more rapidly than water or inorganic ions from the pitcher of Sarracenia. Similarly, Luttge (96) found that L-alanine is absorbed by Nepenthes more rapidly than phosphate or sulfate ions. More recently it has been shown that Drosera will absorb [^{35}S]-methionine intact, although the kinetics of uptake were not investigated (97). After feeding plants with ^{35}S-labelled insects, ^{35}S-labelled methionine and cysteine were found in the leaves, the results being compatible with the absorption of label from the insect in the amino acid form. In a more detailed study, Plummer and Kethley (84) showed that 24 different amino acids could be absorbed from the pitcher of Sarracenia: characteristic differences in their rates of uptake implying the existence of

specific absorption processes. Three dipeptides were also shown to be taken up from the pitcher fluid by the leaf: they appeared to be absorbed intact with subsequent intracellular hydrolysis, seemingly providing the demonstration of peptide transport in a higher plant.

The weight of the evidence clearly points to the existence of specific processes for the absorption of amino acids from the trap of insectivorous plants. Furthermore, when one considers the utilization of peptides (98), and the nutritional utilization of proteins (99,100), it seems probable that they will also be of importance in insectivores. This is a field which would seem to lend itself to further investigation, especially considering the dearth of systems suitable for studying the utilization of extracellular organic nitrogen in higher plants.

Although it has generally been assumed that the transfer of nitrogenous compounds from the endosperm to the embryo of monocot seedlings occurs as amino acids, recent findings have shown that peptide transport may also be of considerable importance (101,102).

A number of earlier reports indicated that peptide transport might be involved. Peptides were detected in barley endosperm (103) and embryo (104), and an endosperm leachate, containing a high proportion of peptides was shown to satisfy the nitrogen requirements of isolated maize embryos (105). The relatively high levels of peptidase activity in the embryo of germinating barley

seedlings, compared with levels in the endosperm, also suggested that peptides may be transported to the embryo prior to hydrolysis (106).

Using a sensitive, fluorescent detection technique Higgins and Payne (100) have extensively characterized a peptide transport system in germinating barley embryo and the general agreement between results obtained using this technique and radiotracers (107) is encouraging. Demonstration of peptide transport was first achieved using the non-physiological peptides Gly-Sar and Gly-Sar-Sar. These peptides have N-methylated peptide bonds, making them exceptionally resistant to enzymic hydrolysis. Both peptides are accumulated intact by embryos against a concentration gradient (99,107,108). The fact that intact Gly-Sar can be detected in the shoots of embryo which have been incubated such that only the scutellar surface has come into contact with the dipeptide solution necessarily requires that the dipeptide is first transported across the scutellum, and this feature together with the extremely high levels of peptide which accumulate, eliminates the possibility that absorption might arise from non-specific surface binding. Furthermore, uptake is prevented by anaerobic conditions and a range of metabolic inhibitor (100). Thus, although the strict criteria for an active process have not been demonstrated, in that it has not been shown that the peptide exists in free solution once absorbed by the embryo, or that it is accumulated against an

electrochemical gradient, it appears that peptide transport is an active process.

The mode of energization for this process is, however, unclear. The absence of a sodium requirement (100,102) implies that, unlike peptide transport in the mammalian gut (109), a sodium-dependent mechanism does not operate. There are, however, indications that a proton gradient may be involved in the energization of transport. Thus, acetate inhibits peptide uptake, and causes exodus of amino acids at pH values below its pK_a. This is usually interpreted with the undissociated form of the acetate molecule acting as a proton shuttle, disrupting the H^+ ion distribution across the plasmalema (100).

The rate of Gly-Sar transport is essentially linear for periods of up to 6 hr, and uptake exhibits saturation kinetics (100,108). Unlike amino acid transport systems in plants, biphasic kinetics have not (yet) been observed, although the limited concentration ranges used may not have revealed the true situation. Transport shows an acidic pH optimum at about pH 3.8, although considerable uptake occurs over the range of pH 3.5-7.0. The pH optimum for uptake corresponds with the pH optimum for the activity of several endosperm proteases (110,111) and also the reported pH of the endosperm (112). The pH optimum for uptake of glycylisoleucine and trialanine is about pH 4 (113). Sopanen *et al.* (102) reported a slightly higher pH optimum for diglycine uptake. Amino acid uptake

by maize embryos also shows an acidic pH optimum (114). Buffer composition has little effect on uptake; interchange of phosphate, tris, and citrate buffer give similar rates of uptake, as does substitution of K^+ for Na^+ (100).

In addition to Gly-Sar, a range of physiological peptides is also taken up from the medium by barley embryos (101,115). Although intracellular peptidase activity is too high to allow detection of these peptides intact within the embryo, a number of results lead to the conclusion that they are indeed absorbed and subsequently hydrolysed intracellularly. Extracellular hydrolysis, followed by amino acid uptake, is precluded for two reasons. First, although peptide uptake is competitively inhibited by a wide range of other peptides, amino acids have no effect (101,102,108). Second, only low levels of free amino acid appear in the medium during incubation with a peptide. This eliminates the possibility of extracellular hydrolysis and amino acid uptake, and the rates of amino acid uptake from free solution are demonstrably too slow to permit this explanation (101). Perhapes the most convincing argument for intact peptide transport concerns the uptake of certain D-amino acid residues; free D-amino acids are not absorbed by barley embryos, yet when they are presented in peptide form they may be taken up at a considerable rate (115). The possibility that vectorially oriented hydrolysis occurs during membrane translocation such that intact peptides never actually appear

intracellularly has been considered in relation to other peptide transport systems (109,126) and must also be considered here. However, the intact absorption of sarcosyl peptides and some peptides containing D-residues (124), clearly show that transport and hydrolysis are not necessary linked. The fact that competitive inhibition is observed between these peptides and a range of physiological peptides implies that all peptides can be transported by this system, which does at least have the ability to mediate transport in the absence of hydrolysis (100).

Use of sarcosyl peptides and a wide range of physiological peptides has shown that the peptide transport system is capable of handling both di- and tripeptides. However, the utilization of larger peptides is still somewhat ambiguous. Only small amounts of Gly-Sar-Sar-Sar are absorbed, which could be accounted for by non-specific uptake. Although some tetra- and pentapeptides are removed quite rapidly from the medium, and also exert competitive effects, the possibility that this is a result of partial extracellular hydrolysis, although unlikely, has not been entirely eliminated (100, 101,108). Competition experiments have shown that di-, tri-, and higher oligopeptides are transported by the same system, and vice versa, indicates that only one transport system exists (100,108). The possibility that several systems exist, each capable of handling di- and tripeptides, or that there is a second system with specific structural requirements, still exists,

although logically this would not be expected. The variety of peptides with very dissimilar amino acid side chains (e.g. proline, glutamic acid, lysine, and leucine) which compete for transport also argues against this.

Peptide transport shows stereospecificity (101,115). The presence of a D-amino acid residue in a peptide generally reduces its rate of transport, often to zero, although the location of the residue in the peptide is important. A D-residue at the N-terminus often has little or no effect, while at the C-terminus of di- or tripeptides it can totally inhibit uptake. This is particularly surprising in the case of tripeptide, and would seem to suggest that, unlike bacteria, oligopeptide transport has a requirement for the C-terminus, but it lacks specificity towards the N-terminus. Some peptides containing D-residues are accumulated intact, showing the transport system and the hydrolases have different specificties.

In the N- and C-terminal requirements for peptide transport, one would not expect a system which will accomodate both di- and oligopeptides to have requirements for both termini (as binding sites aligned for the α-amino and α-carboxyl groups of a dipeptide cannot also be aligned for the same groups in a tripeptide and vice versa), although both seem to be important in barley. It is clear that substitution (as an ester or amide) or complete removal of the C-terminal carboxyl group of a peptide

considerably impairs, although does not totally inhibit, its uptake. Substitution of the N-terminal amino group (by acylation or alkylation) also has a drastic effect on uptake. However, an imino group (proline) can be handled regardless of its position in the peptide.

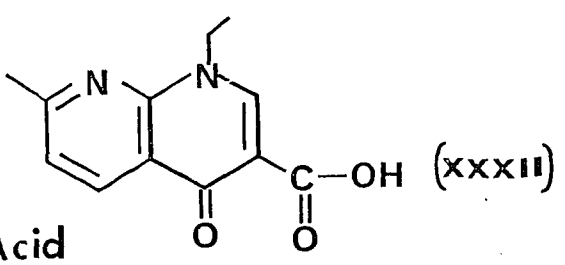
A number of differences have been observed between the peptide transport system(s) of barley and those of other groups of organisms. It is pertinent to consider these in relation to the environment in which the system operates; specific variations between species would be expected to reflect specific biological functions. This is especially relevant in the case of barley embryos which differ from all other systems examined so far, in that they exist in a controlled environment. Thus, the size, amino acid composition, and concentration of peptides is relatively constant and predetermined, unlike the enormous fluctuations that may be presented to bacteria or yeasts in their natural environments. Thus, in barley one could envisage specific peptide transport systems with, say, a requirement for a particular N-terminal residue, reflecting the specificities of protease action within the endosperm.

These studies on peptide transport in barley have shown that peptides may be of considerable importance in certain plant systems and considering these studies, we have tried to use tripeptides in the transport of certain antimetabolites into fruit plants.

1. ANTIBACTERIALS:

Erwinia Amylovora (E. Amy) is a bacteria which causes fire blight disease in fruit plants. At present there is no therapeutic treatment for this disease although streptomycin is used prophylactically. It is known that some gram negative active chemicals, derivatives of the Nalidixic acid family are active against this pathogen in vitro. However, these compounds do not appear to be active in vivo. The reason for the lack of in vivo activity is unknown but may be due to the inability of the compound to migrate within the plant. E. Amy itself has some rather peculiar migration tendencies within a plant, migrating in only an upward direction from the site of infection.

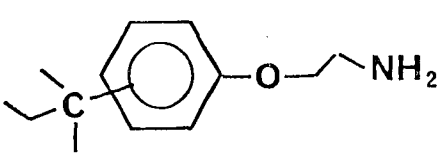
In an attempt to generate an in vivo agent active against Erwinia Amylovora, several in vitro active agents were linked to tripeptids. These included:



(XXXII)

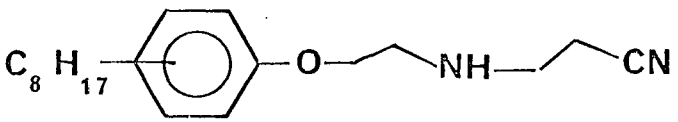
1. Nalidixic Acid

2. Ethanamine; 2-((1,1-dimethylpropyl)phenoxy)-



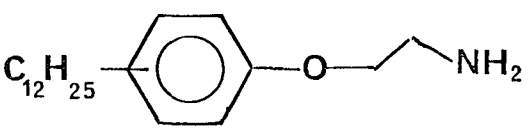
(XXXIII)

3. 3-((2-(4-octylphenoxy)ethyl)amino)propane-nitrile



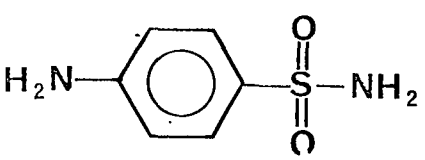
(XXXIV)

4. 2-(Dodecylphenoxy)-ethanamine



(XXXV)

5. sulfanilamide



(XXXVI)

Nalidixic acid (XXXII) has also been used therapeutically with oral administration to treat urinary tract infections. It is

effective against a majority of gram-negative bacteria that infect the urinary tract, especially, E. coli.

Sulfanilamide, used as an anti-infective agent, has a free amino group on the benzene ring and acts as a competitive antimetabolite to block the bacterial synthesis of folic acid from aminobenzoic acid. Execpt in special circumstances (e.g. the high concentrations achieved in treating urinary tract infections), sulfanilamide is bacteriostatic rather than bacteriocidal. It was presumed that p-aminobenzenesulfonamide could be used as a possible cytotoxin on which to attach a transport group. It was proposed that 2-((1,1-dimethylpropyl)phenoxy)-ethanamine (XXXIII), 3-((2-(4-octylphenoxy)ethyl)amino)-propanenitrile (XXXIV), 2-(dodecylphenoxy)-ethanamine (XXXV) and sulfanilamide (XXXVI), each be conjugated with only one chemical vector. This chemical vector is a tripeptide bearing an additional carboxyl function as mentioned in the case of phosphonic acid analogues synthesis, through which attachement of the active agent with an amide linkage is to be performed.

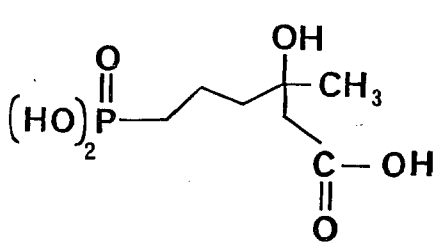
Specifically, as previously mentioned, two tripeptide functions are to be used. First, that bearing a functionalizable carboxylic acid on the amino terminal amino acid, and second with this function at the central amino acid, that is butyloxycarbonyl-L-alanyl-L-asparty1-L-alanine-O-t-butyl ester and butyloxycarbonyl L-asparty1-L-alanyl-L-alanine-O-t-butyl ester

where the additional carboxyl function on the aspartyl amino acid is available for linkage to the amino group of the compound of interest.

For the coupling of nalidixic acid to a tripeptide, serine appears to be the best choice (L-alanyl-L-seryl-L-alanine, XXXVII) which would allow conjugation with the carboxylate function.

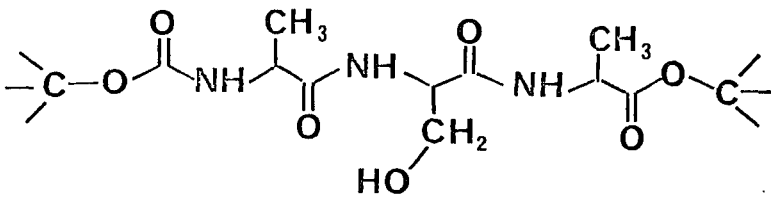
2.INHIBITOR OF STEROL BIOSYNTHESIS:

In addition to the above-mentioned species which serve or are anticipated to serve as antibacterials by inhibition of lipid biosynthesis, an isosteric analogue of 5-phosphomevalonate, 5-carboxyl-4-hydroxy- 4-methylpentyl-1-phosphonic acid (117, XXXVIII)has been found (118,119) to be a potent inhibitor of squalene (and cholesterol) biosynthesis. It is anticipated that linkage to a tripeptide vector will allow entry to mammalian organs , such as intestine, wherin cholesterol biosynthesis during atheriosclerosis is quite significant.



XXXVIII

And the tripeptide vector to be used was chosen as L-alanyl-L-seryl-L-alanine, with coupling of the agent to the



XXXVII

butyloxycarbonyl-L-alanyl-L-seryl-Lalanine-O-t-butyl ester. The extra functionality (hydroxyl group) will allow conjugation with the carboxyl function on the drug [XXXVIII].

EXPERIMENTAL

GENERAL

All chemicals were of reagent quality and used without further purification with the following exceptions: benzene, pentane and hexanes were dried over sodium ribbon, dimethylsulfoxide (DMSO), acetonitrile and pyridine were distilled over calcium hydride, tetrahydrofuran was distilled over lithium aluminum hydride, dimethylformamide (DMF), dioxane and methylene chloride were distilled immediately prior to use and stored over molecular sieves.

Thin-layer chromatography was performed using polygram Sil-N-HR silica gel sheets which were purchased from Brinkmann Instruments, Inc. Silica gel for preparative chromatography was from Baker (60-200 mesh). Palladium on Carbon (5% and 10%) was purchased from Colonial Metals Inc., Maryland.

Infra-Red spectra were measured using Perkin Elmer 237-B and 598 spectrophotometers: NMR spectra were measured using a Varian EM-360 spectrometer. Melting points were obtained on a Meltemp instrument and were uncorrected. The elemental analysis of the

compounds were performed by Galbraith Laboratory, Knoxville, Tennessee. di-tert.Butyl carbonate was purchased from Fluka, AG. Benzyl chloroformate was purchased from ICN Pharmaceutical Inc., Plainview, New York. The optical rotations of the compounds were performed on Perkin Elmer (141) polarimeter.

Dibutyl (S) 3,4-dihydroxybutyl-1-phosphonate , diethyl 4-hydroxy-3-oxobutyl-1-phosphonate and diethyl 5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonate were synthesized as described in the literature (13,21,117).

β-BENZYL ASPARTATE [XXXIX]

Sulfuric acid (10 mL) was added to anhydrous ether (100 mL) followed by benzyl alcohol (100 mL). The ether was removed under vacuum and finely ground L-aspartic acid (13.4 g, 10.52 mmoles) were added, in several portions, while the mixture was stirred magnetically, (there was no change in temp or color of the reaction mixture). The ensuing solution was left at room temperature for 24 hours after which 95% ethyl alcohol (200 mL) was added, followed by pyridine (50 mL), which was added dropwise while the solution was stirred vigorously. The mixture was cooled overnight, the deposit was filtered and triturated with ether. Recrystallization from water containing a few drops of pyridine afforded pure β-benzyl

aspartate (8.9-9.9 g, 40-45% yield) m.p. 214-216° C, Litt.(Ref. 120), m.p. 218-220° C. $[\alpha]_D^{25}$=+27.0 (c=1.4, 1N HCl).

NMR (D_2O) δ 2.9-3.1(d, 2H, CH_2), 4.0-4.1(m,1H, CH), 5.0-5.2(s,2H, $CH_2C_6H_5$), 7.4(s,5H, C_6H_5).

Elemental Analysis: Required: C=59.18 H=5.86 Found: C=58.96 H=6.01

L-ALANINE-t-BUTYL ESTER HYDROCHLORIDE [XL]

Concentrated sulfuric acid (10 mL: 1 mL per gram of the amino acid) was added to a suspension of 10 g (0.112 moles) of L-alanine in 100 mL of dioxane in a pressure bottle. An equal volume (100 mL) of liquid isobutene (isobutene gas was condensed by passing through a trap of dry ice and isopropanol) was added and the mixture shaken overnight. The mixture was poured into an excess of 2N sodium hydroxide and the ester extracted thoroughly with ether. The ether extract was econcentrated to about 70 mL and cooled to 0° C and a dry ethereal solution of hydrogen chloride (this solution was made by bubbling hydrogen chloride gas through cold anhydrous ether) carefully added until there was no precipitation on further addition of hydrogen chloride and an acidic medium was indicated by pH paper (pH 2-3). It was cooled to -20° C overnight. White crystals of L-alanine-t-butyl ester hydrochloride

were filtered and dried under vacuum to yield 8.3 g (41% yield), m.p. 164-167° C (dec.), Litt. (121), m.p. 168-170°C (dec.)

NMR (D_2O): δ 1.2-1.5(1 s, 1 doublet, 12 H, CH_3, $C(CH_3)_3$, 3.7-4.15(m, 1H, CH).

IR ($CHCl_3$)cm^{-1} 3400 (broad), 3000, 1750, 1375, 1250, 1150

BENZYLOXYCARBONYL-L-ALANINE [XLI]

To a solution of 8.00 g (0.0898 moles) of l-alanine in 50 mL of 2N sodium hydroxide, set aside for 20 minutes at 0° C, was added over 30 minutes, 17 g (0.1 mole) of benzylchloroformate and 25 mL of 4N sodium hydroxide simultaneously through two additional funnels attached to a three-necked flask. It was allowed to stir in an ice bath for an additional 10-15 minutes and then acidified with concentrated hydrochloric acid to congo red. The acidified solution, from which an oily compound separated, was cooled in the refrigerator for 4-5 hours. After decanting the aqueous layer, the oil was solidified with cold petroleum ether. The white solid obtained was filtered, washed with ether, dried with anhydrous magnesium sulfate and used without further purification. m.p. 82-83° C, Litt.(122) 84° C. $[\alpha]_D^{25}$=-20 (c. 0.2, MeOH)

NMR ($CDCl_3$) δ 1.0-1.4 (d, 3H, CH_3), 3.9-4.3 (m, 1H,CH), 4.9 (s, 2H, $CH_2C_6H_5$), 5.6 (broad, 1H, NH), 7.1(s, 5H, C_6H_5) , 10.1 (s, 1H, COOH),

IR ($CHCl_3$)cm^{-1}. 3500, 1720, 1510, 1455, 1350, 1075, 915, 695.

BENZYLOXYCARBONYL-L-ALANINE-t-BUTYL ESTER

Benzyloxycarbonyl-L-alanine (15.6 g ,0.07 mole) was dissolved in 250 mL of methyl isobutyl ketone containing 0.8 mL of concentrated sulfuric acid which was saturated with isobutylene. After stirring for three days, it was washed with saturated sodium carbonate solution, water and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residual oil was dried under vacuum for 4-6 hours to yield 11.42 g (58.5%) of [the product]. TLC showed a single spot. Rf=0.54 in chloroform and methanol 8:2.

NMR (CCl_4)δ 1.15-1.4 (1 s and 1 doublets, 12H, CH_3, $C[CH_3]_3$, 3.8-4.3 (m, 1H, CH), 4.95 (s, 2H, $CH_2C_6H_5$), 5.8 (d, 1H, NH), 7.2 (s, 5H, C_6H_5).
IR ($CHCl_3$):cm^{-1}: 3500, 3000, 1745, 1695, 1500, 1450, 1375, 1250, 1150, 1050, 930, 695.

L-ALANINE-t-BUTYL ESTER [XLII]

Benzyloxycarbonyl-L-alanine-t-butyl ester 11.0 g (0.039 moles) was hydrogenated in 50 mL of methanol using 300 mg of Palladium over carbon (10%) as a catalyst at atmospheric pressure. After removing the catalyst by filtration through celite, the filtrate was concentrated on a rotary evaporator to yield 4.0 g (70%) of the compound [XLII]. It was used without further purification. Rf(0.56, EtoAc-hexane 1:1). L-Alanine-t-butyl ester was synthesized by this procedure for further work.

NMR ($CDCl_3$) δ 1.2-1.5 (s, 12H, $C[CH_3]_3$, 1 doublet, 3H, CH_3, CH_3), 3.7-4.1 (m, 1H, CH). IR ($CHCl_3$) cm^{-1}: 3400 (broad), 3000, 1750, 1375, 1250, 1150.

t-BUTYLOXYCARBONYL-β-BENZYL-L-ASPARTATE [XLIII] METHOD A:

di-tert-Butyl-dicarbonate(21.82 g, 0.1 mole) was added dropwise within an hour to a well stirred solution of 22.32 g (0.10 mole) of β-benzyl-L-aspartate and 4.0 g (0.10 mole) of sodium hydroxide in 30 mL of water and 50 mL of tert.butyl alcohol. After a short induction period, the temperature rose to 38° C (without external cooling). The reaction was brought to completion after the addition of a further 50 mL of tert-butyl alcohol and stirring overnight. The turbid solution was diluted with 50 mL of water and

extracted three times with 100 mL pentane. The aqueous phase was acidified to pH 2-3 by the addition of 14 g potassium hydrogen sulfate in the cold and extracted with four 100 mL portions of ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate and filtered. The solvent was removed at 35-40° C under reduced pressure on a rotary evaporator and the residue finally crystallized with benzene and dried in vacuum to a constant weight. (yield 17.04 g, 53%) m.p. 97-98° C, Litt.(123) m.p. 102-103° C. $[\alpha]_D^{25}$=+27 (c 0.2, MeOH).

NMR ($CDCl_3$) δ: 1.2-1.5(s, 9H, $C(CH_3)_3$), 3.0-3.1(t, 2H, CH_2), 5.2 (s, 2H, $CH_2C_6H_5$), 5.7 (broad, 1H, NH), 7.2 (s, 5H, C_6H_5), 9.7 (s, 1H, COOH),

IR ($CHCl_3$) cm^{-1}: 3700-3500 (broad), 1725 (broad), 1475, 1360, 925, 695.

t-BUTYLOXYCARBONYL-β-BENZYL-L-ASPARTATE [XLIV] Method B:

To a solution of β-benzyl-L-aspartate (2.182 g, 10 mmoles) and sodium hydroxide (0.4 g, 10 mmoles) was added dioxane (6 mL) and tert-butoxycarbonyloxyimino-2-phenyl acetonitrile [BOC-ON], (2.71 g, 11 mmoles, Ref. 124) at room temperature. The mixture became homogeneous within one hour and stirring was continued for 2 hours.

After addition of water (15 mL) and ethyl acetate (20 mL), the aqueous layer was separated, washed with ethyl acetate (20 mL), acidified with 5% citric acid solution and extracted with ethyl acetate. The extract was worked up in usual manner to give t-BOC-β benzyl-L-aspartate 2.9 g (91.1%), m.p. 97-99° C, Litt. (123) m.p. 102-103° C. $[\alpha]_D^{25}$=+27 (c 0.2, MeOH).

NMR ($CDCl_3$) δ : 1.2-1.5 (s, 9H, $C[CH_3]_3$), 3.0-3.1 (t, 2H, CH_2), 5.7(broad, 1H, NH), 5.2(s, 2H, $CH_2C_6H_5$), 7.2(s, 5H, C_6H_5),9.8(s,1H, COOH).

IR($CHCl_3$)cm^{-1}: 3200-3700(broad), 1725(broad), 1475, 1360, 925, 695. Elemental Analysis: Required: C= 59.44, H=6.50 Found: C=59.17, H=6.42

tert-BUTYLOXYCARBONYL ALANINE [XLV]

di-tert-butyldicarbonate (120 g, 0.55 moles) was added dropwise within an hour to a well stirred solution of 48.99 g (0.55 moles) L-alanine and 20.0 g (0.50 moles) sodium hydroxide in 50 mL of water and 100 mL of tert-butyl alcohol. After a short induction period, the temperature rose to 42° C (without external cooling). The reaction was brought to completion after the addition of a further 100 mL tert-butyl alcohol and stirring

overnight. The turbid solution was diluted with 250 mL water and extracted three times with 300 mL pentane. The aqueous phase was acidified to pH 2-3 by the addition of 70 g potassium hydrogen sulfate in the cold and extracted with four 400 mL portions of ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate and filtered. The solvent was removed at 35-40° C under reduced pressure on a rotary evaporator and the residue finally dried at 0.5 Torr to constant weight yielding a crystalline material 25 g (93%). m.p. 80-81° C, Litt. (126), m.p. 82-83°C. $[\alpha]_D^{25}$=-34 (c 0.2, MeOH).

NMR ($CDCl_3$) δ : 1.4-1.5 (d, 3H, CH_3), 1.5-1.7(s, 9H, $C[CH_3]_3$), 4.0-4.3(m, 1H, CH), 5.8-6.0(broad, 1H, NH) IR ($CHCl_3$)cm^{-1}: 3300-3600, (broad), 2900, 1710, 1670, 1380

BENZYLOXYCARBONYL β-BENZYL ASPARTATE

β-Benzyl-L-aspartate (4.46 g, 20 mmoles) was dissoled in hot water (300 mL) and the solution was allowed to cool. When the temperature reached 60° C (the compound began to crystallize at 55° C), sodium bicarbonate 3.5 g (50 mmoles) and carbobenzoxy chloride 4.1 g (24 mmoles) were added successively and the solution stirred vigorously for 3 hours. The solution was then extracted twice with ether and the aqueous layer was acidified to congo red

with hydrochloric acid. The oily layer was extracted three times with 100 mL portions of ethyl acetate. The combined ethylacetate extracts were dried over magnesium sulfate and evaporated under reduced pressure on a rotary evaporator to yield a semi-solid material which solidified in the refrigerator in one hour. One crystallization from benzene gave 4.9 g (69%) of the product. m.p. 104-105° C, Litt. (127) m.p. 108° C. $[\alpha]_D^{25}$=+2.5 (c 0.2, MeOH).

NMR ($CDCl_3$) δ 3.0-3.1(d, 2H, CH_2), 4.0-4.2(m, 1H, CH),5.2-5.4(s, 4H, $CH_2C_6H_5$), 5.6-5.7(broad, 1H, NH), 7.4-7.6(s, 10 H, $2C_6H_5$), 9.8(s,1H, COOH).

IR($CHCl_3$)cm^{-1}: 3600-3200(broad), 1725(broad), 1475, 1365, 925, 625.

Elemental Analysis: Required: C=63.86, H=5.35

Found:C=63.47, H=5.42

N-t-BOC-ALANINE PENTACHLOROPHENYL ESTER [XLVI]

t-BOC-Alanine (1.00 g, 0.528 mmoles) was dissolved in dry methylene chloride (50 mL) and dicyclohexylcarbodiimide (1.5 g, 0.729 mmoles) was added to the solution. The mixture was stirred at 0-5° C for

15 minutes. Then pentachlorophenol (1.409 g, 0.529 mmoles) was added to it. Stirring was continued for three hours at 0-5° C and then overnight at room temperature. The excess DCC was decomposed with glacial acetic acid. The dicyclohexyl urea (DCU) was filtered and the solvent of the filtrate was evaporated in vacuo. The residue was dissolved in ethyl acetate, washed successively with 5% sodium bicarbonate, water, 10% citiric acid and water, an dried over sodium sulfate. The solvent was evaporated to yield a white solid which was recrystallized from methanol to yield 1.00 g (44%) of the active ester [XLVI].

m.p. 168-169° , Litt. (128) m.p. 170°C

NMR($CDCl_3$,CD_3OD) δ : 1.3-1.4(d, 3H, CH_3), 3.3(q, 1H,CH), 4.6-4.7(s,2H, CH_2),

4.8-4.9(broad, 1H, NH), 7.3(s, 5H, C_6H_5).

Elemental Analysis: Required: C=43.47, H=2.57

Found: C=43.83, H=2.70

CARBOBENZOXY-ALANYL-β-BENZYL-1-ASPARTATE

To a stirred solution of 0.80 g (1.69 mmoles) N-carbobenzoxy-1-alanine pentachlorophenyl ester and 0.10 g of 2-hydroxy pyridine, as a catalyst, in 80 mL methylene chloride was added in portions over a period of 30 min 10 mL of a suspension of

β-benzyl aspartate (0.38 g, 1.699 mmoles) and 0.21 mL (1.69 mmoles) of dicyclohexylamine (DCA). The reaction mixture after magnetically stirring for 24 hours at ambient temperature, was evaporated in vacuo. The residue was dissolved in 100 mL of ethyl acetate and the resulting solution was treated with 40 mL of 2 N hydrochloric acid, then filtered to remove the insoluble dicyclohexylamine hydrochloride. The aqueous layer was drawn off and the ethyl acetate layer was washed with three 40 mL portions of water. The ethyl acetate layer was extracted with three 15 mL portions of 5% aqueous sodium bicarbonate solution. The bicarbonate extracts were combined and acidified with 1 N hydrochloric acid: the clear solution became cloudy during the acid addition. The acidified suspension was extracted with two 50 mL portions of ethyl acetate which was washed three times with 40 mL portions of water and dried over sodium sulfate. Evaporation of ethyl acetate solution in vacuo left a brown viscous oil which was crystallized from ethyl acetate -ether-petroleum ether (b.p. 30-60°C). The mixture was left at -15°C overnight, then the product,
N-carbobenzoxy-alanyl-β-benzyl aspartate , was collected and dried under vacuum. Yield 0.50 g (69%), m.p. 99-100°C.

NMR($CDCl_3$) δ 0.8-0.9(d, 3H, CH_3), 2.8-3.0(d, 2H,CH_2), 4.0-4.1(m, 1H, CH), 4.9-5.0(d, 4H, CH_2), 7.3-7.4(s, 10H, C_6H_5), 10.0-10.1(s, 1H, COOH).

Elemental Analysis: Required: C=62.44, H=5.88

Found: C=62.30, H=5.69

SYNTHESIS OF CARBOBENZOXY-β-BENZYL-L-ASPARTYL-L-ALANINE-t-BUTYL Ester

To a stirred solution of 3.416 g (9.57 mmoles) of N-carbobenzyloxy-β-benzyl-aspartate in 50 mL of THF at -10° C was added 1.33 mL (9.57 mmoles) triethylamine and 1.33 mL (9.57 mmoles) of isobutylchloroformate. After 30 minutes, triethylamine 1.33 mL (9.57 mmoles) was added to the reaction mixture followed by a stepwise addition over a 30-minutes-period of a suspension of 1.74 g (9.57 mmoles) L-alanine-t-butyl ester hydrochloride in 10 mL THF. The stirred reaction mixture was left at -10°C for four hours, then freed from the solvent by evaporation in vacuo. The residue was triturated with hot ethyl acetate and filtered free from triethylamine hydrochloride. The filtrate was washed three times with 30 mL portions of 1 N hydrochloric acid, water, 5% aqueous sodium bicarbonate and water. The ethyl acetate layer was dried over anhydrous sodium sulfate. The residue obtained after vacuum evaporation of ethyl acetate was an oil which was crystallized in the usual way, (Yield: 3.5 g, 75 %).

NMR ($CDCl_3$) δ 1.0-1.2(d,3H,CH_3), 1.4-1.7(s, 9H, $C[CH_3]_3$), 2.6-2.7(d, 2H), 3.0-3.2(m, 2H, CH, CH_3), 4.8-5.0(broad, 1H, NH), 5.1-5.3(s, 2H, $CH_2C_6H_5$), 7.3(s, 5H, C_6H_5).

IR($CHCl_3$)cm^{-1}: 3300, 2960, 1720, 1670, 1500, 1380, 1150, 960, 650.

Elemental Analysis: Required: C=64.31 H=6.85

Found: C=64.13 H=6.79

SYNTHESIS of ASPARTYL-ALANINE-t-BUTYL ESTER

A suspension of 0.30 g of palladium charcoal catalyst (10%) in absolute ethyl alcohol with a few drops of glacial acetic acid and 3.00 g (6.18 mmoles) N-carbobenzoxy-β-benzyl -asparty1-alanine-t-butyl ester was hydrogenated at atmospheric pressure until no further uptake of hydrogen occurred (overnight). The reaction mixture was filtered free from the catalyst and the filtrate was evaporated under reduced pressure. The residue was crystallized from ethyl acetate and petroleum ether to yield 1.6 g (94%) of the product. NMR shows no benzyl protons.

NMR($CDCl_3$) δ 0.8-0.9(d, 3H, CH_3), 1.3-1.5(s, 9H, $C[CH_3]_3$), 3.4-3.5(d, 2H, CH_2), 4.0-4.1(m,1H, CH), 4.8-5.0(broad, 1H, NH).

Elemental Analysis: Required: C=50.76, H=7.75

Found: C=49.96, H=7.49

SYNTHESIS OF BUTYLOXYCARBONYL-ALANYL-ASPARTYL-ALANINE-O-t-BUTYL ESTER [XLVII]

To a stirred solution of 3.09 g (7.24 mmoles) BOC-alanine pentachlorophenyl ester and 0.20 g 2-hydroxy-pyridine, as a catalyst, in 120 mL of methylene chloride was added in 2mL portions over a period of 30 minutes, 20 mL of a solution containing 2g (7.24 mmoles) aspartyl-alanine-O-t-butyl ester and 1.44 mL (7.24 mmoles) dicyclohexylamine (DCA). The reaction mixture after stirring for 24 hours at ambient temperature was evaporated in vacuo. The residue was dissolved in 100 mL ethyl acetate and the resulting solution was treated with 100 mL of 1 N hydrochloric acid, then filtered to remove the insoluble dicyclohexyl amine hydrchloride. The aqueous layer was drawn off and the ethyl acetate layer was washed with two 50 mL portions of water. The ethyl acetate layer was extracted with three 15 mL portions of aqueous sodium bicarbonate. The bicarbonate extracts were combined and acidified with 1 N hydrochloric acid. The acidified suspension was

extracted with two 50 mL portions of ethyl acetate. The ethyl acetate layer was washed three times with 40 mL portions of water and dried over anhydrous sodium sulfate. The evaporation of ethyl acetate solution in vacuum left a brownish oil which was crystallized from ethyl acetate-ether-petroleum ether. The mixture was kept at -15^{o} C overnight, then the product [XLVII] was collected on a filter paper yielding 1.5 g (48%).
$[\alpha]_D^{25}$=-10 (c0.25, MeOH).

NMR($CDCl_3$) δ : 0.9-1.5(m, 24H, CH_3, $C[CH_3]_3$), 2.9-3.1(d, 2H, CH2), 4.0-4.4(m, 3H, CH), 5.1-5.2(s, 2H, $CH_2C_6H_5$), 5.6(broad, 1H, NH), 7.4(s, 5H, C6H5),
IR($CHCl_3$)cm^{-1}: 3300, 2980, 2960, 1740, 1660, 1500, 1380, 1150, 900.

Elemental Analysis: Required: C=52.20, H=7.40

Found: C=52.04, H=7.27

SYNTHESIS OF BUTYLOXYCARBONYL-ALANYL-β-BENZYL ASPARTATE [XLVIII]

BOC-Alanine (5.00 g, 26.40 mmoles) was dissolved in 80 mL THF and 3.68 mL (26.4 mmoles) of triethylamine. The solution was cooled in an ice-acetone bath. Isobutylchloroformate (3.46 mL, 26.40

mmoles) was dissolved in 15 mL THF, this was added to the solution of BOC-alanine in 1 mL portions with cooling and swirling over a period of 30 minutes. L-Aspartic-β-benzyl ester (5.36 g, 24 mmoles) was suspended in 26 mL of 1.15 N sodium hydroxide and 80 mL dioxane. The reaction mixture was cooled in ice and then added to the anhydride solution. The reaction mixture was left in the cold for several hours and then overnight at room temperature. The reaction mixture was evaporated under vacuum to an oil which was dissolved in water and slowly neutralized with solid citric acid. A solid precipitated which was collected, washed with water and dried under vacuum over phosphorus pentoxide to yield 8.0 g (76%). m.p. 65° C. Rf=0.6 ($CHCl_3/CH_3CO$ 2:1).

NMR($CDCl_3$) δ 0.9-1.1(d, 3H, CH_3), 1.4-1.7(s, 9H, $C[CH_3]_3$), 2.1-2.3(d, 2H), 2.9-3.4(q,1H, CH), 4.0-4.3(broad, 1H), 4.5-4.7(s, 2H, $CH_2C_6H_5$), 4.9-5.2(broad, 1H, NH), 7.2-7.4(s, 5H,C_6H_5), 10.1-10.3(s, 1H, COOH).

IR($CHCl_3$)cm^{-1}: 3500-3300(broad), 2960, 1720, 1690, 1500, 1380, 1150.

Elemental Analysis: Required: C=57.81, H=6.64
Found: C=57.96, H=6.43

SYNTHESIS OF BUTYLOXYCARBONYL-ALANYL-β-BENZYL-ASPARTYL-ALANINE-t-BUTYL ESTER [XLIX]:

Butyloxycarbonyl-alanyl-β-benzyl aspartate [XLVIII] (1.00 g, 2.5 mmoles) dissolved in 25 mL of acetonitrile containing triethylamine 0.35 mL (2.5 mmole) is added to a suspension of N-ethyl-5-phenyl-isoxazolium-3'-sulfonate (Woodward's Reagent) (0.64 g, 2.5 mmoles) in 10 mL acetonitrile and stirred until the reagent is dissolved. Alanine-t-butyl ester hydrochloride 0.46 g (2.5 mmoles) and triethylamine 0.35 mL (2.5 mmoles) were added, and the reaction mixture was stirred overnight at room temperature. The solvent is removed in vacuo, the residue is dissolved in ethyl acetate, washed with water and worked up as described for [XLVII] yielding 0.70 g (58%) of [XLIX]. TLC shows a single spot (Rf=0.72, $CHCl_3$:CH_3OH 7:3). The compound was recrystallized from ethyl acetate and petroleum ether.
$[\alpha]_D^{25}$=-3.5 (c0.3, MeOH).

NMR($CDCl_3$) δ 1.1-1.4(d, 4H,$2CH_3$), 1.4-1.7(s, 18H, $C[CH_3]_3$), 2.7-3.1(m, 2H, CH), 4.6-4.8(broad, 1H, NH), 5.1-5.3(s, 2H), 5.5-5.6(broad, 1H,NH). 7.4(s, 5H, C_6H_5).
IR($CHCl_3$)cm^{-1}: 2960, 1725, 1665, 1500, 1380, 1150

Elemental Analysis: Required: C=59.86, H=7.53
Found: C=59.34, H=7.62

SYNTHESIS OF BUTYLOXYCARBONYL-ALANYL-ASPARTYL-ALANINE-O-t-BUTYL ESTER [XLVII]

A solution of 1.15 g butyloxycarbonyl-alanyl-β-benzyl-asparty1-alanine-O-t-butyl ester in 50 mL of ethyl alcohol was hydrogenated at atmospheric pressure with 100 mg Palladium on carbon as a catalyst. After removing the catalyst by filtration through celite, the filtrate was concentrated on a rotary evaporator to yield 0.8 g (80%) of [XLVII]. TLC shows a single spot (Rf=0.68, $CH_3OH:CHCl_3$, 2:8). The compound XLVII was crystallized as described for XLVII.

NMR($CDCl_3$) δ 1.2-1.4(d, 4H), 1.5-1.8(s, 18 H, $C[CH_3]_3$), 1.9-2.2(d, 2H), 3.4-3.7(m, 2H, $C\underline{H}CH_3$), 5.0-5.2(b, 1H, NH), 6.9-7.0(broad, 1H, NH),

IR($CHCl_3$)cm^{-1}: 3200-3600(broad), 2960, 1735, 1665, 1500, 1380, 1150

Elemental Analysis: Required: C=52.88, H=7.70
Found: C=52.49, H=7.52

SYNTHESIS OF CARBOBENZOXY-L-ALANYL-L-ALANINE-O-t-BUTYL ESTER [L]

A solution of carbobenzoxy-L-alanine (5.225 g, 25.0 mmole) and alanine-O-t-butyl ester hydrochloride (4.54 g, 25.0 mmole) was cooled to $-10^{o}C$. Triethylamine (3.48 mL, 25.0 mmole) and dicyclohexylcarbodiimide (8.25 g, 40 mmoles) and 4-dimethylamino-pyridine (DMAP) (20 mg) were added successively to the stirred mixture which was maintained at $-10^{o}C$ for 2 hours. After warming to room temperature overnight, a few drops of glacial acetic acid were added and after a further 0.5 hr., the DCU precipitate was removed by filtration. The organic layer was washed with 5% sodium bicarbonate solution, cold 1 N hydrochloric acid and water and then evaporated to yield 8.2 g (88%) of dipeptide in white crystals. Rf=0.45 ($CHCl_3$/MeOH 19:1. $[\alpha]_D^{25}=-11$ (c 0.5, MeOH).

NMR($CDCl_3$) δ 1.2-1.4(d,d, 6H, 2 CH_3), 1.5-1.8(s,9H, $C[CH_3]_3$), 4.1-4.7(m, 2H, CH), 5.1-5.3(s, 2H, CH_2), 5.5-5.8(d, 1H, NH), 6.7-6.8(d, 1H, NH), 7.4(s, 5H, C_6H_5).
IR($CHCl_3$)cm^{-1}: 3420, 2990, 2940, 1720, 1670, 1500, 1380

Elemental Analysis: Required: C=61.78, H=7.48
Found: C=61.91, H=7.70

SYNTHESIS OF L-ALANYL-ALANINE-O-t-BUTYL ESTER [LI]

A suspension of 0.2 g of Palladium-charcoal catalyst (10%) in absolute ethyl alcohol with a few drops of glacial acetic acid and 8.0 g of N-carbobezoxy-alanyl-alanine-t-butyl ester was hydrogenated at atmospheric pressure until no further uptake of hydrogen occured (overnight). The reaction mixture was filtered free from the catalyst through celite and the filtrate was evaporated under reduced pressure. The residue was crystallized from ethyl acetate-petroleum ether to yield 5.5 g (97%) of [LI]. $[\alpha]_D^{25}$=-4 (c 0.17, MeOH).

NMR($CDCl_3$) δ: 1.3-1.5(d, 6H, $2CH_3$), 1.5-1.6(s, 9H, $C[CH_3]_3$), 2.0-2.1(s, 2H, NH_2), 3.6-3.8(m, 1H, CH), 4.1-4.4(m, 1H, CH), 7.7-7.8(d, 1H, NH),

IR($CHCl_3$)cm^{-1}: 3340, 2920, 2980, 1650-1740(broad), 1380, 1160

Elemental Analysis: Calculated: C=55.59, H=9.26 Found: C=54.99, H=9.19

SYNTHESIS OF

N-BUTYLOXYCARBONYL-β-BENZYL-L-ASPARTYL-L-ALANYL-L-ALANINE-t-BUTYL ESTER [LII]

t-BOC-l-Aspartate-β-benzyl ester (8.2 g, 25.61 mmoles) and L-alanine-O-t-butyl ester (5.5 g, 25.61 mmoles) were dissolved in 50 mL of dry methylene chloride. The solution was cooled in an ice-acetone bath and treated with 10 g (48.0 mmoles) DCC in 10 milliliters of methylene chloride. The reaction mixture was left at 0^{o} C for 2 hours and then at room temperature overnight. The excess DCU was decomposed by the addition of glacial acetic acid. The reaction mixture was filtered to remove the precipitate of DCU and the filtrate was evaporated under reduced pressure and the residue dissolved in ethyl acetate. The organic layer was washed twice with 5% sodium bicarbonate and water. The solution was dried with anhydrous magnesium sulfate, filtered and evaporated under vacuum. The residual oil was dissolved in a little ethyl acetate and crystallized by the addition of hexane. The product was washed with ether, dried in a dessicator over phosphoprus pentoxide yielding 12 g (89%) of LII. Rf=0.6 ($CHCl_3$/^{i}PrOH 19:1). $[\alpha]_D^{25}$=-7 (0.3, MeOH).

NMR($CDCl_3$) δ : 1.3-1.4(d, 6H, $2CH_3$), 1.4-1.7(s, 18 H, $C[CH_3]_3$), 2.2-2.3(d, 2H), 3.0-3.2(m, 2H, CH), 4.9(broad, 1H,NH), 5.2-5.3(s,2H,CH_2), 5.3-5.5(broad, 1H, NH), 7.4(s, 5H, C_6H_5).

IR($CHCl_3$)cm^{-1}: 3400, 2910, 1650, 1750, 1500, 1380

Elemental Analysis: Required: C=59.86, H=7.53

Found: C=59.92, H=7.60

SYNTHESIS OF BUTYLOXYCARBONYL-ASPARTYL-L-ALANYL-L-ALANINE-t-BUTYL ESTER [LIII]

Hydrogenation of the β-benzyl group was performed in the hydrogenation apparatus using Pd-C (10%) at atmospheric pressure as usual. After removing the catalyst by filtration through celite, the filtrate was worked up in the usual way to yield the acid [LIII] in 90% yield as white crystals under vacuum. Petroleum ether had been added to complete the crystallization. Rf=0.48 ($CHCl_3$/MeOH/Py 90:7:3). $[\alpha]_D^{25}$=-35 (c 0.25, CH_3OH).

NMR($CDCl_3$) δ: 1.3-1.4(d, 6H, $2CH_3$), 1.5-1.7(s, 18 H, $2C[CH_3]_3$),

2.1-2.2(d, 2H), 4.1-4.4(m, 2H, CH), 7.3-7.6(broad, 2H, NH), 10.3-10.4(s, 1H, COOH). IR($CHCl_3$)cm^{-1}: 3200-3450(broad), 2900, 1640-1700(broad), 1500, 1370, 1150

Elemental Analysis: Required: C=52.70 H=7.41

Found: C=52.49 H=7.27

SYNTHESIS OF BOC-ALANYLASPARTYL-(β-(S)-2-HYDROXY-4-DIBUTOXY PHOSPHINYL-1-BUTYL ESTER)-ALANINE-O-t-BUTYL ESTER [LIV]

CARBODIIMIDE METHOD:

A solution of 0.8 g (1.85 mmoles) BOC-alanyl-aspartyl-alanine-t-butyl ester and 0.53 g (1.85 mmoles) dibutyl 3,4-dihydroxybutyl-1-phosphonate in 10 mL of anhydrous pyridine was cooled to 0°C: and to this solution was added 0.40 g (1.93 mmoles) DCC. The reaction mixture was stirred at 0° C for 2 hours and then overnight at room temperature. The excess DCC was decomposed by the addition of glacial acetic acid. The solution was filtered to remove the precipitate of DCU. The filtrate was evaporated under vacuum and the residue dissolved in ethyl acetate. The organic solution was washed with 5% citric acid , once with water, twice with 5% sodium bicarbonate solution and once again with water. The solution was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residual oil was dissolved in a little ethyl acetate, filtered to remove any remaining DCU and crystallized by the addition of petroleum ether. The product was dried under high vacuum yielding

0.6 g (46%) of [LXIX]. Rf=0.59($CH_3CH_2CH_2CH_2OH$, CH_3COOH, 4:1), Rf=0.55($CHCl_3$, C_2H_5OH, 8:2)

NMR($CDCl_3$) δ 0.9-1.1(m, 36H, CH_3, $C[CH_3]_3$, $CH_2CH_2CH_3$), 2.1-2.2 (s, 2H, CH_2), 2.7-2.9(m,2H), 3.9-4.4(m, 4H, CH), 7.4-7.7(broad,1H, NH).

IR($CHCl_3$)cm^{-1}: 3400, 2900, 1720, 1670, 1500, 1380, 1250, 1150

Elemental Analysis: Required: C=53.51, H=8.40

Found: C=53.06, H=8.13

SYNTHESIS OF ALANYLASPARTYL-(β-(S)-2-HYDROXY-4-DIHYDROXY PHOSPHINYL-1-BUTYL ESTER)-ALANINE [LV]

n-Butyl ester linkages were removed to generate the free phosphonic acid by the treatment of the phosphonate ester (30 mmoles) with trimethylchlorosilane (60 mmoles) in the presence of sodium iodide (60mmoles) in acetonitrile with stirring at room temperature for 15 minutes. The exothermic reaction occurred immediately resulting in the precipitation of sodium chloride to afford the corresponding the silyl phosphonate. Treatment of silyl phosphonate with methanol (50 mL) gave free phosphonic acid.

The other protecting groups, t-BOC- and t-butyl groups were removed by the immediate treatment of the above compound with trifluoroacetic acid (15 mL) for 15 minutes at room temperature. The acid was evaporated under vacuum and the residue was triturated with ether. The product was dissolved in water, neutralized with pyridine to pH 6 and precipitated with acetone. The product was filtered, washed with acetone (15 mL) and dried under vacuum in a dessicator at room temperature over phosphorus pentoxide yielding a crystalline material (m.p. 86° C). Rf=0.49(EtOAc/Py/AcOH/H_2O 60:20:6:1). $[\alpha]_D^{25}$=-12 (c0.2, CH_3OH). Spectra and TLC were measured within two hours of reaction using freshly prepared material. Sample for elemental analysis was prepared immediately by drying for 3 hours in an Abderhalden at 56 C. The above material was stored as solid in the deep freeze. NMR($CDCl_3$) δ : 0.9-1.2(d, 6H, $2CH_3$), 1.3-1.6(m, 6H, CH_2), 3.9-4.3 (m, 4H, CH), 7.0-7.3(broad, 3H,NH),

IR($CHCl_3$)cm^{-1}: 3300-3500(broad), 2960, 1720, 1675, 1500, 1250,1150

Elemental Analysis: Required: C=39.35 H=6.13, Found: C=40.09, H=6.07

SYNTHESIS OF BOC-ALANYLASPARTYL-(β-2OXO-4-DIETHOXY PHOSPHINYL -1-BUTYL ESTER)-ALANINE-t-BUTYL ESTER [LVI]

To a stirred solution of BOC-Ala-Asp(OH)-Ala-O-t-Bu [XLVII] 4.00 g (9.27 mmoles) in 10 mL anhydrous dichloromethane is added 30-100 mg dimethyl aminopyridine (DMAP, 81) and diethyl 4-hydroxy-3-oxobutyl-1-phosphonate 2.08 g (9.27 mmoles). Dicyclohexylcarbodiimide 4.20 g (18 mmoles) is added to the reaction mixture at 0^{o} C which is then stirred for 15 minutes at 0^{o} and for 6 hours at 20^{o} C. The excess DCC was decomposed by using a few grams of solid oxalic acid. The precipitated DCU is then filtered and the filtrate evaporated in vacuo. The residue is taken up in ethyl acetate and filtered free of any further precipitated DCU. The organic solution is washed twice with cold 0.5 N hydrochloric acid, saturated sodium bicarbonate solution and then dried over magnesium sulfate. The solvent is removed by evaporation and the ester [LVI] is isolated by crystallization, which was further purified by filtration on a short silica gel column (4.0 g, 67%). Rf=0.54 ($CHCl_3$/MeOH 4:1).

NMR($CDCl_3$) δ : 0.8-1.3(m, 12H, CH_3, C_2H_5), 1.3-1.5(s, 18 H, $C[CH_3]_3$), 1.9-2.2(m, 10H, $5CH_2$), 4.0-4.4(m, 4H, 2CH, CH_2), 7.0-7.2(broad,2H,NH).

IR($CHCl_3$)cm^{-1}: 3400, 3300, 2980, 1720, 1670,1500, 1380, 1150

Elemental Analysis: Required: C=53.66, H=8.13

Found: C=53.26, H=8.01

SYNTHESIS OF ALANYLASPARTYL-(β-2-OXO-4-HYDROXY PHOSPHINYL- 1-BUTYL ESTER)-ALANINE [LVII]

There was treated LVI (2.00 g, 3.14 mmoles) with 6.28 mmoles of trimethylchlorosilane and 6.28 mmoles of sodium iodide in acetonitrile with stirring at room temperature for 15 minutes. The exothermic reaction occured immediately resulting in the precipitation of sodium chloride to afford the corresponding silyl phosphonate. Treatment of the silyl phosphonate with methanol (30 mL) gave the 1.7 g (93%) as the free phosphonic acid analogue .

Immediately the 1.7 g (2.90 mmoles) of the free phosphonic analogue was treated with 15 mL of trifluoroacetic acid for 15 minutes at room temperature. The acid was evaporated under vacuum and the residue was triturated with ether. The product was dissolved in water, neutralized with pyridine to pH 6 and precipitated with acetone. The product was filtered, washed with acetone (15 mL) and dried under vacuum in a dessicator over phosphorus pentoxide yielding 1.00 g (83%) of a yellowish crystalline material [LVII]. Spectra and TLC were measured within two hours of reaction using freshly prepared material. Sample for

elemental anlysis was prepared using freshly prepared material by drying for 3 hours in an Abderhalden apparatus. The above compound was stored as solid in the freezer. m.p. 63° C. Rf=0.67 (^{n}BuOH/Py/AcOH/H_2O 60:10:6:24). $[\alpha]_D^{25}$=-2 (c 0.3, CH_3OH). NMR($CDCl_3$) δ : 0.9-1.2(d, 6H, $2CH_3$), 1.3-1.6(m, 6H, CH_2), 3.9-4.2(m, 3H,CH), 7.0-7.4(broad, 3H, NH). IR($CHCl_3$)cm^{-1}: 3300-3600(broad), 2950, 1715, 1670, 1500, 1250, 1150. Elemental Analysis: Required: C=39.68, H=6.22

Found: C=40.01, H=6.10

SYNTHESIS OF BUTYLOXCARBONYL ASPARTYL-(β-(S)-2-HYDROXY-4-BUTOXY PHOSPHINYL- 1-BUTYL ESTER)-ALANYLALANINE-O-t-BUTYL ESTER [LVIII]

A solution of 2.00 g (4.63 mmoles) BOC-asparty1-L-alanyl-L-alanine-O-t-butyl ester and 1.31 g (4.63 mmoles) of dibutyl (S)-3,4-dihydroxy-1-butyl phosphonate in 30 mL of anhydrous methylene chloride was cooled to 0° C, and to this solution was added successively 300 mg dimethyl aminopyridine and 1.80 g 9.00 mmoles) of DCC. The solution mixture was stirred in cold for 2 hours and then overnight at room temperature. The solution was filtered to remove the precipitate of DCU. The filtrate was evaporated in vacuum and the residue disssolved in ethyl acetate. The organic layer was washed with 5% citric acid, once with water, twice with 10% sodium bicarbonate solution and once again with water. The solution was dried with magnesium

sulfate, filtered and evaporated under reduced pressure. The residual oil was dissolved in ethyl acetate, filtered to remove any remaining DCU and crystallized by the addition of cold petroleum ether. The product was dried under high vacuum over phosphorus pentoxide yielding 1.13 g (45%). TLC showed a single spot both with iodine and molybdate spray. Rf= 0.6 in $CHCl_3$(8):CH_3OH(2)

NMR($CDCl_3$) δ : 0.8-2.0(m, 36H, CH_3, $C[CH_3]_3$, $CH_2CH_2CH_3$), 2.1-2.2 (s, 2H, CH_2), 2.7-2.9(m, 2H), 3.9-4.4(m, 4H, CH), 7.4-7.7 (broad, 3H, NH) IR($CHCl_3$)cm^{-1}: 3400, 2900, 1710, 1675, 1500, 1380, 1250,1150

Elemental Analysis:

Required: C=53.66, H=8.13, Found: C=53.44, H=8.31

SYNTHESIS OF ASPARTYL-(β-(S)-2-HYDROXY-4-DIHYDROXYPHOSPHINYL -1-BUTYL ESTER)-ALANYLALANINE. [LIX]

There was treated (2.00 g, 2.87 mmoles) of LXXIII with 5.74 mmoles of trimethyl silyl chloride and 5.74 mmoles of potassium iodide for half an hour at room temperature. Treatment with methanol (40 mL) gave the free phosphonic acid analogue in good yield (1.4 g, 83%).

This entire material was then immediately treated with trifluoracetic acid (15 mL) for 15 minutes. The acid was

evaporated under vacuum and the residue was triturated with anhydrous ether. The product was dissolved in water, neutralized with pyridine to pH 6 and precipitated with acetone. The amorphous residue obtained (0.8 g, 80%) after filtration, was dried under vacuum in a dessicator over phosphorus pentoxide. Spectra and TLC were measured within one hour of reaction using freshly prepared material. Sample for elemental analysis was prepared by drying for 3 hours in an Abderhalden at 50 C using freshly prepared material. NMR showed no protons for BOC-, t-butyl and n-butyl groups. (Rf=0.56, n-BUOH/Py/AcOH/H_2O 60:10:6:24).

NMR($CDCl_3$) δ : 0.9-1.3(d, 6H, $2CH_3$), 1.4-1.6(m, 6H, CH_2), 3.9-4.2 (m, 4H, CH), 7.0-7.3(broad, 3H, NH).
IR($CHCl_3$)cm^{-1}: 3300-3600(broad), 2970, 1725, 1670, 1500, 1250, 1150.

Elemental Analysis: Required: C=39.35 H=6.12, Found: C=40.12, H=6.10

SYNTHESIS OF BOC-ASPARTYL-(β-2-OXO-4-DIETHOXY PHOSPHINYL-1-BUTYL ESTER)-ALANYLALANINE-t-BUTYL ESTER [LX]

To a stirred solution of Boc-Asp-Ala-Ala-t-Bu LXVIII 4.39 g (10.17 mmoles) in 10 mL anhydrous acetonitrile is added 500 mg 4-dimethylaminopyridine and diethyl

4-hydroxy-3-oxobutyl-1-phosphonate (2.28 g, 10.17 mmoles). N,N'-Dicyclohexylcarbodiimide (4.78 g, 23 mmoles) in 10 mL methylene chloride was added to the reaction mixture at 0^{o} C which was then stirred for 15 minutes at 0^{o} C and 6 hr at 20^{o} C. Precipitated DCU was then filtered off and the filtrate evaporated in vacuo. The residue was taken up in ethyl acetate and filtered free of any further precipitate of DCU. The organic solution was worked up in the usual manner. The purification of the product was done by filtration on a short silica gel column using chloroform and ethanol as eluents. Yield 4.0 g (61%).

NMR($CDCl_3$) δ 0.9-2.1(m, 30 H, CH_3, $C[CH_3]_3$), 2.1-2.1(d,2H,CH_2), 2.7-3.2(q,4H, CH_2), 3.8-4.4(m, 4H, CH, CH_2), 7.0-7.2(broad, 3H,NH). IR($CHCl_3$)cm^{-1}: 3400, 2920, 2840, 1720, 1670, 1500, 1380,

Elemental Analysis:

Required: C=53.66, H=8.13 Found: C=53.19, H=7.96

SYNTHESIS OF ASPARTYL-(β-2-OXO-4-DIHYDROXYPHOSPHINYL-1- BUTYL ESTER)-ALANYLALANINE. [LXI]

There was treated (1.5 g, 2.15 mmoles) of LIII with 4.30 mmoles of trimethyl silyl chloride and 4.30 mmoles of potassium iodide in 25 mL of methylene chloride at room temperature for 2 hr. The usual workup with methanol(30 mL) gave free phosphonic acid analogue, 1.0 g (73%).

This entire amount immediately was treated with 15 mL trifluroacetic acid at room temperature for 15 minutes and the usual workup as described earlier gave a crystalline compound [LXXV] 0.5 g (83%) yield. m.p. 62-63° C. Rf=0.59 (^{n}BuOH/Py/AcOH/H_2 60:10:6:24). Spectra and TLC were measured within one hour of reaction using freshly prepared material. Sample for elemental anlysis was prepared immediately by drying for 3 hours in an Abderhalden at 56 C using freshly prepared material. NMR($CDCl_3$) δ 0.9-1.3(d, 6H, $2CH_3$), 1.4-1.7(m, 6H, CH_2), 3.9-4.2 (m, 3H, CH), 7.0-7.4(broad, 3H, NH). IR($CHCl_3$)cm^{-1}: 3300-3600(broad), 2960, 1775, 1670, 1500, 1150

Elemental Analysis: Required: C=39.35, H=6.13, Found: C=39.66, H=5.92

SYNTHESIS OF BOC-ASPARTYL-(β-ETHANAMIDE-(2-DODECYLPHENOXY))-ALANYLALANINE-O-t-BUTYL ESTER [LXII]

The tripeptide LIII (4.00 g,9.3 mmoles) was dissolved in 80 mL of THF and 1.29 mL (9.3 mmoles) triethyl amine. The solution was cooled in ice-acetone. Isobutyl chloroformate (1.2 mL, 9.3 mmoles) was dissolved in 10 mL of THF: this solution was added to the solution of the tripeptide dropwise with cooling and swirling. 2-(Dodecyl phenoxy)-ethanamine (2.84 g, 9.3 mmoles) was cooled in

ice and then added to the anhydride solution. The reaction mixture was left in cold for several hours and then overnight at room temperature. The reaction mixture was filtered and the filtrate was evaporated under vacuum to an oil. This oil was dissolved in ethyl acetate. The organic layer was washed twice with 10% citric acid, once with water, twice with 5% sodium bicarbonate solution and once more with water. The solution was dried over anhydrous magnesium sulfate, filtered and evaporated under vacuum yielding white crystals 6.0 g (89%). m.p. 78-79° C. $[\alpha]_D^{25}$=-27 (c 0.2, CH_3OH).

NMR($CDCl_3$) δ : 0.5-1.5(broad, 31H, $C_{12}H_{25}$, $2CH_3$), 1.6-1.7(s, 18H, $2C[CH_3]_3$), 2.0-2.3(d, 4H, CH_2-CH_2), 3.3-4.0(m, 4H, CH, CH_2), 5.0-5.2(broad, 2H, NH), 6.8-7.4(m, 4H, C_6H_4).

Elemental Analysis: Required: C=66.63, H=9.46, Found: C=66.41, H=9.30

SYNTHESIS OF ASPARTYL-(β-ETHANAMIDE(2-DODECYLPHENOXY))-ALANYLALANINE. [LXIII]

The protected molecule, LXII, (2.0 g, 2.76 mmoles) was treated with 20 mL trifluoroacetic acid for 15 min at room temp. The acid was evaporated under vacuum and the residue was triturated with

anhydrous ether. The product was neutralized with pyridine to pH 6 and then precipitated with acetone. The product was filtered and dried under vacuum in a dessicator at room temp over phosphorus pentoxide yielding 1.4 g (90%) of LXIII. TLC showed a single spot . Rf=0.42 in PrOH:AcOH:water::7:1:2. Spectra and TLC were measured within one hour of reaction using freshly prepared material. Sample for elemental analysis was prepared immediately by drying in an Abderhalden at 56 C using freshly prepared material.

NMR($CDCl_3$) δ 0.7-1.9(m, 33H, $C_{12}H_{25}$, CH_3, CH_2), 3.9-4.1(m,2H, CH), 5.6-6.7(broad, 2H, CH_2), 6.8-6.9(d,1H), 7.0-7.2(s, 1H),7.9-8.3 (s, 2H).

IR($CHCl_3$)cm^{-1}: 3400, 3200, 2900, 1700, 1660, 1500, 1180

Elemental Analysis: Required: C=64.00, H=8.80, Found: C=63.70, H=8.69

SYNTHESIS OF BOC-ASPARTYL-(β-ETHANAMIDE-2-(1,1-DIMETHYLPROPYL) PHENOXY)-ALANYLALANINE-O-t-BUTYL ESTER [LXIV]

Ethanamine-2-(1,1-dimethyl propyl) phenoxy (0.9 g, 4.64 mmoles) and (2.00 g, 4.64 mmoles) of LIII were dissolved in 50 mL of methylene chloride. The solution was cooled in an ice-acetone bath and treated with 2.0 g (9.69 mmoles) DCC in a few mL of methylene chloride. The reaction mixture was left in the bath for 135 min and

then at room temp overnight. It was filtered to remove the precipitate of DCU. The filtrate was evaporated under vacuum and the residue dissolved in ethyl acetate. The organic solution was washed with 5% citric acid solution, once with water , twice with 5% sodium bicarbonate solution and once with water. The solution was dried with magnesium sulfate, filtered and evaporated under vacuum. The residual oil was dissolved in ethyl acetate, filtered to remove any remaining DCU and crystallized by the addition of cold petroleum ether. The product was dried under high vacuum yielding 2.09 g (74%) of [LXIV]. Rf=0.8 [$CHCl_3$ 1 : CH_3OH 1]. $[\alpha]_D^{25}$=-19 (c 0.3, CH_3OH).

NMR($CDCl_3$) δ 0.6-0.7(d, 6H, CH_3), 0.8-1.2(q, 3H, CH_2CH_3), 1.3-1.4(s, 6H, $2CH_3$), 1.5-1.6(s, 18H, $C[CH_3]_3$), 3.8-4.2(m,2H,CH), 6.7-7.5(m, 4H, C_6H_4).
IR($CHCl_3$)cm^{-1}: 3400, 2960, 2900, 1705, 1660, 1500, 1380, 1210, 1150, 1050 Elemental Analysis: Required: C=61.95, H=8.38, Found: C=62.00, H=8.50

SYNTHESIS OF ASPARTYL-(β-AMIDE-2-((-1,1-DIMETHYLPROPYL) -PHENOXYETHANE)-ALANYLALANINE [LXV]

The protected compound [LXXVIII] (1.00 g, 1.6 mmoles) was treated with 15 mL trifluoroacetic acid for 15 min at room temp. The acid was evaporated under vacuum and the residue was triturated

with anhydrous ether. The product was dissolved in water, neutralized with pyridine to pH 6 and then precipitated with acetone. The product was filtered, washed with acetone and dried under vacuum in a dessicator over phosphorus pentoxide. A crystalline material (m.p. 75-76° C) was obtained (700 mg, 94%). TLC showed a homogeneous compound in PrOH:water:AcOH::7:2:1. Rf=0.52. Spectra and TLC were measured within two hours of the reaction using freshly prepared material. Sample for elemental anlysis was prepared immediately by drying for 3 hours in an Abderhalden at 56 C. The above material was stored solid in the freezer. NMR($CDCl_3$,CD_3OD) δ 0.6-0.8(d, 6H, CH_3), 1.2-1.6(m, CH_2CH_3), 3.8-4.3(m, 2H, CH), 6.7-7.4(m, 4H, C_6H_4)

IR($CHCl_3$)cm^{-1}: 3300-3600(broad), 1740, 1650, 1500, 1450, 1100, 960 Elemental Analysis: Required: C=59.50, H=7.75, Found: C=59.12, H=7.80

SYNTHESIS OF BOC-ASPARTYL-(β-AMIDE-3-(2-OCTYLPHENOXY)PROPANE NITRILE)-ALANYLALANINE-t-BUTYL ESTER [LXVI]

A solution of LVIII (2.00 g, 46.5 mmoles) and propanenitrile: 3-((2-(4-octyl phenoxy)ethyl)amino) (1.4 g, 46.5 mmoles) in dichloromethane (50 mL) was cooled to -10° C and DCC (3.00 g, 14.53 mmoles) was added to the stirred mixture which was maintained

at -10^{o} C for 2 hr. After warming to room temp overnight, a few grams of oxalic acid in methanol were added and after a further 0.5 hr, the DCU precipitate was removed by filtration. The organic phase was washed with water, 5% sodium bicarbonate solution, citric acid and water and then evaporated to yield an oil which was crystallized (2.0g, 59%), m.p. 68-69° C.

NMR($CDCl_3$) δ 0.6-0.7(d, 3H, CH_3), 0.8-1.4(m, 20 H, CH_2), 1.5-1.8 (s, 18 H, $C[CH_3]_3$), 1.9-2.0(d, 2H, CH_2), 3.7-4.3(m, 3H, CH), 6.7-7.6 (m, 4H, C_6H_4).

IR($CHCl_3$)cm^{-1}: 3400, 2960, 1740, 1650, 1510, 1460, 1370, 1150, 1050, 900.

Elemental Analysis: Required: C=63.75, H=8.58, Found: C=63.62, H=8.49

SYNTHESIS OF ASPARTYL-(β-AMIDE)-3-(2-OCTYLPHENOXY ETHYL)-PROPANENITRILE)-ALANYLALANINE [LXVII]

The material LXXX (2.8 g, 3.86 mmoles) was treated with 15 mL of trifluoro-acetic acid for 15 min at room temp. The acid was evaporated under vacuum and the residue was worked up in the usual manner as described earlier yielding 2.00 g (95%) of a light yellow crystalline material. Spectra and TLC were measured within 2 hours of the reaction using freshly prepared material. Sample for

elemental analysis was prepared immediately by drying for 3 hours in an Abderhalden at 56 C. TLC showed single spot, Rf=0.6[^{n}BuOH-AcOH-Water-Pyridine(30:6:24:20)].
$[\alpha]_D^{25}$=-14 (c 0.2, CH_3OH).

NMR($CDCl_3$) δ 0.8-0.9(d, 6H, CH_3), 1.3-1.6(m 21H, CH_2CH_3), 3.7-4.6(m, 3H, CH), 6.7-7.5(m, 4H, C_6H_4).
IR($CHCl_3$)cm^{-1}: 3400, 2960, 1740, 1680, 1650, 1500, 1200, 840. Elemental Analysis: Required: C=62.00, H=8.05, Found: C=60.75, H=8.01

SYNTHESIS OF BOC-ASPARTYL-(β-AMIDE)-SULFANILAMIC-ALANYLALANINE-t-BUTYL ESTER [LXVIII]

To a stirred solution of the tripeptide (4.00 g, 9.27 mmoles) in 50 mL of dichloromethane was added 50 mg DMAP and 1.59 g (9.27 mmoles) of sulfanilamide at -10° C. Then DCC (3.00 g, 14 mmoles) was added to the stirred reaction mixture which was left at 10 C for 5 min and then overnight at room temperature. Precipitated DCU was then filtered off and the filtrate was evaporated in vacuo. The residue was taken up in ethyl acetate and filtered free of any further precipitated DCU. The ethyl acetate solution was washed twice with 5% sodium bicarbonate solution, cold 0.5 N hydrochloric acid and then dried over sodium sulfate. The solvent was then

removed by evaporation and the final product was crystallized as yellow crystals (5.1 g, 94%). TLC shows single spot. Rf=0.55, BuOH-CH_3OH-HCl, 8:1:1. $[\alpha]_D^{25}$=-13 (c=0.4, MeOH).

NMR($CDCl_3$) δ 0.9-1.2(d, 6H, CH_3), 1.3-1.6(s, 18H, $C[CH_3]_3$), 2.6-2.8 (s, 2H, NH_2), 3.9-4.3(m, 3H, CH),5.0-5.3(broad, 1H,NH), 6.0-6.1 (broad, 1H, NH), 7.4-7.7(m, 4H, C_6H_4).

SYNTHESIS OF ASPARTYL-(β-AMIDE)-SULFANILMIC-ALANYL- ALANINE. [LXIX]

The protecting groups were cleaved by treating LXVIII (2.00 g, 3.49 mmoles) with TFA (15 mL) in anhydrous methylene chloride for 15 minutes. The acid was evaporated under vacuum and the residue was triturated with anhydrous ether. The product was dissolved in water, neutralized with pyridine to pH 6 and the water was evaporated. The residue was collected, dissolved in ethyl acetate, dried over sodium sulfate and evaporated to give a dark yellow oil. Further purification was done by column chromatography with silica gel (elution with methanol and chloroform) and a pale yellow crystalline material was obtained (1.2 g, 83%). TLC showed homogeneity in PrOH:water:AcOH::7:2:1. Rf=0.39. Spectra and TLC were measured within 3 hours of reaction using freshly prepared material. Sample for elemental analysis was prepared immediately by drying for 3 hours in an Abderhalden at 56 C.

NMR($CDCl_3$) δ 0.9-1.2(d, 6H, CH_3), 2.6-2.8(s, 2H, NH_2), 3.9-4.3 (m, 3H, CH), 5.0-5.3(broad, 1H, NH), 6.0-6.1(broad, 1H, NH), 7.4-7.7(m, 4H, C_6H_4). Elemental Analysis: Required: C=44.75, H=5.40, Found: C=44.13, H=5.71

SYNTHESIS OF CARBOBENZOXY-LEUCYL-ALANINE-O-t-BUTYL ESTER [LXX]

A solution of Z-leucine (6.19 g, 23.39 mmoles) and L-alanine-t-butyl ester hydrochloride 4.25 g (23.39 mmoles) in 30 mL dichloromethane was cooled to -10^{o} C. Triethylamine (TEA) (3.26 mL, 23.39 mmole) and DCC 6.00 g (29.0 mmoles) and DMAP 30 mg were added to the stirred mixture which was maintained at -10^{o} C for 2 hr. After warming to room temp overnight, a few grams of oxalic acid was added and after a further 0.5 hr the DCU ppt was removed by filtration. The organic layer was washed with 5% sodium bicarbonate solution, water, cold 1 N hydrochloric acid, water and dried over sodium sulfate and evaporated to yield 9.00 g (89.7%) of the dipeptide LXX. Rf=0.64 ($CHCl_3$/MeOH 19:1).

NMR($CDCl_3$) δ 0.8-1.1(d, 6H, CH_3), 1.3-1.4(m, 6H, CH, CH_3), 1.5-1.8 (s, 9H, $C[CH_3]_3$), 4.0-4.6(m, 2H, CH), 5.0-5.2(s, 2H, $CH_2C_6H_5$), 5.5-5.7 (d, 1H, CH), 6.7-6.9(d, 1H, NH), 7.4(s, 5H, C_6H_5).

Elemental Analysis: Required: C=64.26, H=8.21 Found: C=64.07, H=8.12

SYNTHESIS OF LEUCYL-ALANINE-O-t-BUTYL ESTER [LXXI]

A suspension of 0.8 g of Pd-C (10%) in absolute ethanol with a few drops of glacial acetic acid and 6.00 g (16.12 mmoles) of the above dipeptide [LXX] was hydrogenated at atmospheric pressure until no further uptake of hydrogen occurred (overnight). The reaction mixture was filtered through celite and the filtrate was evaporated under reduced pressure yielding an oily residue which crystallizes under high vacuum (3.60 g, 92%). NMR showed no benzyl hydrogens.

NMR($CDCl_3$) δ 0.8-1.1(d, 6H, CH_3), 1.3-1.4(m, CH, CH_3), 1.5-1.7 (s,9H, $C[CH_3]_3$), 4.0-4.6(m, 2H, CH), 5.5-5.7(d, 1H, NH), 6.7-6.9 (d, 1H, NH).

Elemental Analysis:

Required: C=55.81, H=8.24

Found: C=55.74, H=8.50

SYNTHESIS OF BOC-ASPARTYL-β-BENZYL-LEUCYL-ALANINE-O-t-BUTYL ESTER [LXXII]

A solution of BOC-β-benzyl aspartate 6.00 g (19.37 mmoles) and leucyl-alanine-O-t-butyl ester 5.00 g (19.37 mmoles) in 100 mL dichloromethane was cooled in an ice-acetone bath. There were added to the stirred mixture DMAP (300 mg) and DCC (7.8 g) which was maintained at -10^{o} C for 1 hr. After warming to room temp overnight, a 4.5 grams of oxalic acid was added and after a further 0.5 hr the DCU precipitates were removed by filtration. The organic layer was washed with 5% sodium bicarbonate solution, water, cold 1N hydrochloric acid, water and dried over sodium sulfate and evaporated to yield 6.00 g (55%) of [LXXII]. $[\alpha]_{D}^{25}=-11.0$ (c=2.06, MeOH).

NMR($CDCl_3$) δ 0.8-1.1(d, 6H, CH_3), 1.3-1.5(d, 6H, CH_2, CH_3), 1.6-1.8 (s, 18H, t-Bu), 3.9-4.6(m, 3H, CH), 5.1-5.3(s, 2H, $CH_2C_6H_5$), 7.4 (s,5H, C_6H_5).

Elemental Analysis: Required: C=60.96, H=8.22 Found: C=60.74, H=8.17

SYNTHESIS OF BOC-ASPARTYL-LEUCYL-ALANINE-O-t-BUTYL ESTER

The suspension of 500 mg of Pd-C (10%) in absolute ethanol with a few drops of glacial acetic acid and 9.00 g (16.75 mmoles) of the above tripeptide was hydrogenated at room temp at atmospheric

pressure until no further uptake of hydrogen occurred. The reaction mixture was filtered free from the catalyst and the filtrate was evaporated under reduced pressure yielded an oily residue, (6.5 g, 94%). Rf=0.49 (EtOAc/MeOH 9:1).

NMR($CDCl_3$) δ 0.8-1.1(d, 6H, CH_3), 1.3-1.4(m, 6H,CH , CH_2, CH_3), 1.5-1.8(s,9H, t-Bu), 2.0-2.2(d, 2H, NH_2), 3.9-4.4(m, 2H, CH), 7.8-8.3(broad(1H, NH). Elemental Analysis: Required: C=60.04, H=10.07 Found C=59.92, H=10.12

SYNTHESIS OF BOC-ASPARTYL-(β-(S)-2-HYDROXY-4-DIBUTOXYPHOSPHINYL-1-BUTYL ESTER)-LEUCYLALANINE-t-BUTYL ESTER [LXXIII]

To a stirred solution of 2.0 g (4.65 mmole) tripeptide (Boc-Asp-Leu-Ala-O-t-Bu) in 10 mL anhydrous dichloromethane, is added 20 mg DMAP and 1.31 g (4.65 mmole) dibutyl 3,4-dihydroxybutyl-1-phosphonate. Then DCC (1.5 g) is added to the reaction mixture at 0^{o} C, which is then stirred for 5 min at 0^{o} C and 3 hr at 20^{o} C. Precipitated DCU is then filtered off and the filtrate evaporated down in vacuo. The residue is taken up in ethyl acetate and the work-up as described for [LXII] gives the esterified product (2.00 g, 68%). Rf=0.57 in isopropanol-chloroform, 7:3.

NMR($CDCl_3$) δ 0.7-1.1(d, 9H, CH_3), 1.4-1.8(m, 30H, $[CH_3]_3$, $CH_2CH_2CH_3$), 2.0-2.2(m,4H, CH_2), 4.0-4.3(m, 3H, CH), 6.0-6.2(broad, 2H, NH).

Elemental Analysis: Required: C=55.31, H=8.62 Found: C=55.16, H=8.56

SYNTHESIS OF

ASPARTYL-(β-(S)-2-HYDROXY-4-DIHYDROXYPHOSPHINYL-1-BUTYL ESTER)-LEUCYLALANINE [LXXIV]

The protecting groups were removed to give the free phosphonic acid analogue by using trimethylsilyl chloride (60 mmole) and potassium iodide (60 mmole) in acetonitrile (30 mL) at room temp for 2 hr. Treatment with methanol (50 mL) gave the free phosphonic acid analogue in 70% yield. The BOC- and t-butyl groups were removed from LXXIV by treating immediately with trifluoroacetic acid for 15 min at room temp. The excess acid was removed in a rotary evaporator and the residue was triturated with ether. The TFA-salt was dissolved in water and was neutralized to pH 6 with pyridine and was precipitated with acetone. The solid residue was filtered and dried in a dessicator over phosphorus pentoxide. Rf=0.54 (^{n}BuOH/AcOH/Py/H_2O 60:6:10:24). Spectra and TLC were measured within one hour of reaction using freshly prepared material. Sample for elemental analysis was prepared immediately

by drying for 3 hours in an Abderhalden at 56 °C. The above compound was stored as solid in the freezer. NMR($CDCl_3$) δ 0.8-1.1(d, 6H, CH_3), 1.3-1.7(m, 6H, CH_2), 4.0-4.3(m, 3H, CH), 6.0-6.2(broad, 1H, NH), 6.3-6.6(b, 1H,NH). Elemental Analysis: Required: C=42.00, H=6.64 Found: C=41.88, H=6.32

SYSTHESIS OF BOC-ALANYL-SERINE-O-BENZYL ESTER [LXXV]

BOC-L-Alanine (5.00 g, 26.4 mmole) was dissolved in 80 mL THF and (3.67 mL, 26.4 mmole) TEA. The solution was cooled in an ice-acetone bath. Isobutylchloroformate (3.64 mL, 26.4 mmole) was dissolved in 15 mL THF. This solution was added to the solution of BOC-alanine in 1-mL portions with cooling and swirling. L-O-benzyl serine (5.15 g, 26.4 mmole) was dissolved in 26 mL of 1.15 N sodium hydroxide and 80 mL dioxane. This mixture was cooled in ice and then added to the anhydride solution. The reaction mixture was left in the cold for several hours and then overnight at room temp. The reaction mixture was freed from the solvent by evaporation under vacuum. The residue was treated with 100 mL hot ethyl acetate and the resulting solution was treated with 100 mL of 1 N hydrochloric acid. The aqueous layer was drawn off and the ethyl acetate layer was washed with two 50 mL portions of water. The ethyl acetate layer was extracted with three 20 mL portions of aqueous sodium bicarbonate solution which were combined and acidified with 1 N

hydrochloric acid. The acidified suspension was extracted with 50 mL portions of ethyl acetate which were washed three times with 40 mL water and dried over anhydrous sodium sulfate. Evaporation of the solvent left an oil which was crystallized with ethyl acetate and petroleum ether (6.00 g, 62.5%). Rf=0.4 ($CHCl_3$/EtOAc 2:3).

NMR($CDCl_3$) δ 0.9-1.2(d, 3H, CH_3), 1.6-1.9(s, 9H, $C[CH_3]_3$), 2.0-2.2 (d, 2H, CH_2), 4.0-4.3(m, 2H, CH), 4.4-4.7(s, 2H, $CH_2C_6H_5$), 5.1-5.3 (broad, 1H, NH), 5.5-5.8(broad, 1H, NH), 7.4(s, 5H, C_6H_5), 11.0 (s, 1H, COOH).

Elemental Analysis: Required: C=59.00, H=7.15 Found: C=58.81, H=7.71

SYNTHESIS OF BOC-ALANYL-(-BENZYL)-SERYL-ALANINE-O-t-BUTYL ESTER (LXXVI)

A solution of the dipeptide [LXXV], (5.5 g, 15.00 mmoles) and (2.7 g, 15.00 mmoles) alanine-t-butyl ester hydrochloride in dichloromethane (100 mL) was cooled to -10°C. Triethylamine (2.00 mL, 15 mmoles) , DCC (5.00 g, 24 mmoles) and DMAP (20 mg) were added to the stirred mixture which was maintained at -10° C for 2 hr. After warming to room temperature overnight, a few drops of glacial acetic acid were added and after a further 0.5 hr,

the DCU ppt were removed by filtration. The organic layer was washed with 5% sodium bicarbonate solution, water, cold 1 N hydrochloric acid and water and then evaporated to yield 5.00 g (63%) of the tripeptide [LXXVI].

NMR($CDCl_3$) δ 0.8-1.4(m, CH_3, $C[CH_3]_3$), 1.8-2.0(d, 2H, CH_2), 3.8-4.1 (m, 2H, CH), 4.4-4.6(s, 2H, $CH_2C_6H_5$), 5.0-5.2(broad, 1H, NH), 5.9-6.1(broad, 1H, NH), 7.4(s, 5H, C_6H_5).

IR($CHCl_3$)cm^{-1}: 3570, 2990, 2940, 1720, 1670, 1500, 1370, 1300, 1100, 1050, 850.

Elemental Analysis: Required: C=60.83, H=7.96 Found: C=60.964, H=7.52

SYNTHESIS OF BOC-ALANYL-SERYL-ALANINE-O-t-BUTYL ESTER [LXXVII]

A suspension of 0.2 g of Pd-C catalyst (10%) in absolute ethyl alcohol with a few drops of glacial acetic acid and 4.00 g (8.30 mmoles) of LXXVI was hydrogenated at atmospheric pressure until no further uptake of hydrogen occurred. The reaction mixture was filtered free from the catalyst and the filtrate was evaporated under reduced pressure. The residue was crystallized from ethyl

acetate-hexane-pet. ether yielding 3.18 g (95%). Proton NMR did not show any benzyl protons. M.P. 93-94°C. $[\alpha]_D^{25}$=-18 (c 0.3, CH_3OH).

NMR($CDCl_3$) δ 0.9-1.2(d, 6H, CH_3), 1.4-1.8(s, 18H, $C[CH_3]_3$), 3.9-4.1 (m, 3H, CH), 4.7-4.9(broad, 1H, NH). IR($CHCl_3$)cm^{-1}: 3400, 2980, 2920, 1710, 1660, 1500, 1450, 1380, 1250, 1150, 1050.

Elemental Analysis: Required: C=53.28, H=8.24 Found: C=53.06, 8.17

SYNTHESIS OF BOC-ALANYLSERYL-(O-3-CARBOXY-1,4-DIHYDRO-1-ETHYL-7-METHYL-4-OXO-1,8-NAPHTHYRIDINE)-ALANINE-t-BUTYL ESTER [LXXVIII]

To a stirred solution of 0.922 g (3.97 mmoles) of nalidixic acid in 30 mL of anhydrous dichloromethane is added 50 mg DMAP and 1.80 g (3.97 mmoles) of the tripeptide (LXXVII). The DCC (1.2 g) is added to the reaction mixture at 0° C which is then stirred for 5 min at 0° C and overnight at room temp. Precipitated DCU was then filtered off and the filtrate evaporated in vacuo. The residue was taken up in ethyl acetate and filtered free of any further precipitated DCU. The ethyl acetate solution was washed twice with 0.5 N hydrochloric acid , with 5% sodium carbonate solution and then dried over sodium sulfate. The solvent was

removed by evaporation and the ester was obtained as pale yellow crystals. TLC showed a single spot, Rf=0.6 in ethanol. Yield 2.00 g (81.6%).

NMR($CDCl_3$) δ : 0.7-0.9(t, 3H, CH_3), 1.1-1.2(d, 6H, CH_3), 1.4-1.5 (d, 18H, $C[CH_3]_3$), 4.0-4.6(m, 3H, CH), 7.2-7.4(1H, C_6H_2), 8.3-8.6(m, 1H, C_6H_2). Elemental Analysis: Required: C=58.34, H=7.43 Found: C=57.91, H=7.95

SYNTHESIS OF

ALANYLSERYL-(O-3-CARBOXY-1,4-DIHYDRO-1-ETHYL-7-METHYL-4-OXO-1,8-NAPHTHYRIDINE-ALANINE-t-BUTYL ESTER. [LXXIX]

The protected analogue [LXXVIII] (3.00 g, 4.86 mmoles) was treated with 15 mL of trifluoroacetic acid at room temp for 15 min. The acid was evaporated under vacuum and the residue was triturated with ether. The product was dissolved in water, neutralized with pyridine and precipitated with acetone. The product was filtered and dried under vacuum in a dessicator at room temp over phosphorus pentoxide yielding 2.34 g (78%) of LXXIX. Spectra and TLC were measured within two hours of reaction using freshly prepared material. Sample for elemental analysis was preparewd immediately by drying for 3 hours in an Abderhalden at 56°C using freshly prepared material. Rf=0.34: iPrOH: H_2O: NH_3: 7:2:1

NMR($CDCl_3$) δ 0.7-1.0(m, 3H, CH_3), 1.1-1.2(d, 6H, CH_3), 4.0-4.6 (m, 3H, CH), 7.2-7.4(1H, C_6H_2), 8.3-8.6(m, 1H, C_6H_2). Elemental Analysis: Required: C=54.67, H=5.89 Found: C=54.07, H=5.99

SYNTHESIS OF BOC-ALANYLSERYL-(O-3-HYDROXY-3-METHYL-6-DIETHOXYPHOSPHINYL HEXANOYL)-ALANINE-t-BUTYL ESTER [LXXX]

To a stirred solution of 0.9 g (3.30 mmoles) diethyl 5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonate in 10 mL anhydrous dichloromethane was added 50 mg DMAP and 1.7 g of the tripeptide, XCII. The DCC (1.5 g) was added to the reaction mixture at 0° C which was then stirred for 5 min at 0° C and 3 hr at 25° C. Precipitated DCU was then filtered off and the filtrate evaporated in vacuo. The residue is then taken up in ethyl acetate and filtered free of any further precipitated DCU. The ethyl acetate layer was washed twice with 0.5 N hydrochloric acid, twice with 5% sod. bicarbonate solution and then dried over sodium sulfate. The solvent was removed by evaporation and the ester, LXXX, isolated by crystallization.. Crystalline product was further purified on a short silica gel column (1.5 g, 57%).

NMR($CDCl_3$) δ 0.7-0.8(s,3H, CH_3), 0.9-1.1(d, 6H, CH_3), 1.3-1.6 (m, 31H, $3CH_2$, $C[CH_3]_3$), 2.4-2.6(q, 4H,

CH_2), 3.8-4.0(m, 2H, CH), 5.4-5.6(broad, 1H, NH), 6.0-6.2(broad, 1H, NH).

Elemental Analysis: Required: C=50.94, H=7.93 Found: C=50.46, H=7.54

SYNTHESIS OF ALANYLSERYL-(O-3-HYDROXY-3-METHYL-6-DIHYDROXYPHOSPHINYL HEXANOYL)-ALANINE [LXXXI]

Chlorotrimethylsilane (2.1 mL, 16.5 mmoles) was added dropwise to a mixture of the phosphonate analogue, LXXX, (4.00 g, 5.5 mmoles) and anhydrous potassium iodide (2.7 g, 16.5 mmoles) in dry acetonitrile (30 mL). The mixture was stirred at ambient temp for 2.5 hr. The resulting potassium chloride was filtered off rapidly and washed with dry ether (20 mLx2). Upon removing low boiling material from the filtrate with an evaporator, the corresponding silyl ester was obtained as oily residue. This was dissolved in methanol and the mixture was concentrated to give an oil.

This oil was immediately treated with 15 mL TFA for 15 min at room temp. The acid was evaporated under vacuum and the residue was triturated with anhydrous ether. The product, LXXXI, was dissolved in water, neutralized with pyridine to pH 6 and precipitated under vacuum. NMR showed no protons of BOC, t-Bu or ethyl groups. TLC and spectra were measured within one hour of reaction using freshly prepared material. Sample for elemental

analysis was prepared immediately by drying for 3 hours in an Abderhalden at 56°C. Rf=0.52 ($BuOH-H_2O-AcOH-Pyr$, 36:4:25:35)

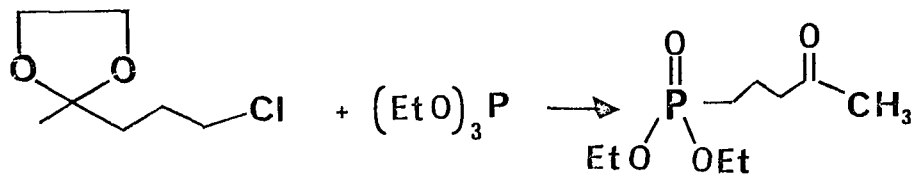
NMR($CDCl_3$) δ 0.7-0.9(s, 3H, CH_3), 1.0-1.2(d, 6H, CH_3), 1.4-1.7(m, 6H, CH_2), 4.0-4.3(m, 2H, CH), 6.0-6.2(broad, 2H, NH).

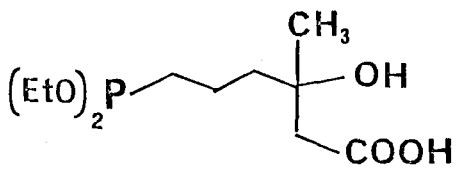
RESULTS AND DISCUSSION

The isosteric analogue of dihydroxyacetone phosphate, diethyl 4-hydroxy-3-oxobutyl-1-phosphononate, , was synthesized by adopting the following procedure. 2-Butyne-1,4-diol was converted into acetoxymethyl vinyl ketone which was further converted into diethyl 1-acetoxy-2-ethoxy- but-2-enyl-4-phosphonate by reaction with triethyl phosphite. Diethyl 1-acetoxy-2-ethoxy but-2-enyl-4-phosphonate was treated with hydrochloric acid to yield diethyl 4-hydroxy-3-oxobutyl-1-phosphonate.

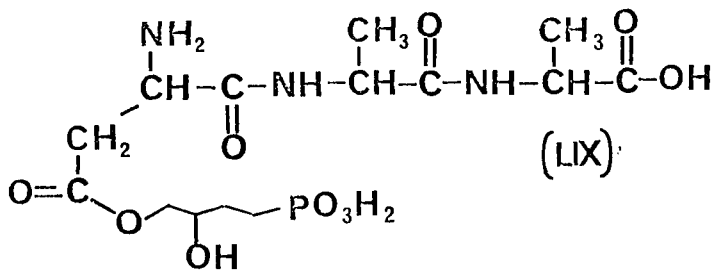
3,4-Dihydroxybutyl-1-phosphonic acid [VI], and 5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonic acid [XXXVIII] were synthesized as described (vide infra) in the literature 21,117).

The generation of compounds which might use the oligopeptide transport system were begun with the synthesis of aspartylalanylalanine as the chemical vector and 3,4-dihyroxybutyl-1-phosphate and 4-hydroxy-3-oxobutyl-1-phosphonate, as the potential antimetabolites. The β-carboxyl functionality on aspartic acid allows conjugation with the hydroxyl groups of the phosphonate analogues giving the conjugates [LIX] and [LXI].

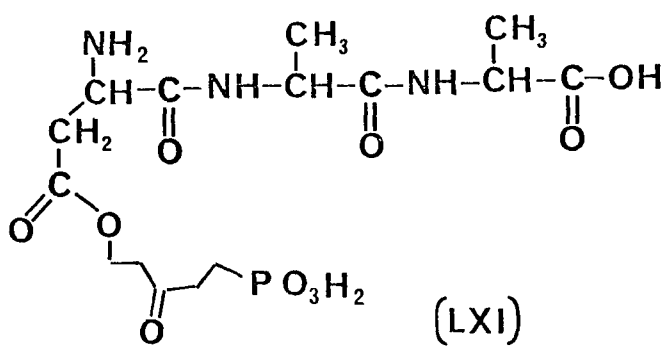




(XXXVIII)

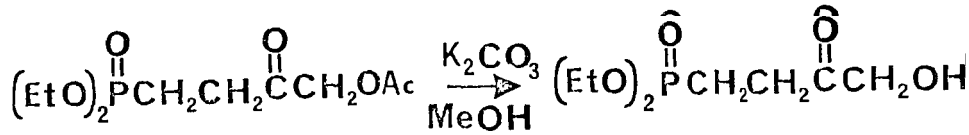


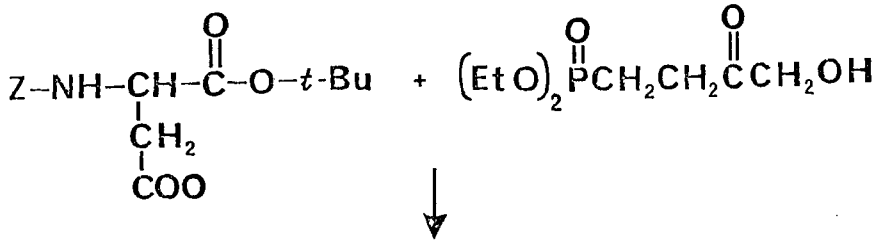
(LIX)

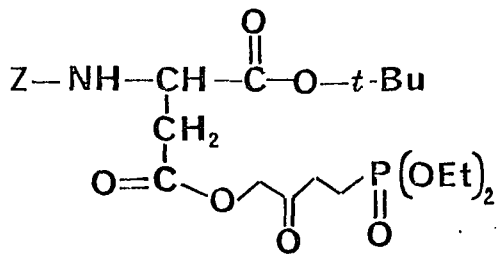


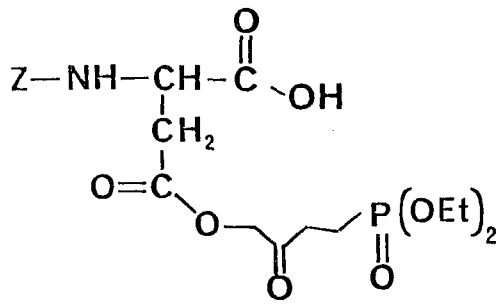
(LXI)

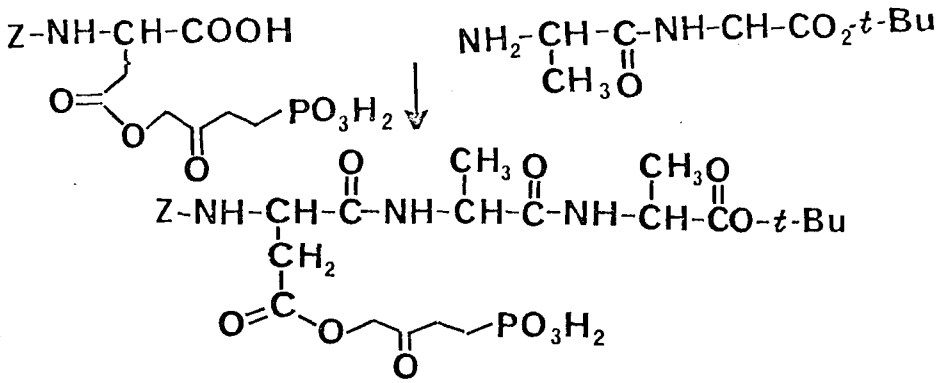
Three approaches for the synthesis of these peptides may be considered. The first of these involves coupling the phosphonate analogue to the aspartate unit at the β-carboxyl position followed by the attachment of a dialanyl unit to the carboxyl function of the adduct as shown:



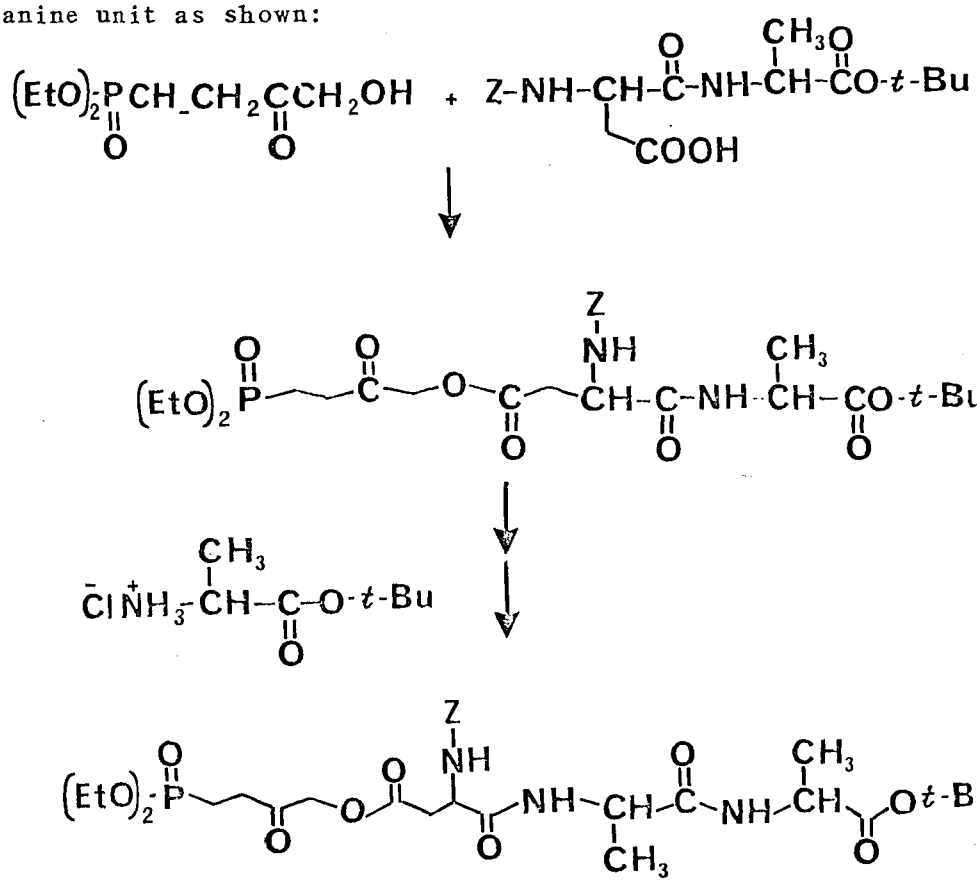




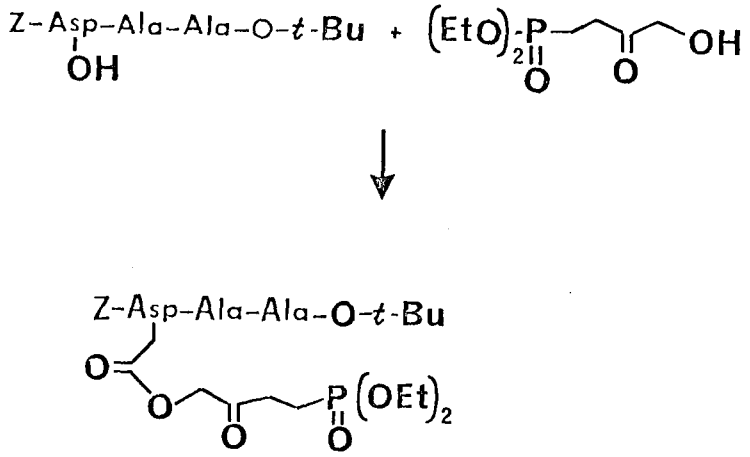




The second approach would be to couple the phosphonate analogue to a dipeptide, aspartylalanyl unit, and then link to it another alanine unit as shown:



The third, and most likely the best approach synthetically, is to synthesize the tripeptide unit first and then couple it to the phosphonate analogue or to other antimetabolites.

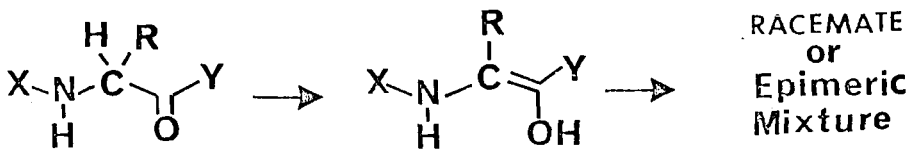


Minor amounts of the undesirable diastereoisomer at an early stage can affect the outcome of the synthesis (126). Racemization can occur during protection of amino acids and also in the procedures used for the removal of protecting groups. The principal concern about racemization involves the step of activation. Conversion of a carboxyl group to a reactive derivative requires the replacement of its hydroxyl by an electron withdrawing substituent. This process should and often does affect the next carbon atoms (α to the carboxyl), which is the center of asymmetry. In the reactive intermediates, reversible removal (of a proton from the α-carbon atom) is generally more facile and racemization can occur.

Two different mechanisms have been put forward to explain the loss of optical purity in reactions involving the carboxyl group of acyl amino acids or peptides: a) the formation of oxazolinone (azlactone) intermediate, which are known to racemize easily

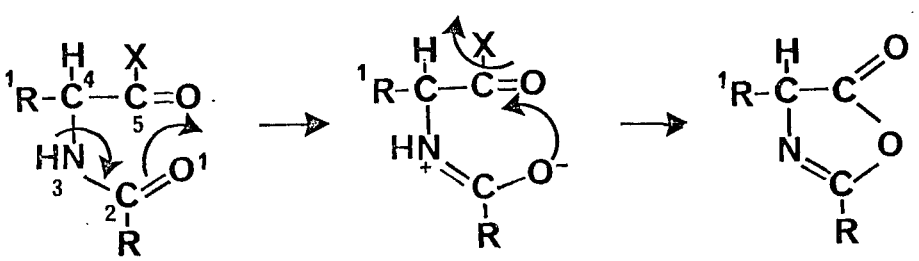
(129-132), and b) the proton abstraction from the asymmetric α-carbon atom.

Perhaps the best understood α-inversion processes are the very slow racemization reactions of simple amino acids and their unactivated amino acid derivatives. These appear to occur via reaction:



simple enolization of the carbonyl, a mechanism that is strongly influenced by the nature of the substituents X, R, and Y, the temperature, and the basicity of the medium.

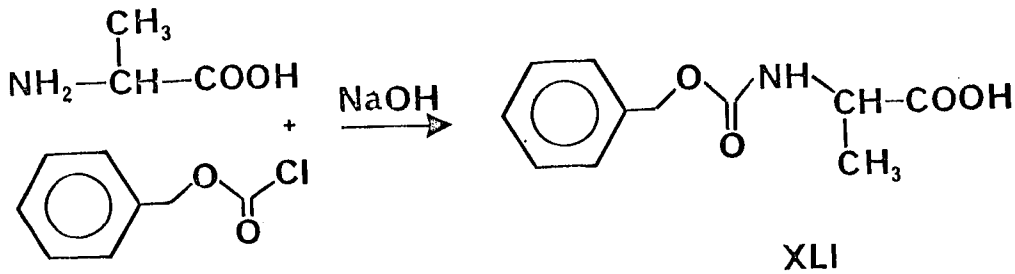
As early as 1928, Bergman and Zervas (133) assumed the formation of azlactones to explain the racemization observed during the acetylation of amino acids in glacial acetic acid with more than the equivalent amount of acetic anhydride. This mechanism can be depicted by the following structures:



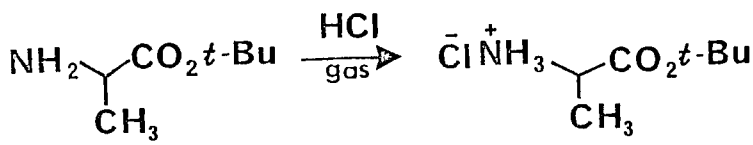
The oxazolinones thus formed have a strong tendency, in the presence of base, to lose the proton at carbon 4. The formation of oxazolinones intermediate is enhanced if R is an electronegative group (phenyl, chloroacetyl, trifluoroacetyl, etc. 134).

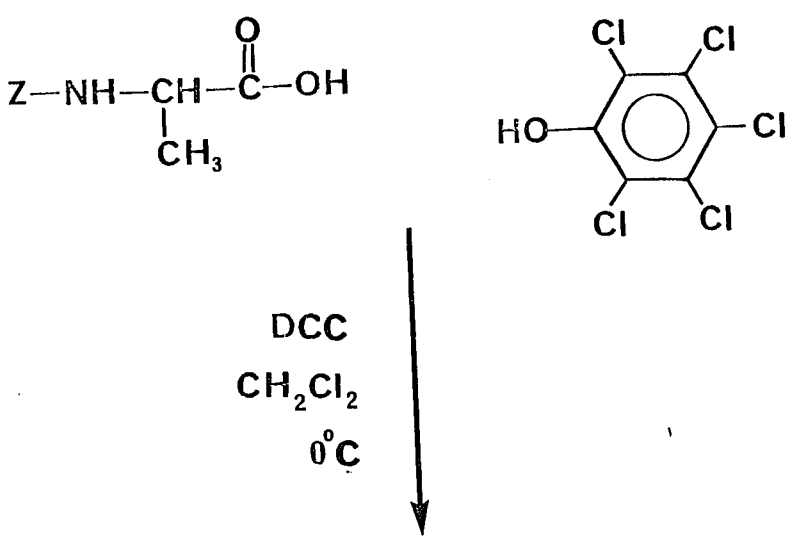
For these syntheses of tripeptides linked to the phosphonate analogues, the carbobenzyloxy group was used initially for the protection of the amino termini (135) and t-butyl esters were used for the protection of carboxyl termini (136). In the latter efforts the butyloxycarbonyl group (137) was used as the amino termini protecting function instead of the carbobenzoxy group since its use and deprotection (along with t-butoxy group) eliminates an additional step. The deprotection of BOC- and t-Bu groups can be performed together and also the preparation of the BOC-amino acid is more facile and proceeds in higher yield.

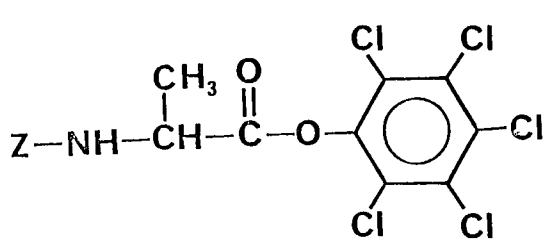
The pentachlorophenyl active esters, which were first reported in the literature in 1961 (138), afford an excellent method for lengthening the peptide chain. These were used as they offer the following advantages: a) they are one of the most active esters (139), b) they are generally higher melting compounds than other active esters which leads to their easy crystallization and purification (140), c) they are conveniently prepared without any significant racemization and in most amino acids protected with a urethane-type protecting group (such as carbobenzoxy group in one case), the tendency of carbanion formation by such direct abstraction of proton from α-carbon is not significant. (141), d) they are stable to controlled hydrogenation conditions and make an excellent combination with N-carbobenzoxy and tert. butyl protecting groups when the incorporation of trifunctional amino acids in the tripeptides is desired (140), Scheme VI).



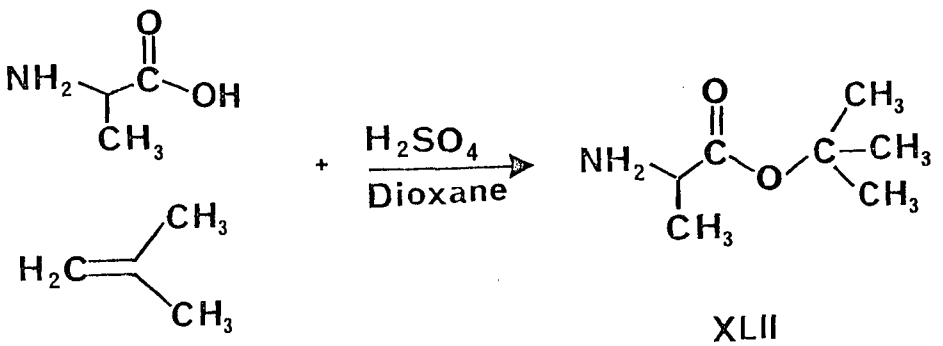
XLI





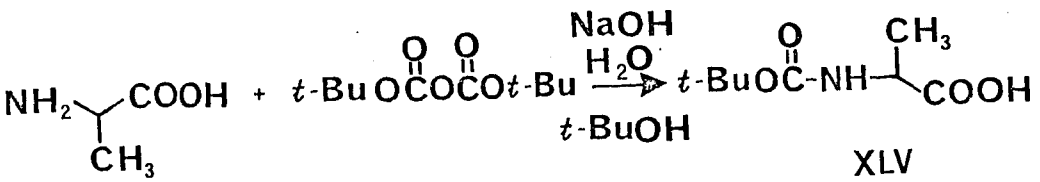


SCHEME VII

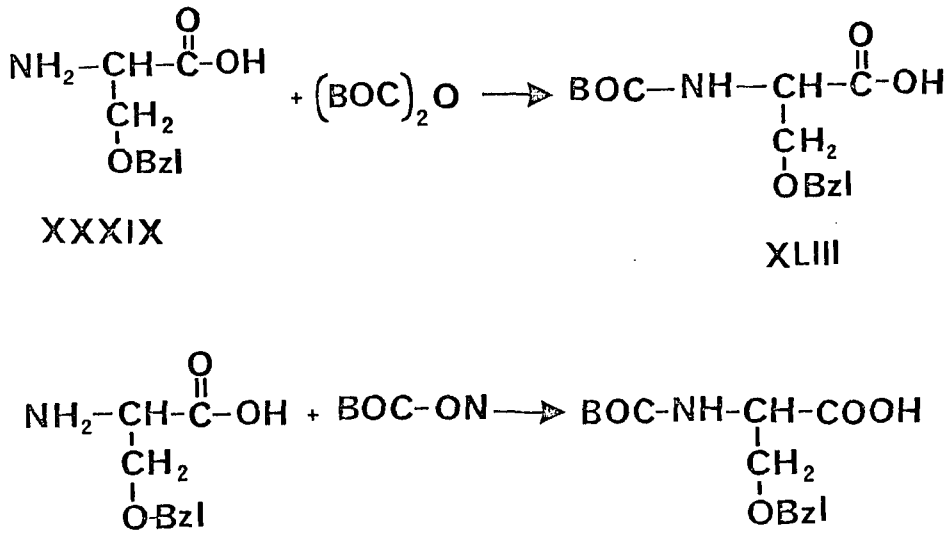


XLII

tert-Butyl carbazate (BOC-azide, (142)), tert-butyl 4,6-dimethylpyrimidyl-2-thiolcarbonate (BOC-S, 143)), tert.butyloxycarbonyloxyimino-2- phenylacetonitrile (BOC-ON, 144)), and Di-tert.butyldicarbonate $(BOC)_2O$ (143) are the most commonly used protecting groups for the amino terminus. Both BOC-ON and BOC-anhydride were used for our purpose but the ideal reagent observed for introducing the t-butyloxycarbonyl protecting group is di-tert-butyldicarbonate because it is a stable, harmless type reagent which reacts smoothly and rapidly in aqueous organic solvent mixtures in outstanding yields, the only by-products being tert.butanol and carbon dioxide (Scheme VII).



XLV



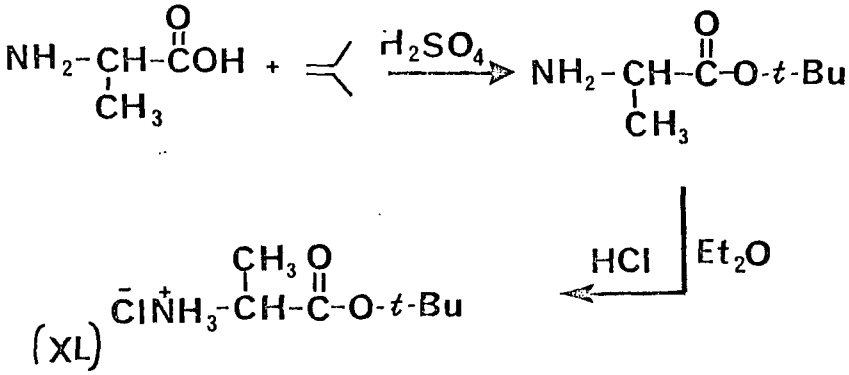
XXXIX

XLIII

SCHEME VIII

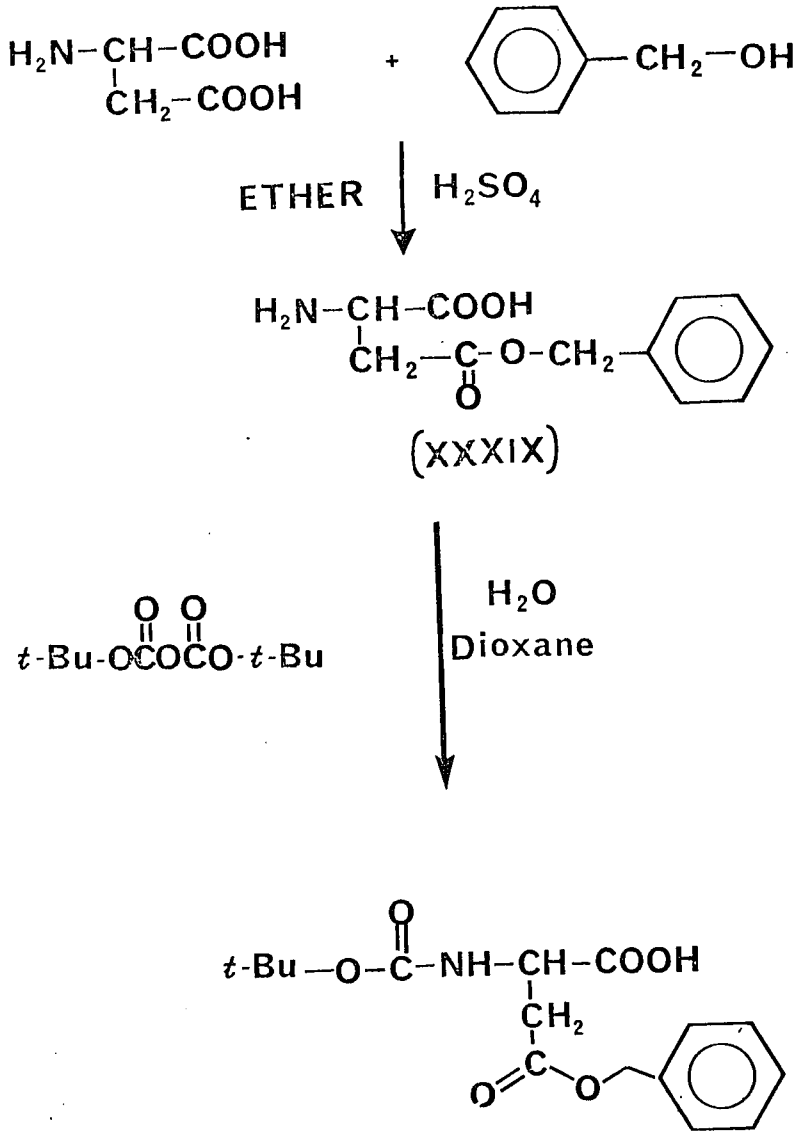
The amino acids protected with a urethane type protecting group, for example, benzyloxycarbonyl group, the tendency of racemization is not significant. Racemization takes place via oxazolones, in which proton abstraction leads to a resonance-stabilized carbanion. The amino acids with benzyloxycarbonyl group and butyloxycarbonyl group, the electron distribution characteristic for urethane prevents the loss of the N-H proton and no azlactone is produced.

A convenient method for protecting the carboxyl group using the t-butyl ester (145), without protecting the amino function, is to allow the amino acid to react with liquified isobutylene in the presence of sulfuric acid in a pressure bottle (146). The desired compound is isolated as the hydrochloride.

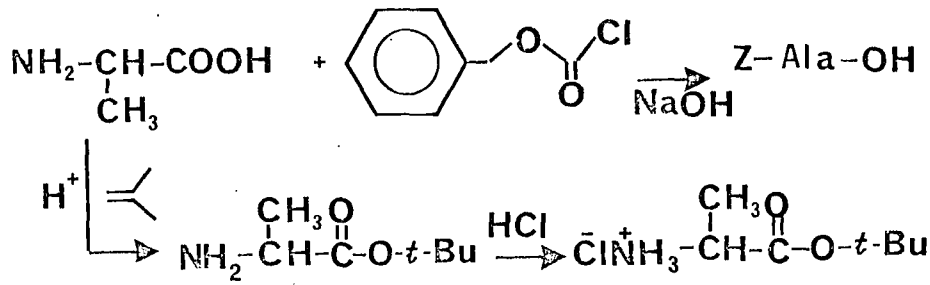


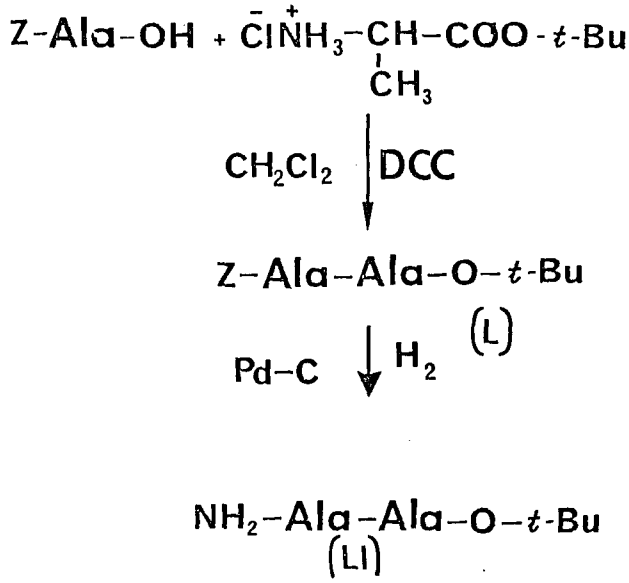
For the synthesis of the tripeptide aspartylalanylalanine starting from the trifunctional amino acid, aspartic acid, the amino function was protected with the BOC group using BOC-ON and BOC-anhydride. Better results were obtained with BOC-anhydride. The β-carboxyl function was protected as the benzyl ester.

The dipeptide, alanylalanine, was synthesised by the coupling of carbobenzoxyalanine and alanine-t-butyl ester hydrochloride by the carbodiimide method in good yield. The dipeptide was subjected to catalytic hydrogenation (147) over palladium on carbon at atmospheric pressure. This catalytic hydrogenation cleaves the benzyloxycarbonyl group in almost quantitative yield without affecting the t-butyl ester.



Butyloxycarbonyl-β-benzyl aspartate and alanylalanine-t-butyl ester were allowed to react in dichloromethane by the carbodiimide method in 89% yield. This procedure has been used to synthesize BOC-aspartylalanylalanine-t-butyl ester for further use with other antimetabolites.With the widely used DCC reagent, racemization increases with the temperature (134) and is more pronounced in polar solvents such as dimethylformamide than in methylene chloride.

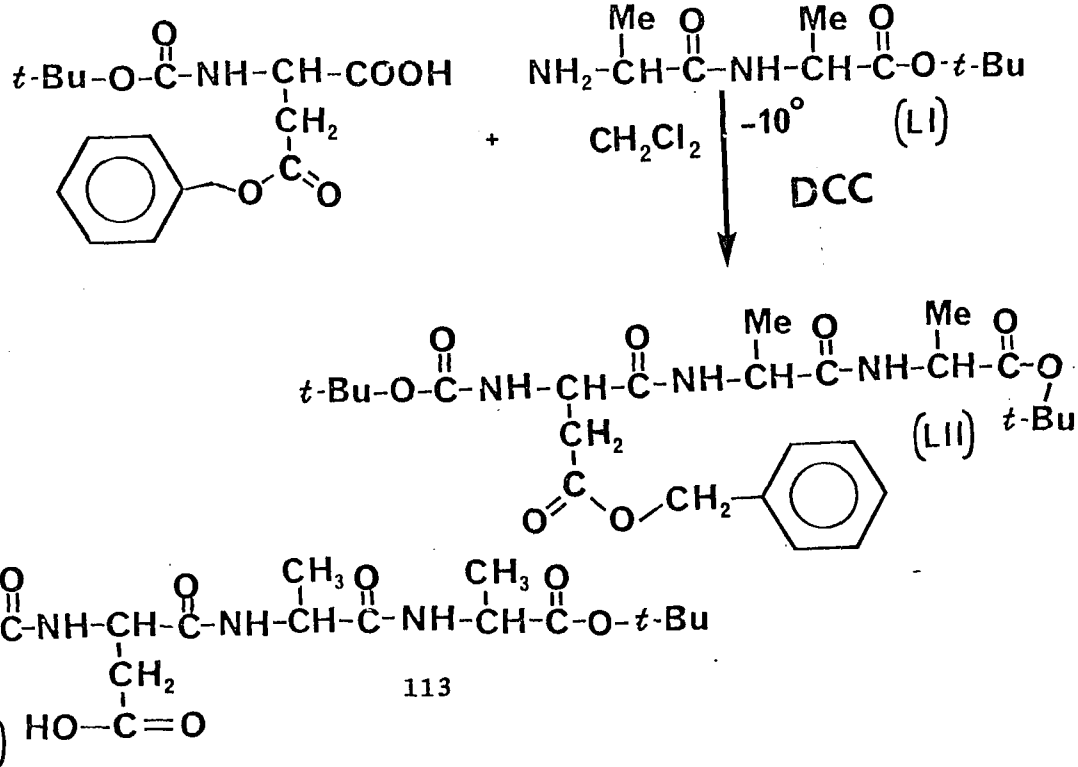


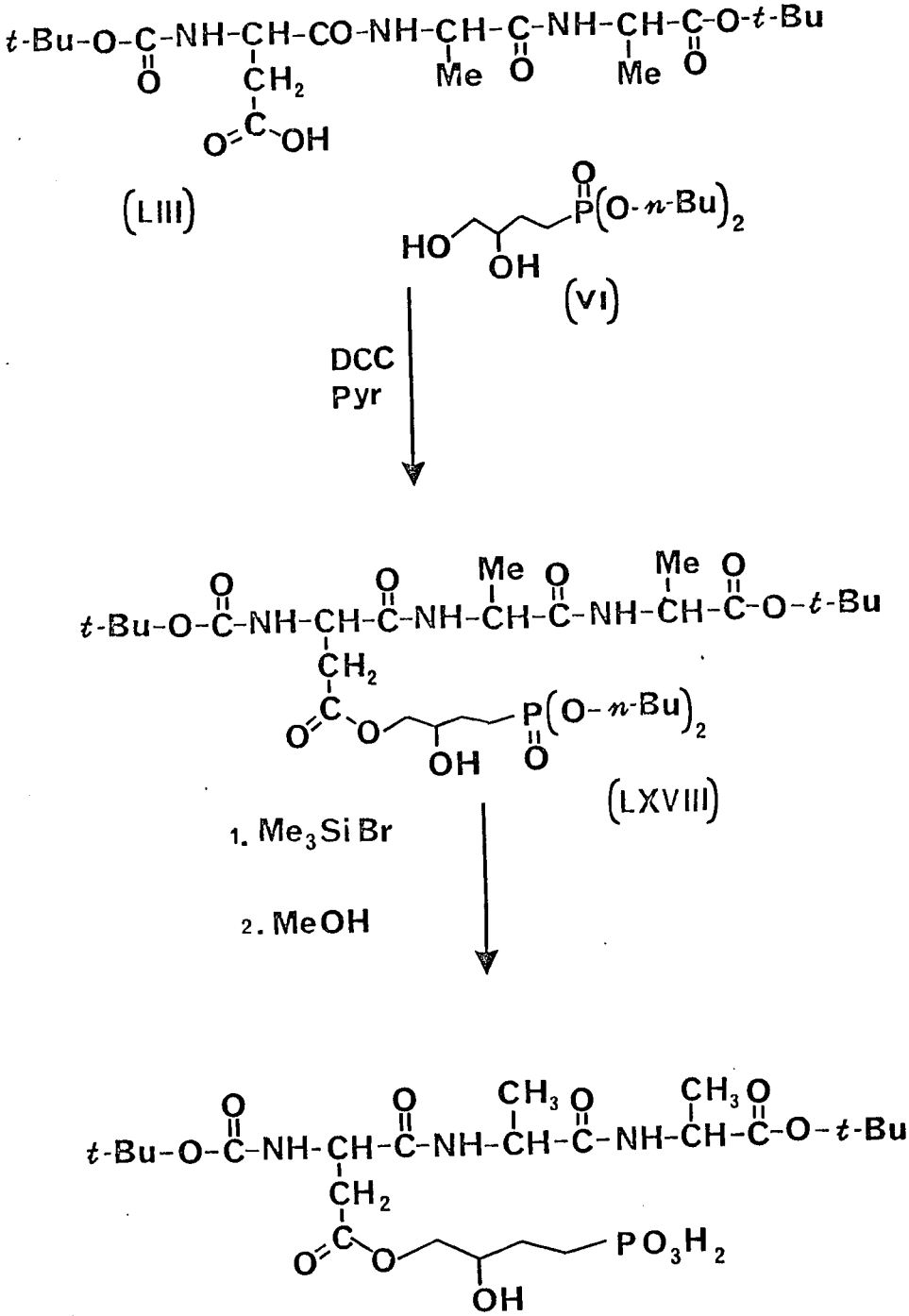


The above tripeptide derivative, BOC-(β-benzyl)-aspartylalanylalanine-t- butyl ester, was hydrogenated catalytically to cleave the β-benzyl group on the aspartate unit. The product was obtained in quantitative yield.

The tripeptide [LIII] was allowed to react with dibutyl 3,4-dihyroxy-1-butyl phosphonate in anhydrous pyridine using N,N'-dicyclohexylcarbodiimide at 0° C giving the coupled compound [LXIX] in 46% yield. The phosphonate esters were cleaved to the phosphonic acid by reaction with bromotrimethylsilane and the subsequent hydrolysis under mild conditions of the resulting bis(trimethylsilyl) phosphonate (148,149). The protecting groups, butyloxycarbonyl and t-butyl groups were cleaved by the treatment with trifluoroacetic acid (TFA) as shown in Scheme IX.

The coupling of the tripeptide [LIII] with diethyl 4-hydroxy-3-oxobutyl-1-phosphonate was performed in anhydrous acetonitrile using DCC in the presence of N,N-dimethylamino

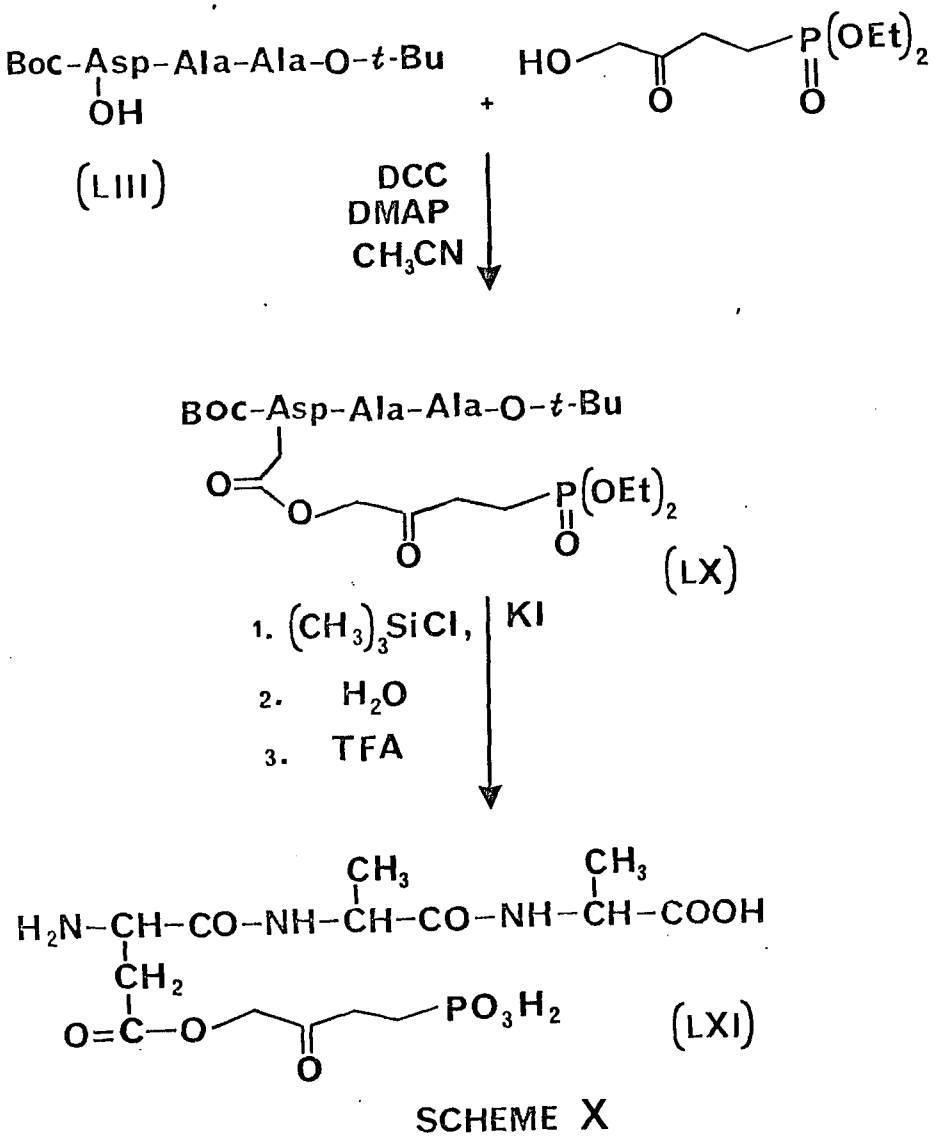




SCHEME IX

pyridine (DMAP) as a catalyst (150) for esterification. The favorable catalytic action of pyridine in such reactions (151) suggested that the 10^4-fold more effective acylation catalyst 4-dimethylaminopyridine might prove to be a useful agent in our present studies on the esterification. Indeed, we found that the addition of 3-10 mole % DMAP accelerates the DCC-activated esterification of carboxylic group in [LIII] with alcoholic group in diethyl 4-hydroxy-3-oxobutyl-1-phosphonate to such an extent that the formation of side products is suppressed and even the strictly demanding ester LX is formed in good yield (61%) at 0^o C. The protecting groups on the phosphonate ester were removed to yield the free phosphonic acid using trimethylsilyl chloride and potassium iodide (152). The silyl ester formed is also easily hydrolysed by the treatment with water to give the corresponding acid. The BOC- and t-Bu ester were removed in the usual way as mentioned earlier, Scheme X.

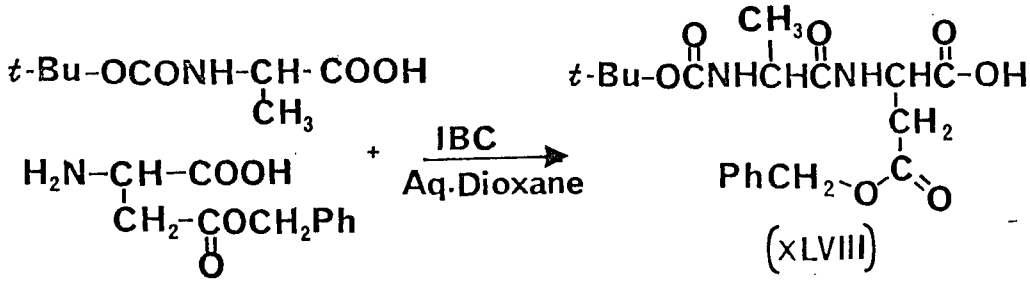
The second chemical vector, alanylaspartylalanine was synthesized using various coupling agents such as isobutyl mixed carbonic anhydride (153), DCC and the Woodward reagent K (154). Carboxylate ($RCOO^-$) reacts rapidly and smoothly with 3-substituted isoxazolium salts under very mild conditions to give the enol esters. Applying this reaction, as a carboxyl-activating step, N-ethyl-5-phenyisoxalium-3- sulfonate (WR) was used.

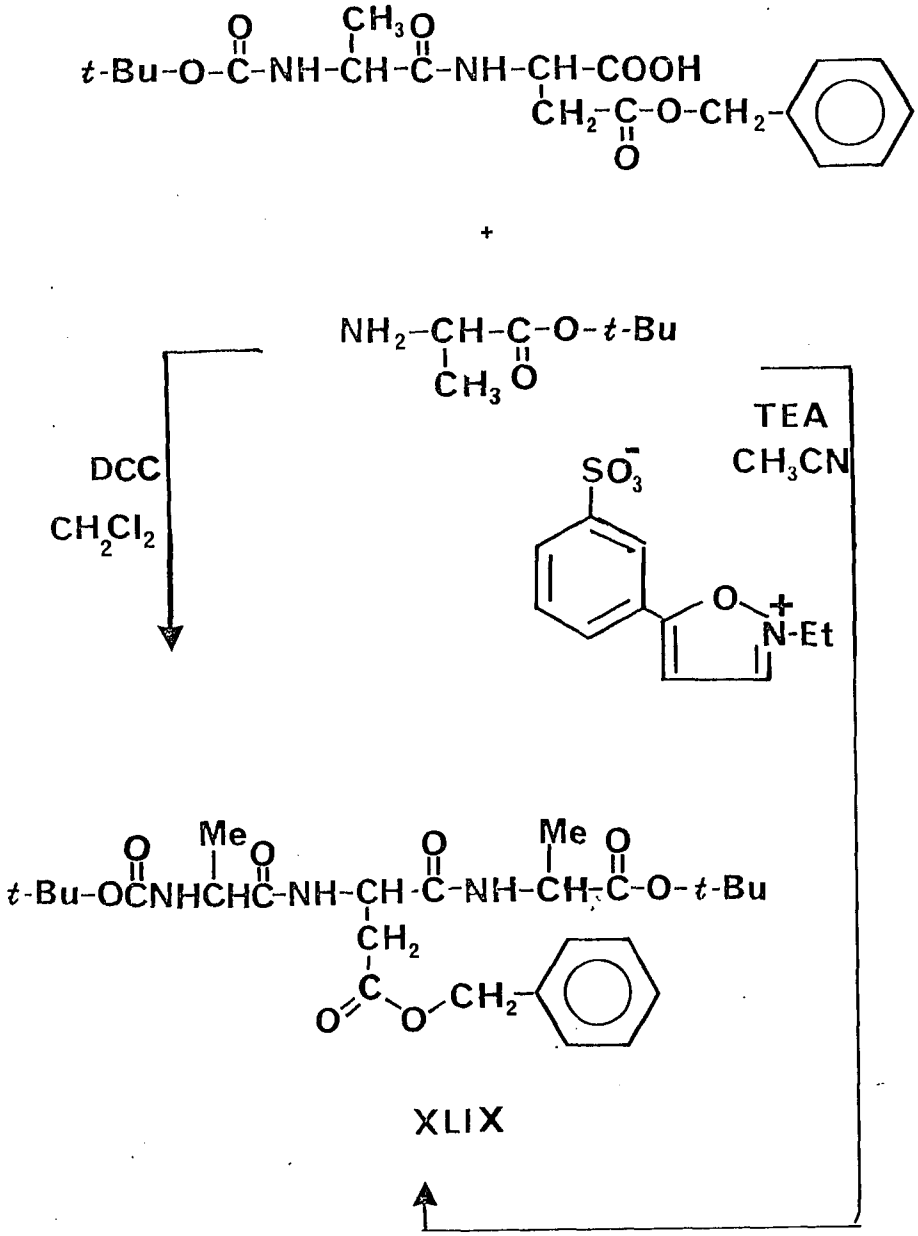


SCHEME X

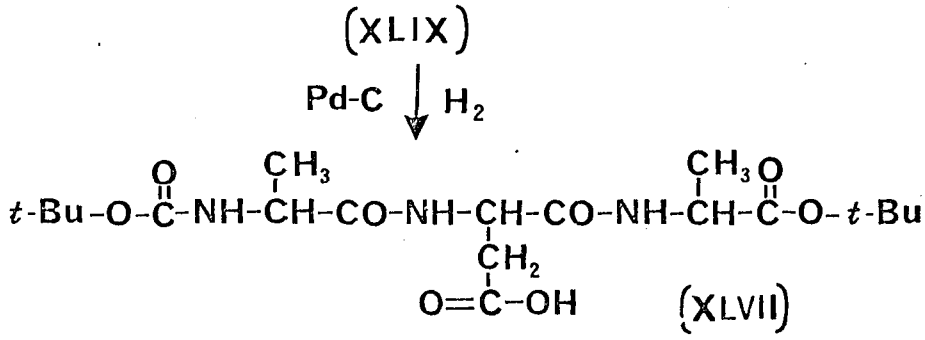
BOC-Alanine was coupled to β-benzyl-l-aspartate (XLVII) by the isobutyl mixed carbonic anhydride (MA) in aqueous dioxane to give a 76% yield of the dipeptide . The mixed anhydride procedure is executed in two stages. First, an alkylchloroformate (usually ethyl or isobutyl) is added to a chilled solution of the peptide acid and an equivalent tertiary amine. Second, this solution is treated with

the peptide amine. Determann and Weiland (155) observed that extensive epimerization occured if the first step is prolonged and that MA formation is complete within a very short time. For this reason a short time (5-10 min) was given after the addition of isobutylchloroformate and the amino component was added. This was coupled to alanine-t-butyl ester with Woodward's reagent to give a fully blocked tripeptide (XLIX) in 58% yield as shown in Scheme XI. The Woodward's reagent K usually give high yields and the carboxyl groups of threonine, serine and tyrosine can be activated without the protection of the side chain hydroxyls. Its disadvantages are the rearrangements of the enol ester to an imide and the limited choice of the solvents. The recommended solvents, acetonitrile or nitromethane are often not applicable to long polypeptide chains. Also, there is an appreciable racemization in the activation of acyl peptides. The activation of acyl peptides is generally accompanied by oxazolinones formation, therefore, the prevention or suppression of racemization during the coupling of the fragments is usually a demanding task. The application of azide coupling will reduce the extent of racemization. The coupling of the dipeptide (XLVIII) with alanine-t-butyl ester by DCC and DMAP yielding the tripeptide (XLIX) proceeded in good yield (68%).





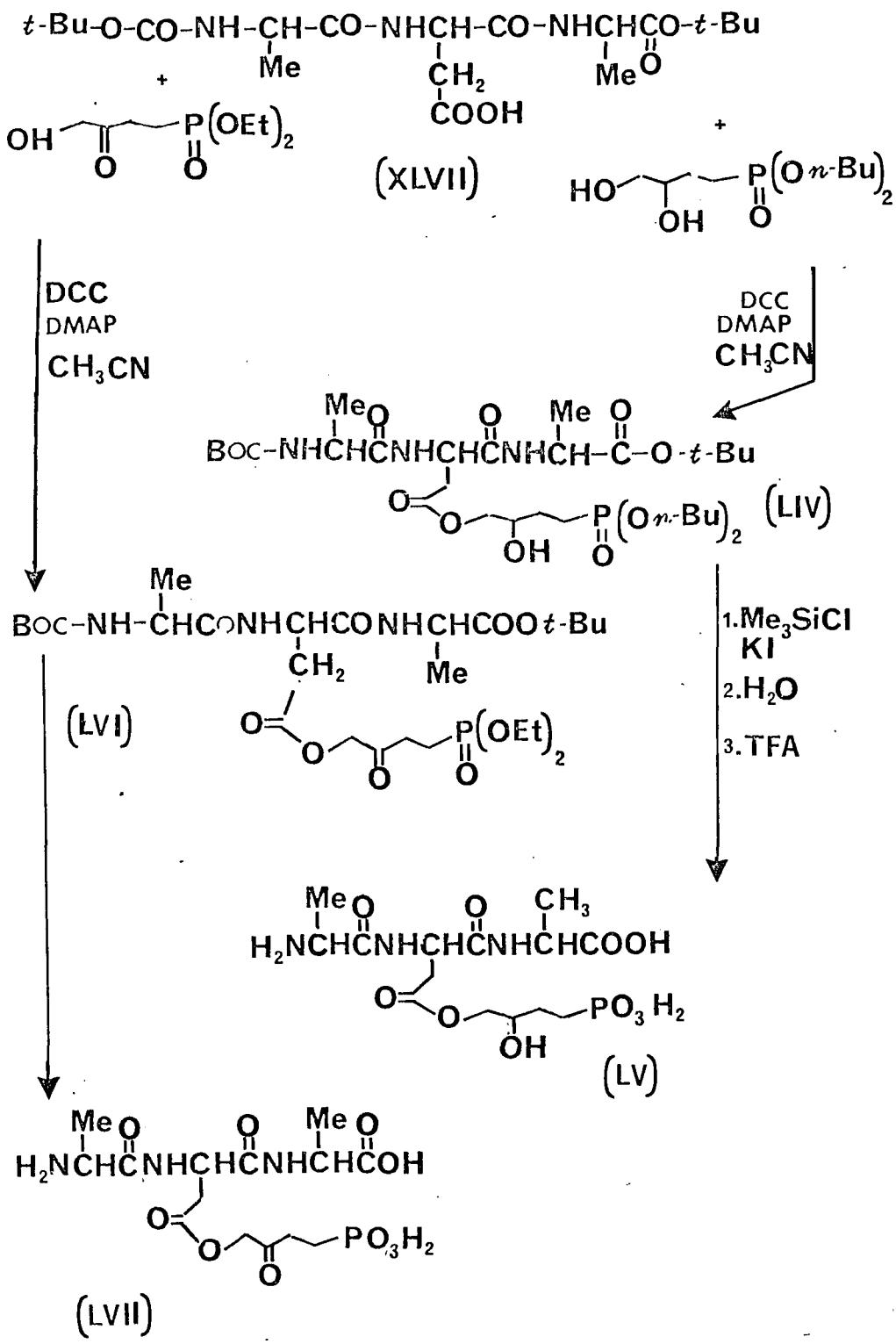
SCHEME XI



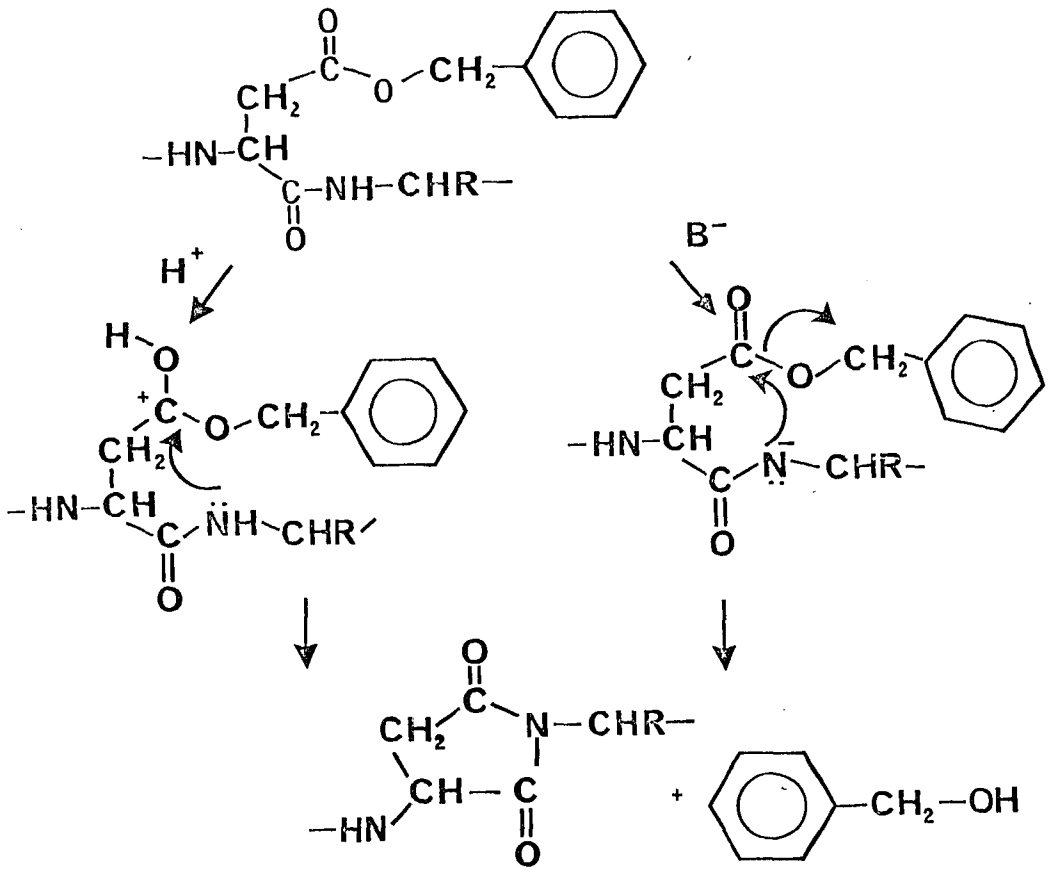
The conjugation of the tripeptide (XLVII) with the analogues of DHBP and DHAP was performed in anhydrous acetonitrile with DCC in the presence of N,N-dimehtylaminopyridine as a catalyst. The coupled products LIV and LVI were obtained in good yield (46% and 67% respectively). The phosphonate esters and the other protecting groups (BOC- and t-Bu) were removed in a similar fashion as described before as shown in Scheme XII.

A point of difficulty in each of these syntheses must be discussed here. It has been noted that significant decomposition of the deprotected conjugated species occurs upon storage under a variety of conditions. This may be understood in terms of a route of peptide cleavage which has been studied (156,157) for aspartate esters under acidic conditions.

The intramolecular displacement of benzyl alcohol in β-benzyl-aspartyl peptides and the concomitant formation of aminosuccinyl derivatives is one of the most disturbing side reactions in peptide syntheses. The cyclization is catalyzed both by acids and bases as shown below.



SCHEME XII

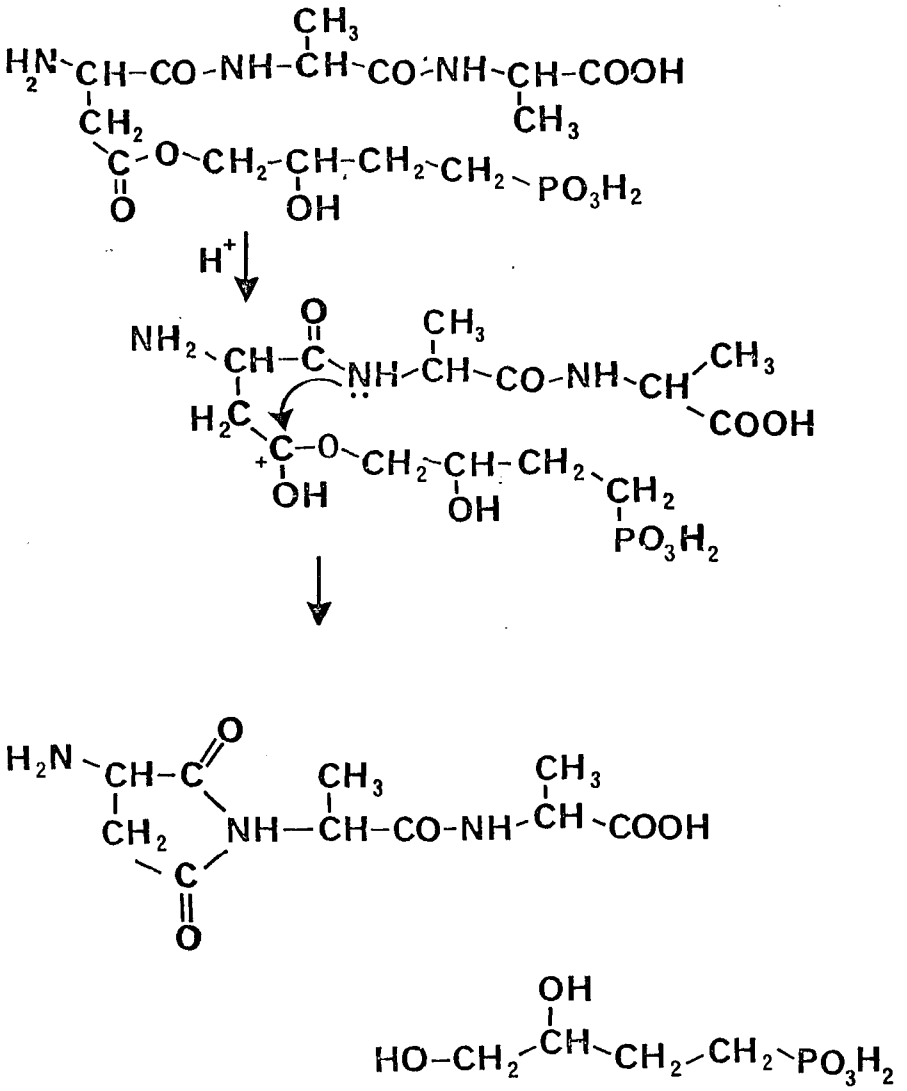


Ring closure that can occur in the case of aspartyl residues with free carboxyl groups in their side chains (158) is enhanced when the β-carboxyls are protected in the form of benzyl esters. Elimination of benzyl alcohol and ring closure is extensive in liquid HF (159), the reagent frequently used for the cleavage of peptides from insoluble polymeric supports (160) and also for the simultaneous removal of all protecting groups.

Acid-catalyzed ring closure in β-benzyl aspartyl residues was investigated under the conditions traditionally applied for the acidolytic removal of the most commonly used amino-protecting

groups. To facilitate a simple, convenient and yet sensitive identification of by products, the model compound t-BOC-β-benzyl-L-aspartylglycine β-naphthylamide was prepared. The β-naphthylamide (NA) group provides the strong U.V. absorption needed for the easy detection on thin layer chromatograms. Exposure of BOC-(Bzl)-Asp-Gly-NA to ca. 2N HBr in acetic acid at room temperature produced 2:1 mixture of Asp-Gly-NA and Asc-Gly-NA.

In the present systems contact of the conjugates, wherein the β-carboxyl is esterified with the agent, with TFA is minimized in the work-up. However, there remains the acidic function of the phosphonic acid, which, even in the monoionic state, can function as an acidic catalyst for the cleavage process. With this occurrence, viability of the coupled agents for biological activity is going to be variable with storage time and condition (161). Evidence of this cleavage process has been noted. The deprotected freshly prepared conjugates were homogeneous on TLC developed by ninhydrin and phosphate sprays. But the species started showing degradation in a couple of days. TLC started showing more than a single spot and NMR gave a very distint peak of succinimide proton at δ 11.0. IR also showed very prominent succinimide carbonyl streching at 5.8 and 6.1 microns.



The promise for *in vivo* antimetabolic activity of DHAP was poor due to the lack of transport mechanism for dihydroxyacetone phosphate, although it was anticipitated that the species would demonstrate activity if a cell could be induced to accept it.

The presently synthesised materials demonstrate *in vivo* the activity desired. It yet remained to be determined biochemically

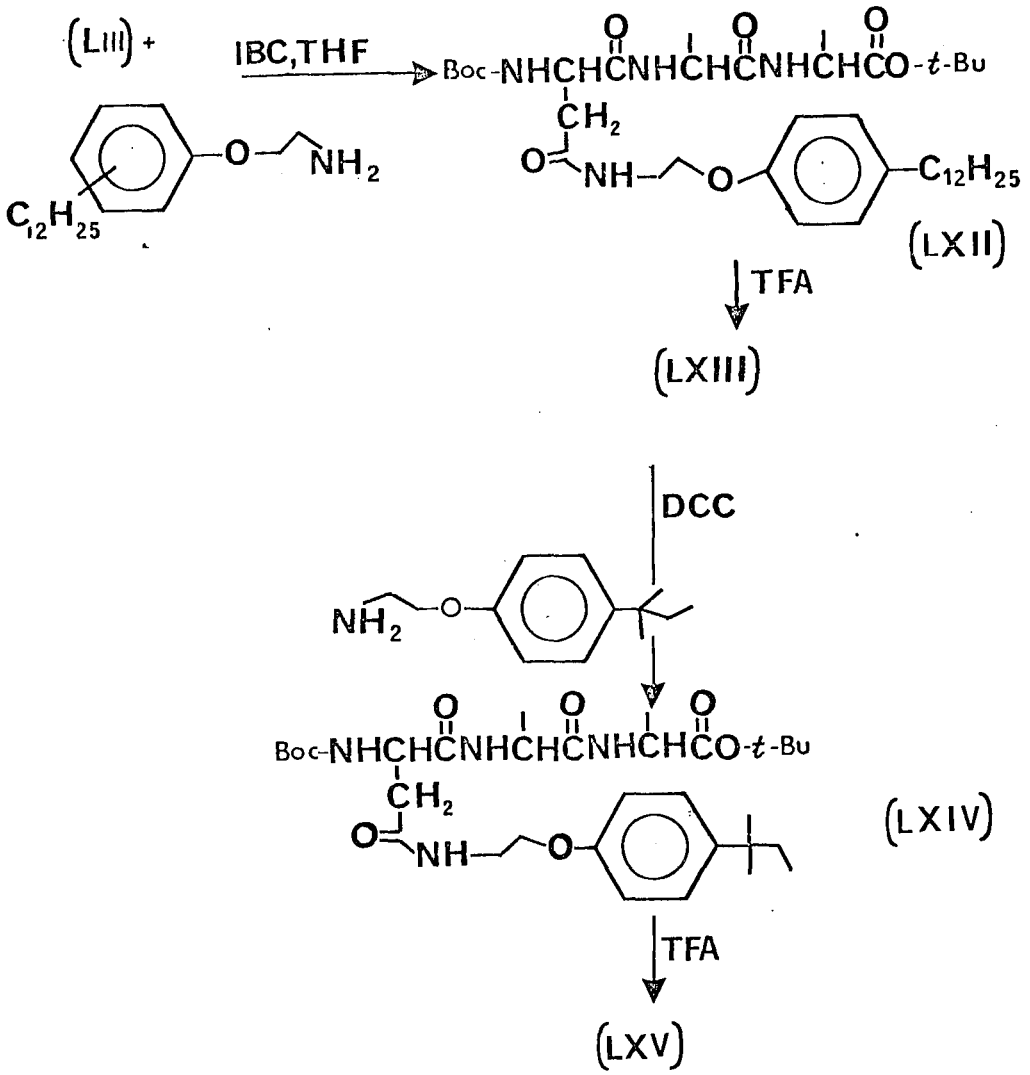
the detailed role of each componenent, the phosphonic acid agent and the peptide, in this activity. The postulated role of the peptide in facilitating transport of the agent needs to be confirmed through bacteriological studies using peptide-transport-negative mutant strains.

Another aspect of the detailed mechanism of *in vivo* activity concerns the structural nature of the agent inside the cell. It is not at present clear if the active agent inside the cell is the peptide-agent conjugate or the free phosphonic acid species, or some other modification. While esterases are certainly present, it is not evident that they are acting on the conjugate prior to its inhibitory performancs. A determination of details in thls regard requires generation of the conjugate with a radioisotopic label, preferably a dual label, ^{14}C in the peptide portion and ^{3}H in the phosphonic acid portion.

2-(Dodecylphenoxy)-ethanamine [XXXV] was coupled with the tripeptide [LIII] using the isobutyl mixed carbonic anhydride method.

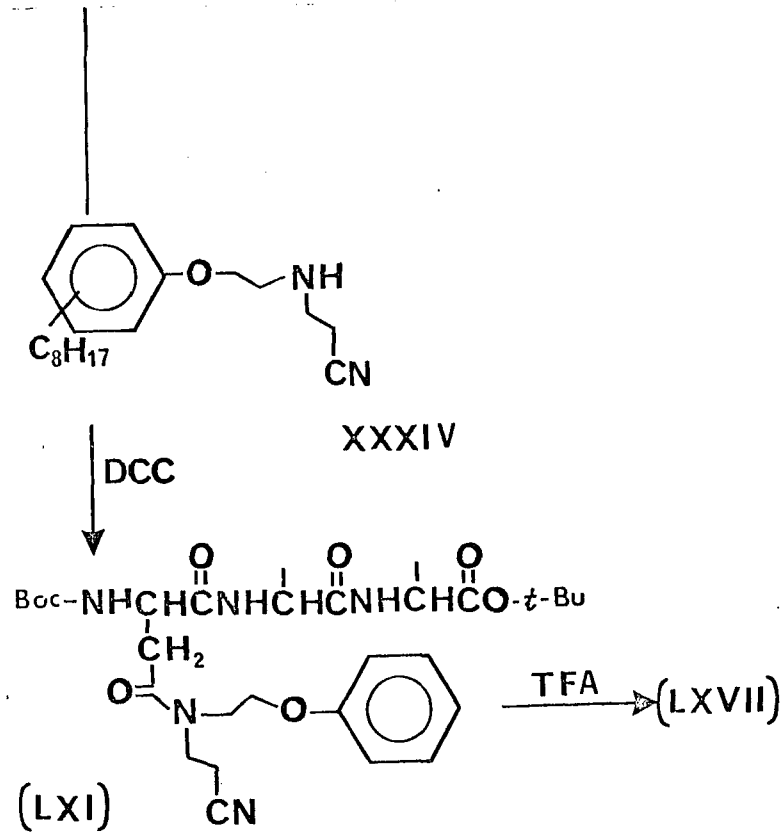
BOC-Asparty1((β-2(dodecylphenoxy)ethanamide)-alanylalanine-O-t-butyl ester [LXII] was obtained as white crystals in 89% yield by precipitation from ethyl acetate with hexane.

The protecting groups (BOC- and t-Bu) were cleaved using the TFA in the ususal manner giving 90% yield of LXIII.



2((1,1-Dimethylpropyl)phenoxy)ethanamine [XXXIII] and 3-((2-(4-octylphenoxy)ethyl)amine) propanenitrile [XXXIV] were conjugated with the tripeptide [LIII] by the DCC method giving [LXV] and [LXVI] in 74% and 59% yield respectively. The protecting groups on the peptide of the molecule were removed in almost quantitative yield in the usual way as described earlier.

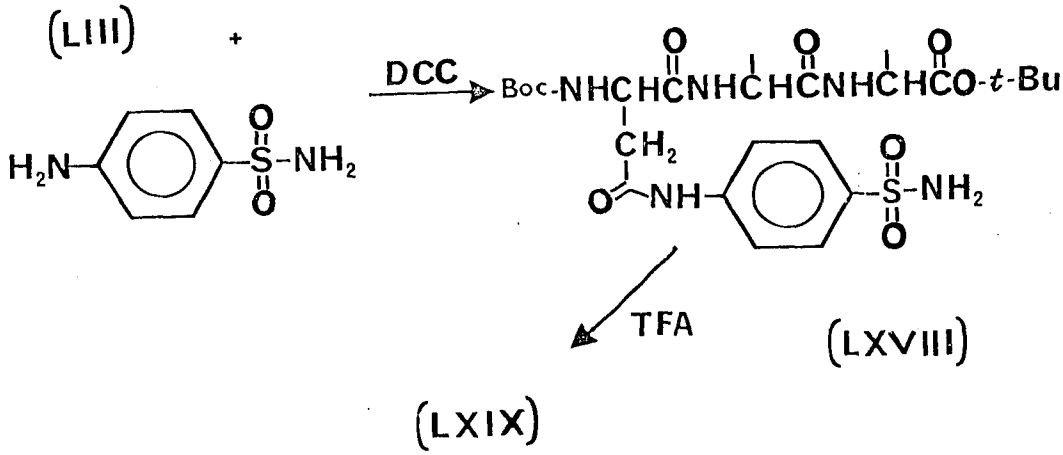
(LIII)



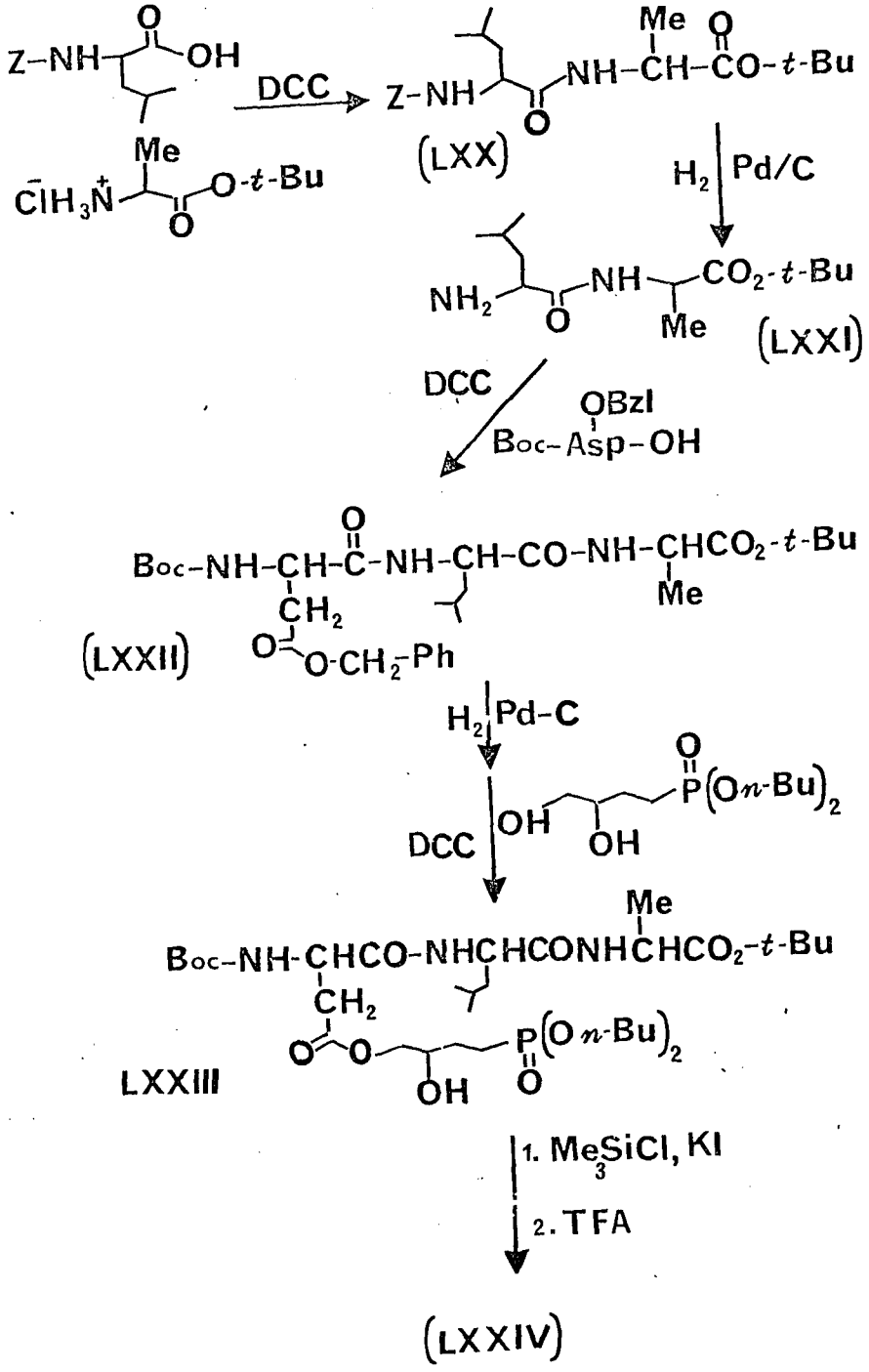
The compound [LXIII, LXV and LXVII] were studied for *in vivo* activity against plant pathogen (*Erwinia Amylovora*), but did not prove to be active against this pathogen.

The sulfanilamide (XXXVI) was conjugated with the usual chemical vector [LIII] using DCC and DMAP in dichloromethane. A crude dark yellow crystalline compound [LXVIII] was obtained in 94% yield. The protecting groups were removed using trifluoroacetic acid and the final compound was purified by column chromatography yielding [LXIX] as a pale yellow crystalline compound.

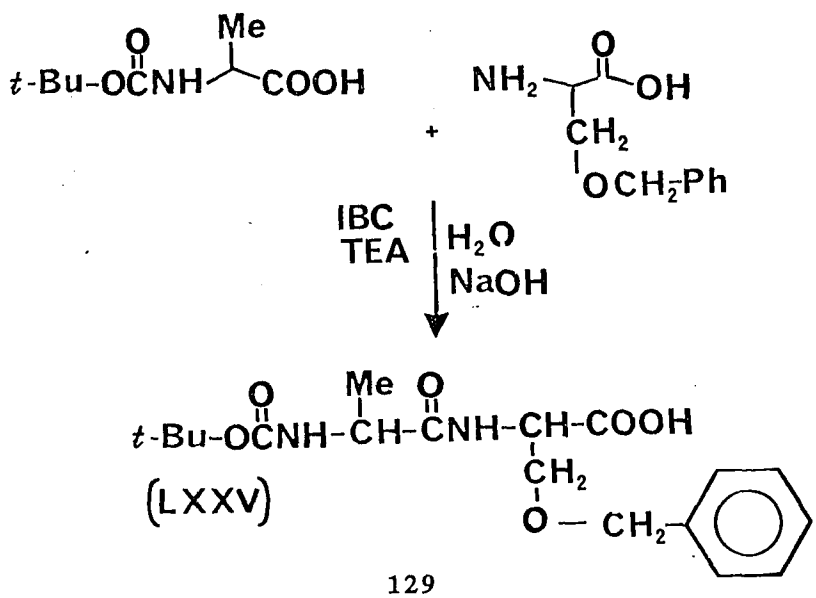
The third chemical vector (aspartylleucylalanine) was synthesized in the usual way. Carbobenzyloxy-leucine was conjugated with alanine-t-butyl ester hydrochloride by DCC in dichloromethane. The CBZ-group was removed by hydrogenation and the dipeptide [LXXI] was coupled with BOC(β-benzyl)aspartate in 55% yield. The β-benzyl group was removed in the usual manner and this tripeptide was coupled with dibutyl 3,4-dihydroxybutyl-1-phosphonate (VI) using DCC and DMAP. It was crystallized from ethyl acetate solution by the addition of petroleum ether. This material again is troubled by the acid catalyzed decomposition process as previously noted. The

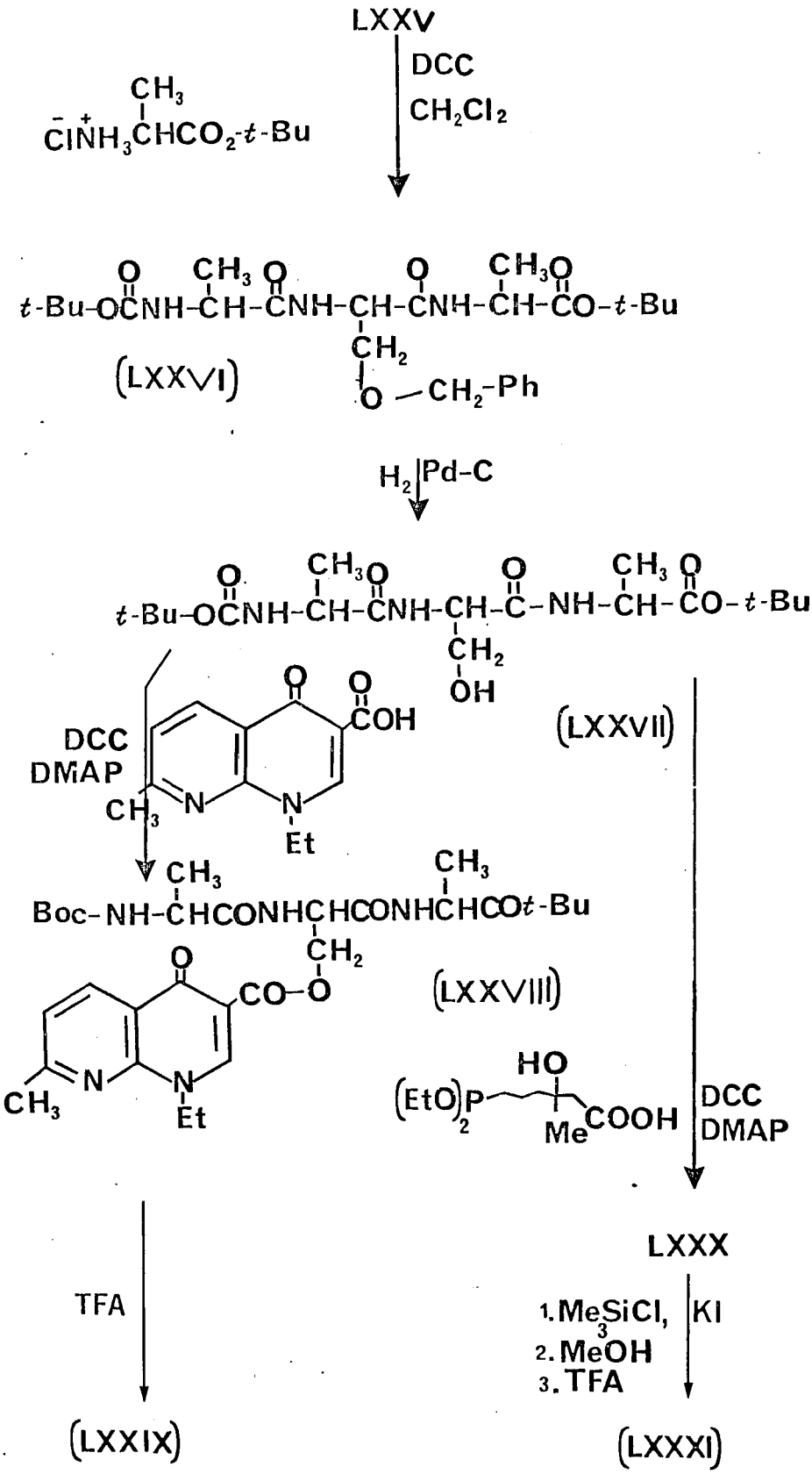


attack by the NH group of the leucine residue on the ester carbonyl is catalyzed by the acidic protons of phosphonte resulting in the aminosuccinimide formation.



The fourth chemical vector, the tripeptide(BOC-alanylserylalanine-O-t- butyl ester) was synthesized as follows. Using the mixed anhydride method, the dipeptide (BOC-alanyl(O-benzyl)serine) was obtained in 63% yield. This dipeptide [LXXV] was coupled with alanine-O-t-butyl ester hydrochloride using DCC in dichloromethane. The benzyl group was removed through hydrogenation giving the tripeptide [LXXVII] in 94% yield. This tripeptide [LXXVII] was conjugated with two agents, i.e., nalidixic acid (yield 81%) and diethyl 5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonate (yield 57%) by the carbodiimide method. The protecting groups on the molecules were removed in the same fashion as described before. A point of difficulty also arises here. It has been noted that significant decomposition of the deprotected conjugated species occurs upon storage. Phosphonic acid group catalyzes the breakdown of the ester group and presumably migration (162) of the phosphonate ester occurs first and ultimately the decomposition of the conjugated species.





SUMMARY AND SUGGESTION FOR FURTHER WORK

The present work has been concerned with the design and synthesis of conjugated forms of phosphonic acids with oligopeptides designed with the intent to facilitate their transport into specific organisms or sites within an organism. The synthesis of tripeptides, aspartylalanylalanine, alanylaspartylalanine, aspartylleucylalanine and alanylserylalanine and their conjugateion with a variety of phosphonic acids, isosteric with natural monophosphate metabolites known to be inhibitors of associated metabolic processes has been accomplished. Many of these have exhibited in vivo and in vitro inhibitory activity against E. coli. It yet remains to be determined biochemically the detailed role of each component, the phosphonic acid agent and the peptide, in this activity. The postulated role of the peptide in facilitating transport of the agent needs to be confirmed through bacteriological studies using peptide-transport-negative mutant strains.

A determination of details in this regard requires generation of the conjugate with a radioisotope label, preferably a dual label, ^{14}C in the peptide chain and ^{3}H in the phosphonic acid portion.

The conjugation of the oligopeptides (mentioned above) with certain gram negative active chemicals (certain amines, nalidixic acid and sulfanilamide) to be tested for use in plant disease treatment applications against E. amy has also been accomplished.

A further interesting investigation in these regards would be the coupling of other species, such as phosphonomycin, to an oligopeptide and the study of its transport capabilities.

The facile decomposition of the aspartate linked conjugates is a major unanticipated problem; prior note of such cleavage had only to do with deprotections involving benzyl esters. It was not anticipated that for the current esters such processes would be so significant. This severly limits the value of the aspartate systems.

Some of the difficulties might be averted by immediate conversion to the dianion form of the phosphonic acid. This is being done in current preparations of the materials. Another approach, obviating these difficulties, is to use glutamate in place of aspartate because there would not be a possibility of succinimide formation as it occurs in the case of aspartate. γ-benzyl ester of glutamic acid, except for the general tendency of glutamic acid derivatives to form pyroglutamyl residues, leads to no major side reactions (156).

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