S-TRIAZOLO[3,4-A]PHTHALAZINES:

IMPLICATIONS FOR C-TERMINAL PEPTIDE SEQUENCING

by

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ABSTRACT

The syntheses of s-triazolo[3,4-a]phthalazines (*15*, 3-R-TAP)
by reaction of hydralazine (*12*, 1-hydrazinophthalazine) with N-protected
amino acids and dipeptides under homogeneous (solution) and heterogeneous
("solid-phase") conditions are reported. Transition metal complexes
containing the TAP ligand were prepared and their spectral properties
investigated. The use of metal-ions and a cation-exchange resin (H^+ form)
were considered for the mild hydrolysis of side-chain amide bonds in TAP
derivatives. The objective of these studies was to determine the feasibi-
lity of reacting the carboxyl groups in amino acids with hydralazine to
afford the TAP derivatives as a method for peptide sequencing from the
C-terminal residue.

Hydralazine reacts with carboxylic acids to form an amide
intermediate which undergoes ring closure with elimination of water to form
the s-triazolo[3,4-a]phthalazine derivative. To promote the initial binding
of hydralazine to the acid, coupling reagents were used to activate the
carboxylate group towards nucleophilic attack.

N-Ethyl-5-phenylisoxazolium-3'-sulfonate (*17*, NEPIS), 1-ethoxy-
carbonyl-2-ethoxy-1,2-dihydroquinoline (*27*, EEDQ), various phosphorus
compounds, carbodiimides, and chloroformates were carboxyl activating agents
used to synthesize TAP derivatives.

In solution studies, the carbodiimides (EDC, *16* and DCC, *51*),
NEPIS (*17*), and a combination of triphenylphosphite with imidazole are the
most successful procedures for TAP synthesis.

In solid-phase studies, the best procedures for activating immobilized amino acids are with isobutyl chloroformate, NEPIS (17), and DCC (51).

Transition metal complexes were synthesized with the general formula: $[M(3\text{-}H\text{-}TAP)_n(H_2O)_{6-n}](ClO_4)_m$ (n = 4, m = 2, M = Co, Ni, Cu; n = 2, m = 2, M = Ni; n = 6, m = 3, M = Co). The infrared and visible spectra of these complexes are reported.

$[Co(trien)(3\text{-}(N\text{-}Ac\text{-}gly)\text{-}TAP)](ClO_4)_2$ was also prepared and under acidic conditions, no hydrolysis of the side-chain amide bond was observed. There was also no significant hydrolysis of the side-chain with free 3-(N-Ac-gly)-TAP in the presence of Co^{2+} and Cu^{2+} under acidic conditions, or when it was eluted through a cation-exchange (H^+ form) column.

The decomposition of hydralazine in non-aqueous media was investigated and a major product of the decomposition was identified as diphthalazinylhydrazine (82).

The implication of our studies is that the modification of amino acids with hydralazine is not yet a viable method for C-terminal peptide sequencing. Improvements are required for improving the yields of the coupled product, and the lack of a mild and selective method for hydrolyzing the C-terminal peptide bond limits the method at present to determination of the C-terminal residue only.

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ABBREVIATIONS

Ac	acetyl
2-AMPy	2-aminomethylpyridine
Ar	aryl
asym.	asymmetric
b	broad
bipy	bipyridine
bipyz	bipyrazine
BOC	butyloxycarbonyl
Bu	butyl
C-	carboxyl
CMC	1-cyclohexyl-3-(2-morpholinyl-4-ethyl)-carbodiimide metho p-toluenesulfonate
CoA	coenzyme A
CPA	carboxypeptidase A
CPG	controlled-pore glass
DABCO	1,4-diazabicyclo[2.2.2]octane
DBu	1,5-diazabicyclo[5.4.0]undec-5-ene
DCC	dicyclohexylcarbodiimide
def.	deformation
DMA	dimethylacetamide
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNS	dansyl
2-DTP	2,2'-dithiodipyridine

DVB	divinylbenzene
EDC	1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride
edda	ethylenediamine diacetate
EEDQ	1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
eee	1,8-diamino-3,6-dithiaoctane
en	ethylenediamine
Et	ethyl
HMPA	hexamethylphosphoramide
HOBt	1-hydroxybenzotriazole
IIDQ	1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline
Imid-H$^+$	imidazolium
IR	infrared
m	medium
Me	methyl
mp	melting point
N-	amino
NEPIS	N-ethyl-5-phenylisoxazolium-3'-sulfonate
nmr	nuclear magnetic resonance
P	polymeric support
Pr	propyl
P$_s$	copolystyrene-divinylbenzene support
PTC	phenylthiocarbamyl
PTH	phenylthiohydantoin
py	pyridine
pyO	pyridine-N-Oxide

s	strong
sh	shoulder
str	stretching
sym.	symmetric
TAP	s-triazolo[3,4-a]phthalazine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
tren	2,2',2"-triaminotriethylene
trien	triethylenetetramine
UV	ultra-violet
v	very
w	weak
WSC	water-soluble carbodiimide
Z	benzyloxycarbonyl

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CHAPTER 1

PROTEIN SEQUENCE DETERMINATION

1.1 INTRODUCTION

Protein sequence analysis has, with justification, been described as one of the most important research activities of today.[1] The information contained in protein sequence is something essentially new. The data represent the covalent chemical structure of the protein molecule and are of immediate use and importance in many different disciplines.

It is certain that the importance of sequence information on proteins will continue to increase in the same way as the body of information itself will increase. Sequence data on proteins are being amassed at an ever increasing rate. This can be seen from the phenomenal growth of the "Atlas of Protein Sequence and Structure", which approximately doubles every year.[2]

The rapid development of appropriate analytical procedures has been one of the factors enabling sequence data to be accumulated at the present rate. However, at the present time, the determination of the amino acid sequence of a protein may still require a major effort and can not be considered a routine matter. In 1943, Synge stated:

> *...it seems that the main obstacle to progress in the*
> *study of protein structure by methods of organic chemistry*
> *is inadequacy of technique rather than any theoretical*
> *difficulty. It is likely that new methods of work in*
> *this field will lead us to a very much clearer understanding*
> *of the proteins.*[3]

Today, thirty-four years later, that statement is still valid.

1.2 PROTEIN STRUCTURE AND NOMENCLATURE

The proteins are chains of long unbranched polymers with
L-α-amino acids as the monomeric units, only twenty of which are commonly
found as the building blocks of proteins. The names and structures of
these common amino acids are given in Table 1.1. The amino acids are
linked together in a head to tail arrangement through amide bonds called
peptide bonds, which arise by elimation of the elements of water from the
carboxyl group of one amino acid and the α-amino group of the next. The
peptide bond is the repeating unit in proteins. The characteristic bond
structure is enclosed in the dotted area:

$$
\begin{array}{ccccc}
\text{H} & & \text{O} & & \text{O} \\
| & & \| & & \| \\
-\text{N}-\text{CHR}- & \text{C}- & \text{N}- & \text{CHR}'- & \text{C}- \\
& & | & & \\
& & \text{H} & &
\end{array}
$$
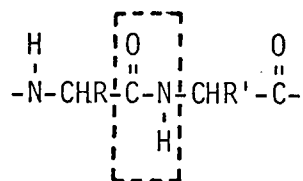
Such polymer chains are called *polypeptides*.

In peptide nomenclature, the amino acid residues are written as
they occur in the chain starting from the free α-amino group, which is
conventionally shown at the left-hand part of the structure. The terminal
residue with the free amino group is referred to as the *N-terminal* residue.
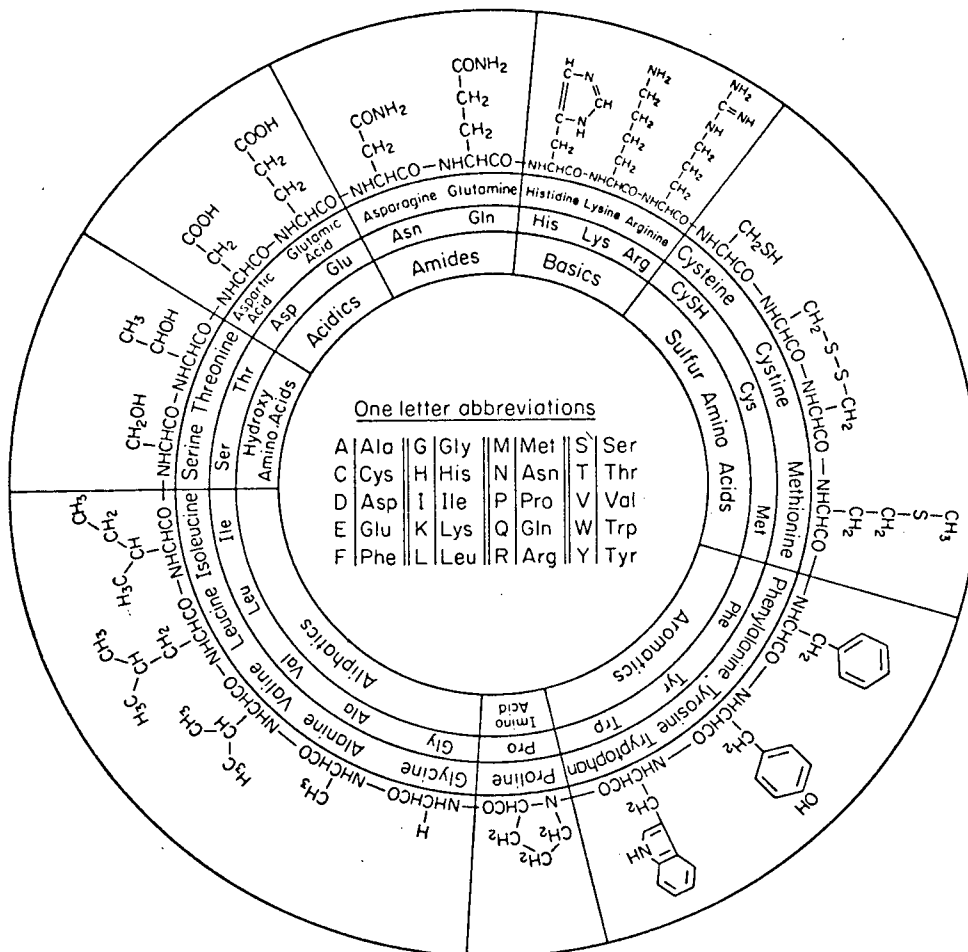
Table 1.1 The "amino acid wheel". The structures of the amino acid residues are shown as they normally occur in a polypeptide chain.

Similarly, the terminal residue with the free carboxyl group is referred to as the *C-terminal* residue. The sequence of the amino acid residues in the polypeptide chains which make up the proteins is frequently called the *primary structure* of the protein.

In general, the polypeptide chains of proteins usually have between 100 to 300 amino acid residues (mol. wt. 12,000 to 36,000). A few proteins have longer chains, such as serum albumen (~550 residues) and myosin (~1800 residues). Usually, any protein having a molecular weight exceeding 50,000 can be suspected to have two or more chains.

1.3 PROTEIN SEQUENCE ANALYSIS

The approach generally used in determining the primary structure of proteins is, in principle, that devised by Sanger in his epoch-making determination of the amino acid sequence of the polypeptide chains of insulin, for which he was awarded a Nobel prize in 1958. Since that accomplishment, many refinements have been added, and new procedures have been developed. Although each protein offers special problems, the following sequence of steps is generally used, though not necessarily in the order shown.[4]

a. If the protein chain contains more than one polypeptide chain, the individual chains are first separated and purified.

b. All the disulfide groups are reduced, and the resulting thiol groups alkylated.

c. A sample of each polypeptide chain is subjected to total hydrolysis, and its amino acid composition is determined.

d. On another sample of the polypeptide chain, the N-terminal

and C-terminal residues are identified.

e. The intact polypeptide chain is cleaved into smaller peptides by enzymatic or chemical hydrolysis.

f. The resulting peptide fragments are separated, and their amino acid composition and sequence are determined.

g. Another sample of the original polypeptide chain is partially hydrolyzed by a second procedure to fragment the chains at points other than those cleaved by the first partial hydrolysis. These peptide fragments are separated and their amino acid composition and sequence determined.

h. By comparing the amino acid sequences of the two sets of peptide fragments, particularly where the fragments from the first partial hydrolysis overlap the cleavage points in the second, the peptide fragments can be placed in the proper order to yield the complete amino acid sequence.

i. The positions of the disulfide bonds and the amide groups in the original polypeptide chain are determined.

A discussion of all the steps involved in the complete structure of a protein is beyond the scope of this thesis. For reviews in these areas, readers are referred to other works.[5,6]

Of direct relevance to our studies are the methods for elucidating the primary sequence of peptides, i.e., determining the amino acid sequence from the N-, and C-termini.

1.4 AMINO TERMINAL PEPTIDE SEQUENCING AND END-GROUP IDENTIFICATION

The general principle for amino end-group determination is based on the introduction of a marker group (colored, fluorescent, UV absorbing, etc.) onto the amino function, followed by the quantitative isolation and characterization of the derivatized amino acid.

In recent years, methods of N-terminal analysis based on dansyl chloride (*1*, 1-dimethylaminonaphthalene-5-sulfonyl chloride, DNS-Cl) have found wide application, largely because of the ease with which one can study minute amounts of peptides and proteins.[7]

Dansyl chloride, which is itself non-fluorescent, gives a strong yellow fluorescence upon sulfonamide formation with the amino group of proteins and peptides as shown in Equation 1.

$$R-NH_2 \ + \ \underset{SO_2Cl}{\overset{NMe_2}{\bigcirc\!\bigcirc}} \ \longrightarrow \ \underset{SO_2NHR}{\overset{NMe_2}{\bigcirc\!\bigcirc}} \ + \ HCl \qquad (1)$$

(*1*)
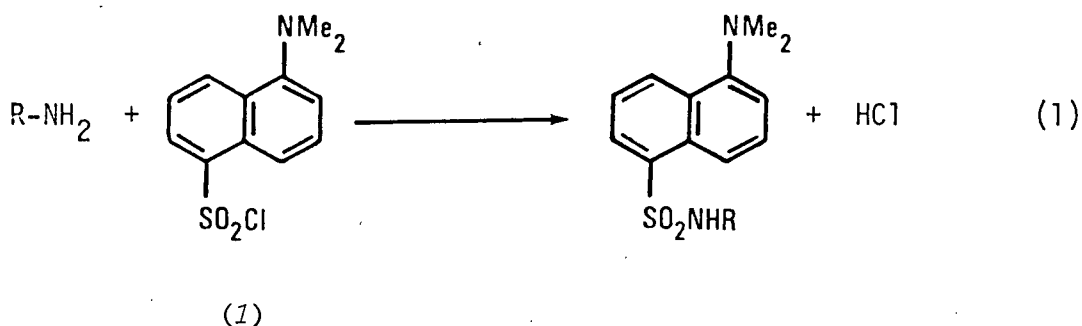
Hydrolysis of the DNS-peptide with acid produces free amino acids and the dansyl derivative of the N-terminal residue. After separation from the untagged amino acids, the dansyl-amino acid may be identified by electrophoresis or thin layer chromatography and visualized by fluorescence.

The most important and widely used labelling reaction for the N-terminal residue of a peptide or protein is the Edman degradation.[8] Since its introduction over 25 years ago, this procedure has undergone

almost continual modification, culminating in the development of two auto-
mated versions for sequence analysis.[9]

The first step of the Edman procedure (Scheme 1.1) involves
reacting the peptide or protein N-terminus with phenyl isothiocyanate (*2*) at
alkaline pH. After removal of excess reagents, the resulting phenylthio-
carbamyl peptide (*3*, PTC-peptide) is treated with acid, which causes cycliza-
tion and cleavage of the N-terminal amino acid as a 2-anilino-5-thiazolinone
(*4*). After separation from the peptide, the thiazolinone is converted to
the isomeric 3-phenyl-2-thiohydantoin (*5*, PTH), which can be identified by a
number of physical techniques. Usually, the Edman procedure is not restricted
to determining the N-terminal residue, and the peptide or protein is subjected
to additional cycles of the Edman degradation, thus effecting a *sequential*
N-terminal analysis.

In favourable cases, one may expect the manual technique to produce
about 30 degradation cycles with clearly interpretable results. To perform
one degradation requires the best part of a working day.

In 1967, Edman and Begg[10] reported the development of an automatic
Edman Sequenator. Since that time, machines based on their design have
become such an integral part of the methodology used for sequence analysis
that less emphasis is now being placed on manual determination of N-terminal
sequences of proteins. The sequence analyzer is ideally suited to degrada-
tion of proteins and large peptide fragments, the optimal size being 100-150
residues. Typically, one may expect the unambiguous elucidation of between
30 and 70 residues of amino acid sequence with the Edman Sequenator, as
opposed to about 15-20 residues with the manual method in not unfavourable
cases.[11]

PhNCS + NH$_2$-CHR-C-NH-----PEPTIDE
                       ‖
                       O

(2)

| pH 8-9

     S   O
     ‖   ‖
PhNH-C-NH-CHR-C-NH-----PEPTIDE

(3) | H$^+$

(4)

H$^+$, H$_2$O

+ NH$_2$-----PEPTIDE

| PhNCS, pH 8-9

| H$^+$

etc.

(5)
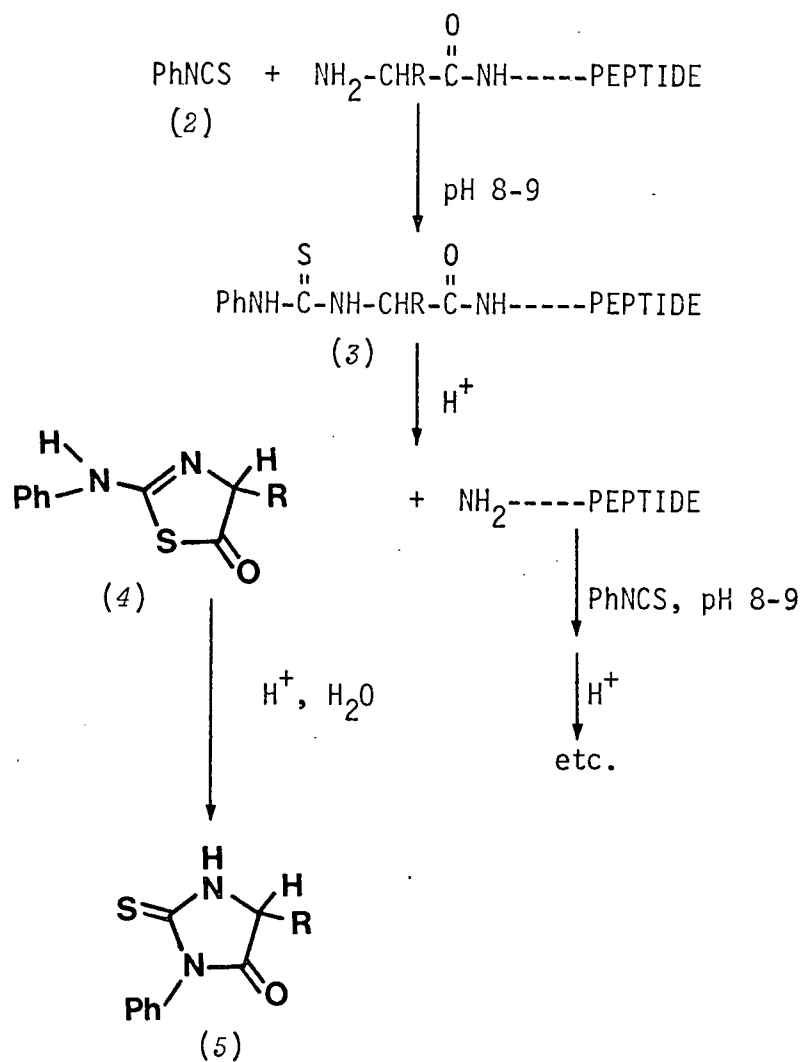
Scheme 1.1   The Edman Degradation

A complementary automatic method is the solid-phase sequencer of Laursen.[12],[13] With this technique, the peptide is attached to a solid support and subjected to the Edman degradation with radioactive phenyl isothiocyanate. The efficiency of the solid-phase method only permits the determination of up to about 30 residues,[11] and in this respect is inferior to the Edman Sequenator. However, the solid-phase instrument may be used with small peptide fragments (up to 30 residues), and is considerably cheaper in cost and maintenance.

The use of enzymes for N-terminal analysis of proteins is still in a fairly incipient state. Two enzymes are commercially available for this purpose: leucine aminopeptidase and aminopeptidase M. These enzymes catalyze hydrolysis of the peptide bond of the N-terminal residue of proteins and peptides, releasing a free amino acid. Hydrolysis proceeds sequentially from residue to residue, for the degradation continually produces a new N-terminus. A partial sequence of a protein may thus be deduced from a kinetic analysis of the amino acids released.

The N-terminal residues of all amino acids are cleaved by amino-peptidase M and leucine aminopeptidase, with the exception of proline for the latter enzyme. Rapid hydrolysis is observed with aliphatic and aromatic amino acid residues, slower rates with all others, with individual differences spanning a range of several orders of magnitude.[14]

1.5 CARBOXY TERMINAL PEPTIDE SEQUENCING AND END-GROUP IDENTIFICATION

While the procedures for N-terminal analysis of proteins and peptides are among the best and most useful methods that have been applied in sequence determinations, methods for the identification of C-terminal residues have been less successful. Only two methods, a chemical and an

enzymatic one, have been applied to any great extent.

The hydrazinolysis method is based on the discovery of Akabori et al.[15] that C-terminal residues of a protein are liberated as free amino acids by treatment of the protein with anhydrous hydrazine. All other amino acids in peptide linkage are converted to amino acid hydrazides as shown in Equation 2.

$$H_2N-CHR_1-CO\cdots NH-CHR_{n-1}-CO-NH-CHR_n-COOH + N_2H_4 \longrightarrow$$

$$H_2N-CHR_1-CONHNH_2 + \cdots + H_2N-CHR_{n-1}-CONHNH_2 + H_2N-CHR_n-COOH \quad (2)$$
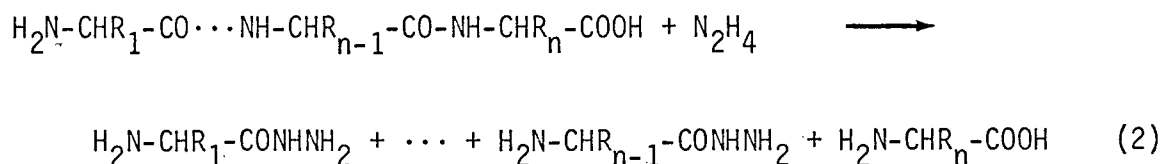
Although the hydrazinolysis method is simple in principle, its application is fraught with difficulties. The yields of C-terminal amino acids are only moderate, necessitating the application of high correction factors. The quantitativeness of the method also suffers from the rather complicated methods for isolating and estimating the free amino acids. The technique of Braun and Schroeder[16], however, appears to be the best improvement of the original procedure. In this method the reaction of the peptide with anhydrous hydrazine is catalyzed by Amberlite CG-50 in the hydrogen form. After removal of catalyst and excess hydrazine, the amino acids are separated from hydrazides on a cation-exchange resin column, and subsequently analyzed by automatic amino acid analysis.

The hydrazinolysis method fails when the C-terminal residue is arginine, cysteine, cystine, asparagine, or glutamine, and may fail when the terminal group is aspartic acid. As with all methods for the identification of end-groups in macromolecules, potential causes of error are sufficiently numerous with hydrazinolysis to justify reservations about any results unless it is corroborated by other methods.

Carboxypeptidases act on proteins and peptides to release L-amino acids one residue at a time from the C-termini of peptides and proteins. Several types of enzymes have been characterized according to their substrate specificities. Carboxypeptidase A (CPA) shows a marked preference for C-terminal residues with an aromatic or branched aliphatic side chain, (Table 1.2).

Table 1.2   Approximate Relative Rates of Release of Amino Acids by Carboxypeptidase A[17]

Rapid Release:	Tyr, Phe, Try, Leu, Ile, Met, Thr, Gln, His, Ala, Val, Homoserine
Slow Release:	Asn, Ser, Lys, $MetSO_2$
Very Slow Release:	Gly, Asp, Glu, $CySO_3H$, S-carboxymethylcysteine
Not Released:	Pro, Arg

Note: The presence of a "very slow" or "not released" amino acid as penultimate residue will generally decrease the rate of release of the C-terminal amino acid.

Carboxypeptidase B (CPB) exhibits a narrower specificity, and cleaves the basic amino acids lysine, and arginine very much faster than any of the other common amino acids. In common with all carboxypeptidases, the rate of release of C-terminal residues is greatly influenced by the structure of the adjacent residue.[18]

Another enzyme gaining popularity for C-terminal analysis of peptides is carboxypeptidase C (CPC).[19] This enzyme has a broad specificity for acidic, neutral and basic amino acids. A great advantage of CPC is its ability to hydrolyze both peptide linkages on either side of proline.

A new commercially available exopeptidase is carboxypeptidase Y (CPY).[20] Like CPC, its specificity is broad for all common amino acids. In general, when the penultimate and/or C-terminal residues have aromatic or aliphatic side chains catalysis is high, but when glycine is placed in the penultimate position, the release of the terminal amino acid is extremely slow. In contrast to CPA and CPB, C-terminal proline is a rather good substrate. However, the rate of splitting of the peptide bond on either side of proline depends extensively upon the structure of the adjacent amino acids.

The enzymes of choice for C-terminus determination of proteins and peptides have traditionally been CPA and CPB. In a typical end-group determination, these enzymes may be incubated separately, or in combination with the protein substrate. However, with the increased availability of CPC and CPY, these latter exopeptidases will find wider use in view of their broader specificities, and their ability to cleave proline.

In practice, the rate of release of amino acids from a peptide by carboxypeptidase is followed by analyzing the entire reaction mixture with an amino acid analyzer. In favorable circumstances, the kinetic measurement can give a fairly reliable indication of the C-terminal sequence. However, the chances of misinterpretation are large, and the results should be confirmed whenever possible.

The method of Stark[21,22] for C-terminal sequencing of peptides and proteins is based on the reaction of the C-terminal amino acid with ammonium thiocyanate and acetic anhydride (Scheme 1.2).

The C-terminal amino acid is activated to nucleophilic attack by formation of a mixed anhydride (6) by reaction with acetic anhydride. Attack by the thiocyanate anion, after cyclization of the product, gives

A. Addition

$$\underset{\text{Peptide}}{\overset{\substack{R\ O\ R'\ O\\|\ \|\ |\ \|}}{NH_2CHCNHCHCOH}} \xrightarrow{\overset{\overset{O}{\|}}{(CH_3C)_2O}} \underset{\text{N-Acetyl mixed anhydride}}{\overset{\substack{O\ R\ O\ R'\ O\ O\\\|\ |\ \|\ |\ \|\ \|}}{CH_3CNHCHCNHCHCOCCH_3}} \quad (6)$$

↓ ⁻NCS

Oxazolinone ⁻NCS→ Mixed anhydride with isothiocyanic acid

Aminoacylthiohydantoin (7)

B. Cleavage

$$\xrightarrow{\overset{\overset{O}{\|}}{CH_3CNHO^-}} \left[\underset{}{\overset{\substack{O\ R\ O\ O\\\|\ |\ \|\ \|}}{CH_3CNHCHCONHCCH_3}} \right]$$

⁻OH or H⁺

$$\underset{}{\overset{\substack{O\ R\ O\\\|\ |\ \|}}{CH_3CNHCHCOH}} \quad + \quad$$ (8)
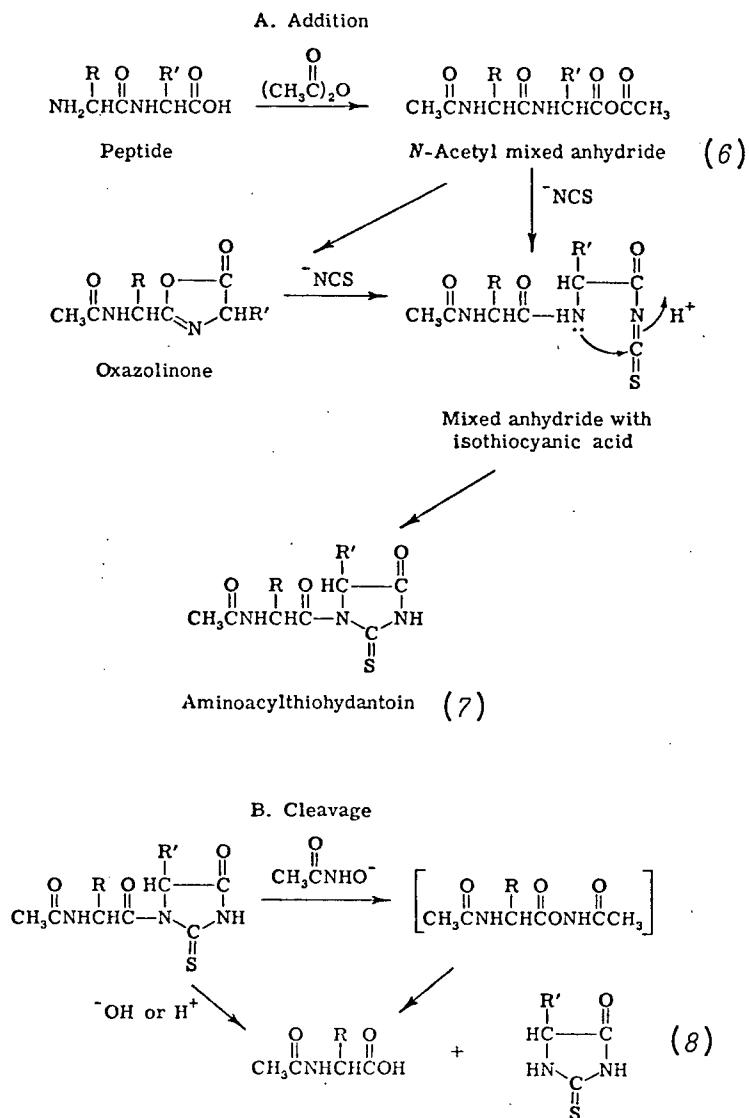
Scheme 1.2 Stark's Method for C-Terminal Sequencing of Peptides and Proteins

the peptidyl thiohydantoin (*7*) which can be cleaved with aqueous acid or base, or with acetohydroxamate. A hydantoin (*8*) is liberated which is characteristic of the C-terminus, and the remaining acetylated peptide or protein possesses a free carboxylate group at the new C-terminus. Degradation of peptides may be followed subtractively by amino acid analysis of the peptide product after each cycle or alternatively, the thiohydantoins may be determined directly by thin-layer chromatography. The thiohydantoin method is somewhat limited in that C-terminal aspartic acid and proline are not removed. The rather extreme conditions required for cleavage of the acyl thiohydantoin (e.g. 12M HCl) limit the method to 2 or 3 cycles for most peptides because of non-specific hydrolysis of internal peptide bonds.

The tritiation method of Matsuo and co-workers[22] for the determination of C-terminal amino acids involves selective exchange of the hydrogen on the asymmetric carbon of the C-terminal amino acid with tritium as in Scheme 1.3

$$R_1 \quad\quad R_{n-1} \quad R_n$$
$$H_2N-CH-CONH----CONH-CH-CONH-CH-CO_2H$$
$$\downarrow Ac_2O$$

$$R_1 \quad\quad R_{n-1}$$
$$AcNH-CH-CONH----CONH-CH$$

$$\downarrow base$$

$$\downarrow {}^3H_2O \text{ (or } {}^2H_2O)$$

$$R_1 \quad\quad R_{n-1} \quad R_n$$
$$AcNH-CH-CONH----CONH-CH-CONH-C-CO_2H$$
$$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad {}^3\underline{H}\,({}^2\underline{H})$$

$$\downarrow hydrolysis$$

$$R_1 \quad\quad R_{n-1} \quad R_n$$
$$H_2N-CH-CO_2H+\cdots+H_2N-CH-CO_2H+H_2N-C-CO_2H$$
$$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad {}^3\underline{H}$$
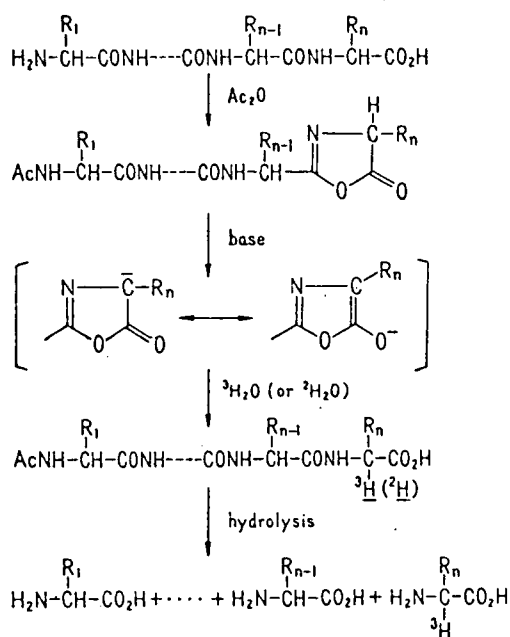
Scheme 1.3   The Tritiation Method for C-Terminal Sequencing
of Peptides and Proteins

In the presence of acetic anhydride, C-terminal amino acids of peptides and proteins selectively undergo cyclization to form oxazalones. The oxazalones contain an active hydrogen and readily incorporate tritium when treated with ^3H_2O and pyridine. Hydrolysis with 18% HCl produces a mixture of amino acids which can be separated by paper chromatography. The tagged amino acid is identified by its radioactivity. Difficulties with the procedure of Matsuo et al.[23] are that C-terminal aspartic acid and proline do not incorporate tritium, and acid-catalyzed conditions must be used. Problems may also arise with serine, threonine,[24] and non-terminal aspartic acid.[25]

A step-wise degradation of peptides from the carboxyl end based on N,O-migration of acyl groups in conjunction with a reduction procedure was described by Bailey.[26] In this scheme, peptide esters are reduced to the corresponding alcohols, which, in the presence of $POCl_3$ or $SOCl_2$, rearrange to yield the β-amino ester, which can be further reduced to give the free amino alcohol and residual peptide in a form prepared for further rearrangement (Scheme 1.4).

$$Y-NH-CHR'-CONH-CHR''-COOR''' \xrightarrow{H} Y-NH-CHR'-CONH-CHR''-CH_2OH$$

$$\downarrow POCl_3$$

$$Y-NH-CHR'-CH_2OH + HO-CH_2-CHR''-NH_2 \xleftarrow{H} Y-NH-CHR'-\underset{O}{\overset{\parallel}{C}}-OCH_2-CHR''-NH_3^+$$
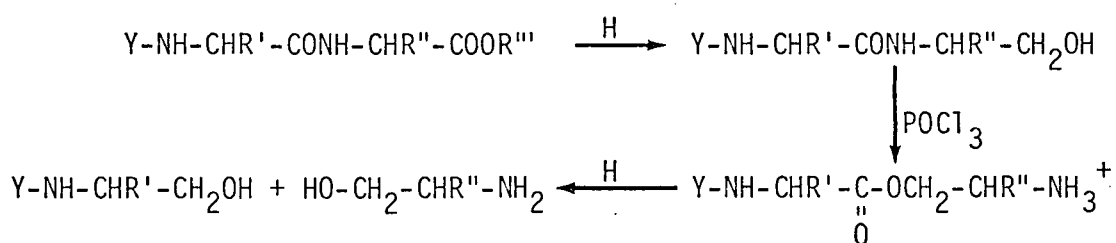
Scheme 1.4 C-Terminal Peptide Sequencing by Reduction

The most stringent requirement in this method is for a mild and selective reduction of the C-terminal ester bond without any accompanying reduction of peptide bonds. Thus, $LiBH_4$ is preferable, while $LiAlH_4$ is unsuitable. Difficulties in establishing optimum reaction conditions for the selective

reduction of ester groups have limited its application in peptide sequencing.

In a modification of Bailey's procedure, Hamada and Yonemitsu[27] recently described the reduction of peptide esters with $NaBH_4$ in aqueous solution followed by hydrolysis of the peptide bonds with 6N HCl. Because of the non-specific hydrolysis of the peptide alcohol, the method is limited to determination of the C-terminal residue only.

A new method for determination of the C-terminal residue in peptides described by Loudon and co-workers[28] involves formation of an O-substituted hydroxamic acid (9) by reaction of the peptide C-terminal carboxyl group with a water-soluble carbodiimide (WSC) and an O-substituted hydroxylamine. The O-substituted hydroxamic acid undergoes, at higher pH, a Lossen rearrangement to the isocyanate (10) leading to degradation of the C-terminal residue (Scheme 1.5).

$$
\begin{array}{c}
\overset{O}{\overset{\|}{Pep\text{-}C}}\text{-NH-CHR-COOH} + H_2N\text{-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-Bu-t} \xrightarrow{\text{WSC}} \overset{O}{\overset{\|}{Pep\text{-}C}}\text{-NH-CHR-}\overset{O}{\overset{\|}{C}}\text{-NH-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-Bu-t} \\
(9)
\end{array}
$$

$$\downarrow OH^-$$

$$
t\text{-Bu-}\overset{O}{\overset{\|}{C}}\text{-}O^- \quad + \quad \overset{O}{\overset{\|}{Pep\text{-}C}}\text{-NH-CHR-N=C=O}
$$
$$(10)$$

$$\downarrow H_2O$$

$$
\overset{O}{\overset{\|}{Pep\text{-}C}}\text{-}NH_2 + RCH + NH_3 \xleftarrow{H^+,H_2O} \overset{O}{\overset{\|}{Pep\text{-}C}}\text{-NH-CHR-}NH_2 + CO_2
$$
$$(11)$$
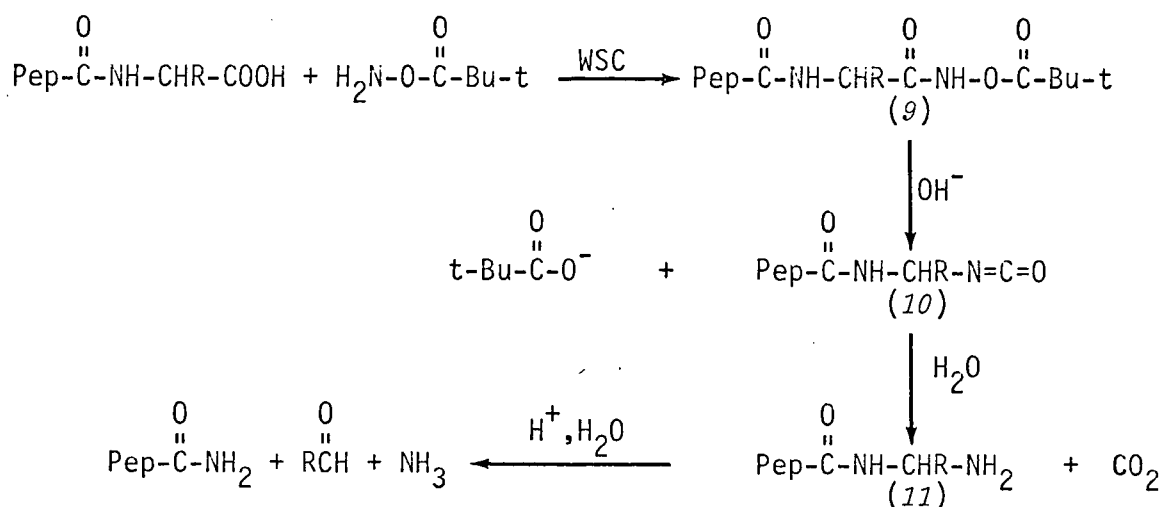
Pep = N-terminal portion of a peptide

Scheme 1.5 Peptide C-Terminus Determination via Lossen Rearrangement of an O-substituted Hydroxamic acid

Hydrolysis of the carboxamide bond to generate a new carboxyl terminus would make a sequential procedure possible. However, the N-aminomethylamide (*11*) formed on degradation of the isocyanate, decomposes under vigorous hydrolytic conditions to the corresponding peptide-amide and aldehyde. Identification of the C-terminal residue depends on the difference in amino acid analysis of the peptide before and after degradation. This restricts the method to relatively small peptides of a size for which this analytical technique is applicable. The method fails when aspartic or glutamic acids are the C-terminal residues. Interferences in the method are the low degradation yields of asparagine and glutamine and the partial loss of internal tyrosine and tryptophan.

1.6 OBJECTIVES AND OUTLINE OF THE PRESENT WORK

From the preceding presentation of methods currently in use, or proposed for determining the amino acid sequence of peptides and proteins, it should be obvious that while some procedures have reached a state of maturity (e.g. Edman degradation), there are others which are as yet unsatisfactory. The statement by Stark seven years ago that *no entirely satisfactory chemical method of carboxyl-terminal analysis exists*[29] still applies. Of the several C-terminal methods that have been proposed (*vide supra*), all suffer limitations, and few have been useful in actual practice. Vigorous conditions, solvent restrictions, and failure at certain amino acid residues have all contributed to their lack of general utility.

It would be highly advantageous to have a method of sequencing peptides from the carboxyl end similar in sensitivity, convenience, and applicability to the Edman degradation from the amino terminus.

The work described in this thesis has as its objectives the investigation of a reaction specific for carboxylates which could be used as a method for C-terminal residue analysis or C-terminal peptide sequencing.

The concept of the method which we envisaged for determination of the C-terminal residue in peptides is shown in Scheme 1.6.

$$\text{Pep-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-NHCHR}_{n-1}\text{CONH-CHR}_n\text{COOH} \quad + \quad \text{(12)}$$

$$\downarrow$$

$$\text{Pep-CONH-CHR}_{n-1}\text{CONH-CHR}_n \quad + \quad 2H_2O$$

(13)

$$\downarrow H_2O$$

$$\text{Pep-CONH-CHR}_{n-1}\text{COOH} \quad + \quad \text{(14)}$$

$$\downarrow$$

Further Degradative Cycles
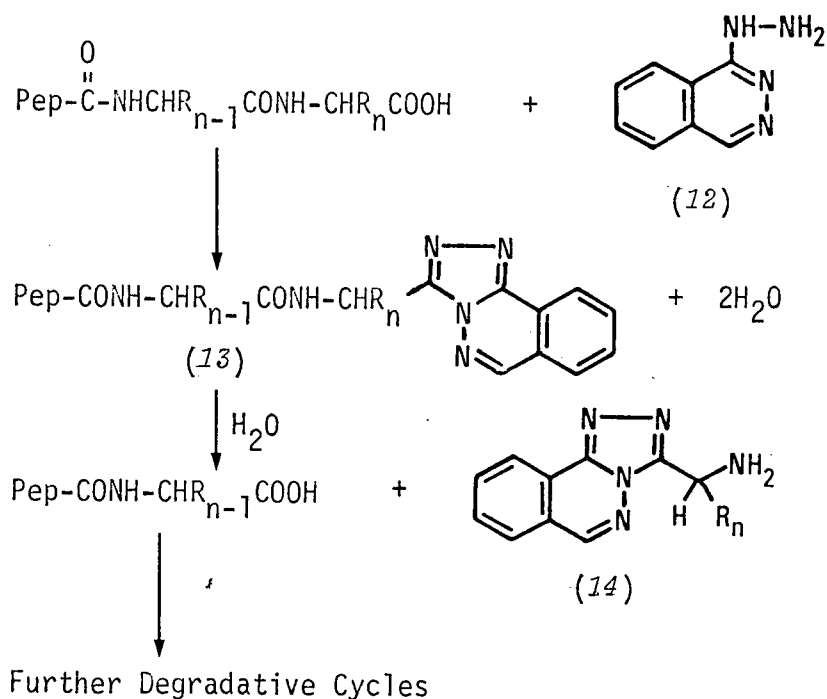
Pep = N-terminal portion of a peptide

Scheme 1.6   Proposed C-Terminal Peptide Sequencing
             via s-Triazolo[3,4-a]phthalazines

The reaction involves the coupling of 1-hydrazinophthalazine (12) with the C-terminal amino acid of peptides to afford a peptidyl-s-triazolo[3,4-a]-phthalazine derivative (13). Hydrolysis of the terminal peptide bond removes the C-terminal residue as a 3-aminomethyl-s-triazolo[3,4-a]phthalazine (14) derivative which is characteristic of the terminal amino acid. The

remaining peptide contains a free carboxylate which can undergo further degradative cycles with 1-hydrazinophthalazine. The choice of 1-hydrazino-phthalazine as the reagent for modification of the C-terminal amino acid arises from several considerations:

a. 1-Hydrazinophthalazine reacts with carboxylates to form an amide intermediate which spontaneously cyclizes with loss of water to form an s-triazolo[3,4-a]phthalazine (TAP). The formation of this cyclic product is specific for carboxyl groups among those functionalities which are commonly found in amino acids, and there can be no ambiguity in results arising from possible reaction of 1-hydrazinophthalazine with other classes of electrophiles.

b. An advantage of the TAP system is that it exhibits a strong blue fluorescence when irradiated with short wavelength ultra-violet light. This property of TAP derivatives should permit identification and detection of the TAP-modified amino acid with high sensitivity, analogous to the Dansyl method for N-terminal amino acids.

c. The essential components for formation of a fused 1,2,4-triazole ring are a carboxyl component and a hydrazino compound with the structure:

$$\begin{array}{c} NH-NH_2 \\ | \\ -C \diagdown_N \\ | \end{array}$$
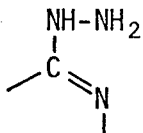
We chose the hydrazino derivative of phthalazine since its chemistry is well known, and in almost all of its reactions with carboxylic acids, the fully cyclized products are formed, unlike those of other ring systems which may stop at the amide product.

The major thrust of this work was in delineating the conditions necessary for coupling 1-hydrazinophthalazine with N-protected amino acids and dipeptides. 1-Hydrazinophthalazine does not react with carboxylic acids under mild conditions, hence we explored several methods of promoting this reaction. In view of the possible application of our reaction for peptide sequencing, we also explored the solid-phase synthesis of TAP derivatives. We considered the use of metal complexes to promote the hydrolysis of peptide bonds. In this context, several transition metal coordination complexes of s-triazolo[3,4-a]phthalazine derivatives were synthesized.

CHAPTER 2

REACTION OF 1-HYDRAZINOPHTHALAZINE WITH N-PROTECTED AMINO ACIDS

2.1 INTRODUCTION

The reactions of hydralazine (12, 1-hydrazinophthalazine,

Apresoline)

(12)
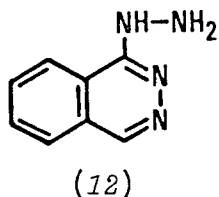
have been studied only since 1950.[30] Studies with this chemical were

prompted by the discovery that in hypertensive man, hydralazine lowers blood

pressure by reducing vascular resistance through direct relaxation of

arteriolar smooth muscle. These characteristics made it an ideal agent for

the treatment of severe arterial hypertension.[31] The effect on blood

pressure was so striking that interest in the drug surged, and within 8

years, there were some 920 references to hydralazine in the literature.

Today, despite the advent of many other drugs, hydralazine is still widely

used for the treatment of high blood pressure, and is frequently administered

in combination with sympathetic-inhibiting and/or diuretic antihypertensive

agents.[32] The cellular mechanism responsible for the relaxation of vascular

smooth muscle remains unknown, but the ability of hydralazine to chelate
certain trace metals perhaps required for smooth-muscle contraction may be
important.[33]

Hydralazine was first synthesized and tested in the Ciba Labora-
tories at Basle by Gross, Druey, and Meir as one of a series of experimental
antihistaminic agents containing a hydrazino group.[30] As it turned out,
this compound was not an antihistamine, but its injection into animals
caused long acting vasodepressor responses unlike those of any hitherto
known drug.

In response to the potentialities of such an agent, studies were
immediately initiated into the chemical and biochemical reactions of
hydralazine with the intention of elucidating its mode of action

Druey and Ringier made the first systematic study of the chemistry
of hydralazine.[34] They found that acylating agents, such as carboxylic
acids, acid chlorides, anhydrides, and esters, did not give the expected
N-acyl derivatives with hydralazine. Instead of the amide product being
isolated, ring closure occurred with elimination of water and a new type of
compound, the s-triazolo[3,4-a]phthalazine (TAP) derivative was always
obtained.
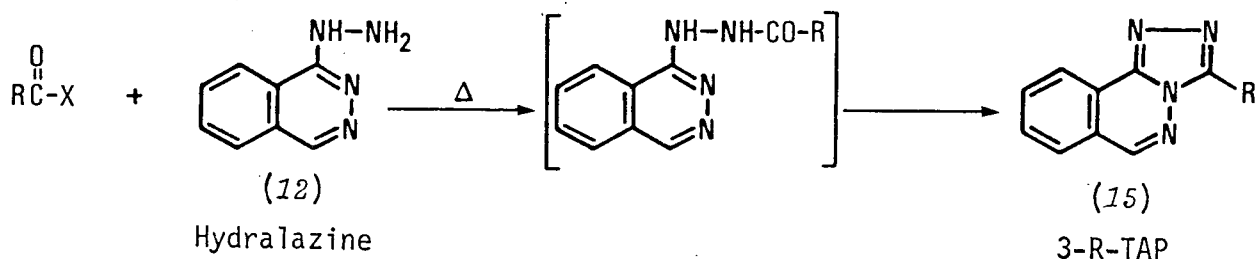
X = -OH, -OR', -OCOR', halide

Subsequently, two reports appeared which identified N-acetyl-hydralazine as a major metabolic product of hydralazine injected into animals.[35,36] These identifications were later shown to be erroneous, and the metabolite isolated was in fact, 3-methyl-s-triazolo[3,4-a]phthalazine (*15*, R=CH$_3$).[37,38] Other metabolites of hydralazine identified since that time include the compounds shown in Chart 2.1.[39-42]

R_1 = H R_2 = H, CH$_2$OH, COOH, CO$_2$C$_6$H$_9$O$_6$
R_1 = OH R_2 = H, CH$_3$

R_1 = OH
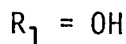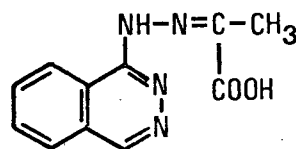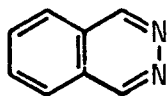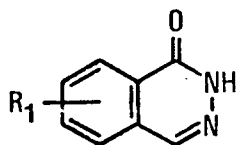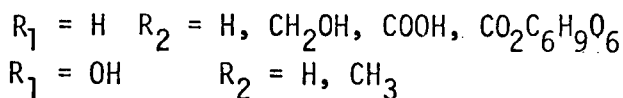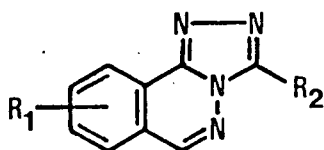
Chart 2.1   Metabolites of 1-Hydrazinophthalazine

The non-enzymatic conversion of hydralazine to 3-CH$_3$-TAP was shown to occur in the presence of acetyl CoA in human plasma ultrafiltrate.[43]

Independently of the pharmacological and clinical studies on hydralazine and its metabolites, Lovelette and Potts investigated the synthesis and chemical properties of various s-triazolo[3,4-a]phthalazine derivatives as part of a general program on the study of bridgehead nitrogen heteroaromatic ring systems.[44,45]

While the work described in this thesis was in progress, Zimmer and co-workers reported their investigations on the reaction between hydralazine and a variety of acylating agents.[46] Their objective was to determine whether the ring closure to give 3-substituted-TAP derivatives was a characteristic of acylation reactions only. In every reaction they studied, the cyclized product was obtained. The range of reactions from which TAP derivatives may be formed are shown in Table 2.1.

The reaction mechanism for the coupling and ring closure suggested by Druey and Ringier is shown in Scheme 2.1.[34]
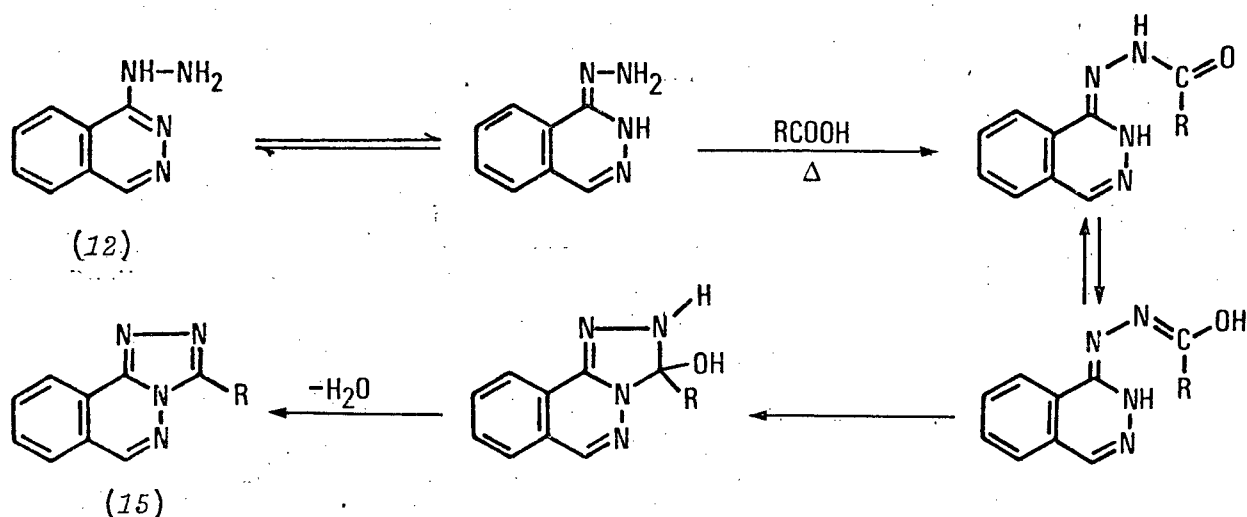
Scheme 2.1   Reaction Mechanism for Formation of s-Triazolo[3,4-a]phthalazines

It has been shown with the 1-hydrazinophthalazine system, that once the acid component was coupled, the amide intermediate cyclized spontaneously, even in those cases where attempts were made to synthesize the acylhydralazine.[38,44,45] The ease by which this cyclization occurs seems to be unique for the phthalazine system. In other cases, e.g. the acylation of 2-hydrazinopyridine, the amide derivatives could be obtained as stable and isolable compounds[47] which cyclize under dehydrative condi-

Table 2.1 Reactions of Hydralazine with Various Carboxylic Acid Derivatives
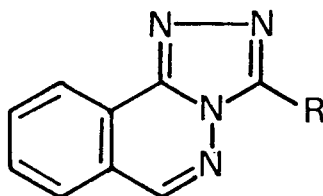
Acid Derivative	R	Reference
HCOOH	H	45
CH_3COOH	CH_3	45
CCl_3COOH	CCl_3	46
$HSCH_2COOH$	CH_2SH	46
PhCH(OH)COOH	CH(OH)Ph	46
COOH COOH	H	45
$(CF_3CO)_2O$	CF_3	46
(succinic anhydride)	$(CH_2)_2COOH$	46
$(CH_3)_3C-COCl$	$C(CH_3)_3$	46
CCl_3CN	CCl_3	46
$HC(OEt)_3$	H	45
$CH_3C(OEt)_3$	CH_3	45
CS_2	SH	44
CNBr	$\overset{+}{N}H_3Br^-$	44

tions, such as, refluxing with phenol or phosphorous oxychloride.[47-49]

The first goal of this work was to delineate the conditions required for coupling hydralazine with carboxylic acids under as mild conditions as possible. Literature methods call for this coupling to be effected at reflux temperatures with neat liquid carboxylic acids, or under melt conditions for solid acids.[45,46]

If the coupling reaction is to be applied to amino acids or to peptides, such severe conditions are not appropriate. The first step in the coupling reaction is the formation of an amide bond from carboxyl and amine components. Amide formation is an endoergic reaction so that energy must be supplied, e.g. in the form of heat. An alternative is that one of the reaction components is introduced in an activated form. Most of the published applications of this reaction are derived from this common technique: they occur through an activated form of the carboxyl group, in general through acyl derivatives such as halides, anhydrides and esters.[50]

2.2 NOMENCLATURE

The following naming and numbering systems, both IUPAC and trivial, are used in this work.

1-Hydrazinophthalazine (Hydralazine)
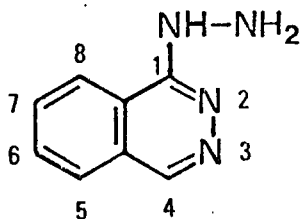
s-Triazolo[3,4-a]phthalazine (TAP)
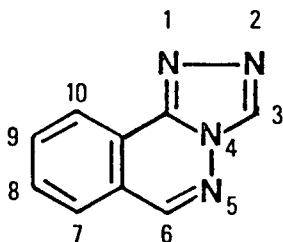
The naming of the side-chain in the 3-position follows normal procedures
when the TAP derivative is derived from hydralazine and a simple carboxylic
acid, e.g.,

Hydralazine + HCOOH ⟶ s-triazolo[3,4-a]phthalazine (3-H-TAP)

Hydralazine + CH$_3$COOH ⟶ 3-methyl-s-triazolo[3,4-a]phthalazine (3-CH$_3$TAP)
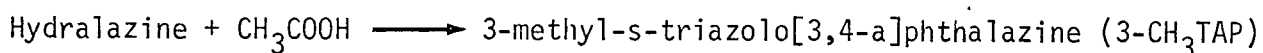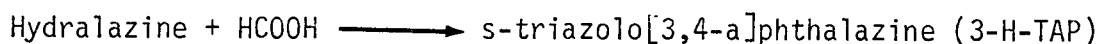
When the TAP derivative is derived from an amino acid, we depart from IUPAC
rules and give the side-chain substituent the same name as the parent
carboxyl component. The naming of TAP derivatives is illustrated in
Table 2.2.

2.3 PRELIMINARY RESULTS

The coupling reaction of hydralazine hydrochloride with neat
glacial acetic acid to give 3-methyl-s-triazolo[3,4-a]phthalazine hydro-
chloride requires up to 4 hours under reflux conditions to be completed.
Carboxylic acids are not always liquids however, and the question arises
whether the coupling can still occur when the reactants are diluted in a
solvent medium. In aqueous acetate buffer solutions (pH 2.9-4.8), no
coupling reaction occurred after 9 hours at 100°C which suggested that the

Table 2.2 Nomenclature for the Side-chains of s-Triazolo[3,4-a]phthalazines

Parent Carboxylic Acid	Accepted Name of 3-Substituent on TAP	Trivial Name of 3-Substituent on TAP	TAP Abbreviation
Formic Acid HCOOH	-	-	3-H-TAP
Acetic Acid CH_3COOH	Methyl	-	3-Me-TAP
N-Acetyl-glycine $CH_3CONHCH_2COOH$	Acetamidomethyl	N-Acetyl-glycyl	3-(N-Ac-gly)-TAP
N-t-Butyloxycarbonylglycine $(CH_3)_3COCONHCH_2COOH$	N-t-Butyloxycarbonylaminomethyl	N-t-Butyloxycarbonyl-glycyl	3-(N-BOC-gly)-TAP
N-t-Butyloxycarbonylalanine $(CH_3)_3COCONHCHCOOH$ $\qquad CH_3$	1-(N-t-Butyloxycarbonyl)amino-ethyl	N-t-Butyloxycarbonyl-alanyl	3-(N-BOC-ala)-TAP
N-Acetyl-methionine $CH_3CONHCHCOOH$ $\qquad (CH_2)_2$ $\qquad SCH_3$	1-Acetamido-3-methylthiopropyl	N-Acetyl-methionyl	3-(N-Ac-met)-TAP

coupling of a carboxylic acid with hydralazine was unfavourable under dilute conditions. The alternative mode of action, that of activating the carboxyl group was then considered.

The approach taken was to generate the activated carboxyl intermediate *in situ* by means of a 'coupling reagent,' and to react the activated species directly with the amine component without prior isolation of the intermediate. The coupling reagent used for exploratory studies was 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride[51] (*16*, EDC).

$$\overset{+}{\text{Et-N=C=N-(CH}_2)_3\underset{\text{H}}{\text{NMe}_2}} \text{ Cl}^-$$

(*16*)
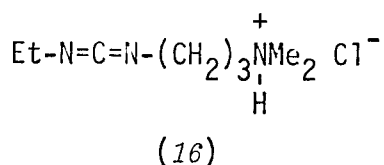
The carbodiimide reacts with carboxylic acids to form an O-acylisourea which is attacked very readily by amine nucleophiles to form the amide product (see Section 2.4.4).

The first trials were performed with hydralazine HCl and aqueous acetic acid in the presence of EDC at ambient temperature. The reaction was monitored by UV spectroscopy (Figure 2.1). The molar absorptivity (ε) of the most intense absorption of 3-CH$_3$-TAP is almost four times greater than that of hydralazine. Thus, as the coupling reaction proceeds, it is possible to monitor the growth of the 231 nm band of 3-CH$_3$-TAP, concomitant with the decrease in intensity of the hydralazine bands.

In aqueous solution, the reaction did proceed with the formation of 3-CH$_3$-TAP. However, the reaction is slow, and after two weeks there was still a significant amount of unreacted hydralazine HCl.

Coupling occurred much faster using dry methanol as solvent, the reaction being essentially complete within two days.
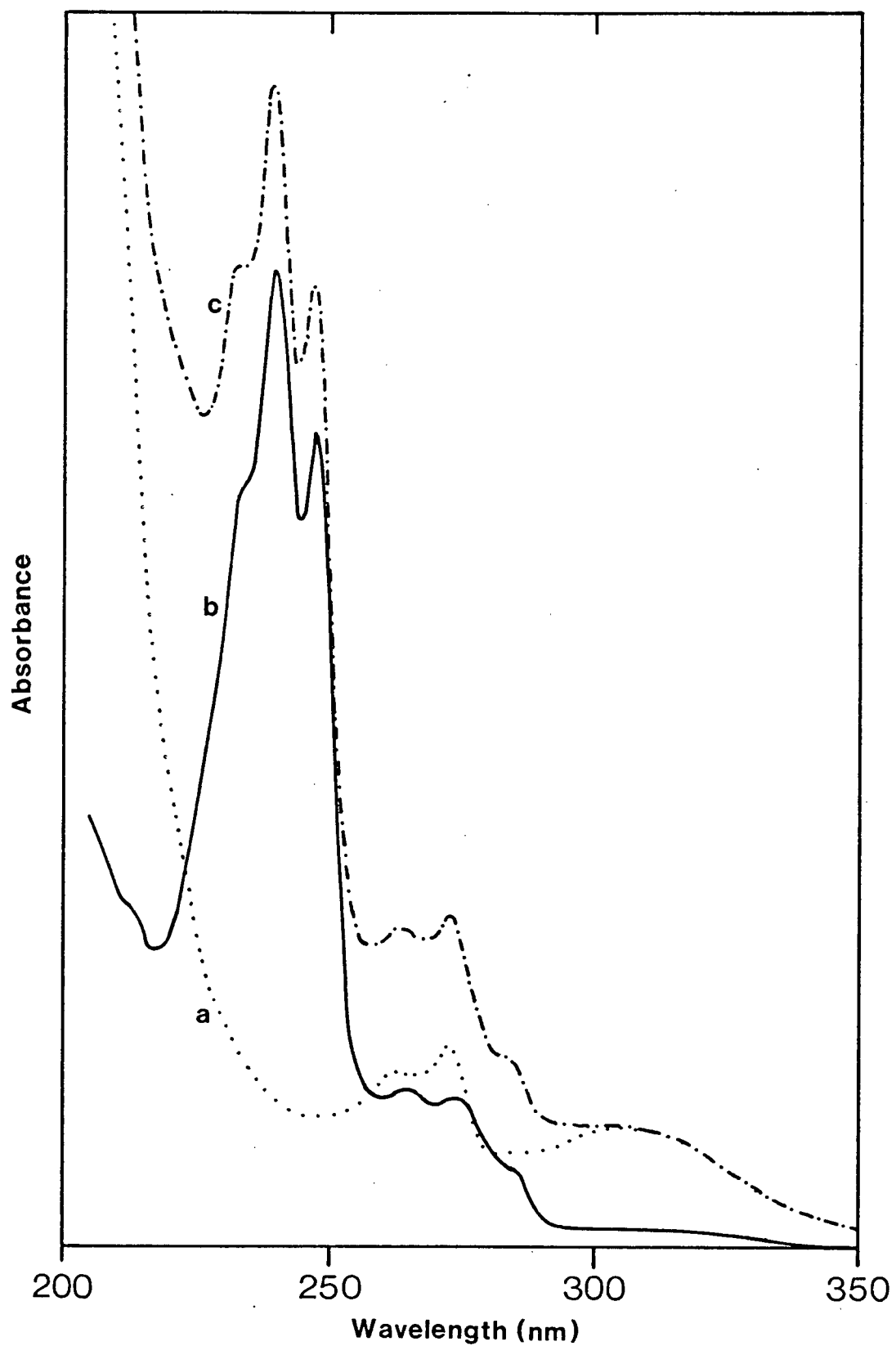
Figure 2.1 Ultraviolet Spectra of Equimolar Amounts of (a) Hydralazine,
(b) 3-Me-TAP, and (c) a Mixture in H_2O Solution

Although the reaction time was not yet satisfactory, these experiments showed that with carboxyl activating agents milder reaction conditions than previous literature methods could be used. This set the stage for the coupling of hydralazine with N-protected amino acids.

In principle, the problems faced in this work were similar to those found in peptide synthesis. The synthesis of peptides requires activation of the carboxyl group of an N-protected amino acid such that it will react with the amino function of another C-protected amino acid molecule to give a new peptide link. In our case, the nucleophile is the amino group of a monosubstituted hydrazine, rather than the amino group of an amino acid. Previous work has shown that once the carboxamide linkage is formed with hydralazine and carboxylic acids, ring closure is immediate to form the s-triazolo[3,4-a]phthalazine derivative.[45,46] One expects therefore, that coupling methods used in peptide synthesis should be applicable to the formation of TAP compounds from hydralazine and amino acids.

Because of the multitude of coupling methods used in peptide synthesis[52-54] it is necessary to be highly selective in the choice of methods for coupling the amino and carboxyl components. In peptide synthesis, the choice of coupling reagents is frequently dictated by the ease of racemization produced by the method. Racemization is unimportant in our system.

In view of the promise of coupling reagents[†] for effecting TAP formation under mild conditions, we directed our attention to finding

---

† In peptide synthesis, coupling reagents are generally considered to be reagents added to the amino and carboxyl components to effect amide bond formation in a one-pot reaction, without isolation of intermediates.

appropriate coupling reagents. The use of coupling reagents simplifies the synthetic procedures considerably, and makes the method more amenable to possible automation of the procedure.

2.4 RESULTS

2.4.1 The Isoxazolium Salt Method

N-Ethyl-5-phenylisoxazolium-3'-sulfonate (*17*, NEPIS, Woodward's Reagent K) was first used as an amide-forming reagent in 1961.[55,56]

(*17*)
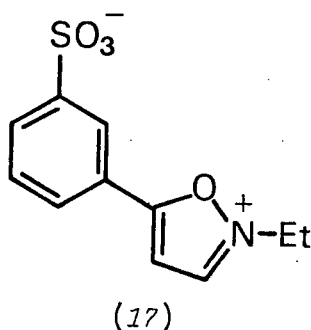
This reagent reacts with carboxyl groups to form active enol esters which condense with amine nucleophiles with amide bond formation as shown in Scheme 2.2.

Reaction is initiated by the base induced abstraction of a proton in *17* with a concerted ring opening rearrangement to form the highly reactive ketoketenimine (*18*). The reaction of this species with free carboxylic acid produces an iminoanhydride (*19b*) which is converted to the enol ester (*20*). This intermediate can react with amine nucleophiles to form the amide product, or it can undergo O ⟶ N acyl migration to yield the unreactive keto-amide, *21*.[57]

The enol ester is a good acylating agent towards nucleophiles since the leaving group is stabilized as the anion of a β-ketoamide. Some additional stabilization of the leaving group might be derived from intra-
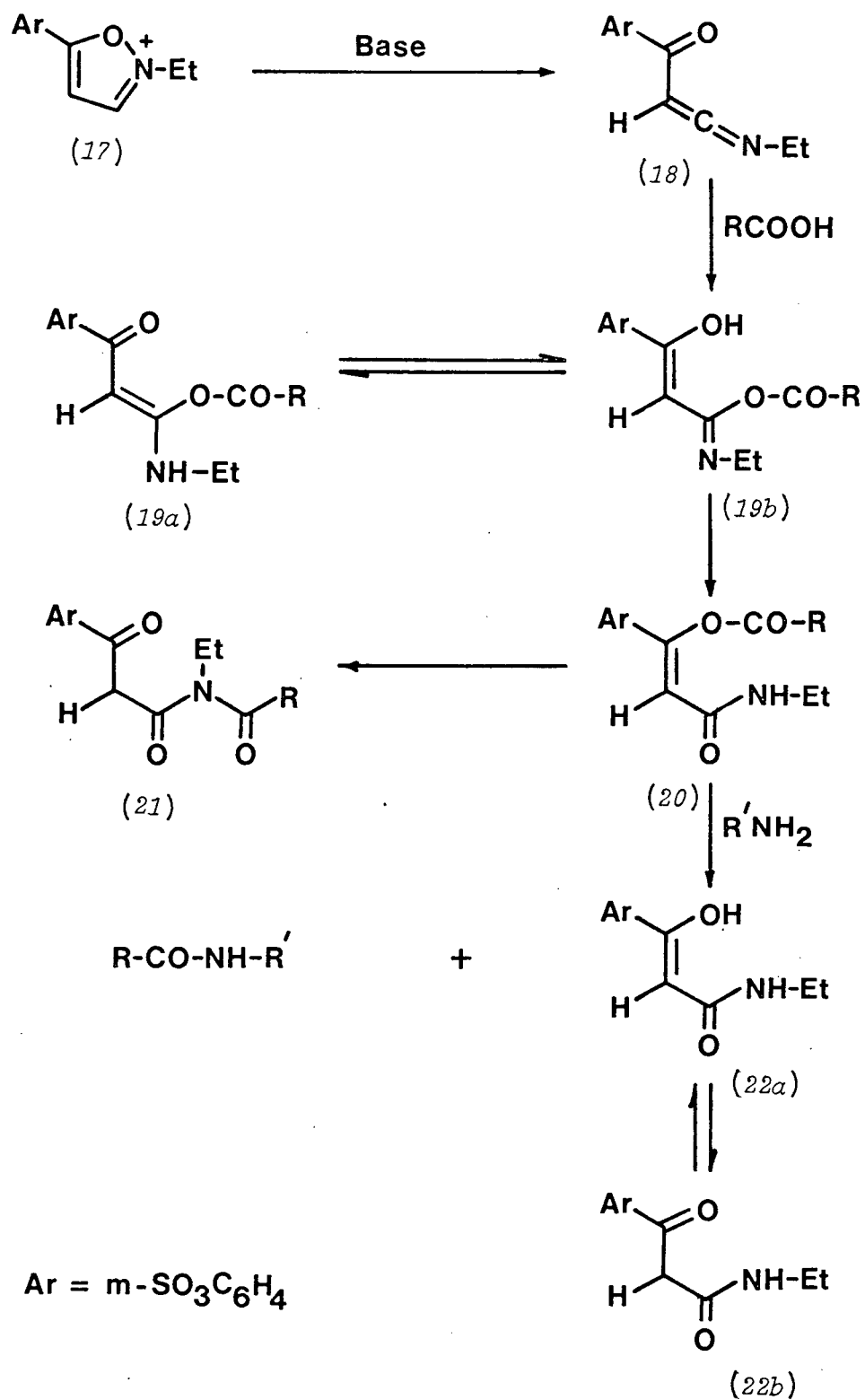
Scheme 2.2   Mechanism of Amide Formation with NEPIS

molecular hydrogen bonding to the forming O-anion during attack by the nucleophile (:N).
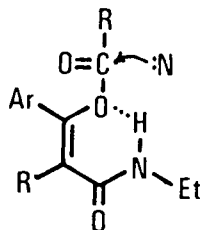
The high reactivity of the active enol ester intermediate together with its facile generation *in situ*, recommended the isoxazolium salt method for facilitating the coupling of hydralazine with amino acids. Another factor in favour of this method was the reputed ease of separation of by-products from the desired coupled product.

NEPIS was designed by incorporating a sulfonate group onto the phenyl substituent to provide an ionic ketoamide by-product (*22*).

(22)
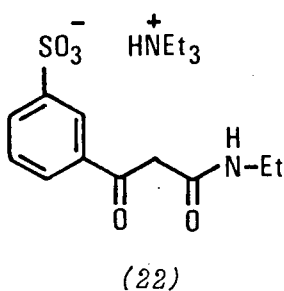
The procedure recommended for product isolation in peptide synthesis involves simple trituration with water to remove the water-soluble impurities.[56] Any unconsumed enol ester or side-reaction products connected to the sulfonated framework would be removed from the coupled product along with *22*.

Literature procedures for synthesis of peptides call for initially suspending the isoxazolium salt with the acid component in the presence of Et$_3$N, in either acetonitrile or nitromethane solvent until the isoxazolium salt dissolved completely. At this stage, the carboxylic acid is assumed to be completely converted to the enol ester. The amine component is then added and the acylation reaction is usually allowed to proceed overnight (15-18 hours) before work-up of the reaction mixture.

For our trials with the isoxazolium salt method, NEPIS was the coupling reagent used. The amine component was hydralazine as the HCl salt because of the instability of the free base (cf. Chapter 5). Initial experiments were conducted with N-acetyl-DL-methionine because of its solubility in acetonitrile and also because, at this early stage of the work, there were few other N-protected amino acids on hand. The reaction of the above components is shown in Scheme 2.3.
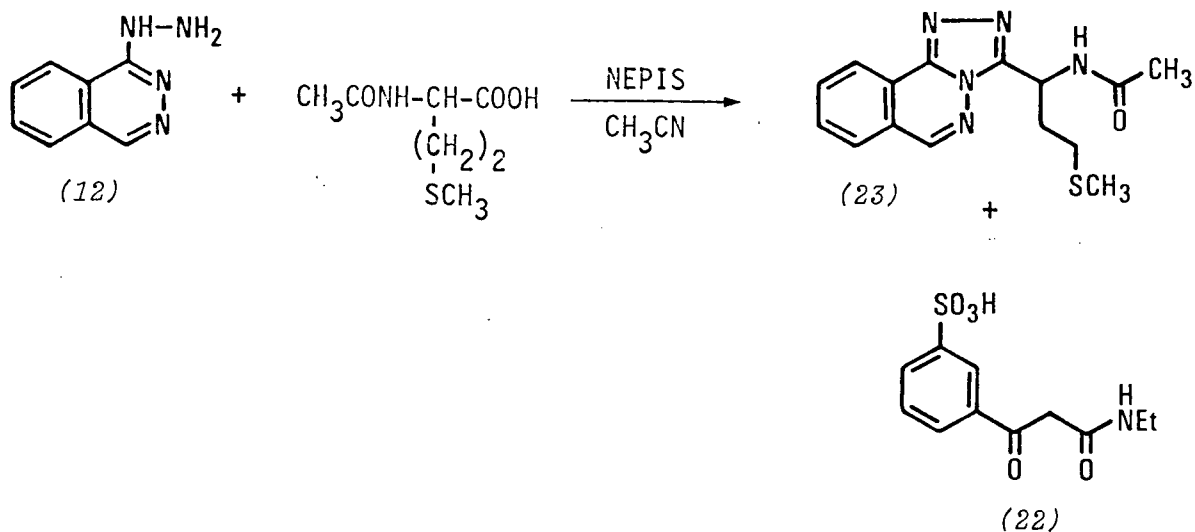
Scheme 2.3 Formation of 3-(N-Ac-met)-TAP with NEPIS

The reaction conditions used initially were similar to those recommended for peptide synthesis, with addition of hydralazine HCl and Et_3N in acetonitrile suspension after formation of the enol ester. However, it became apparent that strict adherence to literature procedures was impractical. In the first instance, high concentrations of all reagents could not be used to promote the coupling reaction because of the low solubility of the hydralazine salt in acetonitrile. A yellow precipitate was formed during the coupling reaction which appeared to be a decomposition product of hydralazine.

Isolation of the TAP derivative was not spectacularly successful. Trituration of the reaction mixture with water after the precipitate was filtered off, and the solvent removed, not only dissolved the sulfonated by-products, but also some TAP product as well. Extraction of a CH_2Cl_2 solution of the reaction mixture with water also resulted in loss of product into the aqueous phase. On an encouraging note however, the CH_2Cl_2 solution after extraction showed clean UV spectra characteristic of the TAP chromophore. The identity of the product isolated from the CH_2Cl_2 solution was confirmed by mass spectrometry to be the desired 3-(N-acetyl-methionyl)-TAP (23).

In an effort to improve the reaction conditions, the next series of experiments were conducted with N-butyloxycarbonylglycine (N-BOC-gly). The expected reaction product is 3-(N-BOC-gly)-TAP (24).
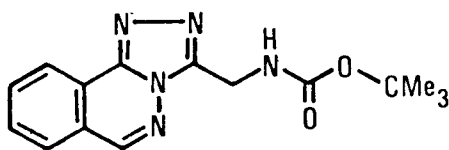
(24)

N-Butyloxycarbonylglycine is very soluble in acetonitrile, and is thus suitable for our needs. Of more importance, the TAP derivative should be relatively insoluble in water, thus facilitating isolation of product.

Coupling reactions were performed under the same conditions as previously, with the product isolated by extraction of CH_2Cl_2 solution with water. As expected, 3-(N-BOC-gly)-TAP showed low solubility in water, and very little TAP was lost in the aqueous phase during extractions. The isolation procedure was also modified by extracting the CH_2Cl_2 solution first with dilute HCl to expedite removal of unreacted hydralazine, and then with aqueous $NaHCO_3$ solution and water. The extractions were usually monitored by recording the UV spectra of the organic and aqueous phases, and extractions were repeated until the UV spectra of the aqueous phases showed no UV absorbing material. However, during the extractions with 5% HCl, some compound remained in the aqueous phase in approximately constant concentration even after most of the sulfonated products were removed. This compound showed a different UV band structure from the TAP chromophore. When an HCl extract was titrated with base, the characteristic TAP band structure was generated. Thus, protonation of one of the ring nitrogen atoms occurred, and the protonated product was more soluble than the basis TAP molecule in aqueous media. When 1N or 1% HCl were used for extractions, no protonation of TAP was observed.

To obtain more homogeneous conditions for amide-bond formation, the solvent system was modified. As before, the enol ester of the N-protected amino acid was prepared in acetonitrile solution. Hydralazine HCl and Et_3N were added in DMF suspension. When the reaction mixture was stirred overnight almost all the hydralazine HCl had dissolved. An attempt was made to isolate the TAP derivative by sonicating the reaction mixture with 0.5N HCl after

organic solvent had been removed. The sonication procedure accelerated the extraction of water-soluble materials into aqueous solution, leaving TAP as a solid.

When this mixture was left standing, a gas was liberated and all solid gradually dissolved. Very little of the dissolved product could be extracted into CH_2Cl_2 or EtOAc. Evidently the work-up procedure deblocked the butyloxycarbonyl group from 3-(N-BOC-gly)-TAP with liberation of CO_2 (Equation 3). The deblocking of the BOC- group under such mild conditions

(24)

(3)
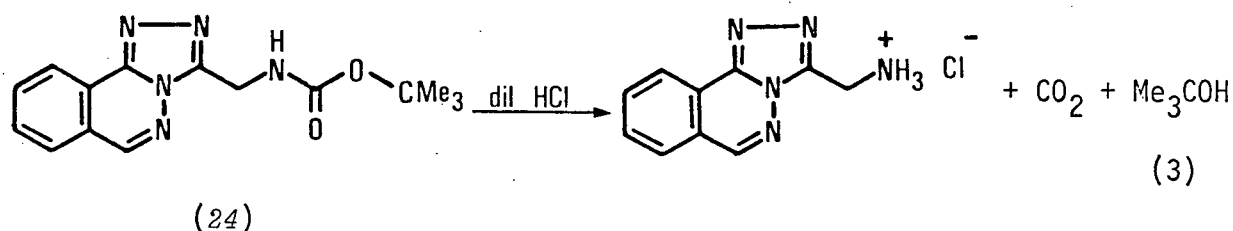
is in contrast with the observed stability in 1N HCl, and the usual deblocking procedure with HBr in glacial acetic acid.[58]

When the coupling reaction was done entirely in DMF solution, all hydralazine HCl dissolved on overnight reaction, but its disappearance was masked by the simultaneous precipitation of $Et_3N.HCl$. Monitoring the reaction by TLC showed significant unreacted reagents after 5 hours, but the reaction appeared to be essentially complete within 22 hours. The product was obtained by extraction with 1N or 2N HCl and H_2O of a methylene chloride solution of the reaction after DMF was removed. This procedure removed almost all impurities. Recrystallization of the product from methanol gave 3-(N-BOC-gly)-TAP pure by TLC criteria.

In contrast to their recommended use in peptide synthesis, acetonitrile and nitromethane were unsuitable for the synthesis of TAP derivatives due to the low solubility of hydralazine HCl. Incomplete dissolution of hydralazine HCl has an obviously detrimental effect on TAP yields, and delayed or prolonged addition of the amine component to the reaction solution encourages competition of rearrangement and side-reactions.[56]

N,N-Dimethylformamide was used as a solvent medium with some reservations in view of its reported unsuitability for carboxamide formation with NEPIS[56] and the instability of hydralazine in DMF solution. Water and ethanol are not recommended as solvents because they react with the ketenimine intermediate (*18*). Low coupling yields in dioxane, tetrahydrofuran (THF) and ethyl acetate are attributed to incomplete enol ester formation. Lowered yields are found with DMF and dimethylsulfoxide (DMSO) probably due to an increase in the rate of ketoamide formation caused by the increased polarity of the solvent.

Our results show that whereas the occurrence of the side-reaction (*20→21*) discourages the use of polar solvents, polar solvents are required to solubilize hydralazine HCl. Indeed, it is possible that the yield-limiting factor for TAP formation in DMF solution is the rearrangement *20→21*. No extensive efforts were made to optimize the reaction conditions, and our maximum observed TAP yield of 73% does not represent an optimum value. Certainly, the yield should be improved with a mixed CH_3CN/DMF system by decreasing the extent of competing O→N acyl migration. Substitution of DMF with dimethylacetamide (DMA) should decrease the extent of hydralazine decomposition in solution (cf. Chapter 5).

Another means of improving the coupling reaction is to use an isoxazolium salt with less tendency to undergo rearrangement of the enol

ester. Woodward and Woodman showed that substituting the N-alkyl substituent with a bulkier group slowed down the rearrangement.[59] They succeeded in blocking the enol ester rearrangement completely in N-t-butyl-5-methyl-isoxazolium perchlorate (25) which bears the bulky N-t-butyl group.

Me $\diagdown$ O $\diagup$ + N-Bu-t   ClO$_4$ $^-$
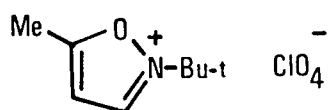
(25)

Unfortunately, the isolable t-butyl ketenimine (26) derived from this cation was so unreactive that it did not easily yield enol esters with peptide acids; the activation step was so slow that side reactions became significant.[60,61]

Me $\diagdown$ O H $\diagup$ C $\diagdown$ N-Bu-t
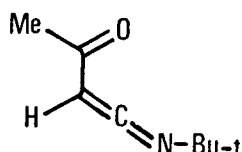
(26)

2.4.2 The EEDQ Coupling Reagent

1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (27, EEDQ) was originally developed by Belleau and his associates[62] as a depressor of the central nervous system. EEDQ was also shown to be an efficient and selective coupling reagent. Belleau and Malek proposed the mechanism for amide bond formation shown in Scheme 2.4.[63]
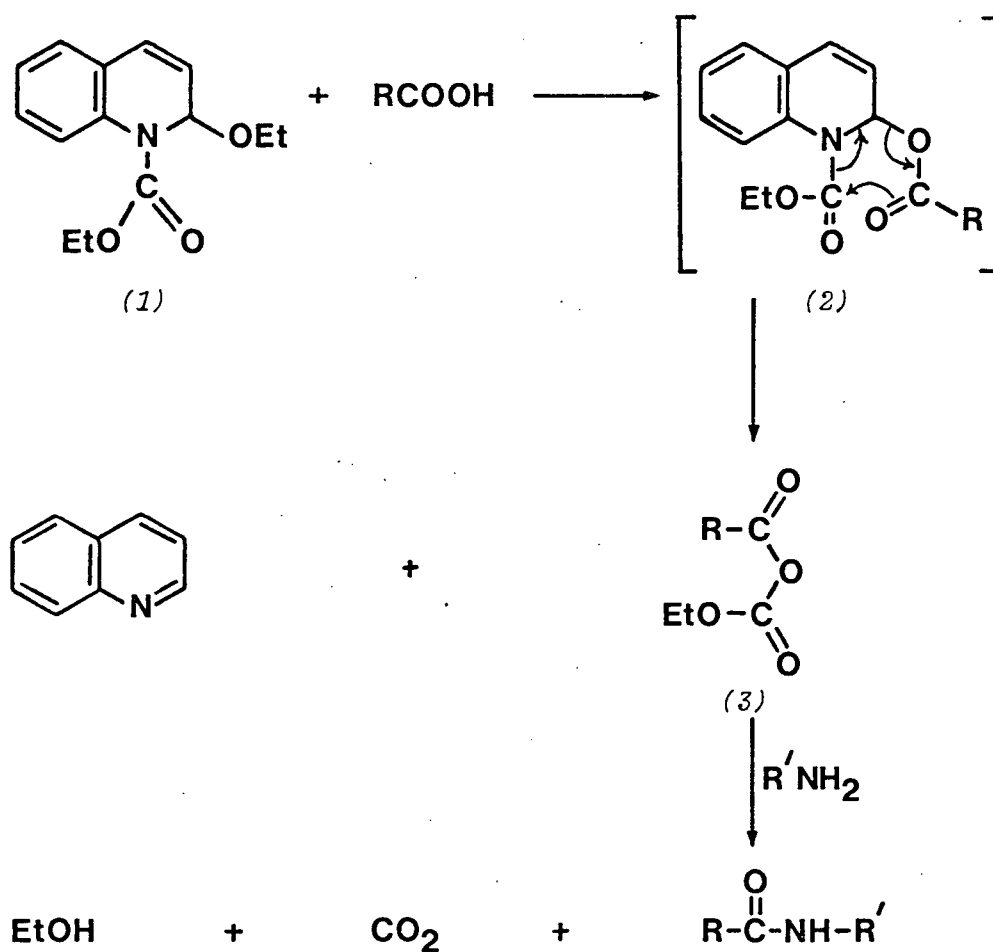
Scheme 2.4 Mechanism of Amide Formation with EEDQ

The carboxylic acid replaces the ethoxy group of EEDQ to form an intermediate (*28*) which is converted to a mixed carbonic anhydride *in situ*. This mixed anhydride (*30*) reacts with amine nucleophiles to form the amide compound. The by-products of the reaction (quinoline, ethanol, and carbon dioxide) are reported to be readily removed by flash evaporation of the reaction mixture. The reaction may be carried out in solvents such as benzene, absolute ethanol, or tetrahydrofuran. An attractive feature of this method is the promise of easy isolation of the desired product.

Trials with EEDQ were conducted with N-acetyl-glycine and hydralazine in methanol solution. UV spectral analysis of the reaction mixture showed very little of the TAP product when the reaction was allowed to proceed at room temperature for up to three days. The presence of quinoline in the reaction mixture indicated that the mixed carbonic anhydride had been formed, but apparently much of the active intermediate was lost through hydrolysis with water present in the solvent.

Other trials in acetonitrile solution were only mildly successful due to the sparing solubility of N-acetyl-glycine in acetonitrile and the tendency of hydralazine to precipitate from solution when EEDQ was added. In spite of these problems, the yield of 3-(N-acetyl-glycyl)-TAP was higher in acetonitrile than in methanol.

Using N-acetyl-L-alanine, which was more soluble in acetonitrile than the glycine derivative, formation of 3-(N-acetyl-L-alanyl)-TAP was much improved for overnight reactions at room temperature. Although some hydralazine still precipitated from solution, the tendency to do so was much less than with N-acetylglycine, notwithstanding the use of more concentrated solutions with the alanine derivative.

Coupling reactions with EEDQ in THF or DMSO solution resulted in little or no TAP formed. Good amide formation was effected with N-BOC-L-

alanine and hydralazine in methylene chloride solution. The product could be isolated by extracting the methylene chloride solution with dilute aqueous acid and base.

2.4.3 Acyloxyphosphonium Cations

Acyloxyphosphonium derivatives (30) are very susceptible to nucleophilic attack at the carbonyl carbon atom (Scheme 2.5), and such species feature as intermediates in several recently developed procedures for amide bond formation.

$$
\begin{array}{ccccc}
& O & & & O & & & R'NH_2 & O & & \\
& \| & & + & & \| & + & & & \| & & + \\
R-C-O^- & + & R_3P-X & \longrightarrow & R-C-O-PR_3 & \longrightarrow & R-C-NHR' & + & {}^-O-PR_3 \\
& & & & (30) & & & &
\end{array}
$$
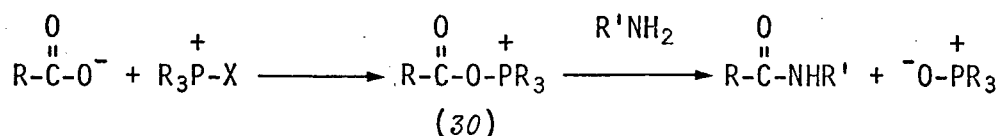
Scheme 2.5   Amide Formation via an Acyloxyphosphonium Intermediate

Much effort has been expended in developing acyloxyphosphonium salts as viable acylating agents in peptide synthesis with encouraging success, and several methods show promise for practical use in peptide synthesis.[54]

Phosphorus-containing reagents were considered for the following reasons:

1. Reaction conditions are generally mild and suitable for 'eintopf verfahren' of TAP derivatives.

2. Reagents are inexpensive and readily accessible.

3. The reagents were developed for peptide synthesis and little is known about their more general applicability in carboxamide synthesis.

In 1969, Kenner and associates reported on the resonance-stabilized acyloxyphosphonium salts derived from hexamethylphosphoramide (*31*, HMPA) (the Kenner-Sheppard Reaction).[64,65] They showed amide formation according to the sequence in Scheme 2.6.

$$(Me_2N)_3PO + TsCl \longrightarrow [(Me_2N)_3\overset{+}{P}-OTs]Cl^-$$
$$(31) \qquad\qquad\qquad (32)$$

$$\downarrow$$

$$[(Me_2N)_3\overset{+}{P}-O-\overset{+}{P}(NMe_2)_3]TsO^-Cl^-$$
$$(33)$$

$$\downarrow RCOOH$$

$$[\overset{O}{\overset{\|}{R}C}-O-\overset{+}{P}(NMe_2)_3]Cl^-(OTs^-)$$
$$(34)$$

$$\downarrow R'NH_2$$

$$\overset{O}{\overset{\|}{R}C}-NHR' + (Me_2N)_3PO$$

$$Ts - = Me-\!\!\left\langle\!\!\bigcirc\!\!\right\rangle\!\!-SO_2-$$
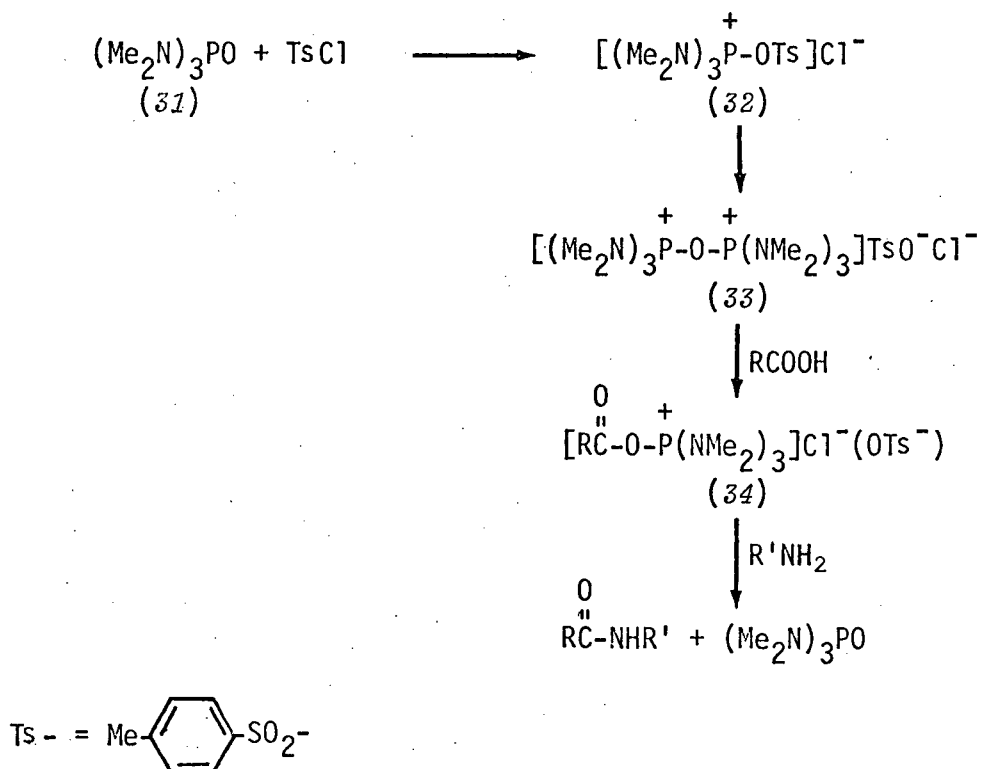
Scheme 2.6   Amide Formation via an Acyloxydimethylaminophosphonium Salt

The addition of tosyl chloride to excess HMPA yields the dication (*33*), presumably by way of the initial adduct (*32*). The dication (*33*) is relatively stable, and the crystalline di-tetrafluoroborate salt has been used as a coupling reagent in peptide synthesis.[66]

Two routes can be visualized for the acylation step. This step can proceed either by direct attack of the amino component at the activated carboxyl group with anchimeric assistance by a hydrogen-bonded cyclic transition state (*35*) or via the addition of the amino component to the

(35)
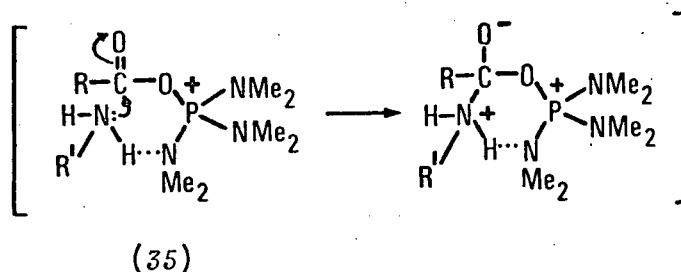
phosphorus atom of *34* to form *36*, which then decomposes to the amide and

(36)
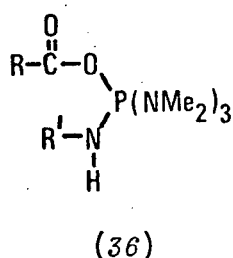
HMPA in a manner not dissimilar to the Wittig reaction.[65] Activation of

HMPA can also be achieved with tosic anhydride or thionyl chloride in place

of tosyl chloride.

In a trial with HMPA and tosyl chloride, we used N-acetyl-glycine

as the carboxyl component and hydralazine HCl with Et_3N as the amine

component with reaction at room temperature. TAP was detected in the reac-

tion mixture within two hours, but after overnight reaction, there still

remained a fair amount of undissolved hydralazine HCl. Efforts at isolating

the TAP product were unsuccessful. Extraction of the HMPA solution with

benzene, petroleum ether, diethyl ether, and methylene chloride was

unsatisfactory because of the high solubility of 3-(N-acetyl-glycyl)-TAP

in HMPA. The high boiling point of HMPA (~290°C) precluded removal of

solvent by distillation.

No further work was done with this system because of the problems of isolating the product from HMPA solution. Also discouraging any reconsideration of this method for TAP synthesis, was a report that HMPA is a potential carcinogen.[67]

Since the Kenner-Sheppard method proved to be unsatisfactory for TAP synthesis, we considered an alternative means of generating acyloxyphosphonium intermediates, i.e. the coupling reagent, azido-tris(dimethylamino)phosphonium hexafluorophosphate (37).[68]

$$[(Me_2N)_3\overset{+}{P}-N_3]PF_6^-$$
$$(37)$$
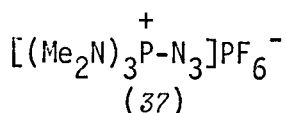
In solution, 37 reacts rapidly at $-10°C$ with triethylammonium salts of carboxylic acids to give the acyl azide (39) via an acyloxyphosphonium azide (38) which is not isolable. The active intermediate reacts with amines to form the carboxamide (Scheme 2.7).

$$[(Me_2N)_3P-N_3]PF_6 + RCOOH + Et_3N \xrightarrow{-10°C} [R-\overset{\overset{O}{\|}}{C}-O-P(NMe_2)_3]N_3 + [Et_3NH]PF_6$$
$$(37) \qquad\qquad\qquad\qquad (38)$$

$$\downarrow$$

$$R-N=C=O + N_2 \longleftarrow [R-\overset{\overset{O}{\|}}{C}-N_3] + (Me_2N)_3PO$$
$$(40) \qquad\qquad\qquad (39)$$

$$\downarrow R'NH_2 \qquad\qquad -10° \downarrow R'NH_2$$

$$R-NH-CO-NH-R' \qquad\qquad R\overset{\overset{O}{\|}}{C}-NHR'$$
$$(41)$$
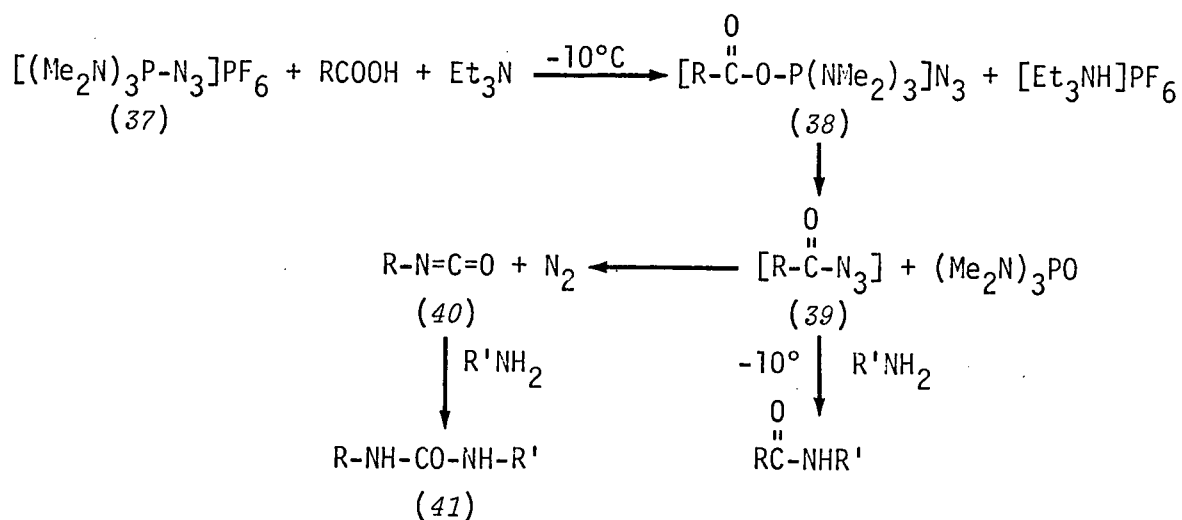
Scheme 2.7   Amide Formation via an Acyl Azide

At higher temperatures, the acyl azide may undergo the Curtius rearrangent to the isocyanate (40) which will condense with the amine component to give the urea (41).

Our efforts with the coupling reagent (37) involved reaction of N-BOC-glycine with hydralazine at -15°C in DMF solution. After one hour, TLC analysis of the reaction mixture indicated only a little TAP present, and UV spectra showed mainly unreacted hydralazine in solution. Overnight reaction at room temperature did not significantly increase the yields of TAP. Coupling reactions in Methyl Cellosolve solution under the same conditions also gave low yields of 3-(N-BOC-glycyl)-TAP.

The coupling reaction with $[(Me_2N)_3P-N_3]PF_6$ was not pursued further because of the low TAP yields and difficulties in maintaining low temperatures to minimize the Curtius rearrangement.

Another coupling method used was the so-called "oxidation-reduction condensation" reaction of Mukaiyama and co-workers,[69,70] in which the dehydration reaction involved in amide synthesis is coupled with oxidation of an aryl phosphine and reduction of a disulfide (Scheme 2.8).
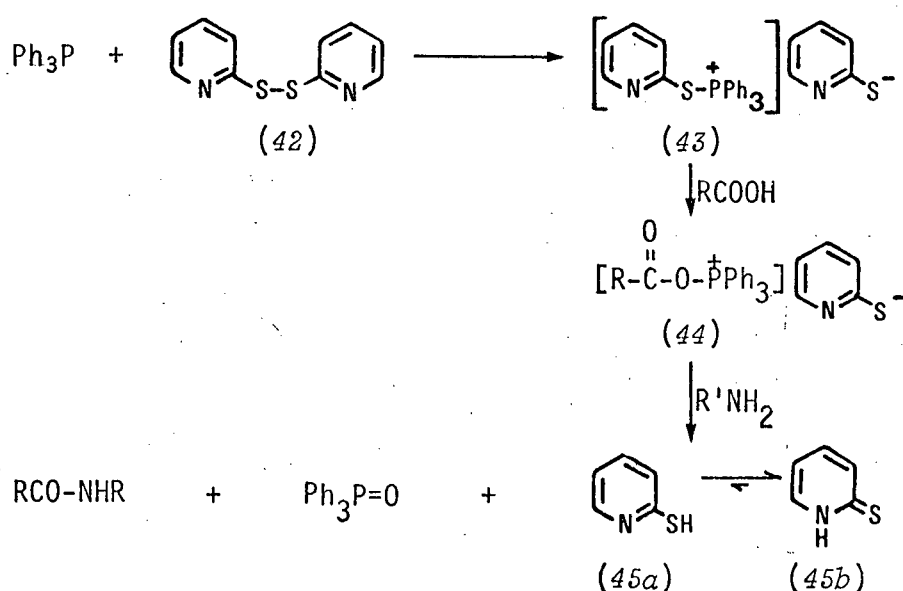
Scheme 2.8 Amide Formation via an Acyloxytriphenylphosphonium Salt

The effectiveness of this combination of oxidant and reductant can be envisaged as arising from formation of an acyloxyphosphonium thiolate (44) as the active acylating intermediate. This salt, by virtue of the electron withdrawing properties of the phosphonium entity, reacts rapidly with incoming amino components to afford amides, triphenylphosphine oxide and the thione as depicted below.
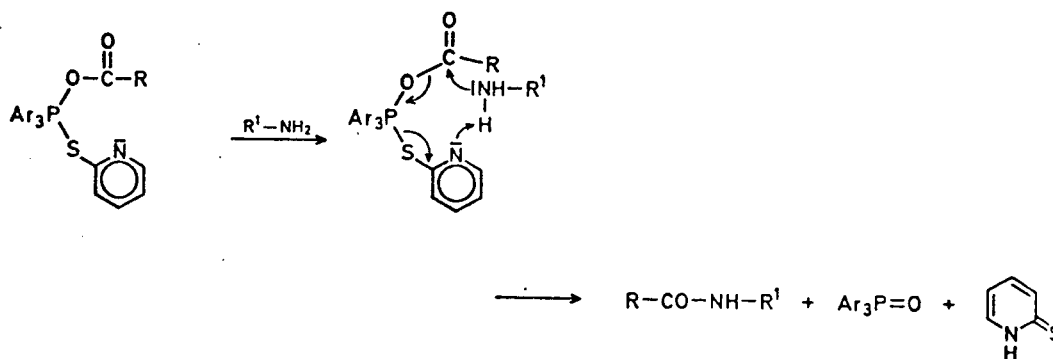
A deciding factor in using 2,2'-dithiodipyridine (42, 2-DTP) as the oxidant is the isomerization in solution of the hydrogenated product (45a) to the thione form (45b). In the absence of this isomerization, the thiol formed would react with the acyloxyphosphonium salt intermediate to give undesirable side products thus necessitating the addition of a thiol scavenger to the reaction mixture.

A coupling reaction with the Mukaiyama procedure was attempted using N-acetyl-glycine and hydralazine HCl (+ Et_3N) in dioxane solution. After overnight reaction at 40°C, UV spectral and TLC analysis of the reaction solution showed only a little of the TAP derivative in solution. A solid precipitate during the reaction showed Et_3N·HCl and a decomposition product of hydralazine.

49

The oxidation-reduction condensation reaction showed little promise for our purposes although the yield of TAP would probably be improved by conducting the reaction in a solvent where hydralazine decomposition is less pronounced. No further action was taken with this system because more encouraging results were being obtained elsewhere.

Continuing our efforts with phosphorus-containing reagents, we considered the method developed by Mitin[71] for the one-step synthesis of peptides from suitably protected amino acids (Scheme 2.9).
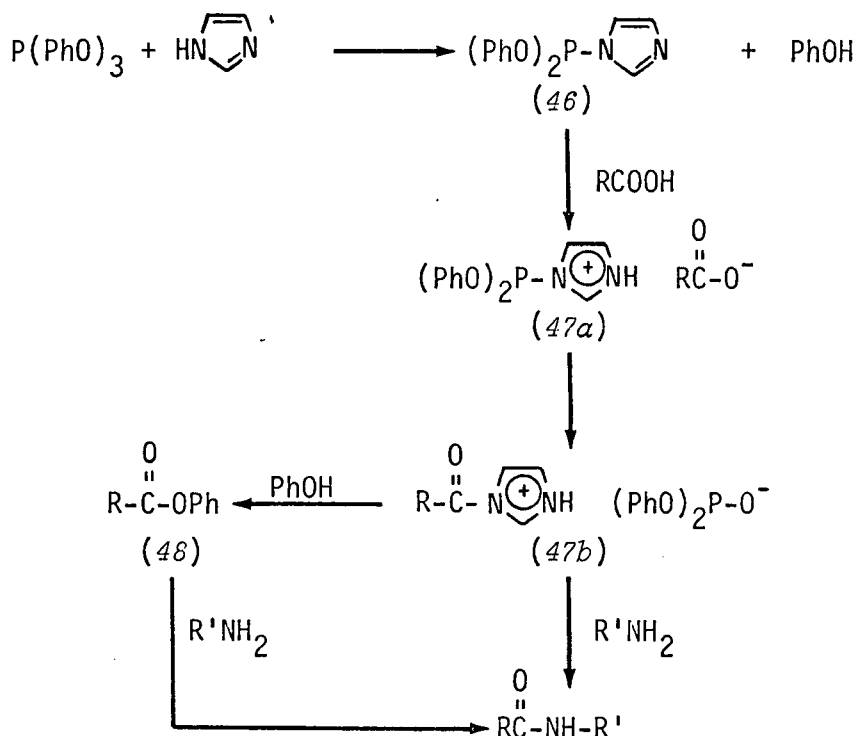
Scheme 2.9   Amide Formation via an Acylimidazolium Salt

On the basis of indirect data only, Mitin et al.[72] assumed the reaction mechanism shown. The reaction sequence is considered to consist of initial formation of imidazolyl diphenylphosphite (46), which on reaction

with the carboxyl component gives the activated compound of the latter (*47a* ⇌ *47b*). The acylimidazolium intermediate (*47b*) reacts with the amino component to afford the carboxamide. In the absence of the amine component, the active intermediate (*47b*) reacts with the phenol formed at the first stage to give the phenyl ester of the carboxylic acid.

Our first experiment with the Mitin procedure involved the coupling of N-BOC-glycine with hydralazine HCl suspended in dioxane with Et_3N added to neutralize the salt. When the reaction was carried out in the presence of triphenylphosphite and imidazole for one day at 40°C, UV spectral analysis of the reaction solution showed a high concentration of the desired TAP product. This result was most gratifying; unfortunately, a significant amount of hydralazine HCl remained undissolved.

The single trial with this procedure showed more promise than any of the previous reactions involving other phosphorus-containing reagents. We therefore set about to optimize the conditions for TAP formation.

The reaction of carboxylic acids with triarylphosphites leads to the formation of aryl esters, but high temperatures (150°-200°C) are required. In the presence of tertiary amines, however, the reaction proceeds rapidly at room temperature.[73]

A series of coupling reactions were performed in which the base was varied. It became apparent that the initial choice of bases in the exploratory experiment was the optimum one. The efficiency of bases in promoting TAP formation follows the sequence:

Imidazole + Et_3N >> DBU, Imidazole, Et_3N >> Morpholine, 2,4,6-Collidine, DABCO, t-Bu_3N, Proton Sponge

where DBU = 1,5-diazabicyclo[5.4.0]undec-5-ene
     DABCO = 1,4-diazabicyclo[2.2.2]octane
     Proton Sponge = 1,8-bis(dimethylamino)naphthalene

One conclusion from the above sequence is that it is not based on base strength alone, since the strongly basic Proton Sponge (pK$_a$ 12.37) is ineffective in the coupling reaction, whilst imidazole (pK$_a$ 6.95) in combination with triethylamine (pK$_a$ 11.0) is satisfactory.

Coupling reactions were performed at ambient temperature ($\sim$23°C), 40°C and 70°C to determine the effect of temperature on the yield of TAP. Not surprisingly, higher yields of TAP were obtained at the higher temperatures. However, some deblocking of the BOC-group from 3-(N-BOC-gly)TAP was observed at 70°C.

The efficiency of TAP formation was highly solvent dependent. A series of experiments was conducted with various solvents using imidazole and triphenylphosphite as the carboxyl activating agents. and Et$_3$N to neutralize the hydralazine HCl. The most suitable of the solvents tried was DMF. Acetonitrile, THF, and dioxane gave good to moderate yields of TAP with dissolved hydralazine, but were limited because of incomplete solution of hydralazine HCl even after one day's reaction. Dimethyl-sulfoxide, water and Methyl Cellosolve were unsuitable even though hydralazine HCl dissolved completely in the solvents.

An expected consequence of slow or incomplete solution of hydralazine HCl is formation of the phenyl ester (*48*) of the N-acylamino acid. In fact, when the amino component is absent, this procedure may be utilized for the synthesis of phenyl esters.[72] Thus, in those solvents where release of hydralazine into solution is slow, acylation of hydralazine via the active phenyl ester should become more prominent.

In the coupling reaction of carboxylic acids with amines, promoted by triphenylphosphite in the presence of imidazole, Mitin *et al.*[71,72] reported the by-products of the reaction to be diphenylphosphite and phenol.

They did not consider the possibility of further coupling reactions with the diphenylphosphite.

Yamazaki and Higashi[74] re-examined the reaction with triphenyl-phosphite and determined the effect of various tertiary bases on the synthesis of an amide. In agreement with our results, they found that imidazole was significantly more effective than other bases. An unexpected result was the reaction with diphenylphosphite, in which pyridine was the superior base. As a consequence, Yamazaki and Higashi explored the use of diphenylphosphite and pyridine for peptide synthesis.[74] They showed that the coupling reaction proceeds as shown in Scheme 2.10

$$\text{HO-P(OPh)}_2 \quad \xrightarrow[\text{py}]{\text{RCOOH}} \quad \overset{\overset{\text{O}}{\|}}{\text{R-C-O-P-H}} \quad {}^-\text{OPh} \quad \xrightarrow{\text{R'NH}_2} \quad \overset{\overset{\text{O}}{\|}}{\text{R-C-NHR'}} + \overset{\overset{\text{O}}{\|}}{\text{H-P-OPh}} + \text{PhOH}$$
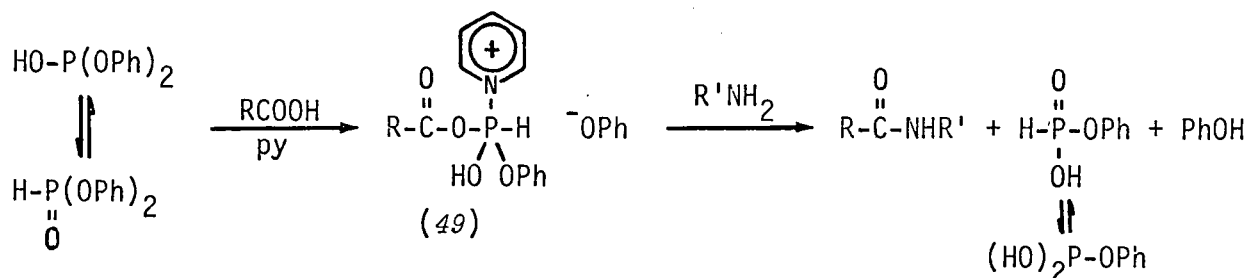
Scheme 2.10   Amide Formation via an Acyloxyphosphonium Salt
from Diphenylphosphite

The acyloxyphosphonium salt of pyridine (49) is formed first, with release of a phenolate anion from the phosphite. In the presence of an amino component, this active ingredient reacts readily to form the amide derivative. When no amino component is present the phenyl ester of the carboxylic acid is formed by interamolecular reaction with the phenolate anion released from the phosphorus atom.

If triphenylphosphite is used in the reaction, then only half an equivalent amount is required, since the pyridinium salt of diphenyl-phosphite (50) is formed which can undergo further reaction (Scheme 2.11).

$$P(OPh)_3 + RCOOH \xrightarrow{py} R-C-O-P-H \xrightarrow{R'NH_2} -O-P-H$$
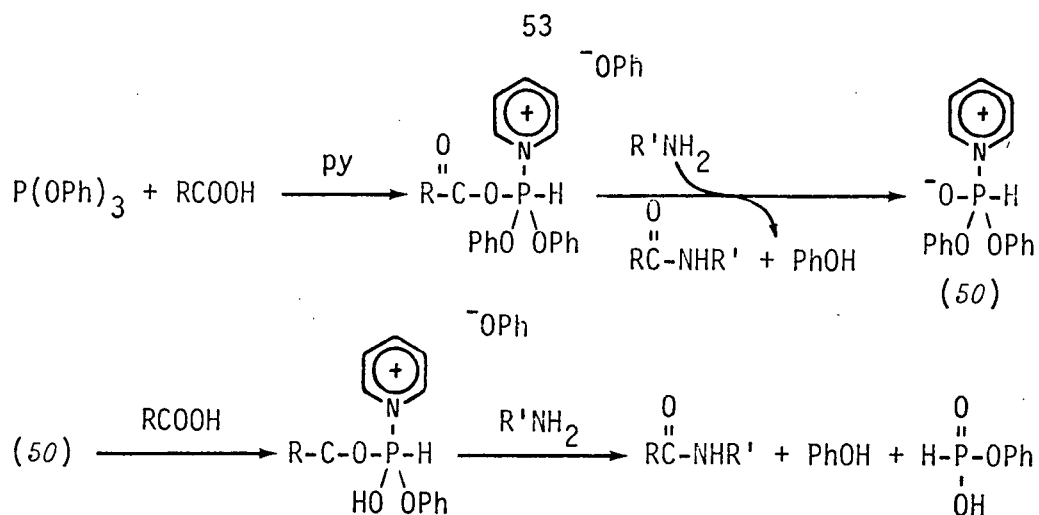
Scheme 2.11   Amide Formation via Acyloxyphosphonium Salts
from Triphenylphosphite

The reaction mechanism proposed by Yamazaki and Higashi for the coupling reaction with phosphites is at variance with that of Mitin, which assumes that the active intermediate is an acylimidazolium salt. It would be interesting to test these assumptions to determine which is the actual active acylating agent: acyloxyphosphonium salt or acylimidazolium salt.

To complete our investigations with phosphorus-containing reagents, we attempted a coupling reaction between N-BOC-alanine and hydralazine HCl with diphenylphosphite in pyridine suspension, following the procedure of Yamazaki and Higashi.[74] After two days, the reaction solution showed no trace of the desired 3-(N-BOC-alanyl)-TAP. The failure of this reaction was attributed partly to the ineffectiveness of pyridine in neutralizing hydralazine HCl. No further trials were attempted under more effective conditions.

2.4.4 Carbodiimides

In 1955, Sheehan and Hess,[75] and Khorana[76] working independently showed that suitably blocked amino acids could be joined through an amide linkage under the influence of dicyclohexylcarbodiimide (51, R=C_6H_{11}, DCC).

Since that time, DCC has become perhaps the most useful and popular coupling reagent in peptide synthesis, and it has also gained great importance as a versatile reagent in organic synthesis.[77]

The accepted mechanism for amide bond formation is shown in Scheme 2.12.[78] The first step of the reaction sequence involves the addition of the carboxyl component to a carbodiimide to form an O-acyl-isourea (52) which is highly reactive. If the external nucleophile is dilatory then internal nucleophilic attack may occur to produce an N-acylurea (53). For amide formation, two pathways are possible:

a. Direct attack of the amino component on the reactive intermediate with formation of the amide bond.

b. Attack by the carboxyl component to give a symmetrical anhydride (54) which in turn acylates the amine.

Delayed addition of the amine component decreases the importance of path (a) and increases the extent of anhydride participation.[79-81]

In contrast to many other methods for carboxyl activation, the carbodiimide reaction is reported to be relatively insensitive to moisture. The coupling reaction may even be carried out in aqueous solution, albeit with lowered yield. The by-product of the reaction, dicyclohexylurea (55, R=C_6H_{11}, DCU), has low solubility in most organic and aqueous solvents, and is usually removed by filtration. However, the dissolved DCU is sometimes troublesome to remove, and to overcome the problems of separating the by-product, the water-soluble carbodiimides, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride, (16, EDC) and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho p-toluenesulfonate (56, CMC) may be used.[51,82] The corresponding urea and acylurea derivatives are

R'COOH + R-N=C=N-R

(51)

$$R-C(=O)-O-C(=N-R)(NHR)$$

(52)

$$R-C(=O)-N(R)-C(=O)-NHR$$

(53)

R'COOH / b

R"NH$_2$ / a

(54)

$$R'-C(=O)-O-C(=O)-R'$$

$$\xrightarrow{R"NH_2}$$

$$R'C(=O)-NHR"$$

+

$$RHN-C(=O)-NHR$$

(55)

+

$$RHN-C(=O)-NHR$$

(55)
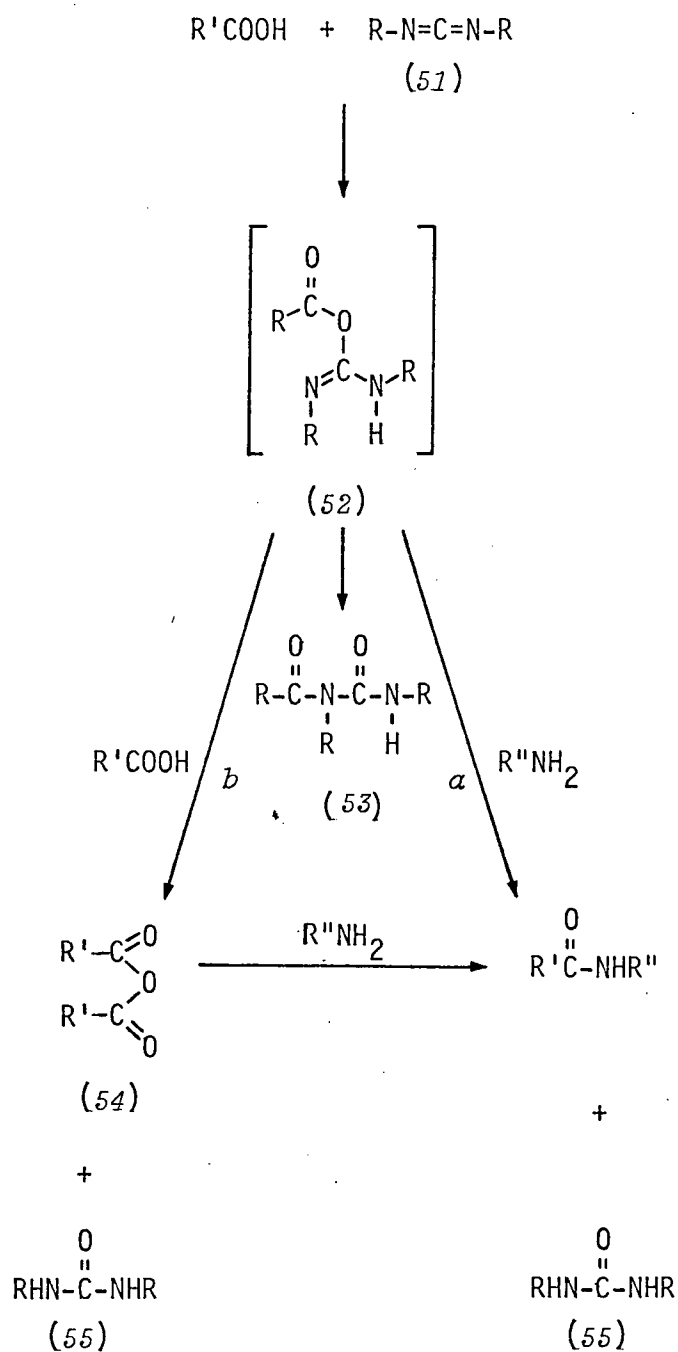
Scheme 2.12   Mechanisms of Amide Formation with a Carbodiimide

$$\overset{+}{\text{Et-N=C=N-(CH}_2)_3\text{NHMe}_2} \quad {}^-\text{Cl}$$

(16)

$$\text{N=C=N-(CH}_2)_2\overset{+}{\underset{\text{Me}}{\text{N}}}\text{O} \quad {}^-\text{OTs}$$

(56)
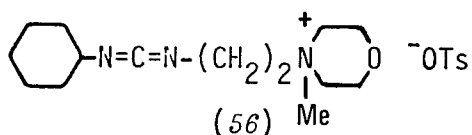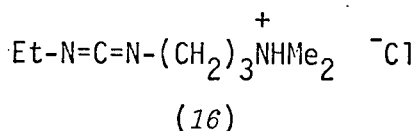
usually soluble in water and may be removed by extraction with dilute acid or water.

The extent of the carbodiimide-mediated coupling of hydralazine with N-protected amino acids was determined from UV spectra and TLC of the reaction mixtures, usually after overnight reaction. Figure 2.1 shows the UV spectra of equimolar amounts of 3-Me-TAP and hydralazine, and a mixture of the two ingredients. The spectrum (c) shows a spectrum of a reaction mixture in which hydralazine has been half converted to the TAP derivative. If no other components in the reaction mixture show significant UV absorptions in the useful wavelength region, then the intensity of the most intense TAP band ($\sim$230-240 nm) relative to those of hydralazine ($\sim$270 nm) can be used as a qualitative guide to the relative proportions of TAP and hydralazine in the reaction mixture. In general, the components of the carbodiimide coupling reaction show little interferences in the UV region of interest. The only significant interferences in the 250-290 nm region arise from degradation products of hydralazine. These decomposition products have higher molar absorptivities than hydralazine itself and can make the coupling reaction appear less complete than it actually is, the extent depending on the degree of hydralazine decomposition.

The first series of experiments with the carbodiimide method involved a comparison of coupling reactions run under identical conditions
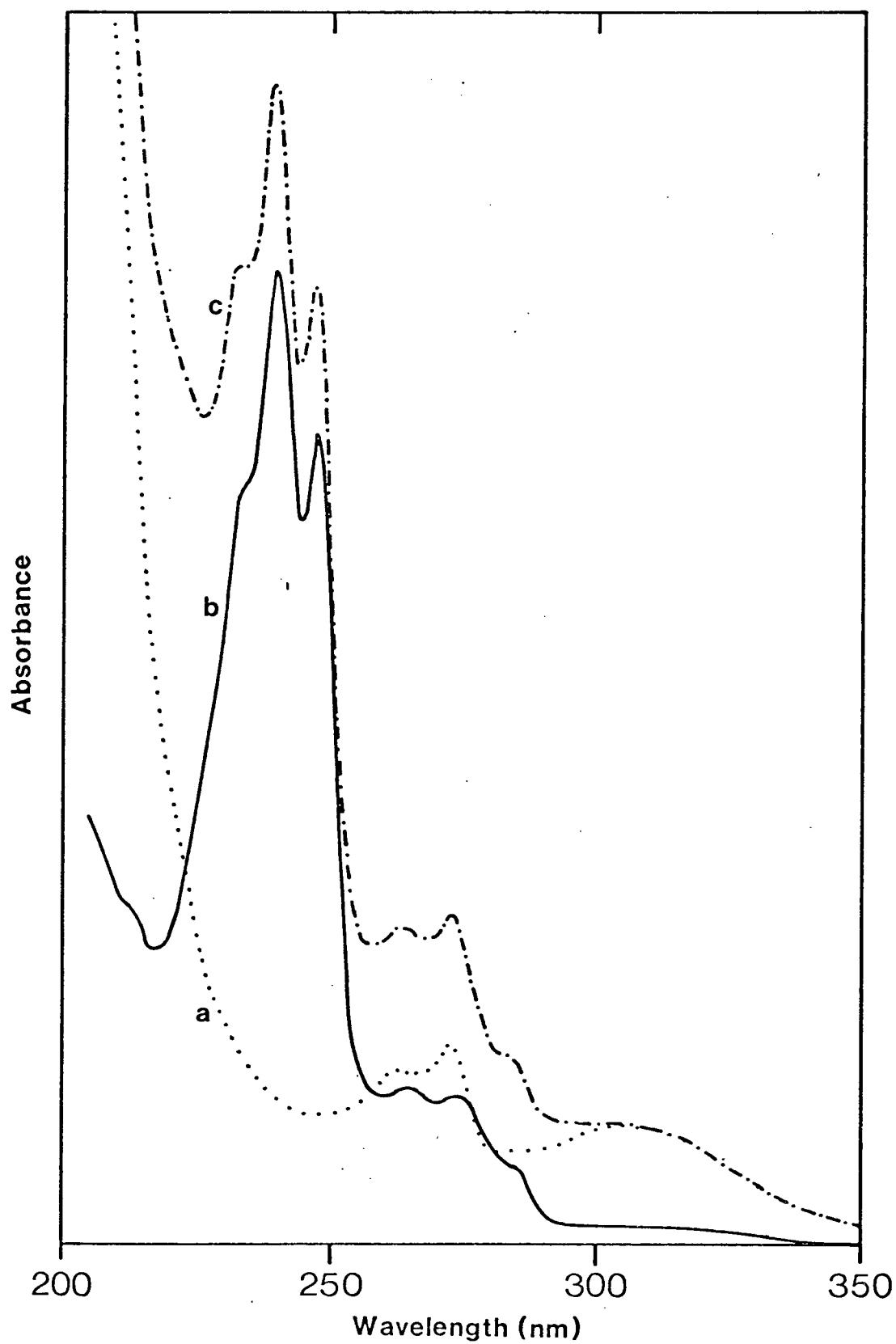
Figure 2.1 Ultraviolet Spectra of Equimolar Amounts of (a) Hydralazine,
(b) 3-Me-TAP, and (c) a Mixture in H_2O Solution

(hydralazine : N-BOC-glycine : DCC = 1 : 1 : 1; 0.05M) except for the solvent used. Six solvents were compared under these conditions. Good yields of TAP were obtained when CH_2Cl_2, $CHCl_3$, CH_3CN, and THF were used. Methyl Cellosolve, dioxane, and DMF gave poor yields of TAP. Other experiments with N-acetyl-glycine as the carboxyl component showed methanol to be capable of mediating good yields of TAP. Water on the other hand, gave poor results, especially under basic conditions, because of the oxidation of hydralazine to phthalazine which was detected in the reaction mixture. Reaction in DMA solution gave a moderate yield of TAP.

These results are consistent with the observations of Sheehan *et al.*[75,83] who found that rearrangement of the O-acylisourea intermediate (*52*) to the inactive N-acylurea (*53*) is suppressed in solvents like methylene chloride and acetonitrile. Khorana[76] showed that when dioxane, tetrahydrofuran, and chloroform were solvents in peptide synthesis, N-acylurea was always obtained. Isdebski,[84] in a study of the formation of N-acylurea in the reaction of N-benzyl-leucine with glycine ethyl ester, found that reaction in DMF gave 22% of the side-product while reaction in THF and CH_2Cl_2 gave 4 and 2% N-acylurea, respectively. In the synthesis of TAP derivatives, we also isolated significant amounts of the N-acylurea from CH_2Cl_2 solution.

Two sets of comparisons were made to determine the effect of elevated temperature on the formation of TAP. The coupling of hydralazine HCl with N-BOC-glycine using DCC in methanol solution in the ratio 1 : 1 : 1 and 1 : 5 : 5 respectively were studied at ambient and at reflux temperatures.

The comparisons indicated that there were little advantages to be gained from pursuing the carbodiimide reaction at high temperature. Indeed, the accepted practice is to initially <u>lower</u> the temperature to 0°C in order to minimize N-acylurea formation.[85] When hydralazine HCl in the presence of

Et$_3$N is the amine component in solvents where the salt has only limited

solubility, there is a significant increase in the rate of TAP formation

at higher temperature, presumably because of increased solubility of

hydralazine HCl. To compensate for the greater probability of N-acylurea

formation at elevated temperature an excess of carboxylic acid and

carbodiimide must be used. Another danger associated with elevated

temperatures is the increased decomposition of free base hydralazine.

In view of the low solubility of hydralazine HCl in most organic

solvents, we wished to determine the effect of varying the proportion of

Et$_3$N in solution. The first series of comparisons involved a 1 : 1 : 1

reaction mixture of hydralazine HCl, N-BOC-glycine, and EDC in methanol,

with either 0, 1, 1.3, 1.6, 3.4, 5.3, 16, or 50 equivalents of Et$_3$N added

to the reaction mixture.

Coupling reactions containing more than 5 equivalents of Et$_3$N

showed little evidence of TAP formation. Inhibition of the reaction by

excess Et$_3$N arises in several ways. In the mechanism for reaction of

carboxylic acids with carbodiimides (Scheme 2.13), the first step involves

formation of the protonated carbodiimide (*51a*) which is added to a

$$R\text{-}N\text{=}C\text{=}N\text{-}R \underset{}{\overset{H^+}{\rightleftharpoons}} R\text{-}\overset{+}{N}H\text{=}C\text{=}N\text{-}R \xrightarrow{R'COO^-} \begin{array}{c} R\text{-}NH\text{-}C\text{=}N\text{-}R \\ | \\ R'COO \end{array}$$

$$\qquad (51) \qquad\qquad\qquad (51a) \qquad\qquad\qquad (52)$$

$$(52) \underset{}{\overset{H^+}{\rightleftharpoons}} \begin{array}{c} R\text{-}NH\text{-}\overset{+}{C}\text{=}NHR \\ | \\ R'COO \end{array} \xrightarrow{R'COO^-} \begin{array}{cc} O & O \\ \| & \| \\ R\text{-}C\text{-}O\text{-}C\text{-}R \end{array} + \begin{array}{c} R\text{-}NH\text{-}C\text{-}NH\text{-}R \\ \| \\ O \end{array}$$

$$\qquad\qquad\qquad (52a) \qquad\qquad\qquad (54) \qquad\qquad (55)$$
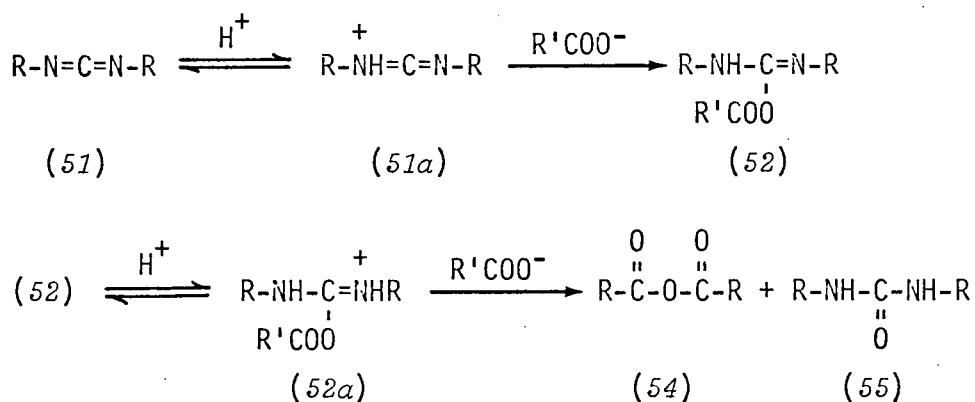
Scheme 2.13   Reaction of Carboxylic Acids with Carbodiimides

carboxylate anion to form the O-acylisourea (52). In the presence of excess acid, a similar protonation equilibrium is set up, and the protonated species (52a) reacts further with carboxylate to give an anhydride (54).[78]

When hydralazine HCl is the amino component in the coupling reaction, it reacts optimally only when its amino group is unprotonated. This requirement conflicts with the initial need to protonate both carbodiimide and the O-acylisourea. As a result, the yield of the coupled product should be very pH-dependent, and addition of a tertiary base will reduce the concentration of the protonated carbodiimide (51a) and the adduct (52a), and thereby reduce the rate of reaction.

Another consequence of excess tertiary base is the increased yield of N-acylurea. For example, reaction of N-protected amino acids with DCC in the presence of pyridine or triethylamine may afford the N-acylurea as the main product.[86,87] We detected substantial amounts of N-acylurea by-products in reaction mixtures containing excess triethylamine.

A distressing feature of reactions conducted in the presence of excess triethylamine is the ready decomposition of hydralazine catalyzed by the tertiary base. This decomposition is characterized in UV spectra by an absorption band centered at 278 nm (cf. Chapter 5).

Carbodiimide coupling reactions performed with 1.3, 1.6, or 3.4 equivalents of triethylamine showed good formation of TAP. In a coupling reaction of N-acetyl-glycine with hydralazine HCl and one equivalent of triethylamine in methanol solution, unreacted hydralazine HCl was isolated from the reaction mixture after 2 days. Thus, in practice, an excess of tertiary base is needed when EDC is the carbodiimide used. The effect of excess triethylamine on the yield of TAP in carbodiimide-mediated reactions is evident in Figure 2.2.
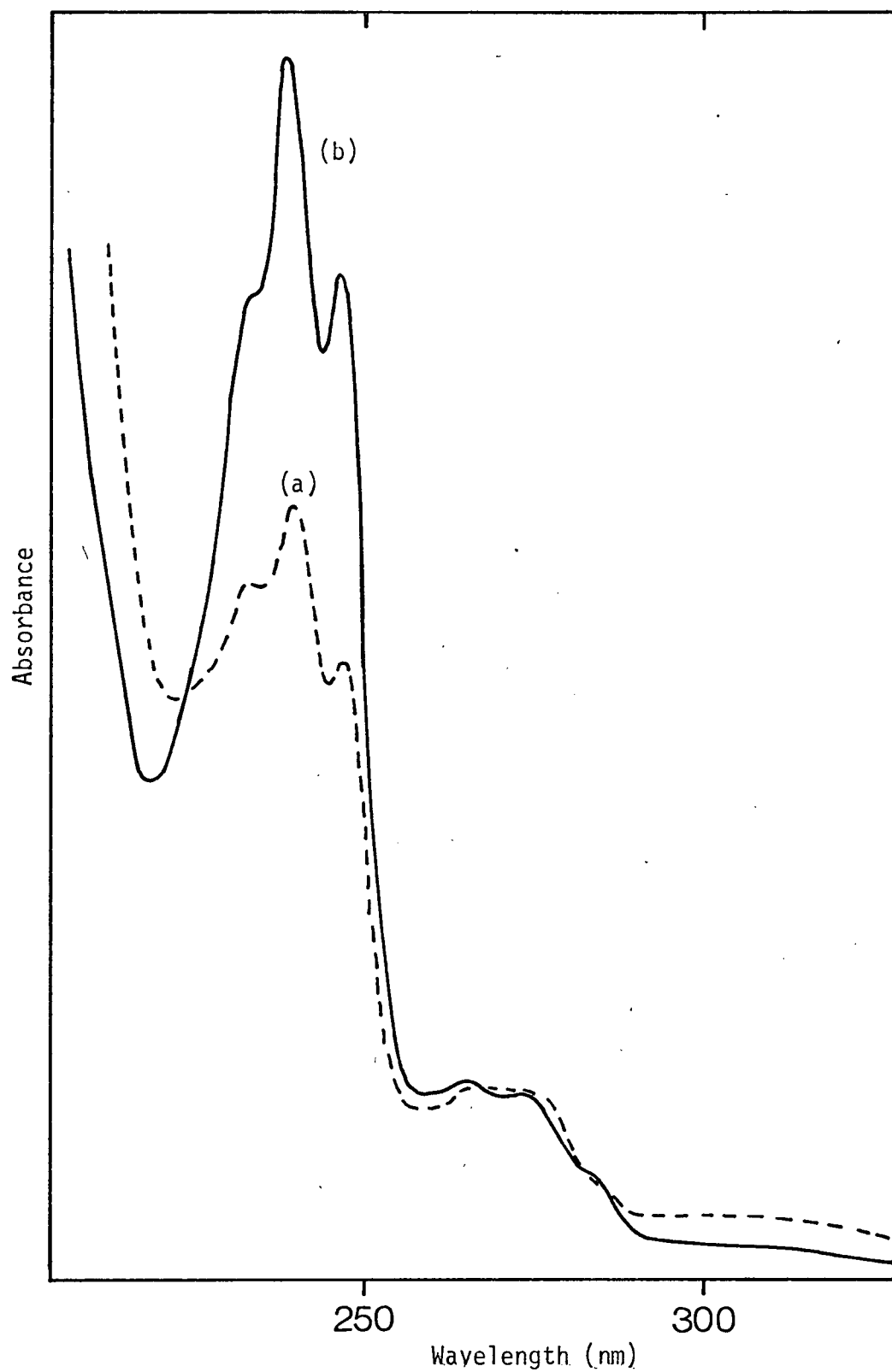
<u>Figure 2.2</u> Effect of Excess Triethylamine on Carbodiimide-mediated
Coupling Reactions. Hydralazine : acid : Carbodiimide : Et_3N
(a) 1:5:5:5, (b) 1:5:5:1.3 Spectra normalized with respect
to bands at 270 nm.

The reaction between hydralazine HCl and N-acetyl-glycine in methanol solution is slower without added triethylamine but is unexpectedly facile considering that hydralazine is present in the protonated form.

The rearrangement of O-acylisourea to the inactive N-acylurea is always a danger in carbodiimide coupling reactions. A consequence of the rearrangement is loss of the carboxyl component to the inactive by-product and hence a lowering of the yield of the desired product. Figure 2.3 shows coupling reactions with stoichiometric and with excess amounts of the carboxyl component and carbodiimide. The superiority of the reaction with excess reagents is evident. Similar results are also obtained in comparison reactions with added triethylamine.

Since the reaction of hydralazine with the N-acylamino acid-carbodiimide adduct (52) is intermolecular, whereas the rearrangement of the adduct to the N-acylurea (53) is intramolecular, keeping the volume of the solution to a minimum favors formation of the desired TAP product. The concentration effect is evident in a comparison of coupling reactions at different concentrations (Figure 2.4).

Since hydralazine was often used as the hydrochloride salt in the carbodiimide coupling reactions, it was interesting to compare the effect of different tertiary bases in the reaction mixture. Two coupling reactions were conducted under identical conditions of reagent concentration and stoichiometry with Et_3N or pyridine as the added tertiary base. Figure 2.5 of UV spectra of the reactions after one day shows definitely superior TAP formation with the triethylamine-containing reaction. Pyridine is a rather ineffective base for neutralizing the HCl salt since the spectral features at 300-320 nm in Figure 2.5(b) arises from protonated hydralazine.
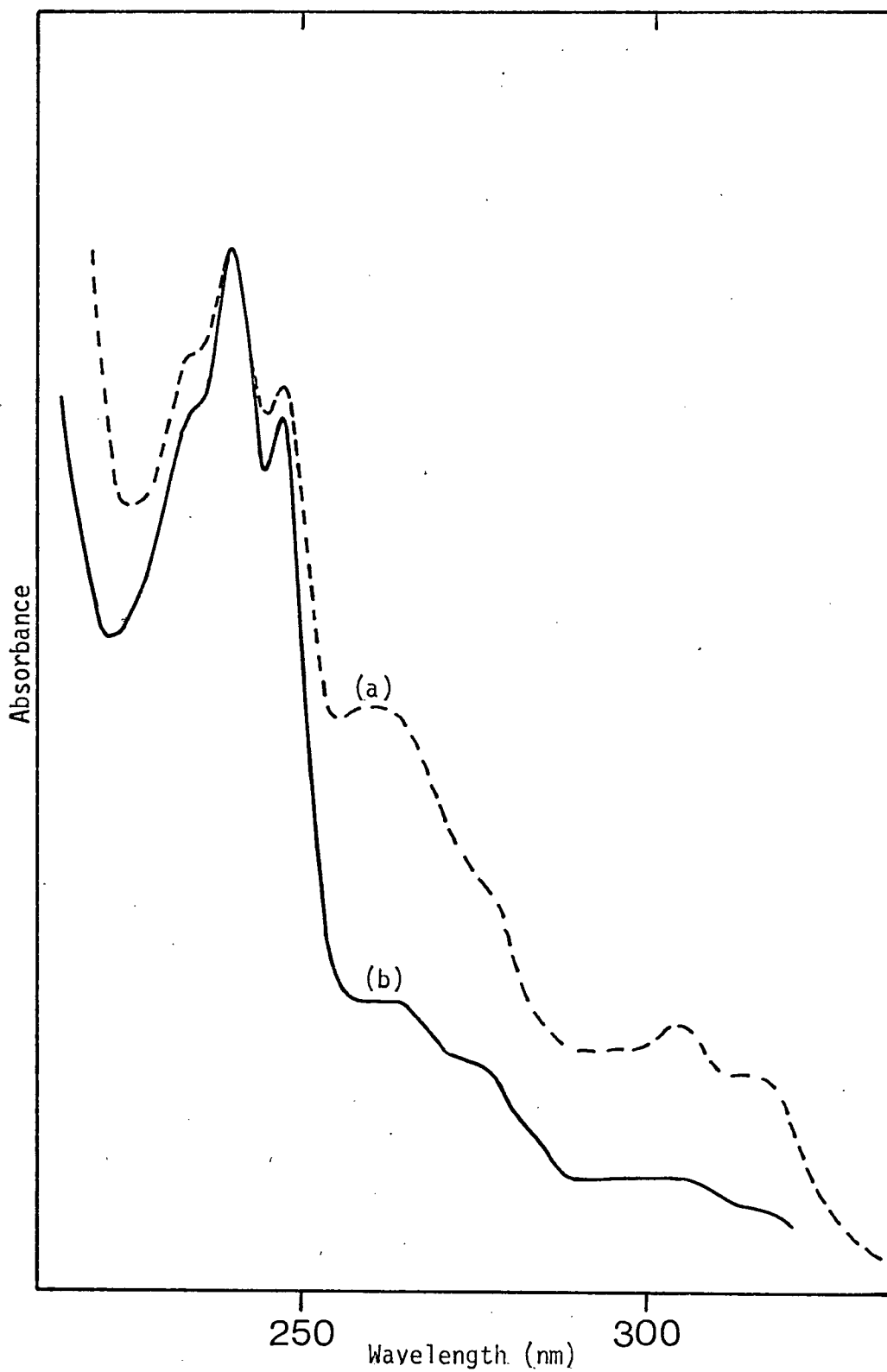
Figure 2.3 Effect of Excess Carboxyl Component and Carbodiimide on
Carbodiimide Mediated Coupling Reactions. Hydralazine : Acid :
Carbodiimide, (a) 1:1:1, (b) 1:5:5. Spectra normalized with
respect to band at 240 nm.
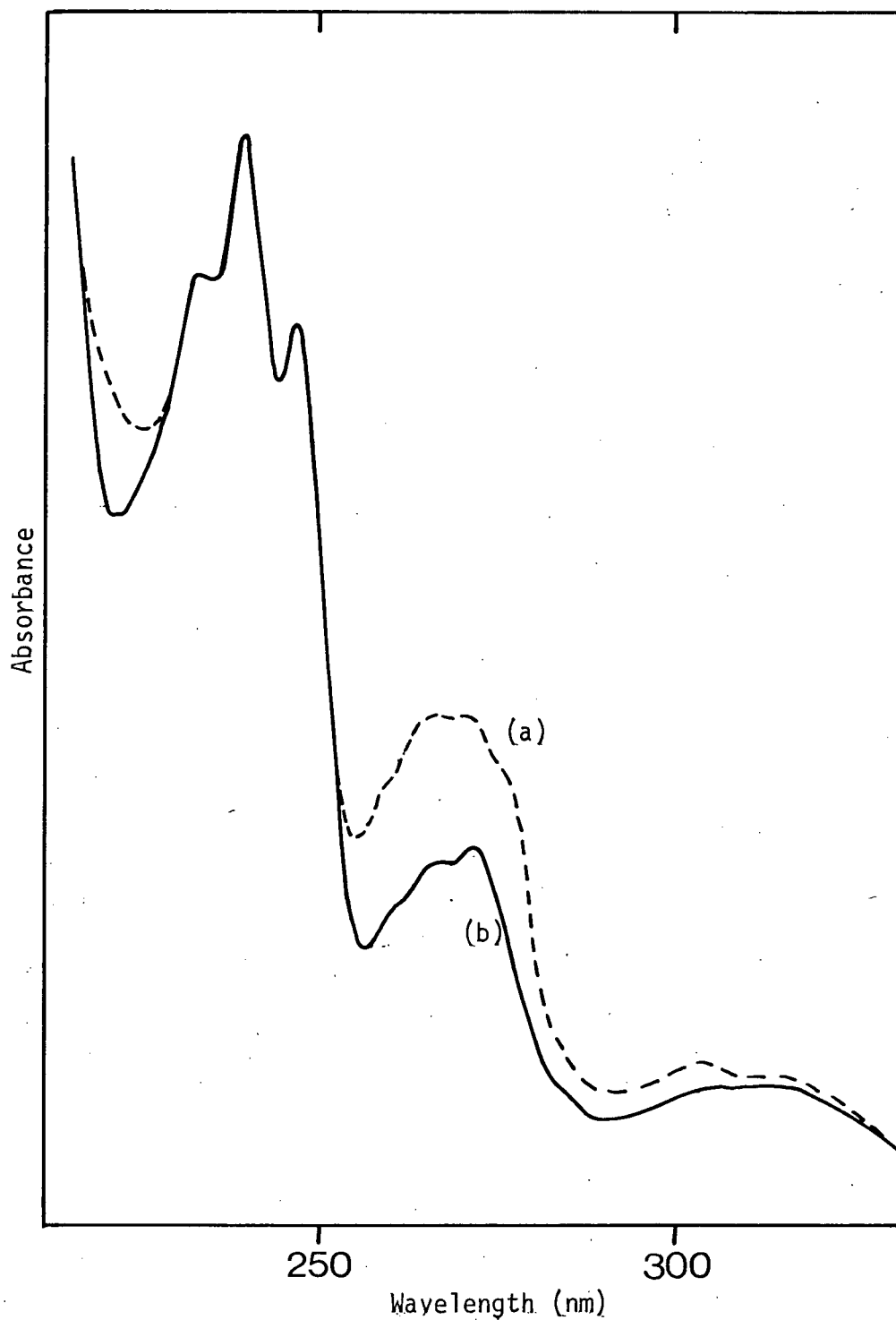
Figure 2.4 Effect of Concentration on Carbodiimide-mediated Coupling
Reactions. Hydralazine.HCl : Acid : Carbodiimide : Et_3N
= 1:1:1:1.5. Concentration (a) 0.016 M, (b) 0.029 M.
Spectra normalized with respect to band at 240 nm.

Figure 2.5 Comparison of Pyridine and Triethylamine in Carbodiimide-
Mediated Coupling Reactions. Hydralazine.HCl : Acid :
Carbodiimide : Base = 1:1:1:1. (a) Et_3N, (b) Pyridine.
Reaction for 1 day. Spectra normalized with respect to
band at 240 nm.
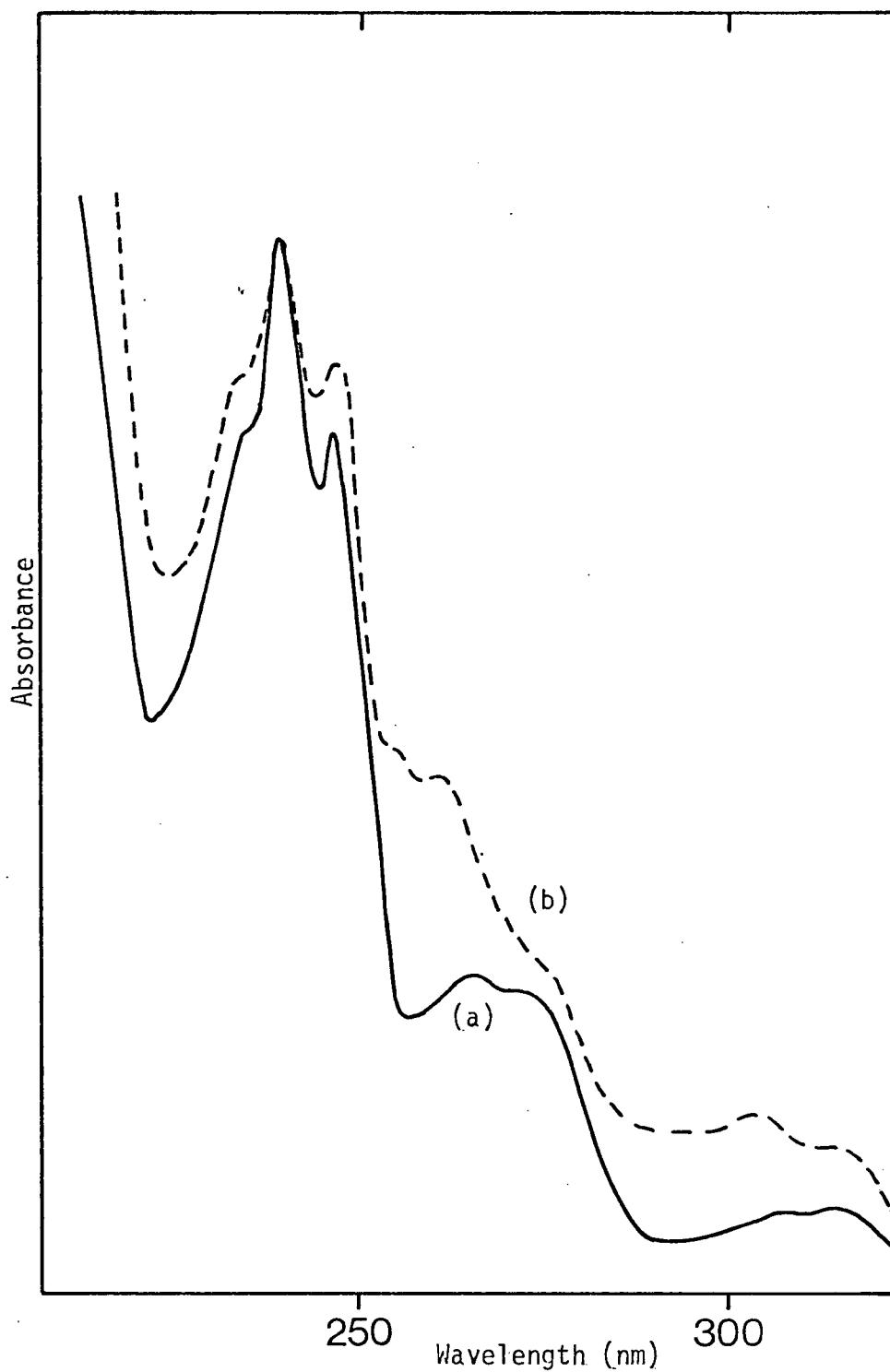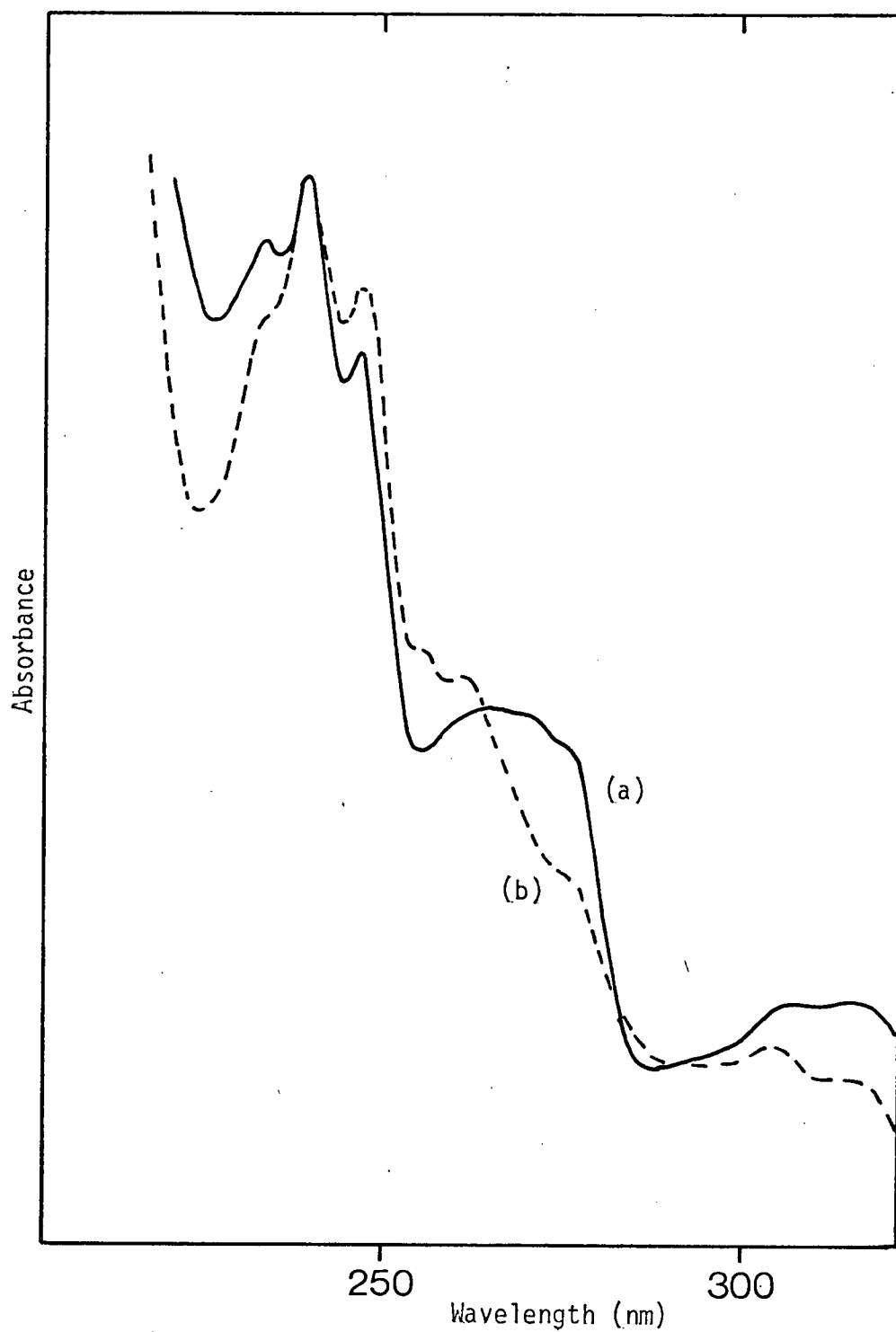
Figure 2.6 Comparison of Pyridine and Triethylamine in Carbodiimide-
Coupling Reactions. Hydralazine.HCl : Acid : Carbodiimide :
Base = 1:1:1:1. (a) Et_3N, (b) Pyridine. Spectra normalized
with respect to band at 240 nm. Reaction for 4 hours.

The yield of TAP after one day can be readily explained by the relative base strengths of Et_3N and pyridine, but the yield after 2 hours of reaction can not. Figure 2.6 of the reaction after four hours shows a different order of reactivity. Reaction with added pyridine is initially faster than with Et_3N. There is little change in the reaction with pyridine between 4 and 24 hours whilst the reaction with added Et_3N shows a slower, more even growth of TAP.

The difference in reaction rates suggests somewhat different reaction mechanisms in both cases. A possible explanation of these observations is formation of an acylpyridinium derivative from the O-acylisourea and pyridine which condenses with hydralazine to form the TAP product. There is good evidence for the acetylpyridinium ion as an intermediate in the pyridine-catalyzed hydrolysis of acetic anhydride.[88] When the carboxyl component is in excess relative to the amine component as in the case with the pyridine reaction, in which hydralazine is relatively inaccessible as the protonated base, then carbodiimide coupling may proceed via the mixed anhydride pathway (Scheme 2.12).

If the above speculations are correct, then this may be the first case, to the author's knowledge, of amide formation proceeding via an acyl-pyridine intermediate. Acylating agents derived from other bases such as imidazole, pyrazole, 1,2,4-triazole, and tetrazole are well known.[89]

Since the substituents on the carbodiimide molecule are expected to influence the reactivity of the O-acylisourea intermediate, we wanted to compare the suitability of some carbodiimides (DCC, EDC, and CMC) for TAP synthesis.

Three pairs of comparison coupling reactions were made, each pair under identical conditions of reagent stoichiometry and concentration. In

the coupling of hydralazine HCl with N-acetyl-glycine in methanol solution, EDC is somewhat more effective than CMC in TAP formation. In the presence of triethylamine, the reaction with CMC is much more susceptible to side-reactions than with EDC. This may arise from increased N-acylurea formation with CMC. Faced with a deficiency of the active O-acylisourea with which to couple, hydralazine showed greater tendency to undergo base-catalyzed decomposition. Comparison reactions with DCC and EDC show that the latter carbodiimide gives superior yields of TAP. The efficiency of the carbo-diimides in the formation of TAP derivatives thus follow the order: EDC > DCC ≳ CMC. A factor to be considered in the choice of carbodiimide is that DCC is allergenic and should be handled with care. Cases of dermatitis have been ascribed to its use.

There is little difference in the rate of formation of TAP whether hydralazine is added to the reaction mixture as the free base, or as the hydrochloride salt with one equivalent of triethylamine. Thus, from that consideration, it matters little which form of hydralazine is used in the reaction-free base or HCl salt with added Et_3N. One caution with the latter system is that a large excess of tertiary base cannot be tolerated due to hydralazine decomposition, but a slight excess of Et_3N is needed to ensure complete neutralization of the hydralazine HCl salt.

An unusual feature of the carbodiimide coupling reaction was observed when N-acetyl-glycine reacted with hydralazine in methanol or aqueous solution. In addition to the expected product, 3-(N-acetyl-glycyl)-TAP, another product was invariably formed which possessed typical charac-teristics of a TAP derivative: blue fluorescence under short wavelength UV light, and similar UV spectrum. The side-product was identified by its TLC behaviour, NMR and mass spectra to be 3-methyl-TAP. Its formation

appears to be favored by EDC relative to DCC and CMC. It forms under acidic and basic conditions apparently simultaneous with 3-(N-acetyl-glycyl)-TAP. Pure 3-(N-acetyl-glycyl)-TAP is itself indefinitely stable in methanol solution. The expected product from hydrolysis of the side-chain amide bond is 3-aminomethyl-TAP and no evidence of this compound was found in carbodiimide coupling reactions. Authentic 3-aminomethyl-TAP appears to be stable in methanol solution.

The following summarizes the results obtained for the reaction of hydralazine with N-protected amino acids using carbodiimide coupling reagents. Appropriate solvents for the coupling reaction are CH_2Cl_2, $CHCl_3$, CH_3CN, THF, CH_3OH, and DMA. The least polar of these solvents are preferred as reaction media in order to minimize formation of the N-acylurea side-product. Low reaction temperatures, high reagent concentrations and a slight excess of carbodiimide and carboxyl component are advantageous for the same reason. If the HCl salt of hydralazine is used as the amino component, then a slight excess of a tertiary base such as Et_3N is needed. However, to avoid complications arising from the presence of excess tertiary base, freshly-prepared, free base hydralazine is preferred. As far as coupling efficiencies and ease of isolation of the TAP product are concerned, EDC is more suitable than DCC as the coupling reagent.

2.5 DISCUSSION

We have shown that hydralazine will couple with N-protected amino acids under a variety of reaction conditions to afford s-triazolo[3,4-a]-phthalazine derivatives. The rate limiting step of this coupling is formation of an amide intermediate which undergoes spontaneous dehydrative cyclization to yield the triazolo compound. We addressed ourselves primarily to the problem of suitably activating the reaction components to give the desired product.
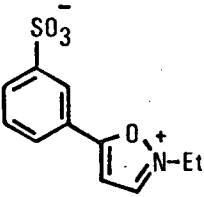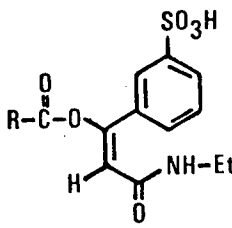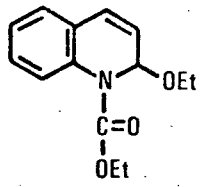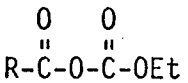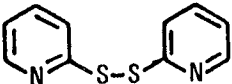
To lend the properties of an acylating agent to an amino acid or peptide, the electrophilic character of the carbonyl carbon of the reacting carboxyl group must be enhanced - usually by substitution of an electron-withdrawing group on the carboxyl function:

$$\begin{array}{c} O \\ \parallel \\ Z-NH-CHR-C \longrightarrow X \\ \delta+ \quad \delta- \end{array}$$
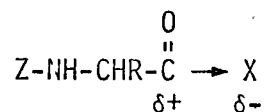
This structural modification of a carboxyl group must raise its reactivity to the 'right' level so that couplings will occur rapidly and completely, without the intervention of inter- or intra-molecular side-reactions. Overactivation may lead to reaction with amino acid side-chains, while low activation leads to low coupling rates. The coupling reagents and their reactive acylating intermediates which were used in this work are shown in Table 2.3. The reactive intermediates include such types as mixed and symmetric anhydrides, activated esters, acyloxyphosphonium salts, acyl azides, and heterocyclic amides.

In mixed anhydrides, the strong electron-withdrawing effect of the carboxyl group activates the carbonyl carbon of the amino acid derivative.

Table 2.3 Coupling Reagents and their Reactive Intermediates used in Amide Synthesis

Coupling Reagent	Reactive Intermediate

1. Isoxazolium Salt

N-ethyl-5-phenylisoxazolium-3'-sulfonate (NEPIS)

2. 1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)

$$\underset{\text{R-C-O-C-OEt}}{\overset{O\quad\ O}{}}$$

3. Acyloxyphosphonium Salts

   (i) $[(CH_3)_2N]_3PO$ + TsCl

   Hexamethylphosphoramide (HMPA) + tosyl chloride

$$R-\overset{O}{\overset{\|}{C}}-O-\overset{+}{P}[N(CH_3)_2]_3 \ TsO^- \ (Cl^-)$$

   (ii) $[(CH_3)_2N]_3\overset{+}{P}-N_3 \ \ PF_6^-$

   Azidotris(dimethylamino)-phosphonium hexafluorophosphate

$$R-\overset{O}{\overset{\|}{C}}-N_3 \quad\quad \text{via}$$

$$R-\overset{O}{\overset{\|}{C}}-O-\overset{+}{P}[N(CH_3)_2]_3 \ N_3^-$$

   (iii) Ph_3P + (2,2'-dithiodipyridine structure)

   Triphenylphosphine + 2,2'-dithiodipyridine

$$R-\overset{O}{\overset{\|}{C}}-O-\overset{+}{P}Ph_3$$

| Coupling Reagent | Reactive Intermediate |

(iv) (PhO)$_3$P + HN⟨imidazole⟩N

$$R-\overset{O}{\overset{\|}{C}}-N\langle\rangle N \quad \text{or}$$

Triphenylphosphite + imidazole

$$R-\overset{O}{\overset{\|}{C}}-O-\underset{PhO\ OPh}{\overset{Imid-H^+}{\underset{|}{P}}}-H \quad {}^-OPh$$

(v) (PhO)$_2$P-OH + ⟨pyridine⟩N

$$R-\overset{O}{\overset{\|}{C}}-O-\underset{HO\ OPh}{\overset{\overset{+}{N}\langle\text{pyridinium}\rangle}{\underset{|}{P}}}-H \quad {}^-OPh$$
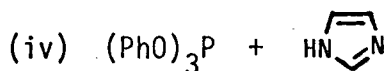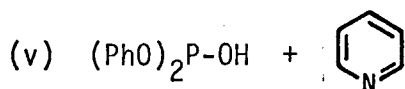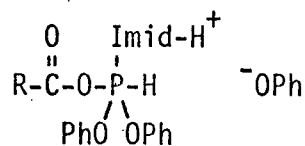
Diphenylphosphite + pyridine

4. Carbodiimide
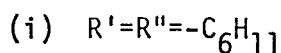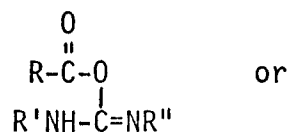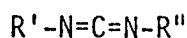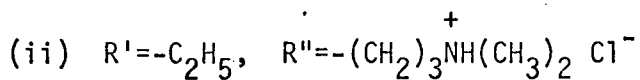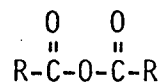
R'-N=C=N-R"

$$R-\overset{O}{\overset{\|}{C}}-O \quad \text{or}$$
$$R'NH-\overset{|}{\underset{}{C}}=NR"$$

(i) R'=R"=-C$_6$H$_{11}$

Dicyclohexylcarbodiimide (DCC)

$$R-\overset{O}{\overset{\|}{C}}-O-\overset{O}{\overset{\|}{C}}-R$$

(ii) R'=-C$_2$H$_5$, R"=-(CH$_2$)$_3\overset{+}{N}$H(CH$_3$)$_2$ Cl$^-$

1-Ethyl-3-(3-dimethylaminopropyl)-
carbodiimide hydrochloride (EDC)

(iii) R'=-C$_6$H$_{11}$, R"=-(CH$_2$)$_2\overset{+}{\underset{Me}{N}}\langle\rangle$O $^-$OTs
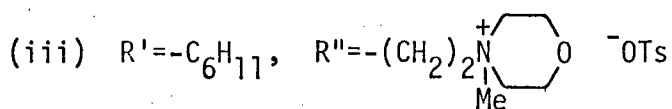
1-Cyclohexyl-3-(2-morpholinyl-4-ethyl)
carbodiimide metho-p-toluenesulfonate
(CMC)

However, there are two electrophilic sites, and nucleophilic substitution at

$$\begin{array}{cc} O & O \\ \| & \| \\ Z\text{-NH-CHR-C-O-C-R'} \\ \uparrow \quad \uparrow \\ a \quad b \end{array}$$
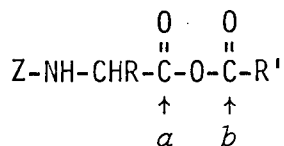
site b will result in the undesired amide product. To minimize this competition reaction, an electron-donating structure is required in the 'partner' acid. The ethoxy moiety in the mixed anhydride derived from EEDQ fulfils this requirement. The isobutyloxy mixed anhydride, derived from 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline (57, IIDQ) has been suggested to be preferable for reducing reaction at the activating group by
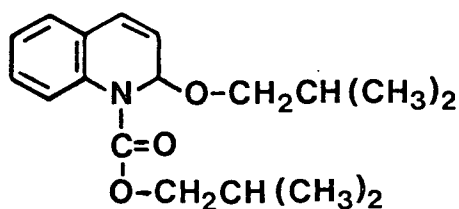
(57)

virtue of the steric hindrance of the isobutyl group.[90] In symmetrical anhydrides, the sites for nucleophilic substitution are identical, and only one amide product is possible.

The ester intermediates derived from carbodiimides and NEPIS receive their activity from the electron-withdrawing nature of the multiple-bonded C=N and C=C attached to the ester oxygen. The migration of electron density from the carbonyl oxygen in acyloxyphosphonium salts is enhanced by localization of a positive charge on the phosphorus atom.

Although the azide group is not a powerful electron-withdrawing group, the reactivity of acyl azides towards amines can be explained by formation of a hydrogen-bonded cyclic transition state which enhances the acylation reaction.[91]

$$
\begin{array}{c}
O \\
\parallel \\
R-C-N \\
\uparrow \quad \quad N \\
\vdots \quad \quad \quad N \\
H-N-H...N \\
\mid \\
R'
\end{array}
$$
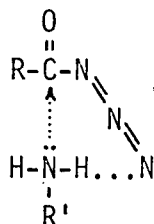
The high reactivity of N-acylimidazole is connected with the aromatic nature of the heterocycle. As a result of participation of the electron-pair on the amide nitrogen in the π-system of the ring, this nitrogen becomes more positive, exerting an attraction on the electrons of the exocyclic bond towards the ring, thus enhancing the rate of nucleophilic reaction at the carbonyl carbon of the acyl group.

Of the ten reagents investigated for activating the carboxyl group of amino acids, four of these were sufficiently successful in TAP synthesis to merit further consideration. These reagents are the carbodiimides - EDC and DCC, the isoxazolium salt - NEPIS, and the combination of triphenyl-phosphite with imidazole.

As we anticipated when this work was initiated, the use of coupling reagents did permit the synthesis of TAP derivatives under relatively mild conditions.

The reaction of hydralazine with amino acids to afford TAP compounds is specific for carboxyl groups, but some care must be taken when reactive side-chains in the amino acids are present. These side-chains show

differing sensitivities to coupling reagents. Thus, the carboxyl groups of serine, threonine, and tyrosine can be activated by NEPIS without previous protection of the side-chain hydroxyl groups. Asparagine and glutamine can be activated without serious problems;[56] this is especially significant since the ω-amide group in these amino acids is the only reactive amino acid side-grouping which cannot ordinarily be blocked.

No problems are observed with histidine and arginine using the triphenylphosphite and imidazole combination, but side reactions may sometimes occur with asparagine and glutamine. The hydroxyl groups of serine and threonine must be protected.[71]

When the carboxyl groups of N-protected asparagine or glutamine are activated by DCC, dehydration of the β- or γ-carboxamide moiety to the corresponding cyano group occurs, asparagine being more susceptible to the reaction. Dehydration does not occur when these amino acids occur within a peptide chain. In addition, the formation of adducts between the imidazole ring of histidine and DCC has been observed.[53] The successful use serine, threonine, and tyrosine in peptide synthesis is noteworthy,[92] however, in aqueous solutions carbodiimides have been reported to react with tyrosine and serine side-chains in proteins.[93]

An unfortunate feature of the methods developed for coupling hydralazine to amino acids is the long reaction times required; in periods of less than 15 hours, coupling reactions were often incomplete. While times of this duration are not unreasonable from a synthetic viewpoint, they do impose limitations on the practicality of the reaction of hydralazine with carboxyl groups as a method of peptide sequencing.

We have isolated pure TAP derivatives from the coupling reactions in yields up to about 80% which are less than ideal for a peptide sequencing method though satisfactory for a new synthetic procedure. Thus, assuming

80% coupling yield in each cycle of a sequencing procedure, the yield of

TAP after 5 cycles is 33%.

Several modes of action are available for improving the hydra-

lazine coupling reaction with amino acids. The coupling methods used in this

work are based on an increase in the reactivity of the carboxyl group of the

carboxyl component due to enhanced electrophilic properties of its carbonyl

carbon. A counterpart of this approach would be enhancement of the nucleo-

philic properties of the amino group of hydralazine. This approach has been

considered in peptide synthesis, but only a few applications are known.

Although the available methods do involve a reactive derivative of the amine,

in the final analysis, they proceed through an activated carboxyl component.

They are, in fact, special cases of the mixed anhydride method. An example

is the phosphazo method which involves reaction of the amino component with

PCl_3 to give the phosphazo intermediate which is present in a dimeric

state,[52] (Scheme 2.14). The reaction of the phosphazo compound with the

$$2PCl_3 + 4R'-NH_2 \longrightarrow \underset{\substack{R'-N \\ \diagdown \\ P-NH-R' \\ \diagup \\ R'-N \\ \diagdown \\ P=N-R'}}{\overset{H}{\diagup}} \xrightarrow{4R''COOH} 4R''CO-NHR' + [PHO_2]_x$$
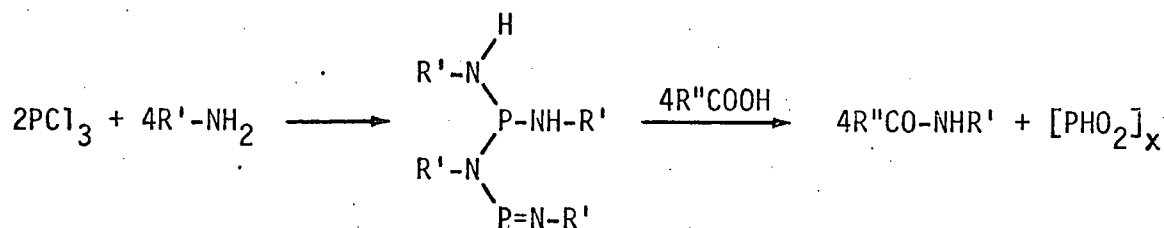
Scheme 2.14 Amide Formation by the Phosphazo Method

carboxyl component leads to formation of the amide product via a mixed

anhydride. The method presents the same problems with regard to possible

applications as direct mixed anhydride methods.

A less direct method of increasing the reactivity of the primary amino group in hydralazine is substitution of a π-electron donating group onto the phthalazine ring system. Considered by itself, the substitution may have only a marginal effect on the reactivity of the hydrazino moiety, but if suitably placed, it may also enhance the coupling reaction by restricting the rotational freedom of the hydrazino group. Thus, placement of a group on the 8-position of the aromatic ring system (58) e.g. alkoxy, directs the hydrazino substituent into a favorable orientation for coupling with carboxylates which is followed by dehydrative cyclization. The hydrazino group can be "frozen" into a single conformation by hydrogen-bonding to the alkoxy oxygen atom.
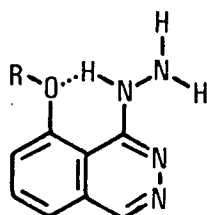
(58)

Cohen has shown that as a result of alkyl substitution in both the aromatic ring and the side-chain (cf. 59), the rate constant for acid-catalyzed lactonization of hydrocoumaric acid (60) is increased by factors as high as 5×10^{10}.[94] The rate-acceleration effects are attributed primarily to a considerable increase in the population of a conformer highly favorable to the lactonization reaction.
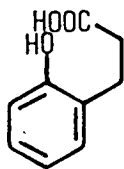
(60)
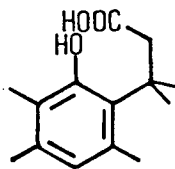
(59)

The procedures we have developed for the synthesis of s-triazolo-[3,4-a]phthalazine derivatives from hydralazine and N-protected amino acids have potential applications in the general synthesis of fused ring s-triazole systems. The overall procedures which we used in this work for phthalazine are applicable to other ring systems as well (Scheme 2.15).
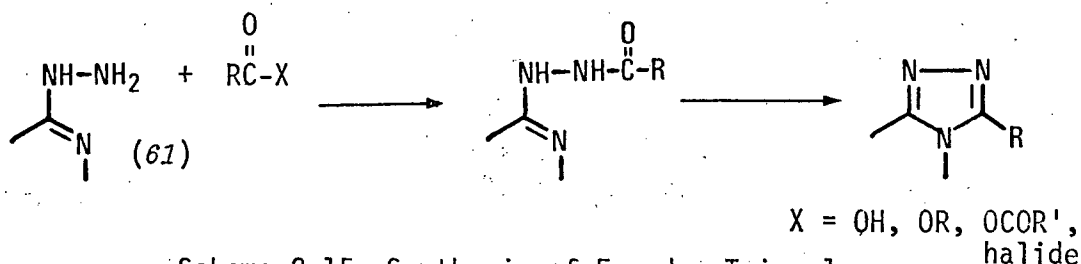
Scheme 2.15 Synthesis of Fused s-Triazoles

X = OH, OR, OCOR', halide

If the carboxyl component is a carboxylic acid, reaction conditions involve heating a mixture of the acid and hydrazino components to high temperatures. If the acid is a solid, melt conditions may be required.

Reactions with other more reactive carboxyl derivatives may also involve elevated temperatures, but these are less common than with acids, presumably because the derivatives are generally less accessible.

As a result of the severe reaction conditions and the low availability of a large variety of carboxyl derivatives, the side-chain substituent in fused s-triazole systems is often limited to groups such as alkyls, haloalkyls and aryls.

Although we have not pursued the applicability of coupling reagents to hydrazino derivatives of heterocycles other than phthalazine, or to carboxyl components other than amino acids, there is no reason why the mild reaction conditions made possible by our procedures should not allow the synthesis of a much wider variety of fused s-triazoles with side chain substituents than is now possible. Thus, a wide variety of carboxylic

acids with, or without other sensitive functionalities can now be used, and this opens up the possibility of side-chains in the triazole with reactive groups (e.g. Scheme 2.16).

(61) + HO-C-CH$_2$-NH-BOC $\longrightarrow$ ... $\xrightarrow{H^+}$ ...
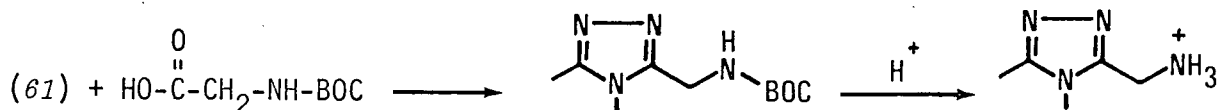
Scheme 2.16 Synthesis of a Fused s-Triazole with an Aminomethyl Side-chain
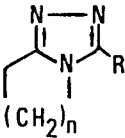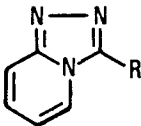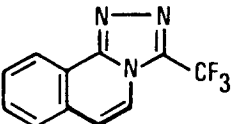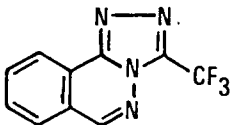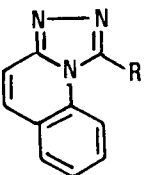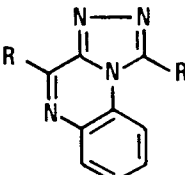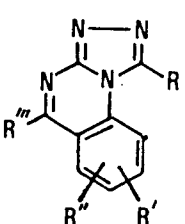
Reaction of a hydralazino compound with N-BOC-glycine can give a s-triazole derivative with an aminomethyl side-chain after removal of the BOC-group. We have done this with 1-hydrazinophthalazine.
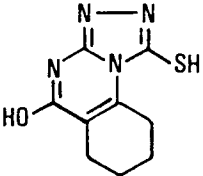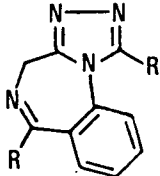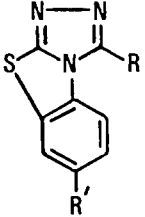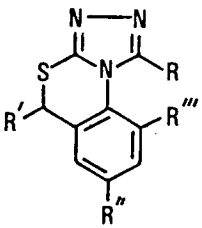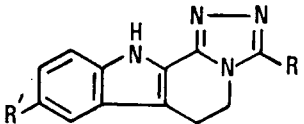
One might well ask what is the significance of extending the scope of s-triazole synthesis, beyond that of a purely academic achievement. The uses and potential uses of triazole derivatives are many and varied, and even limiting our consideration to fused s-triazoles only, the number of applications remains considerable. Table 2.4 shows a sample of condensed s-triazole systems and their properties and uses, taken from the recent literature (mostly 1976-1977).

In many of the examples shown in Table 2.4 the triazole ring was formed by coupling a hydrazino derivative with a carboxyl compound and subsequent cyclization of the resulting carboxamide. Other methods involve a preformed s-triazole which bind to reactive groups in the molecule to form the multicyclic structure.

The triazole derivatives in Table 2.4 show properties and uses which include applications as fungicides, in cancer chemotherapy, and a wide variety of pharmacological uses. A significant aspect of these uses is that most of them are described in the patent literature. Evidently, the

Table 2.4   Fused s-Triazoles - Properties and Uses

	Fused s-Triazole	Properties	Reference
62		Central nervous system and respiratory system stimulants	95
63		Anti-convulsant and tranquil-izer. Anti-inflammatory agent	96
64		Anti-inflammatory, analgesic, and anti-pyretic activities	97
65		Anti-inflammatory agent	98
66		Control of plant-pathogens	99a
67		Control of plant-pathogens	99b
68		Anti-inflammatory, analgesic and anti-viral activities	100

Fused s-Triazole	Properties	Reference
69	Anti-tumor and anti-cancer activity	101
70	Anti-depressive and anxiolytic activities. Hypothermia antagonist, tranquilizer, sedative, muscle relaxant	102
71	Control of plant-pathogens	103
72	Control of fungal foliar pathogens	104
73	Sedative and analgesic activities. Anti-hypotensive action	105

synthesis of these condensed s-triazoles are sufficiently important to warrant protection of the procedures and applications under the patent laws.

An integral part of any screening program for biological activity of a compound is the synthesis of a series of compounds within the same general class. Variation of the side-chain substituent on the triazole ring is a common denominator in most of the examples shown in Table 2.4. Our work would permit the synthesis of triazoles with a wider range of side-chains than previously prepared, and hence, possibly fused triazoles with a broader spectrum of activities.

Only a few of the biological properties of the condensed s-triazoles have been described in any detail. 3-Trifluoromethyl-s-triazolo[3,4-a]isoquinoline (*64*) shows a pharmacological profile which suggests that it would be useful in the treatment of edema, pain and fever associated with inflammatory diseases, such as rheumatoid arthritis. The minimal gastric activity in the rat indicates that it causes less gastric distress than the antiinflammatory agents now in use. Its analgesic activity in mice is greater than that of acetylsalicylic acid.[97]

3-Trifluoromethyl[3,4-a]phthalazine (*65*) was recently reported to have a higher antiinflammatory effect than the corresponding isoquinoline derivative (*64*).[98]

s-Triazolo[4,3-a]quinolines (*66*) are used for the control of plant pathogenic organisms. Thus, the methyl derivative (R=CH$_3$) controlled anthracnose of cucumbers and rice blast of rice.[99a] Also active for the control of rice blast are s-triazolo[4,3-a]quinoxaline (*67*), the parent triazole (R,R$_1$=H) being most effective against *Piricularia oryzae*,[99b] and s-triazolo[3,4-b]benzothiazoles (*71*).[103]

1-Mercapto-5-hydroxy-6,7-tetramethylene-s-triazolo[3,4-b]pyrimidine
(*69*) prevents metastasis of human epidermoid carcinoma and exhibits antitumor
activity against primary human epidermoid carcinoma and other tumors, such
as adenocarcinoma and sarcoma.[101]

CHAPTER 3

SOLID-PHASE SYNTHESIS OF S-TRIAZOLO[3,4-A]PHTHALAZINES

3.1 INTRODUCTION

The process of assembling a peptide chain in a step-wise manner while it is attached at one end to an insoluble support is known as the "solid-phase" method for polypeptide synthesis. The technique was first used by two groups operating independently. The first application was by Merrifield in 1963 to the synthesis of a tetrapeptide.[106] Soon after, Letsinger reported the synthesis of a dipeptide on a "popcorn" polymer support.[107] The major difference in the two methods was that Merrifield attached the polymer to an amino acid as the carboxylate ester while Letsinger bound the amino acid via the amino group as an amide.

Since its introduction, the solid-phase method has been success-fully applied to peptides of increasing size. These developments have been crowned by the solid-phase preparation of ribonuclease A (124 residues)[108] and human growth hormone (188 residues),[109] both with a significant degree of enzymic activity.

After the original successes of the solid-phase method in polypeptide synthesis, parallel activity developed in the application of

polymer attachment to non-peptide organic syntheses. Polymer supports have now been applied to the synthesis of carbohydrates and their derivatives, and to polynucleotides.[110,111] Only very recently have solid supports been used in general organic synthesis unrelated to repetitive 'sequential-type' syntheses of polypeptides, polynucleotides, and polysaccharides. It has now been found that insoluble polymers can be used for many purposes to solve specific synthetic problems.

We considered the coupling of hydralazine with N-protected amino acids on an insoluble support for two primary reasons. The first stems from synthetic advantages of the solid-phase method. The most appealing advantage within the context of this work is that the solid-phase method allows excesses of reagents to be separated from the reaction product by simple filtration, thus avoiding tedious chromatographic or solvent extraction procedures. For example, we have shown in Chapter 2 that s-triazolo[3,4-a]-phthalazine (TAP) derivatives can be obtained by a variety of coupling methods. Difficulties in some of these methods arose from isolating the desired product from solution. Thus, efforts to isolate the TAP product were frustrated when HMPA + tosyl chloride were used as a coupling method (cf. Section 2.4.3).

The other reason for considering the solid-phase method is connected with the possible application of the hydralazine reaction with carboxylate groups of peptides as a C-terminal peptide sequencing method. Several benefits are obtained by anchoring the peptide onto a polymeric support:

a. The peptide material, once attached to the solid support, is not lost during the degradative procedures. This permits sequencing to be performed with small amounts of peptide samples or further into the peptide chain.

b. After each reaction step, excess reagents may be readily removed by filtering and washing the support.

c. The solid-phase method is amenable to automation because of the repetitive nature of peptide sequencing.

The first application to the step-wise degradation of peptides by the solid-phase method was that of Stark,[112] whose procedure involved attaching the peptide at its N-terminus to an insoluble Edman reagent, polystyryl isothiocyanate, cyclizing the adduct to form the thiohydantoin, isolating and analyzing the residual peptide, and reattaching the peptide to the support (Scheme 3.1).
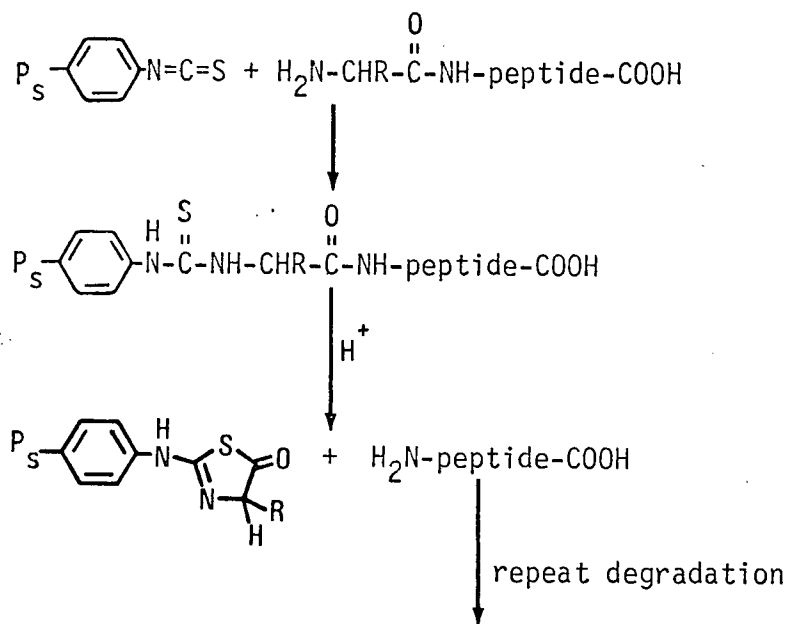
P$_S$ = copolystyrene-divinylbenzene support

Scheme 3.1   Stark's Method for Subtractive N-terminal Peptide Degradation

Using a somewhat different approach, Laursen attached the peptide by its C-terminal amino acid to a polystyrene support and then performed the degradation in the usual manner with phenyl isothiocyanate. The liberated thiazolinone is removed in each cycle by filtration. After the thiazolinone is converted to a phenylthiohydantoin it is identified by a number of procedures (Scheme 3.2).[13] In keeping with the success of solid-

$$
\begin{array}{c}
\overset{\displaystyle O}{\underset{\displaystyle \|}{}} \\
\text{X-NH-CHR-C-NH-peptide-COOH}
\end{array}
$$

$$\Big\downarrow \quad P_s\text{-NH}_2$$

$$
\begin{array}{c}
\overset{\displaystyle O}{\underset{\displaystyle \|}{}} \\
\text{X-NH-CHR-C-NH-peptide-CONH-}P_s
\end{array}
$$

$$\Big\downarrow \quad \text{deblock}$$

$$
\begin{array}{c}
\overset{\displaystyle O}{\underset{\displaystyle \|}{}} \\
\text{NH}_2\text{-CHR-C-NH-peptide-CONH-}P_s
\end{array}
$$

$$\Big\downarrow \quad \text{PhNCS}$$

$$
\begin{array}{c}
\overset{\displaystyle S}{\underset{\displaystyle \|}{}} \qquad \overset{\displaystyle O}{\underset{\displaystyle \|}{}} \\
\text{PhNH-C-NH-CHR-C-NH-peptide-CONH-}P_s
\end{array}
$$

$$\Big\downarrow \quad \text{H}^+$$

 $+ \quad \text{NH}_2\text{-peptide-CONH-}P_s$

$$\Big\downarrow \quad \text{repeat degradation}$$
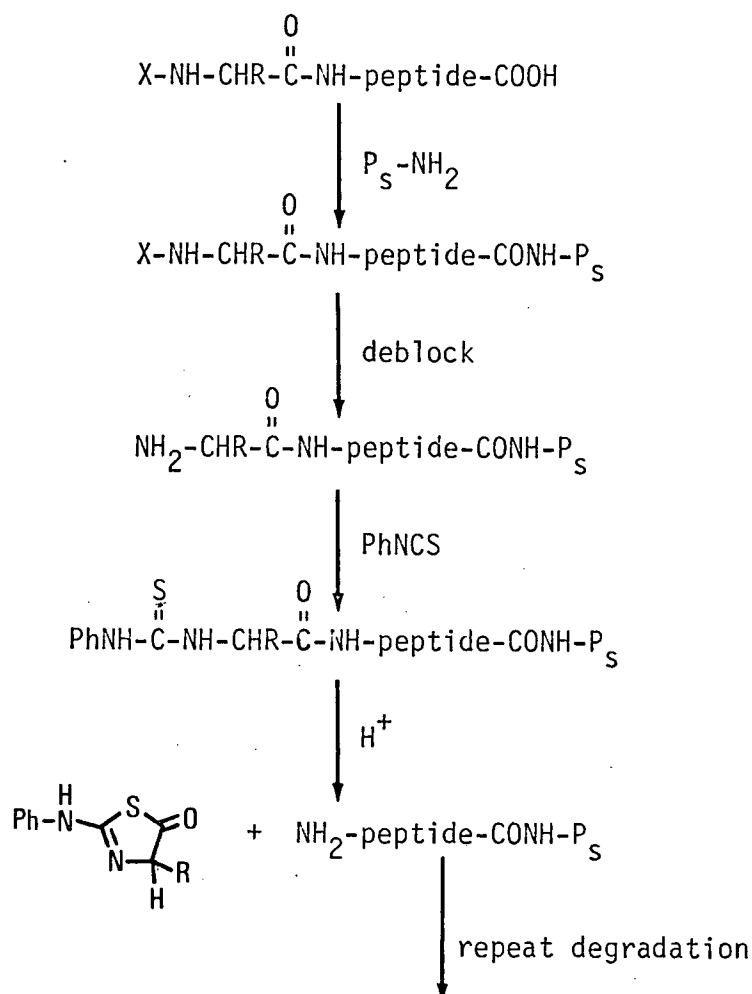
P_s = copolystyrene-divinylbenzene support

Scheme 3.2   Laursen's Method for N-Terminal Peptide Sequencing

phase peptide synthesis, peptide sequencing by the Laursen method has been automated and commercial versions of the solid-phase sequencer are available.[113]

Although the principles of determining the amino acid sequence of a peptide attached to an inert support were described by Laursen over 10 years ago,[114] the method is still not as commonly used as the liquid-phase techniques of Edman and Begg.[10] The reasons for this arise mainly from the development of only a few suitable supports, and the problems associated with the procedures for coupling peptides to supports. Some progress is being made in overcoming these problems, and the solid-phase sequencing method is rapidly increasing in popularity.

While solid-phase N-terminal peptide sequencing is still in its infancy, sequencing from the C-terminus is barely past the embryonic stage. During the time that this work was in progress, two C-terminal solid-phase methods were reported based on Stark's thiocyanate reaction and using copoly(styrene-divinylbenzene) or porous-glass supports (Scheme 3.3).[114,115]

$$
\underset{\text{O}}{\overset{\text{O}}{\|}}\text{P-C-NH-peptide-}\underset{\text{O}}{\overset{\text{O}}{\|}}\text{C-NH-CHR-COOH}
$$

acetic anhydride

NH_4NCS

$$
\text{P-C-NH-peptide-C—N} \cdots R
$$

H^+

$$
\text{P-C-NH-peptide-COOH}\ +
$$

repeat degradation

P = polymeric support
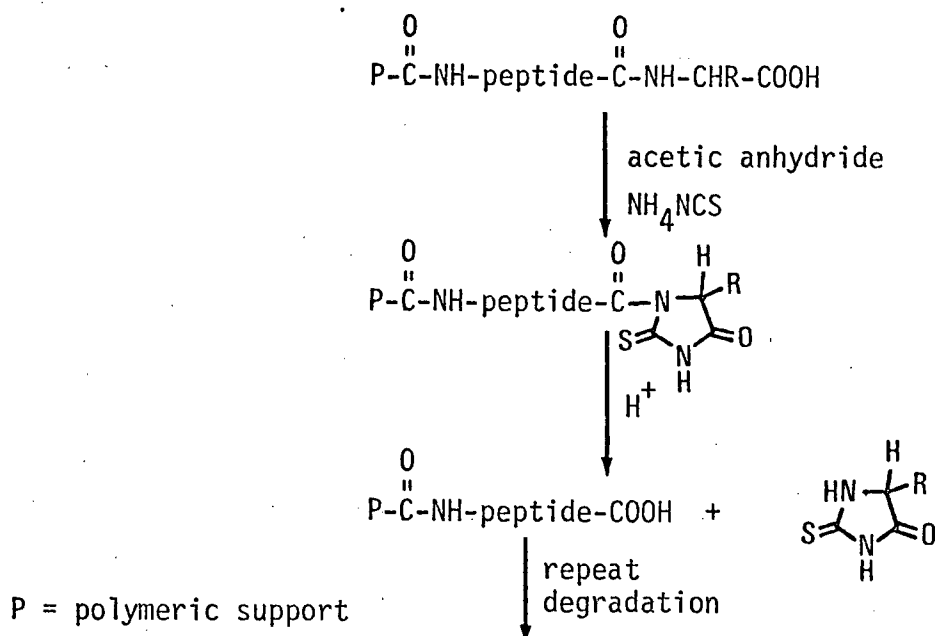
Scheme 3.3 Solid-phase Peptide Sequencing by Stark's Thiocyanate Reaction

3.2 RESULTS

The choice of an appropriate support material and the means of attaching the substrate onto the support in the solid-phase method of organic synthesis are major decisions affecting the success of the synthesis. In spite of the enormous polymer technology which has developed in recent years, a very limited amount of polymer types have been examined for use in solid-phase synthesis. The most widely used polymer, and the one used in this work, is the copolymer of styrene and divinylbenzene (DVB).[106] The resin[†] used was a polymer in the form of small (200-400 mesh) beads with 1% cross-linking by divinylbenzene. The low degree of cross-linking allows the polymer to swell in non-polar solvents and permits penetration of reagents in solution to react with substrate molecules bound to internal surfaces of the polymer.

For the synthesis of s-triazolo[3,4-a]phthalazine derivatives on insoluble supports, either hydralazine or the amino acid may be anchored to the support. If hydralazine is attached to the support, the hydrazino group must remain free for reaction with amino acids. The attachment is most readily achieved via a functionality introduced onto the aryl ring of the phthalazine moiety. However, synthesis of ring-substituted hydralazine suitable for solid-phase synthesis is not a trivial undertaking, and the more direct alternative of immobilizing the amino acid via its α-amino group is preferred.

Several options are available for anchoring amino groups onto a polymeric matrix. The method used by Letsinger,[107] and by Merrifield[116]

---

† The solid-phase terminology which has become established for derivatives of copolystyrene-DVB and for various linkages with amino acids or peptides.

was adopted in this work. Commercial chloromethylated copolystyrene-1% DVB was treated with potassium acetate. The product was converted to the hydroxymethylated resin by saponification with aqueous sodium hydroxide. Treatment of this resin with phosgene in benzene afforded the methyl-chloroformylated resin (Scheme 3.4). By this procedure, complete conversion

$$P_s\text{-CH}_2\text{Cl} \xrightarrow{\text{KOAc}} P_s\text{-CH}_2\text{OAc} \xrightarrow{\text{NaOH}} P_s\text{-CH}_2\text{OH} \xrightarrow{\text{COCl}_2} P_s\text{-CH}_2\text{O}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-Cl}$$
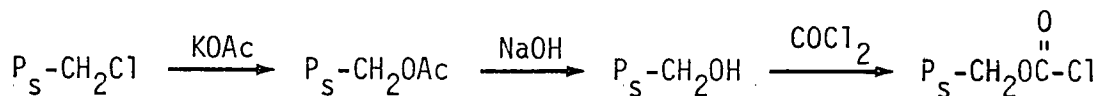
P_s = copolystyrene-divinylbenzene support

Scheme 3.4 Preparation of Methylchloroformylated Resin

of chloride to acid chloride groups was obtained to give a resin capacity of 1.36 meq chloride per gram dry resin.

The functionalized polystyrene resin thus obtained can couple readily to the amino groups of C-protected amino acids via an amide bond which is normally stable to synthetic operations with the resin. Solvents such as benzene, pyridine, DMF, and chloroform penetrate the polymer effectively and are therefore good reaction media, whereas with water, methanol, and ether, regions of the polymer appear to be inaccessible since there is little swelling in these solvents.

Dahlmans suggested the use of a polystyrene resin containing sulfonyl chloride groups which would bind amino groups by means of a Schotten-Bauman reaction.[117] A benefit of this method is the high stability of the P_s-SO_2-NH bond. The resin may be split off from the bound amino acid by treatment with phosphonium iodide in trifluoroacetic acid. This method however, has found very limited use in solid-phase synthesis.

In our first efforts at coupling amino acids to the carboxyl activated resin we used the procedure of Letsinger and Kornet.[107] The methylchloroformylated resin was stirred with excess glycine ethyl ester hydrochloride and triethylamine in dry DMF. The product was saponified with base in methanol-acetone solution. The final resin product, expected to be immobilized glycine ethyl ester, showed strong infra-red bands at 3580 cm^{-1} and 3440 cm^{-1} which were attributed to free and hydrogen-bonded O-H groups, respectively. After ester hydrolysis, the lower wavenumber ν(O-H) band shifted to 3390 cm^{-1}. The carbonyl bands were broad for both products, and exhibited a shift from 1724 cm^{-1} to 1718 cm^{-1} upon resin hydrolysis. By comparison, free glycine ethyl ester hydrochloride exhibits a sharp carbonyl stretching band at 1746 cm^{-1}, and glycine shows a very broad band about 1582 cm^{-1}. Letsinger and Kornet observed a shift of similar magnitude from 1730 cm^{-1} to 1724 cm^{-1} on ester hydrolysis of P_s-CH$_2$O-leucine ethyl ester. Hydrolysis of a sample of the acid chloride resin with sodium **hydroxide** in methanol-acetone showed ν(O-H) bands similar to those obtained in the attachment reaction. However, there were no carbonyl bands indicating that any acid which might have been formed by hydrolysis of the acid chloride had decomposed further to the hydroxymethyl resin. If hydrolysis of the acid chloride resin was carried out in the presence of excess pyridine, infra-red spectra of the product showed bands at 3575 and 3360 cm^{-1}, and 1738 cm^{-1}, suggesting that both P_s-CH$_2$OCOOH and P_s-CH$_2$OH were formed. On the basis of infra-red spectra, the coupling reaction of the acid chloride resin with glycine ethyl ester was interpreted as giving a mixture of P_s-CH$_2$OCOOH, P_s-CH$_2$OH, and the desired P_s-CH$_2$OCO-glycine ethyl ester. The low intensity of the ν(O-H) bands in the spectra of the glycine ethyl ester resin from the initial attachment reactions suggested low substitution yields. Further trials using chloroform in place

of DMF as the reaction medium appeared to give somewhat better yields, and this solvent was used in subsequent immobilization reactions.

The conditions ultimately used for attaching amino acids to the support were stirring the methylchloroformyl resin (1.36 mmol Cl/g) with two-fold excesses of the amino acid ethyl ester hydrochloride and triethylamine in dry chloroform at room temperature for one day. After filtering and washing, the resin was resuspended in chloroform and reacted with excess diethylamine to block any unreacted acid chloride groups. The resin thus obtained was saponified with potassium hydroxide in methanol-acetone solution. Substitutions of up to 0.63 mmoles of amino acid per gram of resin were obtained with alanine and glycine ethyl ester hydrochlorides. A substitution of 0.19 mmol/g resin was obtained with glycylglycine ethyl ester hydrochloride. These results compare with a substitution of up to 0.50 mmol L-leucine BOC-hydrazide/g resin from P_s-CH$_2$OCOCl (0.72 mmol Cl/g) by Felix and Merrifield.[116] Substitution capacities up to about 0.6 mmol dipeptides/g were obtained by Darbre and Rangarajan.[115]

We focussed our attention on utilizing the immobilized substrate once conditions for attaching amino acids to the support were established. A search of the literature showed that the number of carboxyl activation methods used in solid-phase synthesis was quite limited.

The first efforts at coupling hydralazine with immobilized glycine were with the carbodiimide method (cf. Section 2.4.4). The glycine resin was stirred with 2.5-fold excesses of hydralazine HCl, triethylamine, and dicyclohexylcarbodiimide in dimethylacetamide suspension under an argon atmosphere. After reaction overnight, the reaction products were cleaved from the polystyrene support with HBr/CF$_3$COOH. The desired product should be obtained as the HBr of 3-aminomethyl-s-triazolo[3,4-a]phthalazine (Scheme 3.5).

$$P_s\text{-}CH_2O\overset{O}{\overset{\|}{C}}\text{-}NH\text{-}CHR\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH \quad + \quad \text{[hydralazine: 1-hydrazinophthalazine]} \quad \xrightarrow[\cdot\ DMA]{DCC} \quad P_s\text{-}CH_2O\overset{O}{\overset{\|}{C}}\text{-}NH\text{-}RHC\text{-}\text{[s-triazolophthalazine]}$$

$$\Big\downarrow \text{HBr} \mid \text{TFA}$$

$$P_s\text{-}CH_2Br \quad + \quad CO_2 \quad + \quad \text{[s-triazolophthalazine]}\text{-}C(\text{NH}_2\text{HBr})(H)(R)$$
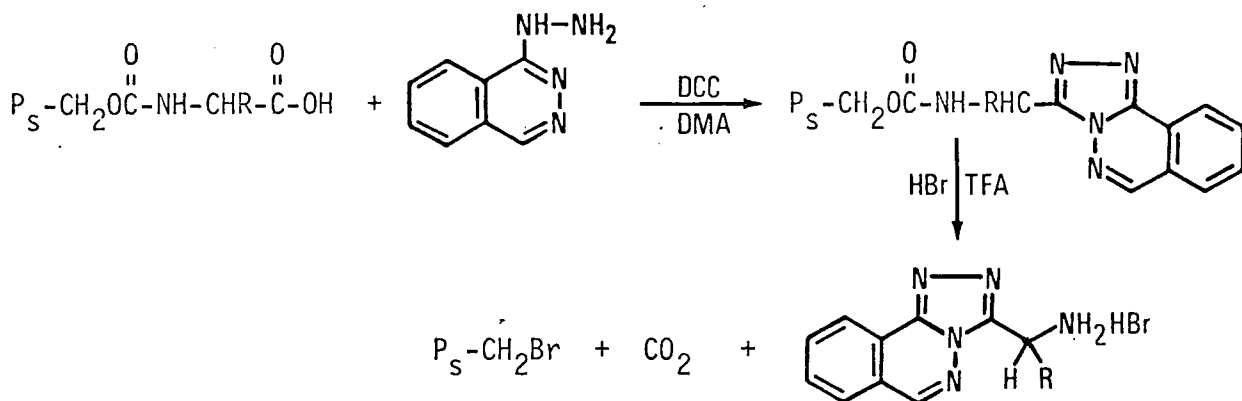
Scheme 3.5   Solid-phase Synthesis of s-Triazolo[3,4-a]phthalazines

Monitoring the progress of the reaction is a difficult problem, as it is in solid-phase peptide synthesis. In a developmental situation it is important to know, at least qualitatively, the rate of reaction and when the reaction is completed. Unfortunately, we did not find any entirely satisfactory monitoring method.

In principle, infra-red spectroscopy should be useful. Reaction of hydralazine with the immobilized amino acid should show a disappearance of the carbonyl band arising from the carboxylic acid. In practice, the overlap of strong bands arising from amide carbonyls of the linkage bonds makes any changes in shape and/or frequency of the broad carbonyl bands of dubious diagnostic value. Infra-red bands arising from the tri-cyclic TAP product are superimposed on the rather crowded spectrum exhibited by the copolystyrene-DVB matrix. Furthermore, in our hands, polystyrene beads almost invariably did not give translucent KBr discs.

A straightforward monitoring procedure is to measure the decrease of hydralazine in solution by ultra-violet spectroscopy. This procedure however, is inaccurate and unreliable by virtue of the decomposition of hydralazine, especially in the presence of triethylamine.

In view of the difficulties, little effort was made to follow the rate of the coupling reaction, although IR spectra of the resin, and UV spectra of the solution were recorded routinely. Coupling reactions were usually terminated after one day and the products examined for the presence of TAP product.

Initial efforts with the carbodiimide method were totally unsuccessful in detecting any TAP products. This is not surprising as ample opportunities were available for failure at every stage of the solid-phase procedure. A potentially serious difficulty with the carbodiimide methods is the probability that some of the active O-acylisourea intermediate might be shunted off to the inert N-acylurea (Scheme 2.12). In solid-phase peptide synthesis the side-reaction is rapid in DMF solution[118] and this may be a contributing factor to failure of the hydralazine coupling reaction in DMA. Chloroform was used in subsequent reactions since the side-reaction is not favored in this solvent. However, the coupling reaction remained relatively unsuccessful, presumably because of the low solubility of hydralazine HCl in chloroform.

Confronted with the lack of success with the carbodiimides, salvation was sought with the mixed-anhydride method used by Letsinger and Kornet.[107] The mixed anhydride intermediate may be prepared *in situ* by the reaction of isobutyl chloroformate with the carboxyl component, and then coupled directly with the amino reagent (Scheme 3.6).[119]

$$C_4H_9O\overset{\overset{O}{\|}}{-}C-Cl \ + \ RCOOH \ \longrightarrow \ \left[\begin{array}{c} R-C\diagup^O \\ \diagdown O \\ C_4H_9O-C\diagdown_O \end{array} \right] \ \xrightarrow{R'NH_2} \ RCONHR' \ + \ C_4H_9OH \ + \ CO_2$$
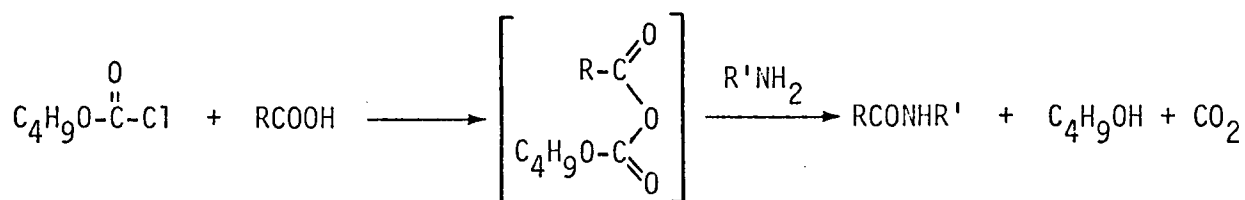
Scheme 3.6   Amide Formation with Isobutyl Chloroformate

Isobutyl and other alkyl chloroformates react through similar intermediates as the EEDQ and IIDQ coupling reagents (Section 2.4.2). In a solid-phase peptide synthesis by extension from the C-terminus, Tilak and Hollinder[120] reported that mixed anhydride coupling with isobutyl chloroformate proceeded faster than with DCC and required shorter coupling periods.

Mixed-anhydride coupling was effected by reacting an excess of isobutyl chloroformate with the amino acid resin in chloroform suspension for an hour at -10°C. After the amino acid anhydride was freed of excess chloroformate, the resin was immediately resuspended in a chloroform solution of hydralazine HCl and triethylamine. The product was cleaved from the support after the reaction mixture was allowed to react for about 18 hours at room temperature.

Two undesired side-reactions are associated with amide formation by the mixed-anhydride method. First, anhydride (74) can disproportionate to yield a symmetrical anhydride (75) and a dialkylpyrocarbonate (76) (Scheme 3.7). The latter can irreversibly block the amino component

$$
\begin{array}{c}
\overset{O}{\underset{\|}{}} \ \overset{O}{\underset{\|}{}} \\
R\text{-}C\text{-}O\text{-}C\text{-}OC_4H_9
\end{array}
\longrightarrow
\left\{
\begin{array}{l}
(1) \quad \overset{O}{\underset{\|}{R}}\text{-}C\text{-}O\text{-}\overset{O}{\underset{\|}{C}}\text{-}R \; + \; C_4H_9O\text{-}\overset{O}{\underset{\|}{C}}\text{-}O\text{-}\overset{O}{\underset{\|}{C}}\text{-}OC_4H_9 \\
\qquad\qquad (75) \qquad\qquad\qquad (76) \\[2mm]
(2) \quad C_4H_9O\text{-}\overset{O}{\underset{\|}{C}}\text{-}NHR' \; + \; R\text{-}COOH
\end{array}
\right.
$$

R'NH₂
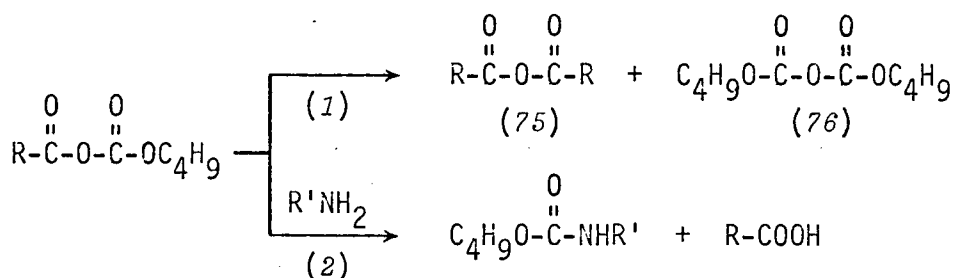
Scheme 3.7 Side-reactions of Mixed-anhydride in Amide Synthesis

(route 1). In solid-phase synthesis, aminolysis of the symmetrical anhydride results in lowered yield of the coupled product since half of the carboxyl

component is lost as the free acid. This disproportionation reaction is minimized by activating the amino acid at low temperature. Second, wrong side aminolysis of the anhydride (74) can result in a mixture of products (route 2). This reaction is made less favorable by using a bulky alkoxy group, e.g. isobutyloxy.

We were finally successful with the mixed-anhydride method in preparing the TAP derivatives by coupling hydralazine with immobilized glycine. Only one fluorescent product was isolated indicating that nucleophilic attack by hydralazine at the isobutyloxy carbonyl carbon atom (route 2) was insignificant. The product of the coupling reaction was purified by preparative TLC on silica gel, and its identity was confirmed by mass spectrometry. Coupling of hydralazine with alanine resin under similar reaction conditions also gave the desired TAP product in moderate yield.

Buoyed by the success with isobutyl chloroformate, we next tried the coupling reagent, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, Section 2.4.2). EEDQ generates an ethoxy mixed carbonic anhydride on reaction with carboxylic acids. Glycine resin in THF suspension was allowed to react with excess EEDQ at room temperature for 45 minutes under a nitrogen atmosphere, and then filtered free of excess reagents. After the resin had reacted with hydralazine in THF solution for about 18 hours, the product was cleaved from the resin with HBr/HOAc. UV spectral analysis of the product showed that TAP was successfully formed, but in relatively low yield.

In solid-phase peptide synthesis and in this work, the usual linkage of amino acids to the polystyrene resin is a benzyl ester bond. For a long time, the standard procedure for cleaving this bond was treatment

of the resin in trifluoroacetic acid suspension with HBr. However, tri-
fluoroacetic acid swells the polystyrene resin very poorly, and infra-red
spectra of the resin (after 4 hours treatment) showed incomplete cleavage
of product. This has also been one of the more aggravating disadvantages
of the HBr/TFA cleavage in peptide synthesis.[121] When trifluoroacetic acid
was replaced by acetic acid, swelling of the polystyrene support was
improved, and the cleavage reaction appeared to be more complete by IR
criteria (absence of ν(C=O) bands).

The experiments with dicyclohexylcarbodiimide as a coupling
reagent were performed at an early stage of this work when experience with
the solid-phase procedure, and with interpretation of infra-red spectra of
the resins were limited. A coupling reaction in chloroform solution with
DCC was repeated under the same conditions as described previously except
for the change in cleavage procedure to treatment with HBr/HOAc. UV spec-
tral analysis of the product showed the desired 3-aminomethyl-s-triazolo-
[3,4-a]phthalazine! Thus, the apparent failure of the carbodiimide method
in earlier efforts probably resulted not only from the coupling step, but
from incompleteness of the cleavage reaction as well.

3.3 DISCUSSION

One facet of our studies on the solid-phase synthesis of
s-triazolo[3,4-a]phthalazine derivatives that is clearly evident is the
interdependence of the various factors: support material, attachment,
coupling, and cleavage methods, in determining the success of the solid-
phase method for chemical synthesis.

The insoluble support is the backbone of the solid-phase method
itself. A good support should possess certain desirable physical and

chemical characteristics. The support should be in the form of homogeneous, relatively rigid, porous beads. An appropriate porosity permits rapid diffusion of reagents into the reactive sites and easy removal of reagents by filtration and washing. It also provides for a large effective surface area which is desirable for obtaining a high degree of modification.

Ideally, the support should have good mechanical strength and chemical stability toward extremes of temperature and pH, and toward organic solvents. The support should also be highly insoluble in the solvent media of reaction.

The support must possess chemical characteristics which allow introduction of reactive functionalities under mild conditions and permit activation of the support without destroying its structural integrity.

Last, but not least, a good support should be reasonably priced, otherwise large-scale syntheses would be economically prohibitive. Availability from either supply houses or from simple chemical synthesis is advantageous. The cost factor will be minimized if the active support can be regenerated after use.

The support material which we used in this work was a copolymer of styrene and divinylbenzene which is an amorphous gel with a random network of loosely crosslinked polystyrene chains. We preferred the 1% crosslinked polymer over the 2% crosslinked resin used in the majority of solid-phase reactions. Copolystyrene-1% DVB swells appreciably more in non-polar solvents than polymers with higher crosslinking, and can lead to more complete reactions in peptide synthesis.[122] In highly crosslinked copolystyrene-DVB polymers, the beads are too rigid to permit easy penetration of reagents, and slower and less complete reactions may result. The crosslinked polystyrene beads are not a "surface support". Due to their
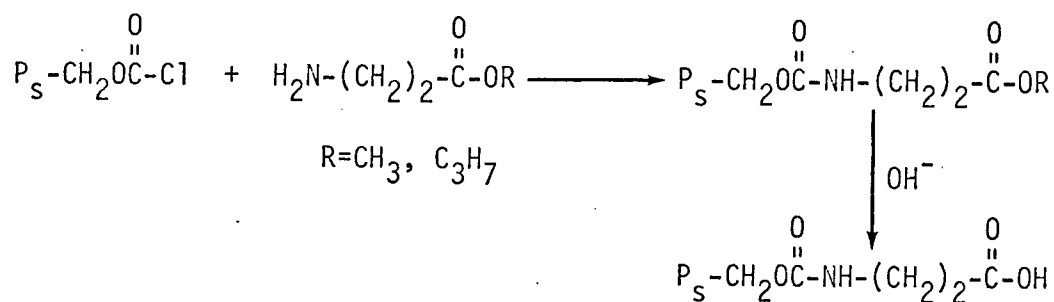
swelling in organic solvents they are freely permeable to reagents. Merrifield and Littau[123] showed by autoradiography of beads containing tritiated peptides that the distribution is quite uniform throughout the bead. Recent studies with polymer-bound transition metal catalysts and complexes strongly suggest that the degree of swelling of the polymer lattice is an important factor in determining the chemical reactivity of immobilized molecules, and therein lies one of the main deficiencies of crosslinked polystyrene.[124,125] Supports are needed which are more compatible with polar solvents, or are less affected by the swellability of the resin.

In N-terminal peptide sequencing with an insoluble Edman reagent, Dowling and Stark[112] synthesized a copolystyrene-0.25% DVB resin with covalently linked glucosaminol to increase its hydrophilic character. Rangarajan and Darbre[115] introduced methylthiocarbamoyl groups into the benzene rings of copolystyrene-2% DVB to achieve the same end. This modified polymer approximately doubled its swelling in aqueous media and showed improved attachment yields of tetra- and hexapeptides to the support. The improvement was ascribed to the increased polarity of the polymer allowing easier accessibility of reactive sites.

Affinity chromatography has in common with solid-phase synthesis the use of insoluble supports for immobilizing a ligand. In the former application, it is frequently advantageous to attach the ligand to the support at a distance from the surface. Spacer arms provide more efficient interaction between the immobilized ligand and the solute molecules due to the increased steric availability of the ligand to the solute. The length of the spacer is chosen empirically and must be optimized for each use.

The same principles also apply to the solid-phase reaction of amino acids with hydralazine. The bifunctional molecules β-alanine methyl

ester HCl and β-alanine isopropyl ester HCl were therefore synthesized to be used as spacer arms as shown in Scheme 3.8.

$$P_s-CH_2O\overset{O}{\overset{\|}{C}}-Cl \quad + \quad H_2N-(CH_2)_2-\overset{O}{\overset{\|}{C}}-OR \quad\longrightarrow\quad P_s-CH_2O\overset{O}{\overset{\|}{C}}-NH-(CH_2)_2-\overset{O}{\overset{\|}{C}}-OR$$

$$R=CH_3, \quad C_3H_7 \qquad\qquad\qquad\qquad \Big\downarrow OH^-$$

$$P_s-CH_2O\overset{O}{\overset{\|}{C}}-NH-(CH_2)_2-\overset{O}{\overset{\|}{C}}-OH$$

Scheme 3.8   Modification of Polystyrene with a Spacer Arm

The advantages of a spacer arm were also apparent to Rangarajan and Darbre[115] who attached a γ-amino-n-butyric acid methyl ester spacer arm to a carboxylate polystyrene support. An improvement in the yield of peptide attachment was attributed to arise partly from this polymer modification.

In a similar vein, Sparrow[126] modified a Merrifield resin by introducing a long spacer arm for use in peptide synthesis. A three-fold

$$P_s-\!\!\!\left\langle\bigcirc\right\rangle\!\!\!-CH_2-[NH-CO-(CH_2)_{10}]_2-NH-CO-CH_2-\!\!\!\left\langle\bigcirc\right\rangle\!\!\!-Br$$
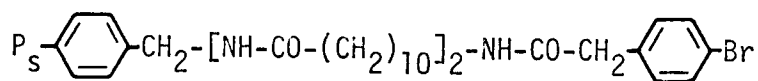
improvement in the overall yield of a 19-residue peptide was realized with this support. EPR studies of a spin-label attached to the N-terminus of a peptide on this resin suggested an increased mobility of the N-terminal residue over the unmodified commercial resin, and hence decreased peptide-resin interactions.

In spite of modifications to the usual polystyrene matrix, the inherent hydrophobicity and dependence on swelling by solvents of styrene

based polymers limit their usefulness in peptide sequencing. An ideal

support should be capable of coupling effectively all sizes of peptides,

including proteins, and the functional groups should be accessible for

chemical reaction in a variety of aqueous and organic media.

Polyacrylamide appears to be an excellent matrix for solid-phase

synthesis. The beads are mechanically and chemically stable, and are

relatively hydrophilic. The neutral polyacrylamides are entirely synthetic

gels formed by copolymerization of acrylamide with the bifunctional cross-

linking agent, N,N'-methylene-bis-acrylamide. The ratio of the concentra-

tion of acrylamide in the reaction mixture to that of the crosslinking

agent can be varied to give an infinite series of insoluble gel products

which differ in their average pore size.[127] Figure 3.1 shows part of a

polyacrylamide matrix. The principal advantage of polyacrylamide is that

$$
\begin{array}{l}
-CH-CH_2-CH-CH_2-CH-CH_2-CH- \\
\quad CONH_2 \qquad\qquad CONH_2 \quad CONH_2 \\
\qquad HN-CO \\
\qquad CH_2 \\
\qquad HN-CO \\
-CH-CH_2-CH-CH_2-CH- \\
\quad CONH_2 \qquad\qquad OC-NH \\
\qquad\qquad\qquad CH_2 \\
\qquad\qquad OC-NH \\
-CH_2-CH-CH_2-CH-CH_2-CH- \\
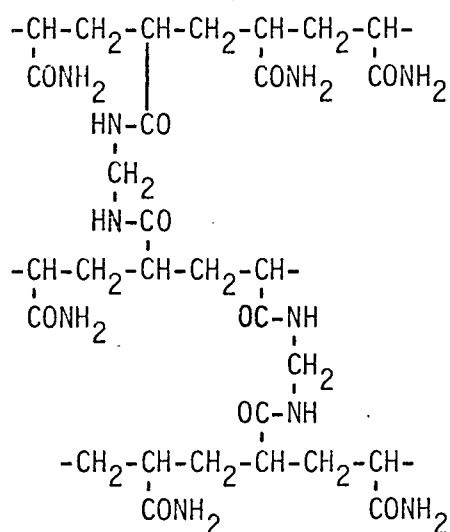\qquad CONH_2 \qquad\qquad CONH_2
\end{array}
$$

Figure 3.1 Partial Structure of a Polyacrylamide Matrix

it possesses a very abundant supply of modifiable carboxamide groups which, together with a versatility in derivatization techniques, allows the covalent attachment of a variety of functionalities.[127] Limitations of polyacrylamide as an insoluble matrix are the low degree of porosity of the beads currently available, and the shrinkage observed during the chemical modifications required for attachment of functional groups.

Polyacrylamide gels have found their most frequent applications in the immobilization of proteins and other bioorganic materials in affinity chromatography, immunosorbent synthesis, etc.[127-130] Applications in synthetic organic chemistry are limited to the recent use of a polydimethyl-acrylamide support in peptide synthesis.[131] A notable feature of this support is the large swelling of the resin in polar media such as DMF, HOAc, and H_2O. Very much less swelling occur in methylene chloride and less polar organic solvents. These properties are the reverse of those of poly-styrene-based resins. In peptide synthesis, the polydimethylacrylamide support gave results clearly superior to those obtained with the customary polystyrene support. In N-terminal protein sequencing, polyacrylamide-based supports are just beginning to attract attention. Preliminary results show significant advantages over polystyrene resins.[132,133] Clearly, polyacryl-amide supports show sufficient promise to warrant further investigations in a wider range of applications.

Bio-Gel CM-2 is a fully carboxylated polyacrylamide matrix avail-able from Bio-Rad Laboratories with a capacity of 5 meq/gram. We used this material to prepare the acid-chloride resin by reaction with thionyl chloride, with the intentions of modifying the support with a β-alanine ester spacer arm, and using this support to evaluate its suitability for the solid-phase synthesis of TAP derivatives. These experiments however, remain incomplete, and they deserve consideration by other workers.

Other types of supports are in use for solid-phase protein sequencing and prominent among them are those based on controlled-pore glass (CPG). The term controlled-pore refers to a rather narrow pore distribution. This type of support was first introduced for Edman degradation of peptides and proteins by Machleidt and co-workers in 1973.[134] Porous glass is an attractive support material for a number of reasons. It is rigid, with-stands organic solvents, is regenerable, and is resistant to microbial attack.

Porous glass may be activated by silanization with γ-aminopropyl-triethoxysilane.[135] The amino groups of the resulting alkylamine glass may be succinylated to give an extension arm approximately 10 Å long. The carboxyl glass may be converted to the acyl chloride with thionyl chloride.

$$-\overset{|}{\underset{|}{Si}}-(CH_2)_3-NH-\overset{O}{\overset{\|}{C}}-(CH_2)_2-\overset{O}{\overset{\|}{C}}-OH$$
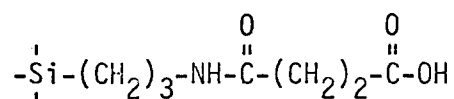
This product can be used directly for coupling with the amino groups proteins. The succinylated CPG is commercially available from Pierce Chemical Company, as are the N-hydroxysuccinimide and *p*-nitrophenyl esters. For an excellent review on CPG, the reader is referred to the Pierce General Catalog.[136]

Recent C-terminal peptide and protein sequencing studies have used N-hydroxysuccinimide CPG beads.[114,115] Good attachment yields of lysozyme and ribonuclease to the porous glass support were obtained. Williams and Kassell[114] achieved average attachment yields varying from 49 to 85% with eight different peptides possessing between two and five amino acids.

Porous glass possesses many of the requisites for a good support material, and it is gradually replacing the conventional polystyrene-based support in protein sequencing. There are several inherent limitations in

the support, however. The Si-O bonds which hold the ligand to the glass
are not completely stable to an acid (particularly aqueous acid) and small
amounts of peptide tend to be lost in each degradative cycle. Erosion of
the glass, especially at high pH is also a problem, and severe ligand
leakage can be encountered. A coat of zirconium oxide stabilizes the glass
but this raises further the cost of an already expensive material. The
Corning CPG/N-hydroxysuccinimide ester distributed by Pierce costs $60/25
ml.[136] A further liability of CPG is its rather low binding capacity
(CPG/N-hydroxysuccinimide, ~0.036 meq/ml)[136] which makes it somewhat
impractical for preparative scale syntheses.

At the present state of development, there are many deficiencies
in the solid-phase coupling reaction of hydralazine with amino acids.
Using dicyclohexylcarbodiimide as a coupling reagent, the desired TAP
product is formed, but yields are unsatisfactory. Perhaps one of the most
serious problems with the carbodiimide method is the possibility of forma-
tion of the inactive N-acylurea. A tactic used to reduce the amount of
N-acylurea is the addition of N-hydroxy compounds such as 1-hydroxybenzo-
triazole (HOBt)[137] (77) and 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine
(78).[138] These reagents are used primarily for the suppression of racemiza-

(77)   (78)
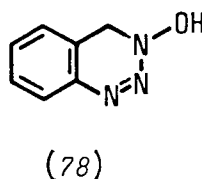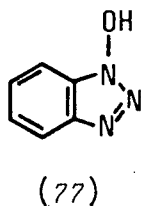
tion in peptide synthesis with DCC. They combine rapidly with the carboxyl
component to form highly reactive esters which in turn couple with the
amino component. Formation of a complex between the amino component and

the HOBt active ester was proposed to explain the properties of these
additives[139] (Scheme 3.9).
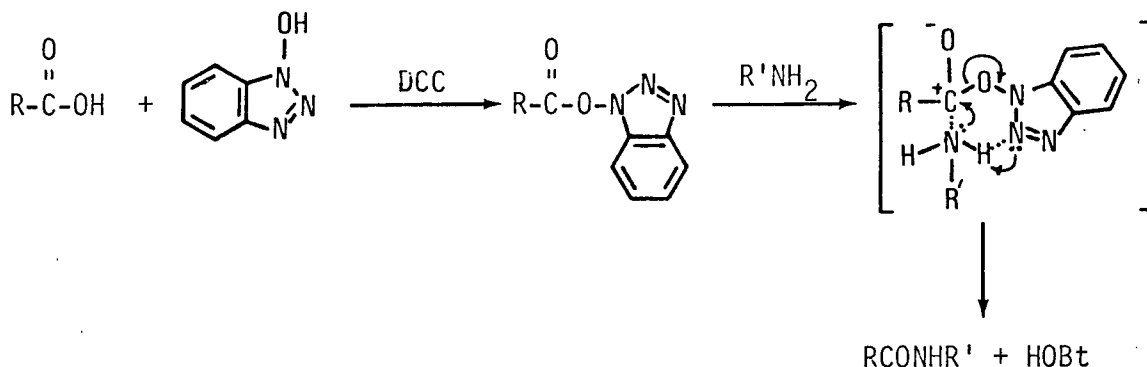
Scheme 3.9   Amide Formation with DCC/HOBt

N-hydroxy additives are good nucleophiles and they compete with
the amino component for the acyl group of the O-acylisourea intermediate to
form active esters. Furthermore, the presence of the amine and of the
additive results in a higher concentration of nucleophiles, thus reducing
the lifetime of the O-acylisourea, and hence decreasing the probability of
intramolecular rearrangement. Single coupling reactions were attempted with
the HOBt additive for both solid-phase and solution reactions. In both
cases, results were inconclusive and no definite improvement in coupling
yields could be demonstrated.

The best coupling yields were obtained when isobutyl chloroformate
was used to activate the immobilized amino acid. This suggested that EEDQ
and IIDQ (cf. Section 2.4.2) which also react via mixed-anhydride inter-
mediates should also be successful. Thus, Yajima and co-workers[90] obtained
nearly quantitative coupling of a hexapeptide with H-Gly-Ala-P_s after 48
hours reaction with IIDQ in DMF. Good coupling yields with EEDQ were also
obtained by Sipos and Gaston in solid-phase peptide synthesis.[140]

The N-ethyl-5'-phenylisoxazolium-3'-sulfonate (NEPIS), (cf. Section 2.4.1) coupling reagent was successfully applied to the synthesis of TAP derivatives under conventional liquid-phase conditions. However, this reagent has attracted very little attention for solid-phase reactions, and it would be worthwhile to investigate its efficiency in solid-phase TAP synthesis.

Of the other reagents used in this work for coupling hydralazine with N-protected amino acids in homogeneous solution, only the "oxidation-reduction" reagents of Mukaiyama et al. (cf. Section 2.4.3) appear to have been used in solid-phase synthesis. This method is particularly relevant to our studies since there are very few examples in the literature for carboxyl activation of an amino acid or peptide attached to a support at its amino group. These studies in peptide synthesis by chain elongation from the N-terminal amino acid use isobutoxy mixed anhydride (from isobutyl chloroformate)[107] and azide[116] intermediates. Matsueda and co-workers succeeded in synthesizing porcine luteinizing hormone-releasing hormone (LH-RH, 10 residues) by coupling three fragments with excess 2,2'-dithio-dipyridine and triphenylphosphine.[141] This synthesis was accomplished without the necessity of protecting the side chains of tryptophan, glutamine, histidine, tyrosine and serine. In a further demonstration of the utility of the oxidation-reduction method, a 24 residue segment of adrenocortico-tropin (ACTH) was synthesized in good yield by elongation from the N-terminus in five fragment coupling steps.[142]

The superiority of the oxidation-reduction process certainly lies in the advantage it possesses of minimizing side-reactions. By comparison the carbodiimide method, which is by far the most widely used in solid-phase peptide synthesis, is susceptible to (a) N-acylurea formation, and

(b) nitrile formation from side-chain carboxamide groups during activation of glutamine or asparagine derivatives.

While we were unsuccessful with the oxidation-reduction method for synthesis of TAP derivatives in solution, perhaps this was due to the experimental conditions used, and not to the method itself. The oxidation-reduction method certainly deserves some consideration for the solid-phase coupling of hydralazine with immobilized amino acids.

We have shown new avenues for further development of our solid-phase studies, but with the present system, a modicum of changes in the reaction conditions can bring about significant improvements in the reaction efficiencies observed. The first change which suggests itself is the use of free base hydralazine rather than hydralazine HCl with triethylamine. The hydrochloride salt requires polar solvents for dissolution whereas poly-styrene-based supports are most efficiently swelled by non-polar solvents. With freshly-prepared hydralazine non-polar solvents such as methylene chloride can be used. In addition, hydralazine decomposes rapidly in the presence of excess Et_3N. This is especially a problem when the dissolution of hydralazine HCl is slow, and there is a low concentration of hydralazine in solution relative to Et_3N. If hydralazine decomposition is extensive, any of the decomposition products containing primary or secondary amine groups may couple with the amino acid to give undesired side-products. Our observation of a yellow colour in the resin during coupling reactions may be evidence of this side-reaction.

Several approaches are available for improving the coupling efficiency of any given coupling method. Reaction times of 15-18 hours were generally used in these studies. Even these extended reaction times may be insufficient. In solid-phase peptide synthesis, the standard

reaction time is two hours. However, the application of increasingly longer reaction times has been a noticeable trend. In some cases, reaction times have been extended to 24 hours or greater.[121] One of the intrinsic problems with the solid-phase procedure is that the phase separation may often result in slower reaction.

Application of larger excesses of hydralazine and coupling reagent would seem to be an obvious remedy for incomplete coupling. We have generally used a two- to three-fold excess of these reagents in the coupling reaction. In a solid-phase synthesis of cytochrome C, Sano and Kuihara used amounts of 30- to 70-fold excess of reagents in a routine manner through the final eighteen steps of their synthesis.[143] In these cases, excesses of reagents would be filtered off and reused.

The reactivity of any peptide carboxylate group is somewhat dependent on the nature of the amino acid side-chain and the adjacent amino acid sequence. This variability in reactivity advises against a standard reaction time for hydralazine coupling with amino acids and peptides. There must therefore be some means of monitoring the progress of the reaction. The difficulties associated with application of infra-red spectroscopy to polymeric materials have been previously discussed (*vide supra*). Feasible alternatives include cleavage of the peptide from the support and subjecting it to amino acid analysis to determine the extent of TAP modification. The amount of TAP present in a hydrolyzate of the peptide under study may also be determined by quantitative fluorescence analysis of the TLC isolated product. The extent of peptide modification by hydralazine can also be monitored by microanalysis of the peptide resin. However, low substitution of peptide on the support, and the presence of the support itself, can make this a somewhat insensitive method.

If the solid-phase method is to be used as a purely synthetic procedure, then a cleavage of the benzyl ester bond is required by which the TAP derivative is attached to the support. We have found that scission of this bond is satisfactory under acidic conditions with HBr/HOAc. However, if serine and threonine are present in the peptide chain, the hydroxyl groups may be acetylated under these conditions.[118] This side-reaction is avoided if acetic acid is substituted by anhydrous trifluoro-acetic acid, but extended reaction times may be required since we observed incomplete cleavage with HBr/TFA. Alternatively, anhydrous, liquid hydrogen fluoride may be used.[118]

Peptide sequencing from the carboxyl terminus by the solid-phase method requires attachment of the N-terminal amino acid to the support at the α-amino group. A frequently used method for producing peptides from proteins is treatment of the protein with trypsin. This enzyme cleaves peptide bonds at the C-end of lysine and arginine. A significant number of peptides used in peptide sequencing may therefore contain lysine. If such a peptide is attached to a support, binding will occur at the N-terminus (α-amino group) and at the C-terminus (ε-amino group). C-terminal sequencing of the peptides thus anchored will show gaps in the amino acid sequence at the N-terminus and at lysines since these residues will remain bound to the resin. Their identities may be deduced from the difference between the amino acid composition of the peptide and the TAP derivatives detected, or by hydrolysis and analysis of the support after degradation.
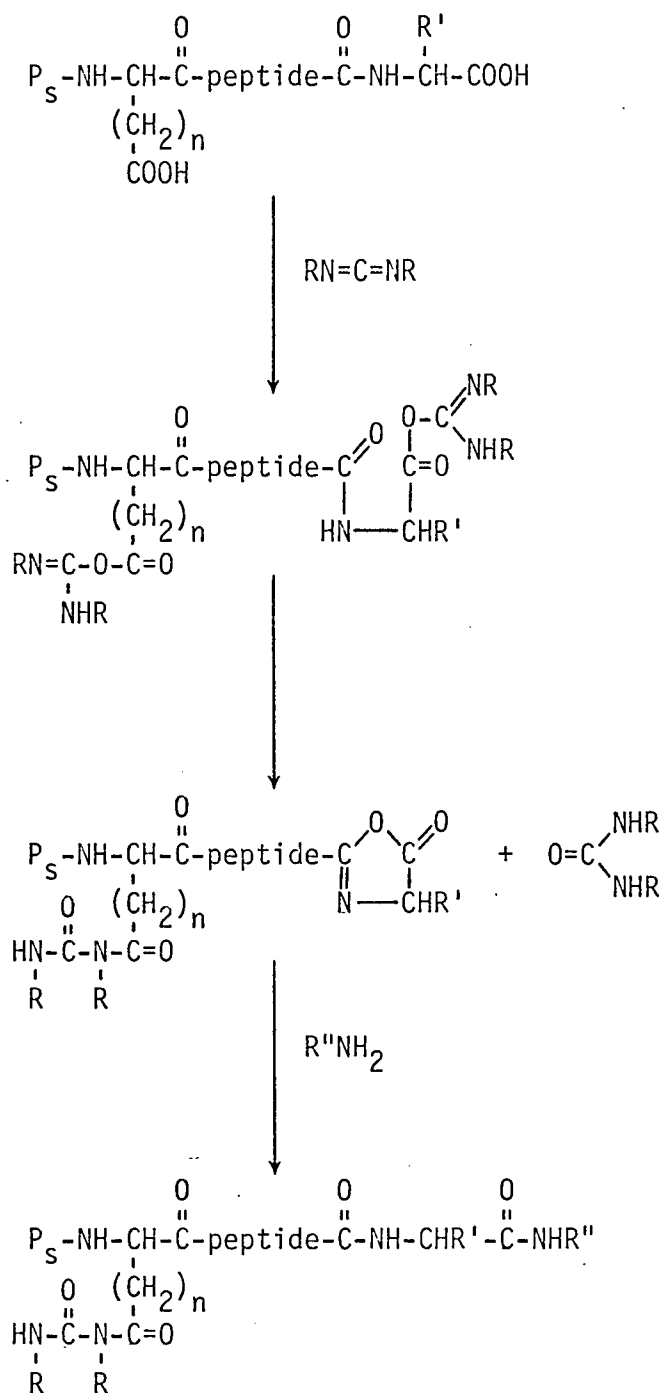
If basic amino acids are present in the peptides, attachment of the peptide at the amino side-chains may be prevented by carrying out one step of Edman N-terminal degradation on the free peptide.[114] This procedure protects ε-amino groups of lysine as their phenylthiocarbamyl (PTC) deriva-

tive, and at the same time permits identification of the N-terminal residue. After this step, the α-amino group of the second residue is the only amino group free for attachment to the support.

A possible complication in C-terminal residue analysis is the reaction of hydralazine with side-chain carboxyl groups of aspartic and glutamic acids. This side-reaction may create difficulties where the hydralazine modification reaction is used for C-terminal determination only. Total hydrolysis of the modified peptide will yield TAP derivatives from both the C-terminal amino acid and internal amino acids. The situation may be more complex in the case of aspartic acid; the β-carboxyl group after activation by coupling reagent can react with a neighbouring amide nitrogen to form a β-lactam which may stop peptide degradation completely.

Several solutions to the side-chain carboxyl group problem are possible. Previero *et al.*[144] have shown that carbodiimides can effect both protection of side-chain carboxyl groups and activation of the C-terminus under suitable experimental conditions (Scheme 3.10). Peptides containing a carboxyl group only at the C-terminal reach a degree of activation which remains constant as a function of time, while peptides containing a side-chain carboxyl show an initial maximum of activation which subsequently decreases. Thus, acidic peptides which were incubated with 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (EDC) for 90 minutes at 40°C in the absence of nucleophiles before reacting with the amino component showed coupling with only the C-terminal amino acid. During the incubation period, the O-acylisourea intermediate isomerizes to the inert N-acylurea derivative at the side-chain carboxyl, while the C-terminus remains activated as an oxazolinone which subsequently reacts with the amino component.

$$\underset{P_s-NH-\underset{\underset{COOH}{\overset{|}{(CH_2)_n}}}{\overset{|}{CH}}-\overset{\overset{O}{\|}}{C}-peptide-\overset{\overset{O}{\|}}{C}-NH-\underset{}{\overset{\overset{R'}{|}}{CH}}-COOH}{}$$

$$\downarrow RN=C=NR$$

$$P_s-NH-\underset{\underset{NHR}{\overset{|}{RN=C-O-C=O}}}{\overset{|}{\underset{(CH_2)_n}{\overset{|}{CH}}}}-\overset{\overset{O}{\|}}{C}-peptide-\underset{HN---CHR'}{\overset{O}{C}}\overset{O-C}{\underset{C=O}{}}\overset{NR}{NHR}$$

$$\downarrow$$

$$P_s-NH-\underset{\underset{\underset{R\quad R}{\overset{|}{}\quad\overset{|}{}}}{\overset{|}{HN-\overset{\overset{O}{\|}}{C}-N-C=O}}}{\overset{|}{\underset{(CH_2)_n}{\overset{|}{CH}}}}-\overset{\overset{O}{\|}}{C}-peptide-\overset{O}{C}\underset{N---CHR'}{\overset{O}{C}} \quad + \quad O=C\overset{NHR}{\underset{NHR}{}}$$

$$\downarrow R''NH_2$$

$$P_s-NH-\underset{\underset{\underset{R\quad R}{\overset{|}{}\quad\overset{|}{}}}{\overset{|}{HN-\overset{\overset{O}{\|}}{C}-N-C=O}}}{\overset{|}{\underset{(CH_2)_n}{\overset{|}{CH}}}}-\overset{\overset{O}{\|}}{C}-peptide-\overset{\overset{O}{\|}}{C}-NH-CHR'-\overset{\overset{O}{\|}}{C}-NHR''$$

Scheme 3.10   Selective Amide Formation at the C-Terminus of Peptides

A different approach to this problem is to esterify all the carboxyl groups in the peptide, and then to liberate the C-terminal α-carboxyl group with trypsin, which can act as a fairly specific esterase. Mross and Doolittle have suggested converting peptide carboxyl groups to amides and then cleaving the C-terminal amide selectively with trypsin.[145]

3.4 SUMMARY

We have investigated the reaction of hydralazine with amino acids attached to insoluble supports under a variety of conditions. Using a copolystyrene-1% divinylbenzene matrix substituted with methylchloroformyl groups, amino acid and peptide esters can be attached to the support via their α-amino groups. The immobilized amino acids react with hydralazine to form the s-triazolo[3,4-a]phthalazine (TAP) derivatives when isobutyl chloroformate, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), and dicyclohexylcarbodiimide (DCC) are used for carboxyl activation. The TAP derivatives may be cleaved from the resin under acidic conditions with HBr-HOAc.

The work on solid-phase reactions is still in a preliminary state of development, but we have demonstrated the feasibility of using the solid-phase method for the synthesis of TAP derivatives. Under the present reaction conditions, the products of the coupling reaction are contaminated with unreacted amino acids, and they require chromatographic techniques for purification. The coupling reaction of hydralazine with amino acids is not yet practical for solid-phase C-terminal sequencing of peptides because of the unacceptably low coupling yields.

CHAPTER 4

TRANSITION METAL COMPLEXES WITH
THE S-TRIAZOLO[3,4-A]PHTHALAZINE LIGAND

4.1 INTRODUCTION

Perhaps the most serious limitation which prevents the extension of most peptide C-terminal amino acid determination methods to sequential analysis is a satisfactory method of selectively and quantitatively hydrolyzing the terminal peptide bond under mild conditions.
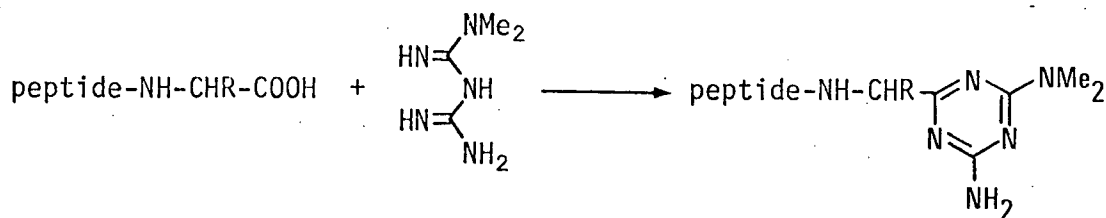
In the thiocyanate method of Stark,[21,22] (Scheme 1.2) the thiohydantoin group which is formed from the C-terminal amino acid is cleaved by nucleophilic catalysis with acetohydroxamate, or acid catalysis with 12M HCl. The use of highly acidic conditions for cleavage of the thiohydantoin group is a risky proposition, and cleavage of internal peptide bonds is also likely. The probability of this occurring prompted Yamashita[146] to look for a milder, more selective cleavage method. He found that shaking the peptidylthiohydantoin with the acidic form of a cation-exchange resin was successful in hydrolyzing the bond between the peptide and thiohydantoin, but not the other peptide bonds. With this modification, Yamashita was able to determine the amino acid sequence up to around 10 residues from the C-termini of polypeptides.[147]

The very nature of the hydrazinolysis method[15] precludes its use as a C-terminal sequential procedure since on treatment of peptides with hydrazine, the peptide chain is split and all the internal amino acids are converted to hydrazides, except the C-terminal amino acid which remains as the free amino acid.

The tritiation method of Matsuo *et al.*[22] (Scheme 1.3) and the reduction method of Bailey[26] (Scheme 1.4) rely on concentrated hydrochloric acid to hydrolyze the terminal peptide bond. These methods suffer from the same limitations as the Stark method.

In the method described by Loudon and co-workers[28] (Scheme 1.5) the N-aminomethylamide formed from the C-terminal residue degrades under hydrolytic conditions (6N HCl) to the peptide-amide which prevents further C-terminal amino acid analysis of the peptide.

In another C-terminal amino acid analytical method, Maekawa and Kumano[148] converted peptides to the corresponding peptide-triazines by reacting the carboxyl groups with dimethylbiguanidine. However, in the absence of a mild, selective method for cleaving the terminal bond they resorted to total hydrolysis of the peptide with *Streptomyces griseus* protease, and identification of the modified C-terminal derivative in the hydrolyzate.

$$\text{peptide-NH-CHR-COOH} \ + \ \begin{array}{c} \text{HN}= \\ \\ \text{HN}= \end{array}\!\!\!\!\Big\langle\begin{array}{c} \text{NMe}_2 \\ \text{NH} \\ \text{NH}_2 \end{array} \longrightarrow \text{peptide-NH-CHR}$$

From the above exposition, it is clear that the lack of a suitable method of hydrolyzing the C-terminal peptide bond is an impediment

which is common to most of the C-terminal methods. The approach which we considered was the metal-ion assisted hydrolysis of the peptide bond.

The role that metal ions can play in catalyzing the hydrolysis of peptide bonds was long utilized by Nature in incorporating a zinc(II) ion into the active site of carboxypeptidase A (CPA). This enzyme, as its name implies, catalyzes the hydrolysis of the peptide bonds at the carboxyl end of a polypeptide substrate. The zinc ligands have been identified as two histidines, glutamic acid, and water in distorted tetrahedral coordination[149] In the presence of a peptide substrate, the carbonyl group of the C-terminal peptide bond displaces the water ligand from the zinc ion in the complex. A nearby arginine side-chain binds the terminal carboxyl group present in the substrate.

A possible mechanism for the action of CPA given by Lipscomb and co-workers,[150] is shown in Scheme 4.1.
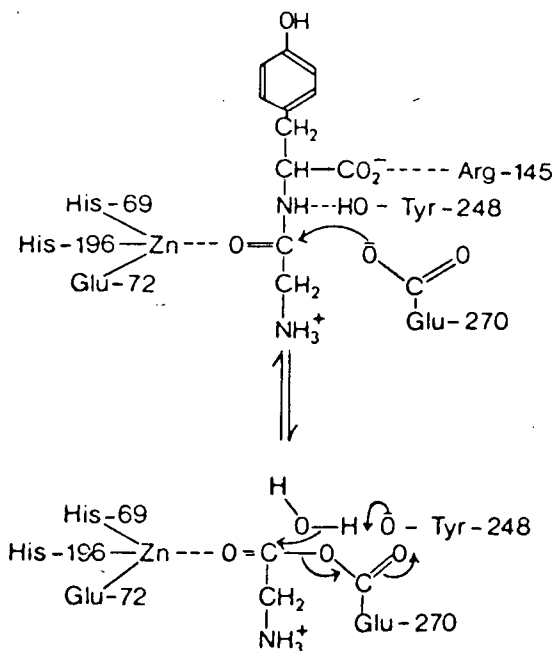
Scheme 4.1 Possible Mechanism for the Carboxypeptidase A-
Catalyzed Hydrolysis of Glycyl-L-tyrosine

Lipscomb has suggested that coordination of the metal ion by the carbonyl oxygen atom of the substrate results in polarization of the C=O bond of the carbonyl group, rendering the carbon atom more susceptible to nucleophilic attack.

In addition to the 'zinc-carbonyl' mechanism, the 'zinc-hydroxide' mechanism can also be considered, in which a zinc-bound hydroxide ion acts as a nucleophile. The two mechanisms are illustrated in Figure 4.1. Structural evidence from X-ray studies however, favors the zinc-carbonyl mechanism.

(a)   (b)
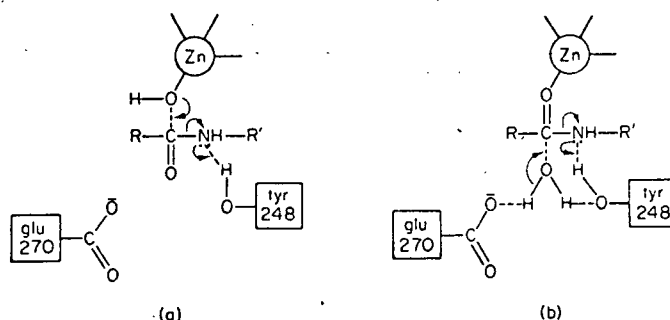
Figure 4.1   Diagrammatic Illustration of the Mechanism of Carboxypeptidase-Catalysed Peptide Hydrolysis: (a)   Zn-hydroxide Mechanism and (b)   Zn-carbonyl Mechanism

The effect of metal ions on the hydrolysis of amide bonds has been known for over twenty years. Lawrence and Moore[151] found that $CoCl_2$ almost doubled the rate of acid hydrolysis of glycylgycine. Meriwether and Westheimer[152] examined the effects of copper(II), cobalt(II), and nickel(II) ions on the hydrolysis of glycinamide and phenylalanylglycinamide. Copper(II) ions were the most effective catalysts, and between pH 7.9 and 9.25 at 75°C, they increased the rate of hydrolysis of glycinamide by a factor of thirty over the uncatalyzed hydrolysis. The mechanism of these hydrolysis reactions is not yet established, but it is likely that the catalytically active species is the carbonyl-bonded complex.

The copper(II)-catalyzed hydrolysis of glycylglycine attains a maximum rate at pH 4.2.[153] The decrease in rate at higher pH values is associated with the formation of a catalytically inactive complex produced by ionization of the peptide hydrogen atom (Figure 4.2).[154]
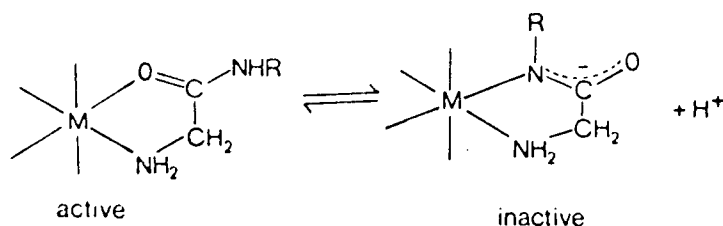

active

inactive

Figure 4.2 Ionization of Peptide Amide Hydrogen

Extensive work, mainly by the Buckingham and Sargeson groups in Canberra, with kinetically inert cobalt(III) complexes has greatly clarified the mechanistic pathways in metal-assisted amide hydrolysis.

It has been found that a number of complexes of the type $[CoL_4(OH)(H_2O)]^{2+}$ $(L_4$ = 2en, trien, tren, edda, eee)[†] stoichiometrically and specifically cleave the N-terminal amino acid from peptides.[155,159]

Buckingham et $al.$[155] were the first to demonstrate the selective N-terminal hydrolysis of simple peptides by stoichiometric reaction with the β-$[Co(trien)OH(H_2O)]^{2+}$ ion. The proposed mechanisms for the hydrolysis are shown in Scheme 4.2.

The rate determining step involves the replacement of a coordinated water molecule by the terminal amino group of the peptide. Then either the carbonyl group is attacked by the adjacent coordinated hydroxide group (path A) or the carbonyl group becomes activated to attack by external

---

† en = ethylenediamine, trien = triethylenetetramine, tren = 2,2',2"-tri-aminotriethylamine, edda = ethylenediamine-diacetato, eee = 1,8-diamino-3,6-dithiaoctane
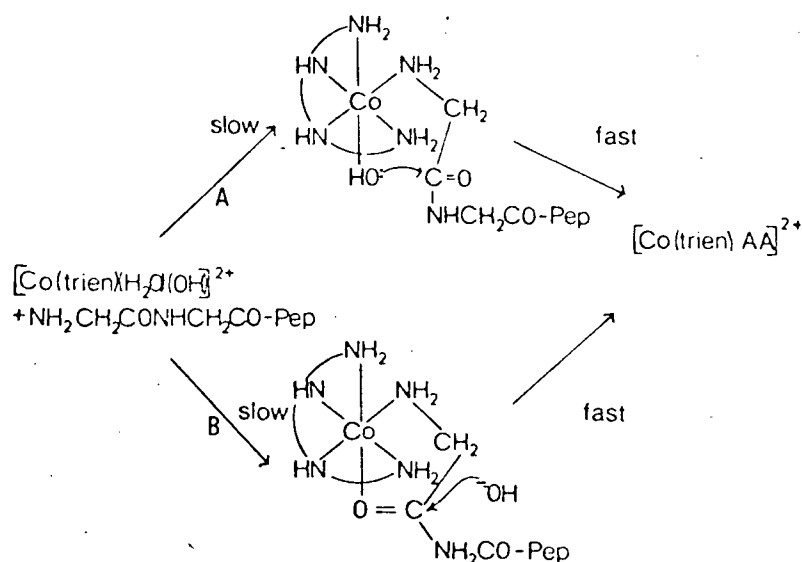
Scheme 4.2   Proposed Mechanisms for Peptide Hydrolysis

hydroxide through prior coordination of the carbonyl oxygen (path B).
These mechanisms are analogous to the "zinc-carbonyl" and "zinc-hydroxide"
mechanisms proposed for carboxypeptidase A. Buckingham, Sargeson, and their
collaborators have shown that both of these mechanisms contribute signifi-
cantly to the hydrolysis reactions although the exact extent of their
contribution may depend on the reaction conditions employed.[160,161]

The acceleration of amide and peptide hydrolysis provided by
direct polarization of the coordinated carbonyl function by metal ion
(carbonyl mechanism) is $10^4 - 10^5$ over that found in the absence of the
metal. Intramolecular attack of bound hydroxide (hydroxide mechanism), in
the absence of buffers is somewhat less effective at pH 7 ($10^3 - 10^4$), but
buffers (e.g. HPO_4^{2-}) result in a tremendous rate enhancement, $10^{10} - 10^{11}$ at
pH7. Under slightly more acidic conditions (pH 4-5) where the bound aquo
group is involved, there is a similar rate increase (10^{11}). Such rates match,
or exceed, the turn-over number found in carboxypeptidase and trypsin

proteases under identical conditions of pH and temperature.[162]

Cis-β-cobalt(III) complexes are appealing as catalysts for N-terminal peptide sequencing because of the amino acid specificity and the large rate enhancement effect. Indeed, the hydrolysis of peptides and proteins by cis-β-[Co(trien)OH(H$_2$O)]$^{2+}$ has been developed into an N-terminal amino acid determination and peptide sequencing method by several workers.[163-167] Comparisons of the efficiency of various cis-[CoL$_4$(OH)(H$_2$O)]$^{2+}$ species indicated the order of L$_4$ for peptide hydrolysis:[164]

$$trien \ > \ tren \ > \ 2en$$
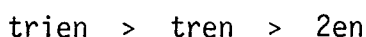
In a comparison of trien and edda, Oh and Storm[158] found that the rates of hydrolysis of dipeptides were somewhat slower for edda complexes, but that there was a smaller rate variation between different peptides.

The success of cobalt(III) complexes in selectively promoting the hydrolysis of peptide bonds prompted us to consider the feasibility of using metal complexes to facilitate the hydrolysis of s-triazolo[3,4-a]phthalazine modified peptides. The overall scheme envisaged for peptide sequencing with hydralazine and cobalt(III) complexes is shown in Scheme 4.3.

In this scheme, we anticipate displacement of the aquo and hydroxo ligands in cis-β-[Co(trien)OH(H$_2$O)]$^{2+}$ by the N$_2$-nitrogen of the TAP ligand and the deprotonated amide nitrogen of the C-terminal peptide bond. Acid hydrolysis of the exocyclic imide bond should liberate the peptide minus the C-terminal residue.
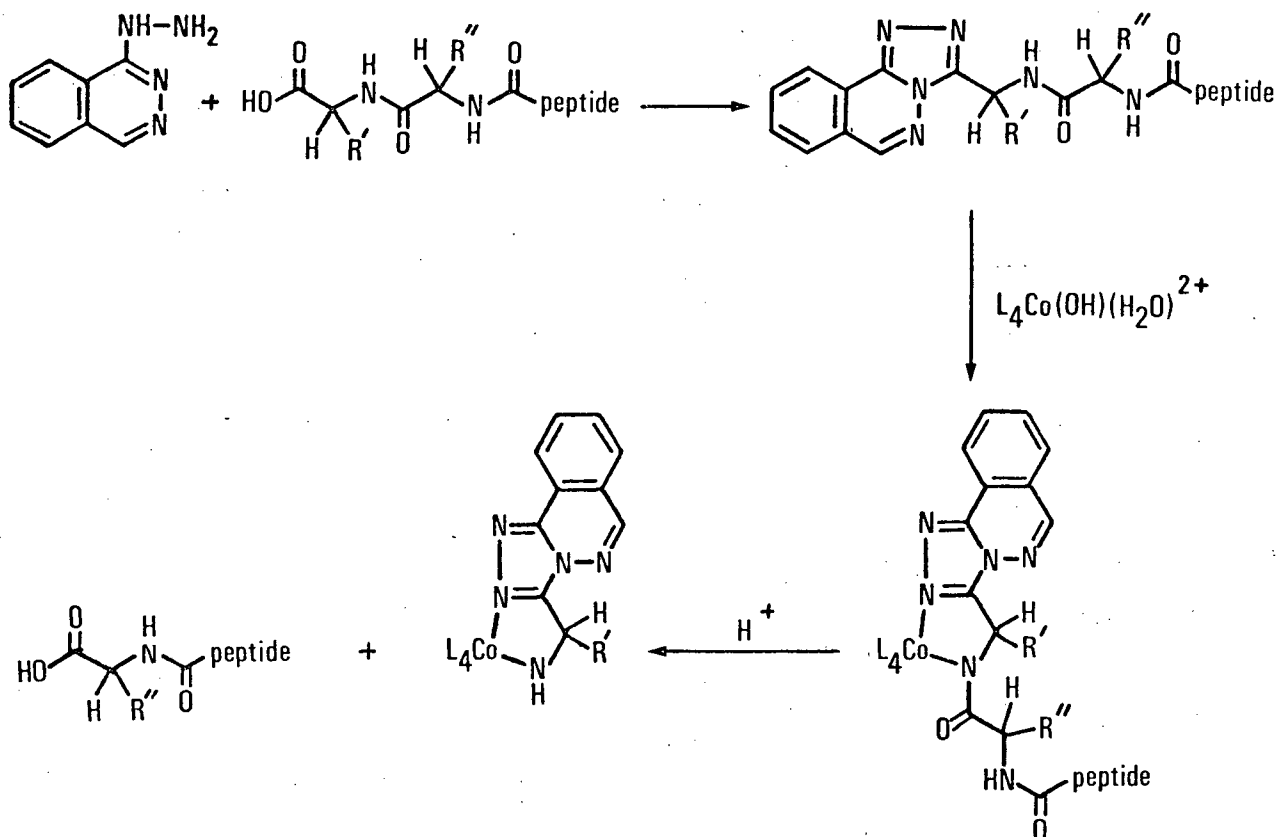
Scheme 4.3. Proposed Peptide Sequencing with Peptidyl-TAP and Cobalt(III) Complexes

There are two likely sites in the TAP group at which the TAP-peptide can coordinate to the matal ion: N-2 of the triazole ring (I), or N-5 of the phthalazine ring (II). CPK space-filling models of both coordination possibilities show that the t-o complexes are feasible and there is no undue steric strain involved. Alkylation of s-triazolo[3,4-a]phthalazines give only the N-2 alkyl product[45] indicating that N-2 is the most basic nitrogen atom and hence the most likely to form a strong coordinate bond.

(I)

(II)
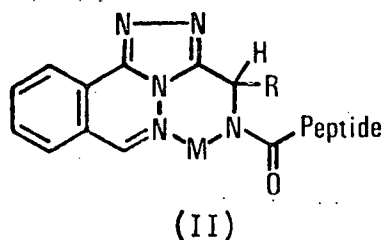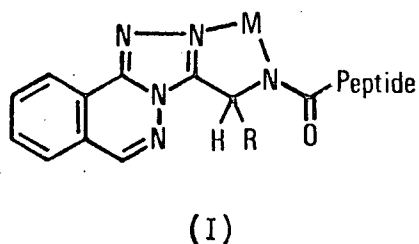
Since our envisaged scheme for peptide sequencing involves metal complexes with TAP ligands, we saw our first task as studying the coordination properties of the TAP ligand since these are totally unknown. The next priority was then to prepare metal complexes with a suitable TAP derivative to determine whether the presence of the metal ion actually aided in hydrolyzing amide bonds in the TAP side-chain.

4.2 RESULTS

The following transition metal complexes containing the s-triazolo-[3,4-a]phthalazine (3-H-TAP) ligand were synthesized:

$[M(3-H-TAP)_n(H_2O)_{6-n}]$ $(ClO_4)_2$ (n = 4, M = Co, Ni, Cu; n = 2, M = Ni) and $[Co(3-H-TAP)_6]$ $(ClO_4)_3$.

$[Co(3-H-TAP)_6]$ $(ClO_4)_3$ was prepared from a 6:1 molar mixture of s-triazolo[3,4-a]phthalazine and sodium tris(carbonato)cobaltate(III) in an ethanol suspension containing dilute perchloric acid. $Na_3[Co(CO_3)_3]$ is a convenient intermediate for the synthesis of cobalt(III) complexes since it avoids the difficulties associated with other methods of synthesis which involve *in situ* oxidation of cobalt(II) to cobalt(III).[168] The hexakis-(s-triazolo[3,4-a]phthalazine)cobalt(III) perchlorate complex is formed as a light brown amorphous solid.

The octahedral spin-paired d^6 Co^{3+} ion is suitable for nmr studies of the coordinated ligand without interferences from paramagnetism of the metal ion. $[Co(3-H-TAP)_6]$ $(ClO_4)_3$ was synthesized primarily for nmr studies. On coordination of 3-H-TAP, perturbation by the metal ion of the electron density about the protons is expected to be most evident in the chemical shifts of protons adjacent to the locus of coordination on the ligand. Thus, in 3-H-TAP, coordination of metal to N-2 should deshield
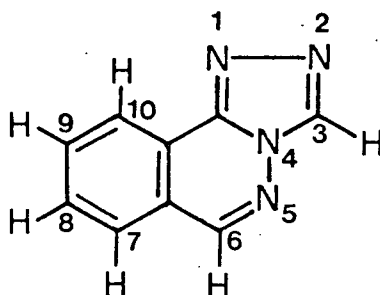
the proton at the C-3 position which would show up in a shift of the nmr
resonance to lower field. There may be a smaller deshielding effect at
other positions arising from an inductive effect.

^{1}H-nmr studies of $[Co(3\text{-H-TAP})_6](ClO_4)_3$ are hindered by its low
solubility in suitable organic solvents. A Fourier-transform nmr spectrum
of a dimethylsulfoxide-d_6 solution of the complex is shown in Figure 4.3.
It is identical in appearance with that of free 3-H-TAP. The sharp singlet
at lowest field is assigned to H-3 and the adjacent sharp resonance at
higher field arises from H-6. The chemical shifts of selected resonances
of free, complexed, and protonated 3-H-TAP are listed in Table 4.1.

Table 4.1 NMR Spectral Data for s-Triazolo[3,4-a]phthalazine in DMSO-d_6

	δ_3	δ_6	δ_{10} [b]		$\delta_{7,8,9}$ [c]
			Chemical Shifts, ppm [a]		
3-H-TAP	9.58	9.07	8.53	8.44	8.06
3-H-TAP.DCl	9.80	9.21	8.68	8.60	8.14
3-H-TAP.HCl	9.71	9.17	8.63	8.56	8.15
$[Co(3\text{-H-TAP})_6]^{3+}$	9.77	9.16	8.54	8.46	8.12

a   Chemical shifts for δ_{10} and $\delta_{7,8,9}$ are given for resonances indicated in Figure 4.3.
b   Doublet with superimposed fine structure.
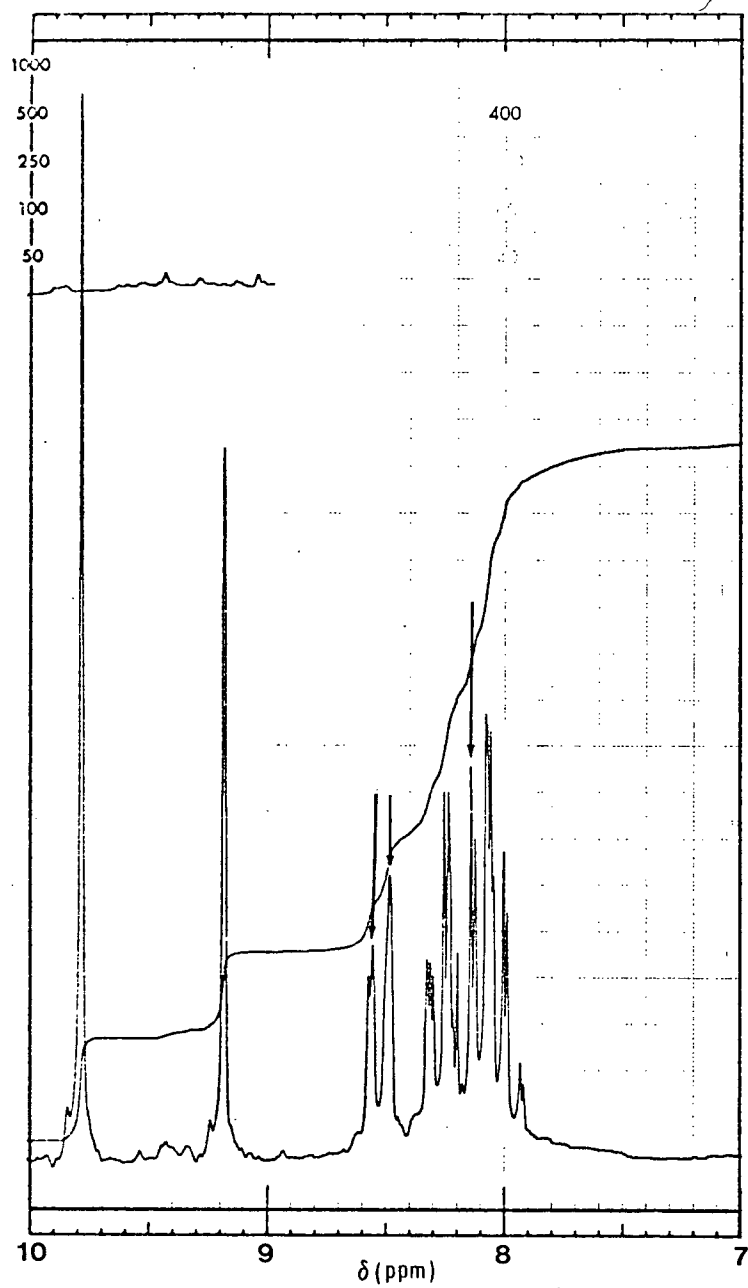
c   Multiplet

Figure 4.3   100 MHz Fourier-transform NMR Spectrum of
[Co(3-H-TAP)$_6$](ClO$_4$)$_2$ in DMSO-d$_6$

It is apparent from Table 4.1 that the greatest change in chemical shifts on complexation and protonation of 3-H-TAP occurs for H-3. This suggests that complexation and protonation do occur at the N-2 site. We cannot state unequivocally that coordination did not occur at N-5, but the small shift of δ_6 to lower field indicates that this is not very significant. Small changes in the chemical shifts may arise from an inductive or concentration effect.

$[Co(3-H-TAP)_6](ClO_4)_3$ is not completely stable in DMSO or methanol solution since free ligand crystallizes from a saturated solution of the complex . The dissociation of ligands is not evident in nmr spectra of the complex which were obtained within one hour of solution preparation.

For a spin-paired d^6 ion in an octahedral field, two electronic transitions are expected: $^1T_{1g} \leftarrow {}^1A_{1g}$ and $^1T_{2g} \leftarrow {}^1A_{1g}$. The diffuse reflectance spectrum of $[Co(3-H-TAP)_6](ClO_4)_3$ is shown in Figure 4.4. Only one d-d transition is resolved in the visible spectrum, and this is superimposed on an intense background. A nujol mull absorption spectrum of the complex has the same appearance as the diffuse reflectance spectrum. The band at 17.1 kK (584 nm) is assigned to the $^1T_{1g} \leftarrow {}^1A_{1g}$ transition. An indication of the higher energy transition is apparent in Figure 4.4, but an estimate of the energy of band is not possible due to the intense background absorption. For comparison, the absorption spectrum of the $[Co(en)_3]^{3+}$ ion in aqueous solution shows bands at 21.4 kK (467 nm) and 29.4 kK (340 nm).[169]

The tetraaquobis(3-H-TAP) and diaquotetrakis(3-H-TAP) complexes were all prepared by the same procedure. The hexaquo metal(II) perchlorates (metal = cobalt, nickel, copper) were dehydrated with 2,2-dimethoxypropane[170] according to the equation:
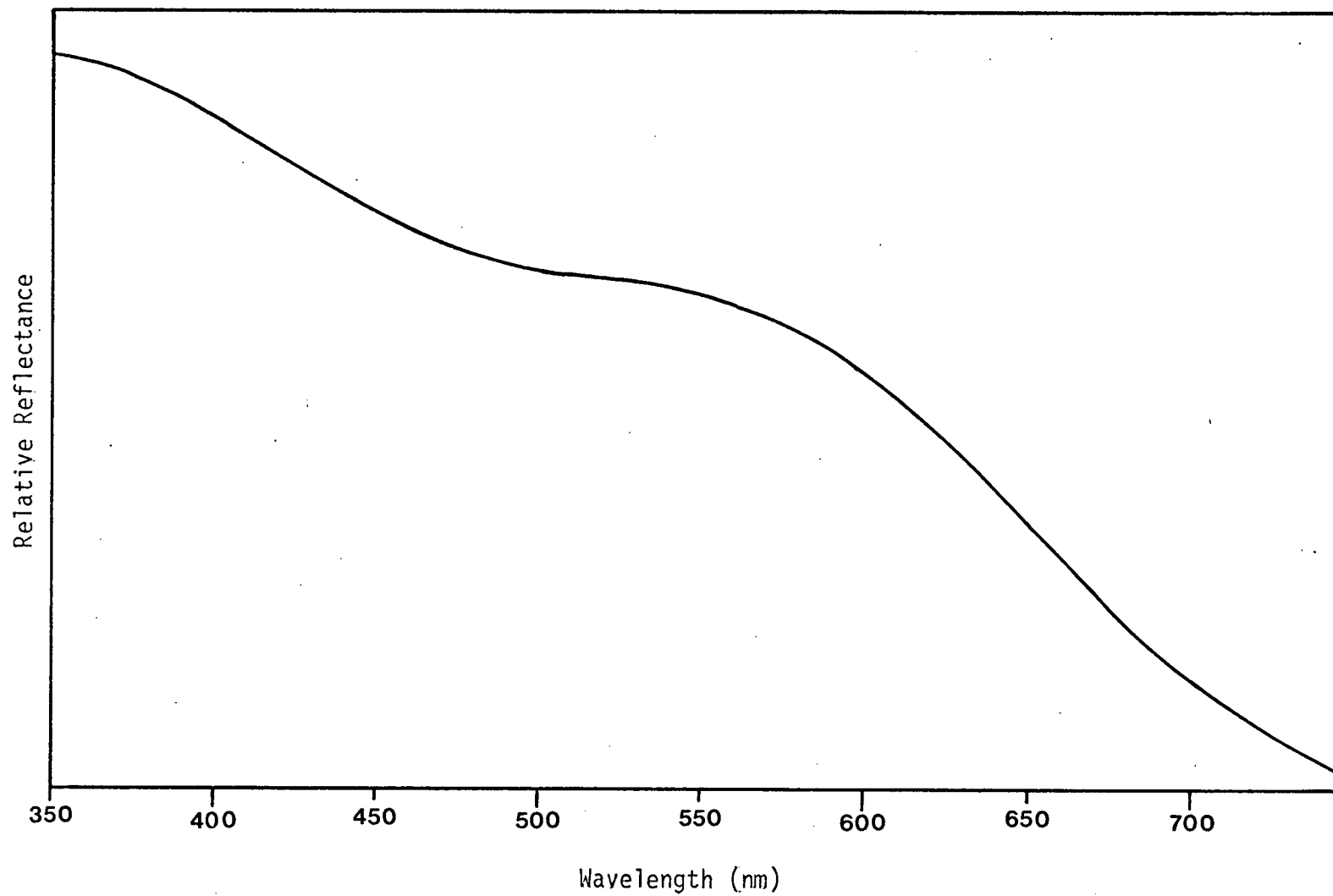
$$(CH_3O)_2C(CH_3)_2 + H_2O \longrightarrow 2CH_3OH + CH_3COCH_3$$

Figure 4.4   Diffuse Reflectance Spectrum of $[Co(3-H-TAP)_6](ClO_4)_3$

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The solutions of the solvated metal perchlorates thus obtained were used directly for reaction with the stoichiometric amount of 3-H-TAP.

Infrared spectra of the 3-H-TAP complexes are shown in Figure 4.5. In general, the infrared spectra of the complexes are not particularly different from those of the free s-triazolo[3,4-a]phthalazine (allowing for the peaks due to water and the anion). However, some of the TAP bands change intensities on coordination or are split. A number of the vibrational modes of TAP show significant shifts to higher frequencies, and these may be characteristic of coordination of the TAP ligand. These bands are the C-H stretching modes $ca.$ 3120-3030 cm^{-1}, and also the bands at 1530, 1476, the doublet 1356 and 1352, 1184, 776, and 625 cm^{-1}.

The two absorption bands between 3600 and 3400 may be attributed to the antisymmetric and symmetric O-H stretching modes of coordinated and lattice-bound water. Analytical evidence supports the presence of lattice water in all of the complexes except $[Cu(3-H-TAP)_4(H_2O)_2](ClO_4)_2$. The broad band about 1640-1600 cm^{-1} may be assigned to the HOH bending mode. Other vibrations arising from coordinated water are not visible because of the superimposed TAP vibrations. The band structure about 630 cm^{-1} in $[Ni(3-H-TAP)_2(H_2O)_4](ClO_4)_2$ may arise from overlap of a Ni-OH$_2$ wagging vibration on a TAP ring deformation mode and the ClO$_3$ bending modes of perchlorate.

The free perchlorate ion possesses tetrahedral symmetry and belongs to the point group T_d having nine vibrational degrees of freedom distributed between four normal modes of vibration. The assignments of these modes are listed in Table 4.2.[171]

In the solid state infrared spectra of perchlorates, the non-degenerate frequency, v_1, which should be infrared inactive usually occurs
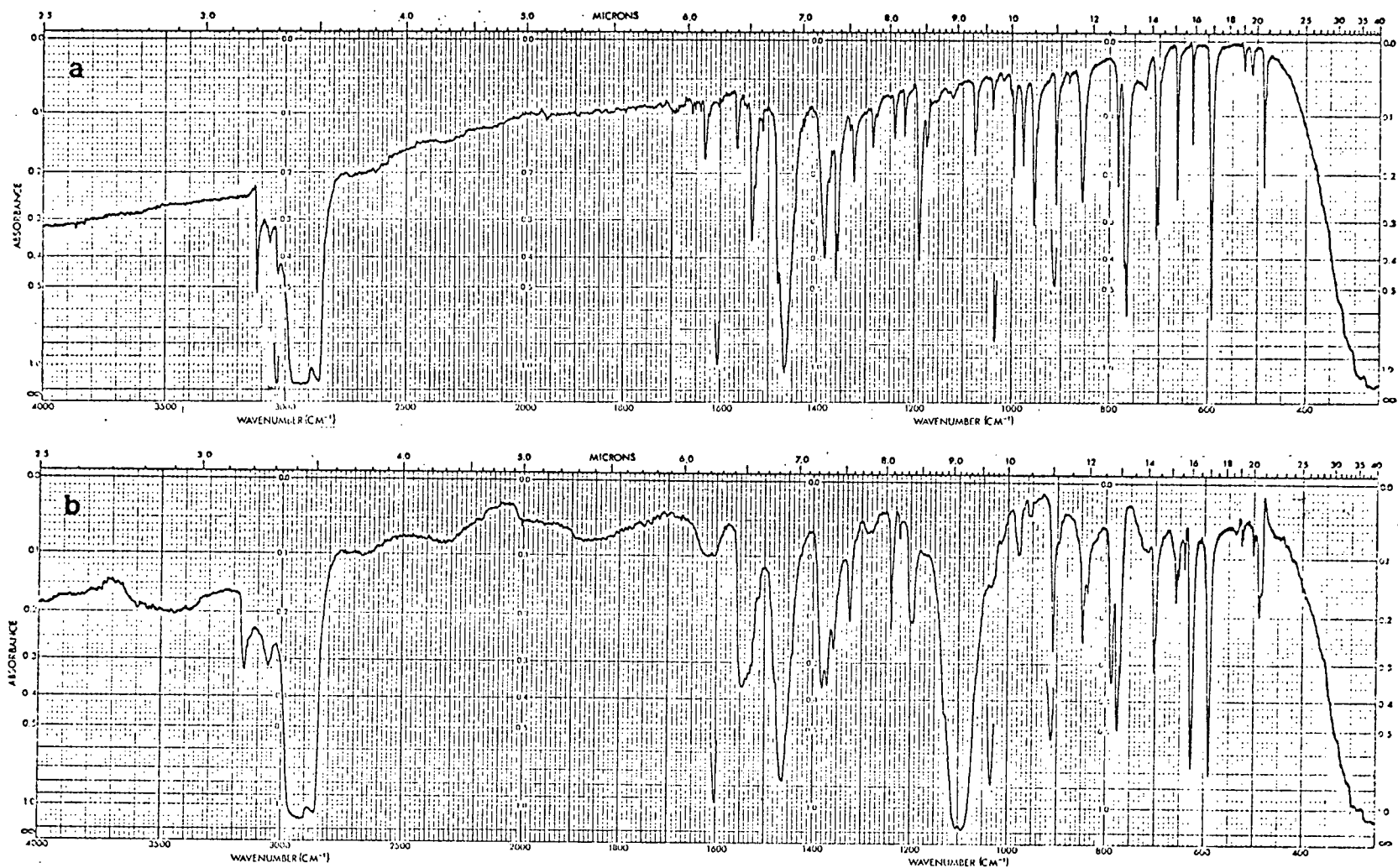
Figure 4.5 Nujol Mull Infrared Spectra of 3-H-TAP and Metal Complexes
(a) 3-H-TAP, (b) $[Co(3H-TAP)_6](ClO_4)_3 \cdot H_2O$

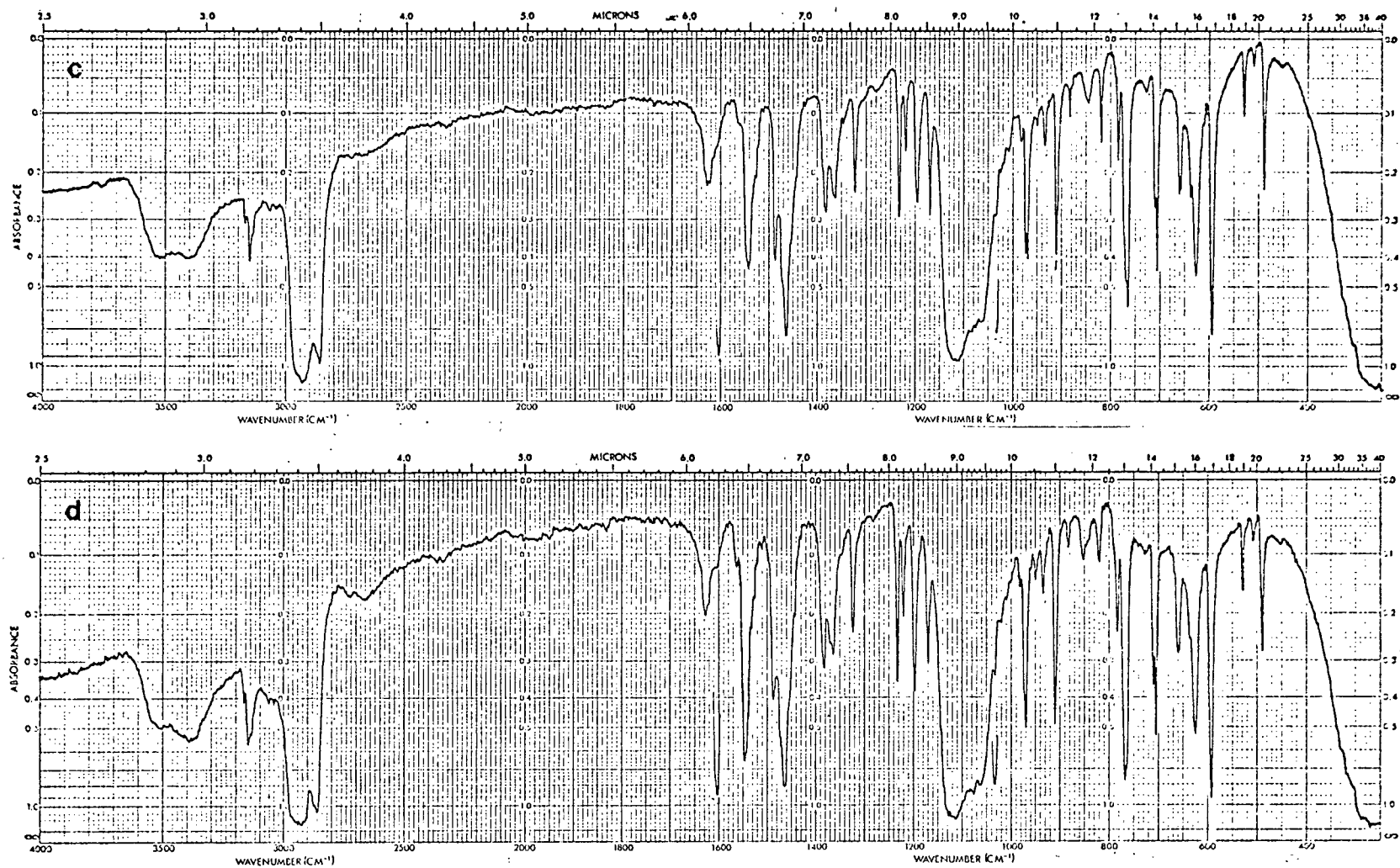Figure 4.5   IR Spectra.   (c) $[Co(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$, (d) $[Ni(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$
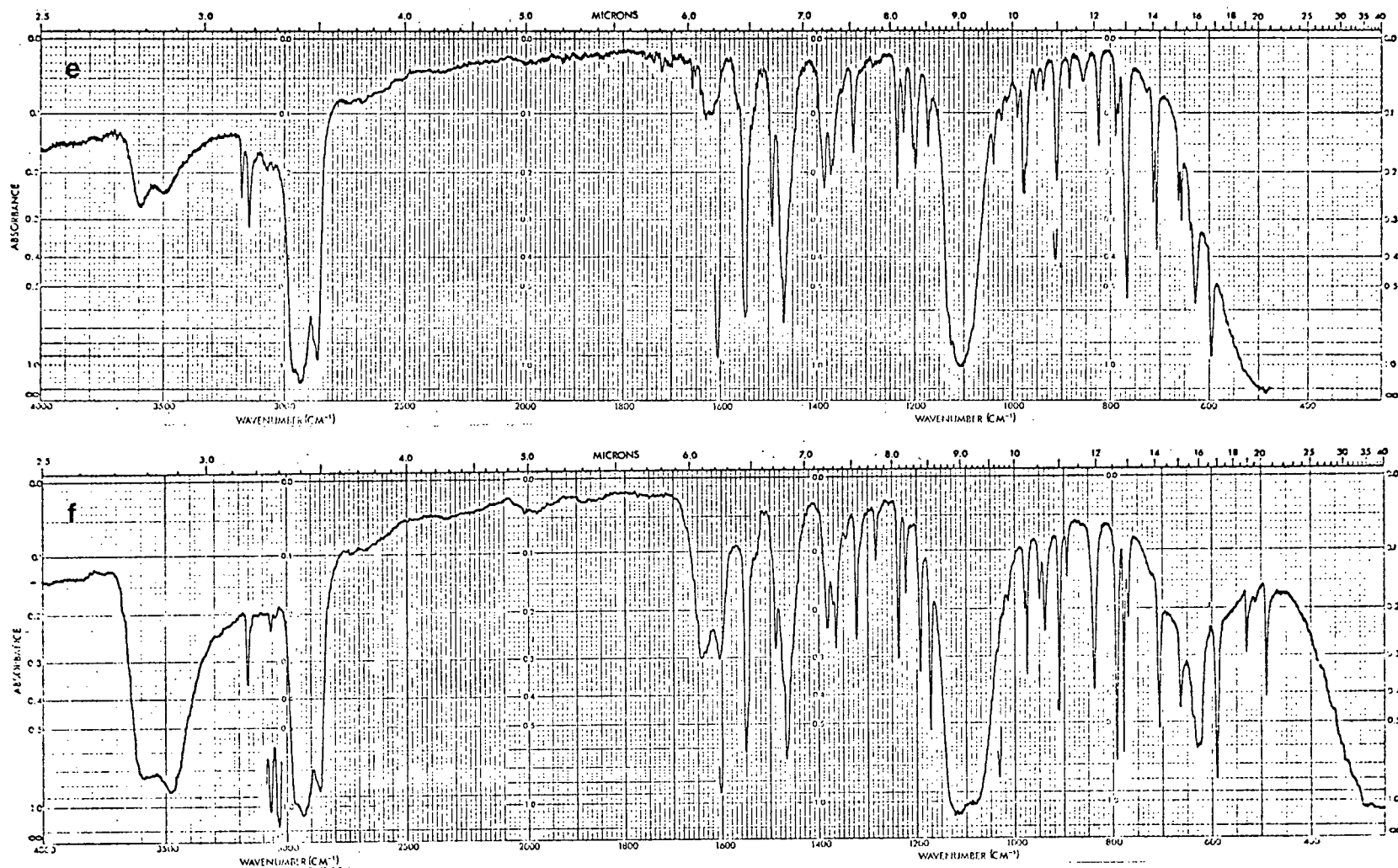
Figure 4.5   IR Spectra.   (e) $[Cu(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$, (f) $[Ni(3-H-TAP)_2(H_2O)_4](ClO_4)_2$

Table 4.3 Infrared Spectra of Perchlorate Groups (cm^{-1}) *

	ν_3		ν_1	ν_4		ν_2
[Cu(3-H-TAP)$_4$(H$_2$O)$_2$](ClO$_4$)$_2$	1100vsb		931w	621s		-
[Co(3-H-TAP)$_6$](ClO$_4$)$_3$	1093vsb		950w	622s		448vwb
[Co(3-H-TAP)$_4$(H$_2$O)$_2$](ClO$_4$)$_2$	1110vsb	1070vsb	929w	623s		446vw
[Ni(3-H-TAP)$_4$(H$_2$O)$_2$](ClO$_4$)$_2$	1110vsb	1070vsb	928w	619s		446vw
[Ni(3-H-TAP)$_2$(H$_2$O)$_2$](ClO$_4$)$_2$	1110vsb	1080vsb	935	626s	620s	-
[Co(py)$_4$(ClO$_4$)$_2$] [172]	1137s	1034s	931m	631m	617m	425w
ClO$_4^-$ [171]	1110		932	626		460

* Band assignments in terms of T_d symmetry
  vs, very strong. s, strong. m, medium. vw, very weak. w, weak.
  b, broad.

frequencies with those of ionic ClO$_4^-$ indicates that perchlorate is uncoordinated in the complexes studied. The lowering of the perchlorate group symmetry from T_d to C_{3v} probably arises from distortion of the anion by the crystal lattice or from interaction with water molecules with which the ClO$_4^-$ ion may form hydrogen-bonds. These effects are common in the spectra of metal perchlorates.[173] By comparison, in the tetragonally distorted [Co(py)$_4$(ClO$_4$)$_2$] where the perchlorate groups are coordinated, there is clear splitting of the ν_3 (103 cm^{-1}) and ν_4 (14 cm^{-1}) bands unlike the complexes studied here which show incomplete resolution of the split ν_3 band, and removal of degeneracy of the ν_4 band is observed in only one case.

The electronic spectra of the bis and tetrakis(3-H-TAP) complexes in the solid state were obtained by diffuse reflectance and from nujol mulls at room temperature. Solution spectra were not obtained because of the limited solubility of the complexes in appropriate solvents.

$[Co(3\text{-}H\text{-}TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$ is obtained as an amorphous tangerine solid. Nujol mull visible spectra of the complex are characterized by a weak, very broad band centered at 10.5 kK[†](950 nm) and a stronger asymmetric band at 21.1 kK (475 nm) with a shoulder about 19.1 kK (523 nm), (Figure 4.6).

Three spin-allowed transitions are expected for high-spin d^7 cobalt(II) complexes in an octahedral field, and the observed absorptions in the visible spectrum are given the assignments:

$$^4T_{2g}(F) \leftarrow {}^4T_{1g}(F) \qquad 10.5 \text{ kK} \qquad \nu_1$$

$$^4T_{1g}(P) \leftarrow {}^4T_{1g}(F) \qquad 21.1 \text{ kK} \qquad \nu_3$$
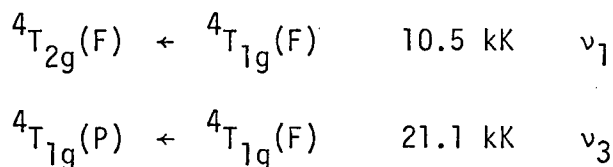
The shoulder about 19.1 kK probably arises from spin-forbidden transitions to doublet states or from low symmetry splitting of the $^4T_{1g}(P)$ term. The $^4A_{2g} \leftarrow {}^4T_{1g}$ transition is not normally observed.[174]

By application of the Tanabe-Sugano diagram and Transition Energy Ratio diagram for ions with the T_1 ground state (Figure 4.7) to the electronic spectrum, it is possible to calculate the ligand field splitting parameter, Dq, and the Racah electron repulsion parameter, B.[175] The ratio of the upper and lower bands, $\nu_3/\nu_1 = 2.0$, when fitted to a plot of transition energy ratio vs. Dq/B corresponds to

$$Dq/B = 1.50 \qquad \text{and to}$$

$$\frac{^4T_{2g}(F) \leftarrow {}^4T_{1g}(F)}{B} = 13.5$$

$$= \frac{10,500}{B}$$

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† kK = kilokayser = 10^3 cm^{-1}
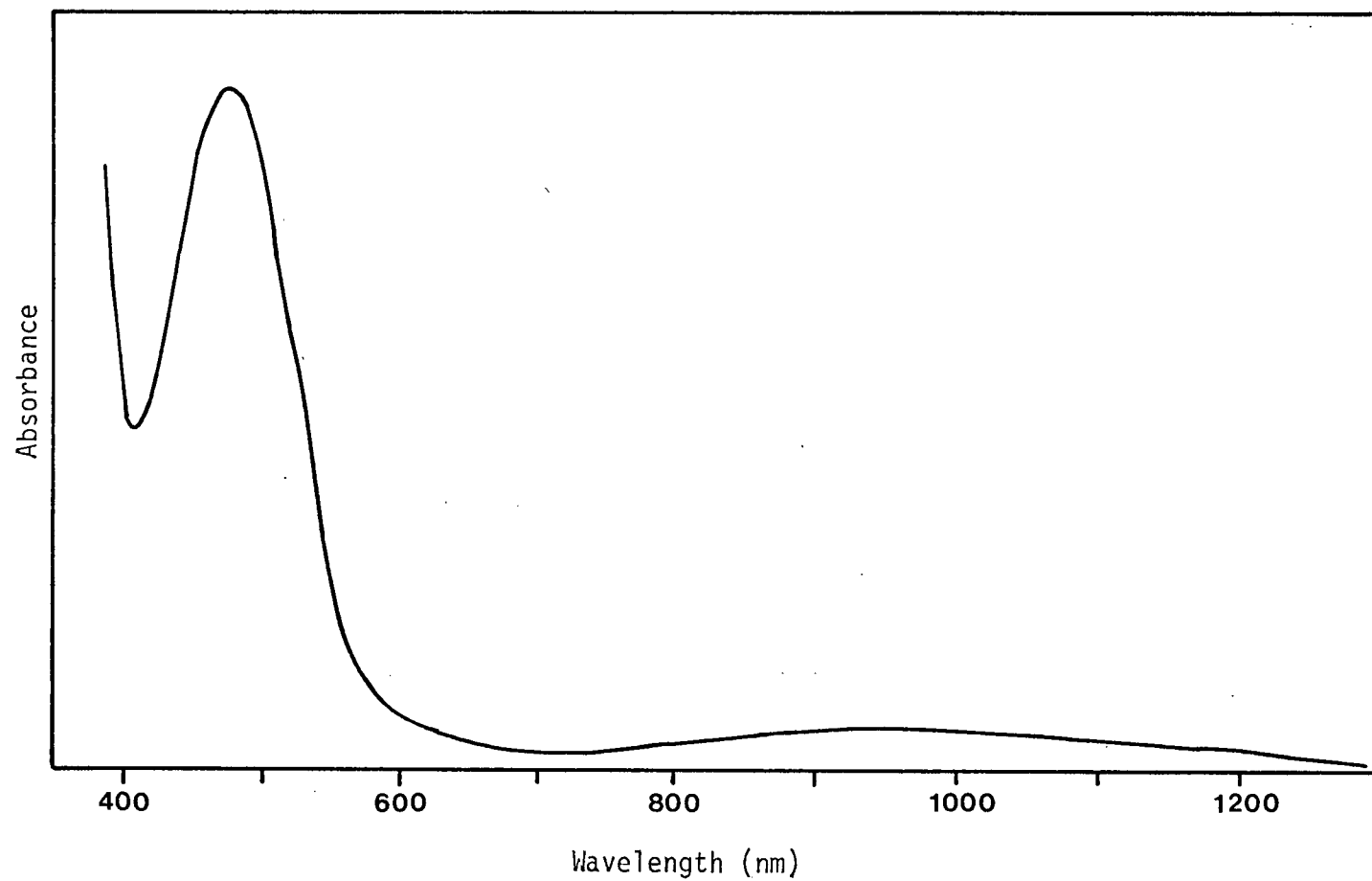
Figure 4.6   Nujol Mull Visible Spectrum of $[Co(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$
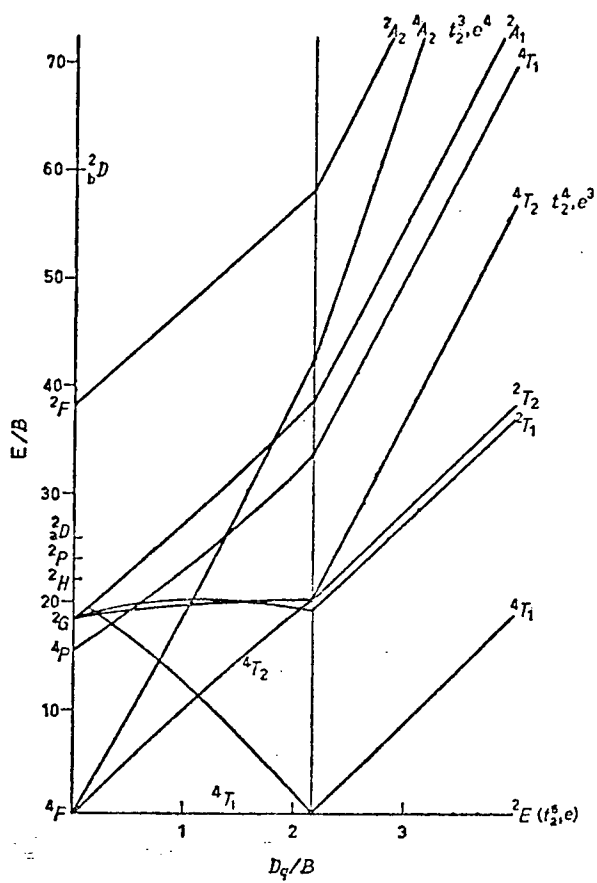
Figure 4.7(a) Energy Level Diagram (Tanabe-Sugano) for d^7 Ions in a Octahedral Field
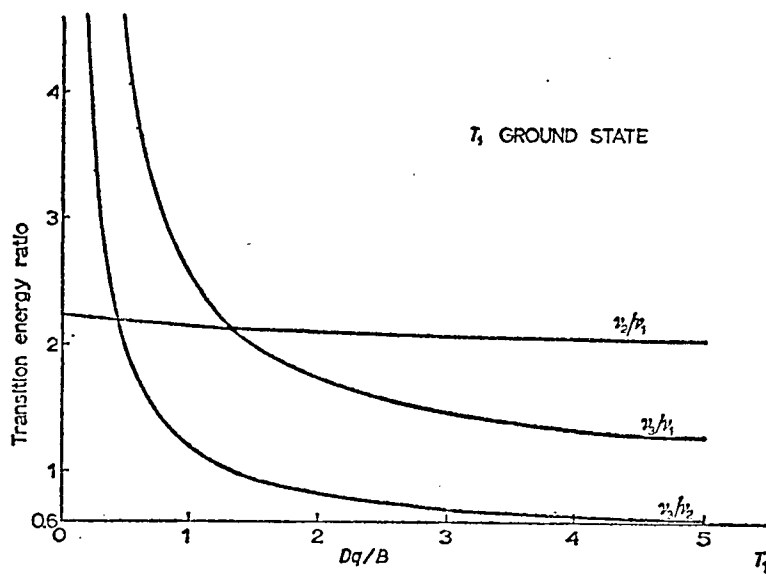
Figure 4.7(b) Transition Energy Ratio Diagram for T$_1$ Ground State

Whence, for $[Co(3-H-TAP)_4(H_2O)_2](ClO_4)_2$:

$$B = 780 \text{ cm}^{-1}$$

$$10 \text{ } Dq = 11,800 \text{ cm}^{-1}$$

Using the calculated values of B and 10 Dq the fit of the upper and lower band to the Tanabe-Sugano diagram predicts that the $^4A_{2g}(F) \leftarrow$ $^4T_{1g}(F)$ transition should be at 22.1 kK (452 nm). This transition is not observed in the visible spectrum because of the overlapping $^4T_{1g}(P) \leftarrow$ $^4T_{1g}(F)$ transition in this region.

The Racah electron repulsion parameter, B, is a function of ligand, central ion, and stoichiometry. The larger the metal ion, the smaller is the mutual interelectronic repulsion. Since the size of the ion is related to the effective nuclear charge experienced by the d-electrons, B is not only a measure of size, but also of effective nuclear charge. A comparison of the B value for the Co(II) complex with that of the free ion $(B_o = 971 \text{ cm}^{-1})$ shows a reduction in B upon complexation. A mechanism responsible for this effect is covalency in the metal-ligand bond. Thus, the greater the reduction in B, as represented by the ratio

$$\frac{B \text{ in complex}}{B \text{ in free ion}} = \frac{B}{B_o} = \beta$$

the greater the covalency in the metal-ligand bond. The series obtained for β with different ligands is called the nephelauxetic series. The ligand field and nephelauxetic parameters for octahedral cobalt(II) complexes are shown in Table 4.4.

Table 4.4 Ligand Field and Nephelauxetic Parameters for
Octahedral Cobalt(II) Ions *

Complex	$10\ Dq(cm^{-1})$	$B(cm^{-1})$	β
$[Co(3\text{-}H\text{-}TAP)_4(H_2O)_2]^{2+}$	11,800	780	0.80
$[Co(H_2O)_6]^{2+}$	9,200	825	0.85
$[Co(NH_3)_6]^{2+}$	10,200	885	0.91
$[Co(DMSO)_6]^{2+}$	8,480	824	0.85
$[Co(PyO)_6]^{2+}$	10,195	766	0.79
$CoCl_2$	7,640	775	0.80
$CoBr_2$	6,490	786	0.81

* Parameters for all complexes, except those of the TAP complex are taken from reference 176.

The covalency of the metal-ligand bonds in $[Co(3\text{-}H\text{-}TAP)_4(H_2O)_2]^{2+}$ thus appear to be similar to complexes containing Cl^-, Br^-, and pyridine-N-oxide.

The ligand field parameter, $10\ Dq(\Delta)$ is a measure of the splitting of the t_{2g} and the e_g d-orbitals of a metal ion in an octahedral field. For a given metal, and stereochemistry, a spectrochemical series of the ligands can be established. There may be a number of factors such as polarizability, dipole moment, ligand charge or electronegativity which make up the 'strength' of a ligand, and this makes it difficult to rationalize the order of ligands in the series. However, the ligand field parameter for the TAP complex probably reflects the extent of π-bonding of the ligand with the metal. In a mixed ligand complex such as $[Co(3\text{-}H\text{-}TAP)_4(H_2O)_2](ClO_4)_2$ it is appropriate

to apply the law of average environment, which states that the 10 $Dq(\Delta)$ value is given by the weighted average of the 10 Dq values for each of the individual liquids. Thus, for the complex [MA$_4$B$_2$] we have

$$\Delta = 1/6[4\Delta^A + 2\Delta^B]$$

Using the appropriate Δ values from Table 4.4 we obtain a Δ for [Co(3-H-TAP)$_6$]$^{2+}$ of 13,100 cm^{-1}. When comparing the parameters derived from the visible spectrum of the TAP complexes, it must be remembered that the symmetry is not O_h, but actually closer to D_{4h}, and thus the ligand field and nephelauxetic parameters are only approximations.

The pale blue [Ni(3-H-TAP)$_4$(H$_2$O)$_2$](ClO$_4$)$_2$ shows two clearly resolved bands in the nujol mull electronic spectrum at 11.2 kK (890 nm) and 17.7 kK (564 nm) (Figure 4.8). The nickel(II) ion has a 3d^8 valence electron configuration which gives rise to a ^{3}F ground term. In an octahedral crystal field, the degeneracy of the ^{3}F term is removed, and the visible spectrum involves three spin allowed transitions from the ^{3}A$_{2g}$(F) state to the ^{3}T$_{2g}$(F), ^{3}T$_{1g}$(F), and ^{3}T$_{1g}$(P) levels. The bands observed are assigned as follows:

$$^3T_{2g}(F) \leftarrow {}^3A_{2g}(F) \qquad 11.2 \text{ kK} \qquad \nu_1$$

$$^3T_{1g}(F) \leftarrow {}^3A_{2g}(F) \qquad 17.7 \text{ kK} \qquad \nu_2$$
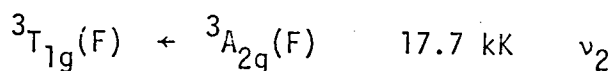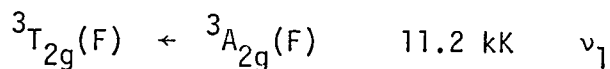
The highest energy transition (ν_3) ^{3}T$_{1g}$(P) $\leftarrow$ ^{3}A$_{2g}$(F) appears as a shoulder about 27.8 kK (360 nm). A weak absorption at 13.7 kK (730 nm) probably arises from a spin-forbidden transition to the ^{1}F$_g$ level.
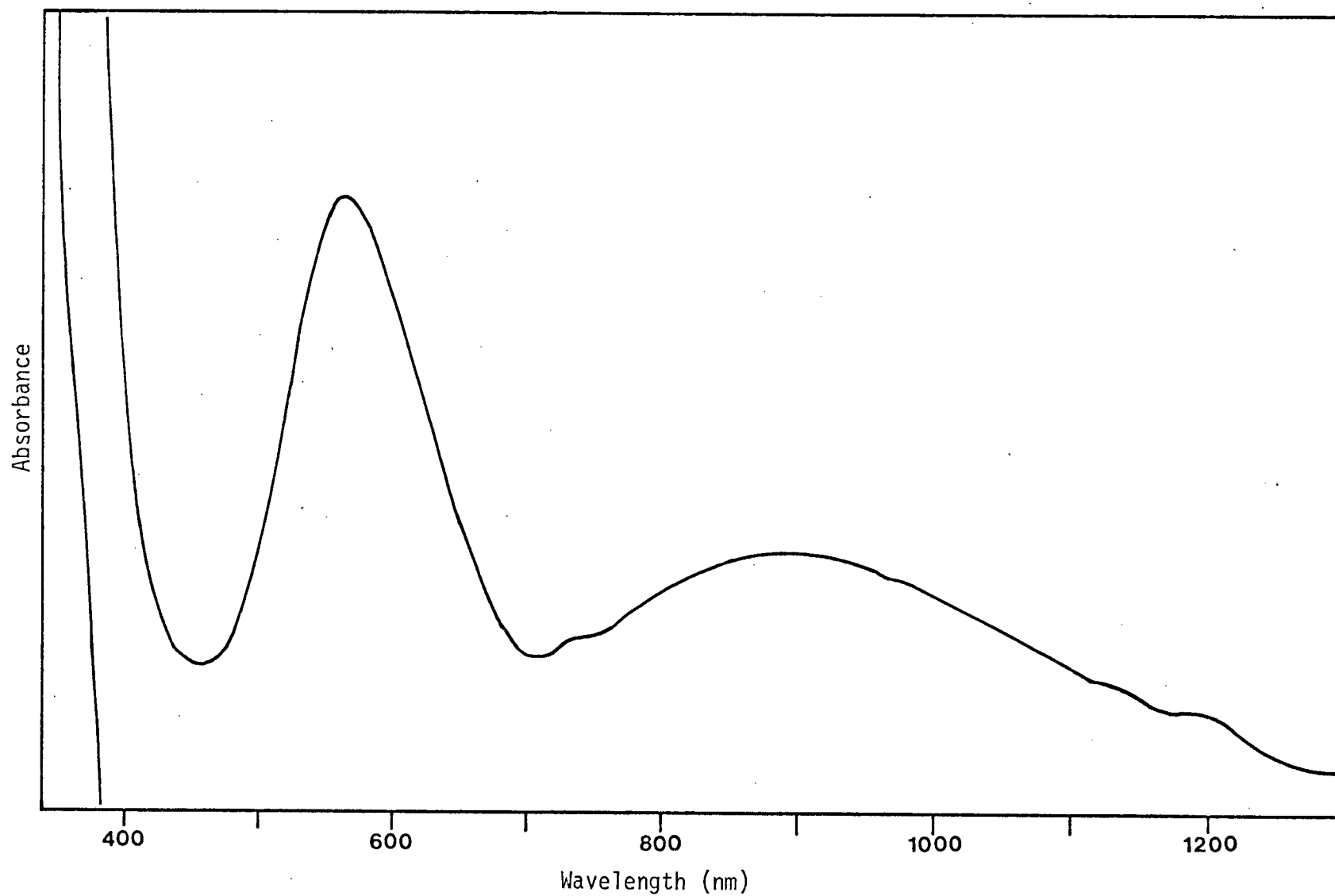
Figure 4.8   Nujol Mull Visible Spectrum of $[Ni(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$

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The accuracy of the nephelauxetic parameter, B, calculated from incomplete spectral data may be suspect. Thus, using the Tanabe-Sugano diagram for A_2 ground state ions and the appropriate transition energy ratio diagram (Figure 4.9)[175] and the ν_1 and ν_2 bands, we obtain $B = 826$ cm^{-1} and predict that ν_3 [$^3T_{1g}(P) \leftarrow {}^3A_{2g}(F)$] should appear at 28.4 kK (352 nm). This shows reasonable agreement with the shoulder about 27.8 kK observed in the visible spectrum. A problem with using the ν_1 and ν_2 bands to calculate the electron repulsion parameter is that a comparatively small error in measuring the band maxima can cause quite a large change in the calculated value of B. In a mixed ligand complex such as [Ni(3-H-TAP)$_4$(H$_2$O)$_2$] (ClO$_4$)$_2$ the lowered symmetry from O_h may be reflected in a splitting of the ν_1 band, and possibly of the ν_2 and ν_3 bands as well. These splittings are another source of error. However, the visible spectrum of the TAP complex shows little splitting of these absorption bands, indicating that distortion from O_h symmetry is small. By comparison, in the D_{4h} molecules [Ni(py)$_4$(ClO$_4$)$_2$] or [Ni(py)$_4$(SO$_3$F)$_2$], definite splitting of all absorption bands are observed.

The crystal field parameter, 10 Dq, is equal to the energy separation between the $^3A_{2g}(F)$ and the $^3T_{2g}(F)$ levels. The 10 Dq values for various nickel(II) complexes are compared in Table 4.5.

Applying the law of average environment using the data from Table 4.5 the calculated 10 Dq for [Ni(3-H-TAP)$_6$]$^{2+}$ is 12,550 cm^{-1}.

The nujol mull electronic spectrum of [Cu(3-H-TAP)$_4$(H$_2$O)$_2$] (ClO$_4$)$_2$ consists of a single asymmetric band centered at 18.3 kK (546 nm) which spans the entire visible region (Figure 4.10).

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Figure 4.9(a)   Energy Level Diagram (Tanabe-Sugano) for d^8
Ions in an Octahedral Field
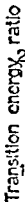
Figure 4.9(b)   Transition Energy Ratio Diagram for Ions with the A$_2$
Ground State
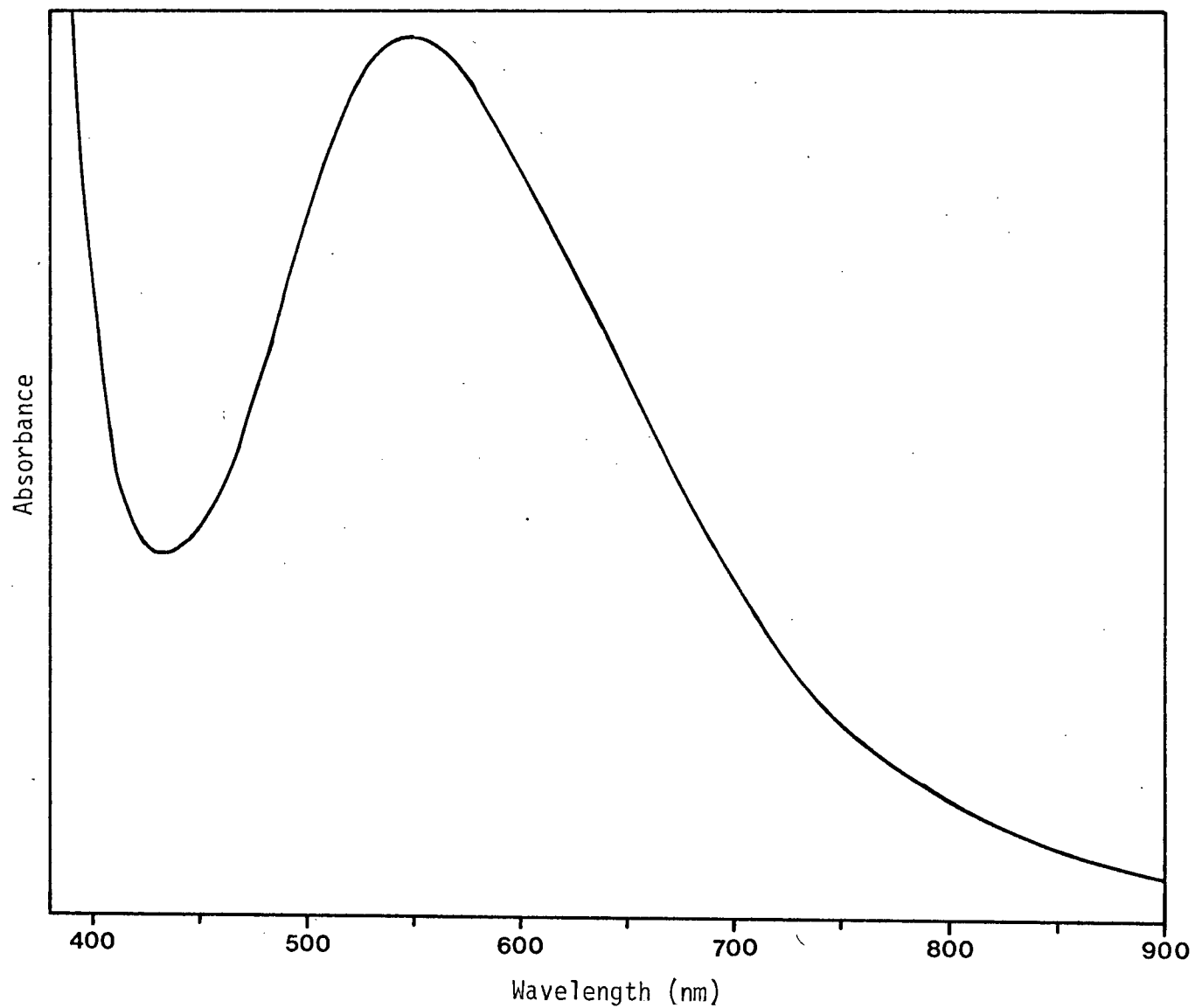
Figure 4.10   Nujol Mull Visible Spectrum of $[Cu(3-H-TAP)_4(H_2O)_2](ClO_4)_2$

Table 4.5 Crystal Field Parameters for Octahedral Nickel(II) Derivatives *

Complex	$10\ Dq(cm^{-1})$
$[Ni(3-H-TAP)_4(H_2O)_2]^{2+}$	11,200
$[Ni(H_2O)_6]^{2+}$	8,500
$[Ni(CH_3OH)_6]^{2+}$	8,430
$[Ni(NH_3)_6]^{2+}$	10,800
$[Ni(DMSO)_6]^{2+}$	7,730
$[Ni(PyO)_6]^{2+}$	8,400
$[Ni(CH_3NH_2)_6]^{2+}$	10,000
$[Ni(CH_3CN)_6]^{2+}$	10,700
$[Ni(Bipy)_3]^{2+}$	12,650
$[Ni(Bipyz)_3]^{2+}$	12,900

* Values taken from ref. 178 except for 3-H-TAP complex.

In an octahedral cubic ligand field, the five-fold degenerate 3d-orbitals split into the lower t_{2g} orbitals and the upper e_g orbitals. The single unpaired electron in d^9 copper(II) could be in either of the components of the e_g state thus giving rise to a single transition $^2E_g \leftarrow \, ^2T_{2g}$ in the absorption spectrum. The Jahn-Teller effect requires any non-linear system with a degenerate ground state to undergo such a distortion as will remove the degeneracy. For copper(II), the degeneracy is often removed by elongated tetragonal distortions of the octahedron.

A single asymmetrical absorption band is frequently observed for copper(II) compounds because the relative energies of the d-d transitions involved generally occur within 5.0 kK of each other.[179]

Hathaway[180] has observed that the $^2E_g \leftarrow \,^2B_{1g}$ transition is the most intense transition, and lies at the higher energies in tetragonal compounds. The band maximum at 18.3 kK (545 nm) for $[Cu(3\text{-}H\text{-}TAP)_4(H_2O)_2]$ therefore probably represents a close approximation to the $^2E_g \leftarrow \,^2B_{1g}$ transition with the remaining transitions hidden on the low energy side of the band to give an asymmetric band shape.

The facile synthesis and spectral properties of transition metal complexes containing the 3-H-TAP ligand indicated that the ligand possesses a fairly strong and specific coordination to metal ions. We therefore turned our attention to the bidentate coordination of the heterocyclic N-2 nitrogen of TAP and the amide nitrogen of a side-chain at the C-3 position. In Scheme 4.3 we envisaged complexation of a metal ion with a TAP-modified peptide to give after hydrolysis, a complex with the general structure:
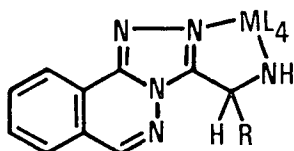
As a model for the bidentate TAP ligand, we considered that 2-amino-methylpyridine (2-AMPy) would possess similar coordination properties. Both ligands possess heterocyclic and primary amine nitrogens similarly oriented for metal coordination. By using the 2-AMPy ligand we were able to conserve the precious supply of TAP ligand during preliminary studies on establishing the protocol for the complexation reaction and isolating the product from the reaction mixture.

The reactions studied involving formation of a cobalt(III) - 2-AMPy
complex is shown in Scheme 4.4. The procedures of Bentley and Creaser[165]

$$\beta\text{-}[Co(trien)CO_3]^+ \xrightarrow{H^+} \beta\text{-}[Co(trien)(H_2O)_2]^{3+} \xrightarrow{OH^-} \beta\text{-}[Co(trien)OH(H_2O)]^{2+}$$

$$\downarrow \text{2-AMPy}$$
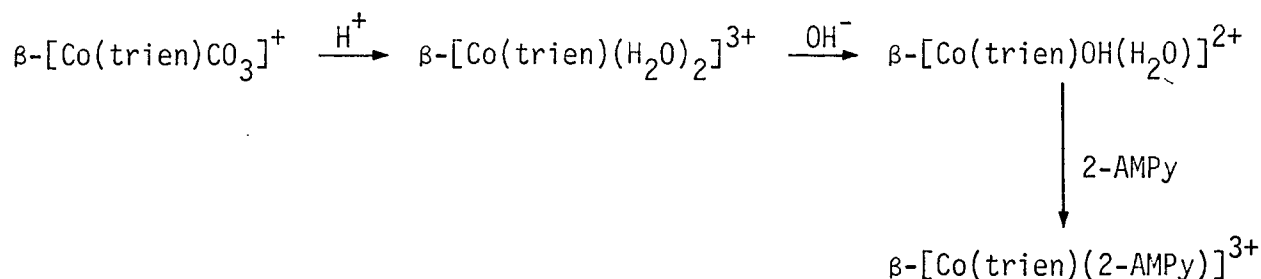
$$\beta\text{-}[Co(trien)(2\text{-}AMPy)]^{3+}$$

Scheme 4.4 Synthesis of $\beta\text{-}[Co(trien)(2\text{-}AMPy)]^{3+}$

for the formation of $[Co^{III}(trien)(amino\ acid)]$ complexes were modified to
our purposes. A solution of $[Co(trien)CO_3]ClO_4$ was acidified to form the
red diaquo complex which was then converted to the hydroxoaquo complex.
After addition of 2-aminomethylpyridine to the solution and heating to 60°C
or being left at room temperature for a couple of hours, the reaction mixture
turns orange. This is evident in absorption spectra by shifts of the two
bands visible for the hydroxoaquo-cobalt(III) complex to lower wavelength.
On heating the solution, an intense purple complex precipitates which
dissolves in chloroform, acetone, and methanol to give a blue solution, and
in water to give a blue-violet solution. Visible spectra of this complex in
various solvents are shown in Figure 4.11. No effort was made to identify
the purple complex.

After removing the purple precipitate by filtration, the components
of the reaction mixture were separated by ion-exchange chromatography on
Carboxymethyl Sephadex CM-25, eluted with sodium perchlorate solution
(0.1-1.5M). Four components were isolated by the cation-exchange column.
Visible spectra of these components are shown in Figure 4.12.
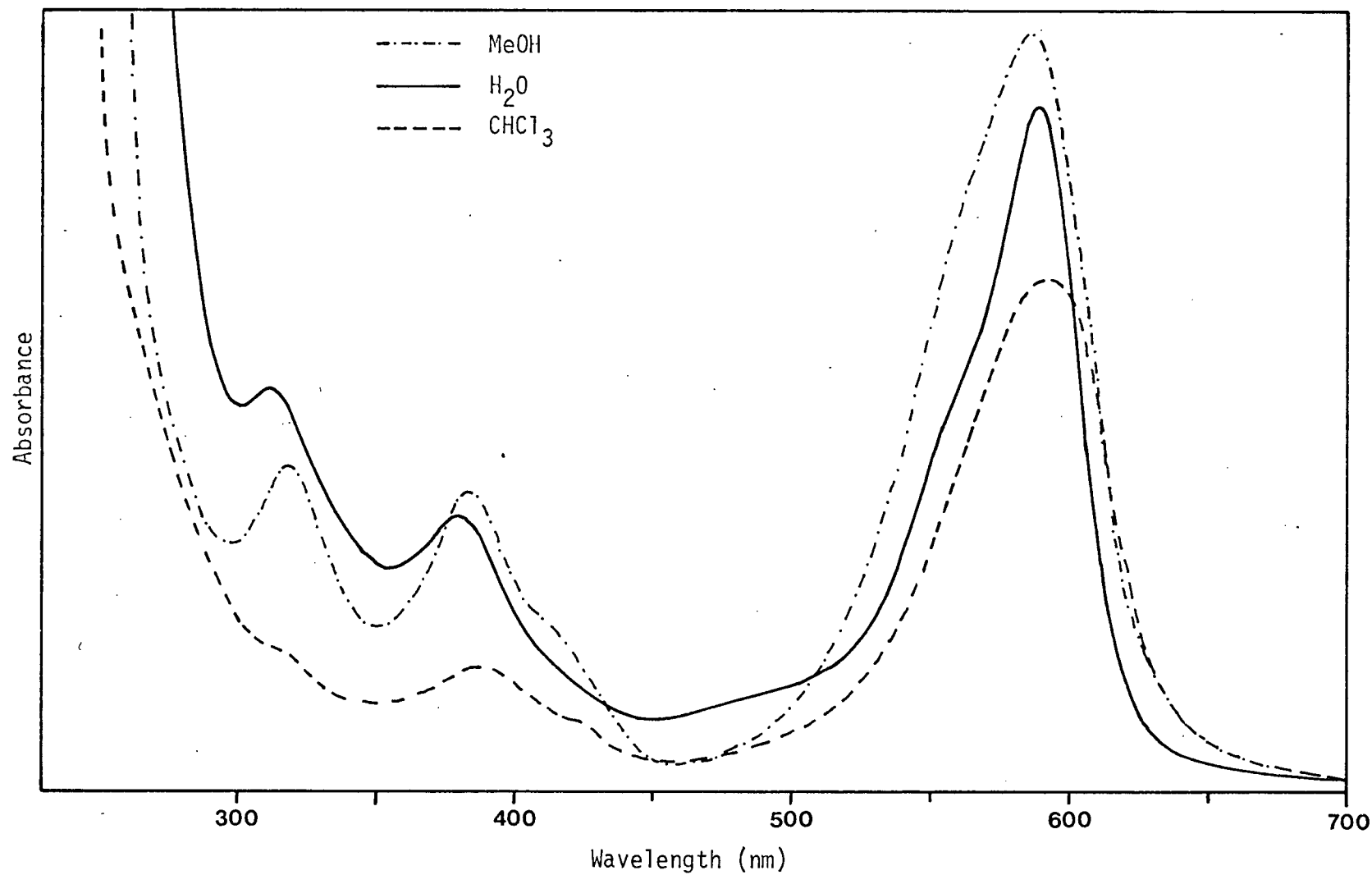
Figure 4.11   Absorption Spectra of Purple Complex from $[Co(trien)(2-AMPy)]^{2+}$ Preparation
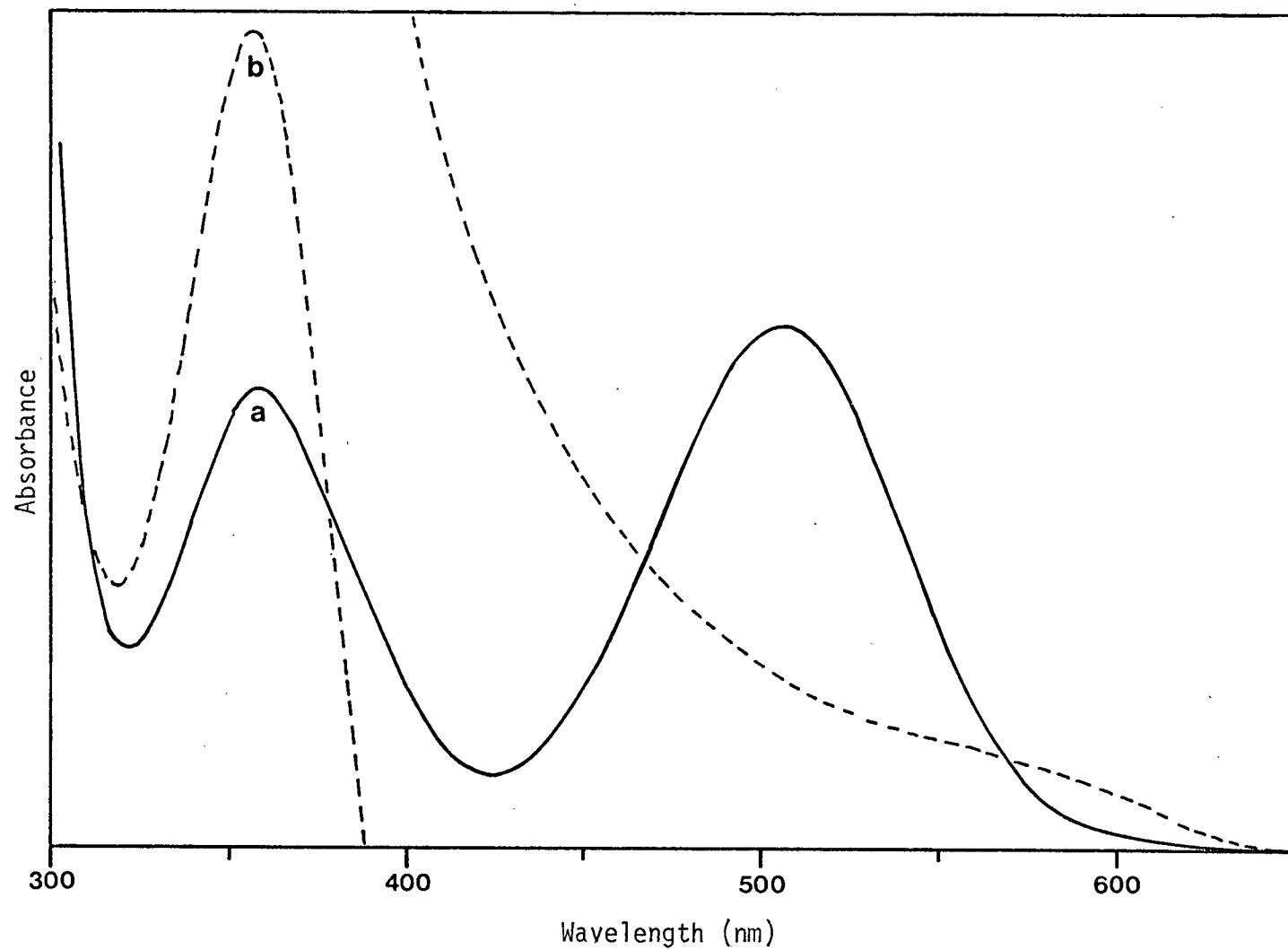
Figure 4.12   Components Separated from the Preparation of [Co(trien)(2-AMPy)]$^{3+}$ by
Ion-exchange Chromatography   (a) Red, (b) Brown
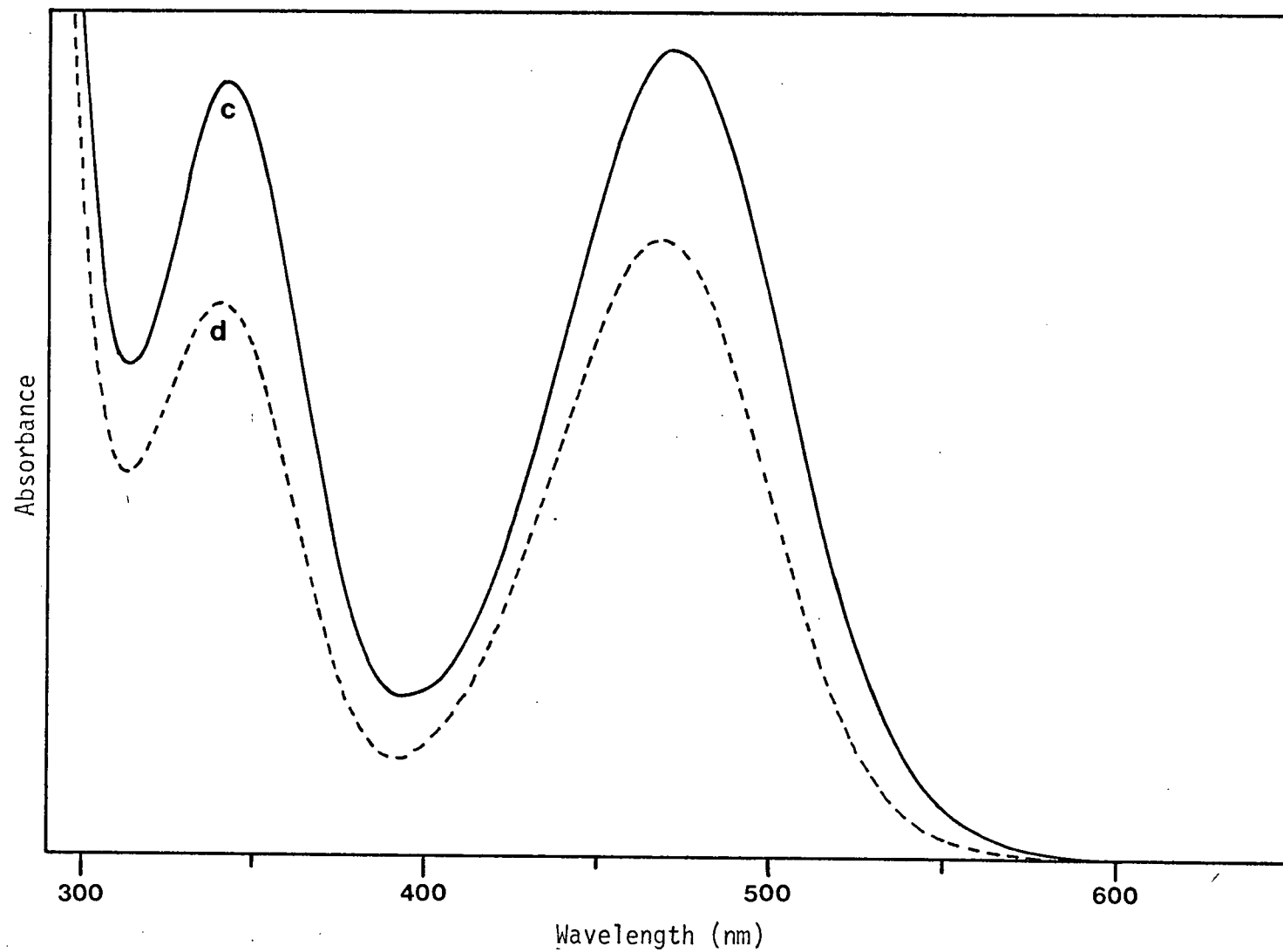
Figure 4.12 (continued). (c) 1st Orange, (d) 2nd Orange

The first complex eluted from the column as a red band with absorptions at 28.0 kK (357 nm) and 19.8 kK (505 nm) in the visible spectrum. This was probably β-[Co(trien)OH(H$_2$O)]$^{2+}$. The next component of the reaction mixture was a brown complex with a single absorption band at 28.2 kK (355 nm) in the visible spectrum. Its identity was not determined, but by its elution behaviour appears to be a 3+ charged species. Closely following the brown complex was a major orange band in the ion-exchange column which split into two well resolved components. The two orange components showed similar visible spectra, the first orange complex eluting exhibited absorption band at 29.2 kK (342 nm) and 21.2 kK (471 nm); the other orange complex showed bands at 29.5 kK (339 nm) and 21.4 kK (467 nm). These complexes were taken to be the two isomers of the β-[Co(trien)(2-AMPy)]$^{3+}$ ion:

Infrared, UV, and mass spectra of the complexes indicated the presence of both trien and 2-AMPy ligands in the orange complexes.

Chelation of 3-(N-Ac-gly)-TAP to β-[Co(trien)OH(H$_2$O)]$^{2+}$ was effected in a manner similar to that of 2-AMPy, the main difference in procedure being the addition of alcohol to the aqueous suspension to dissolve 3-(N-Ac-gly)-TAP completely. The reaction components were separated after overnight reaction by ion-exchange chromatography.

On developing an ion-exchange column with 0.1M NaClO$_4$, a red band was rapidly eluted which appeared to contain two metal components and

uncomplexed TAP. Well separated from the red bands were two orange components which were eluted with 0.2-0.3M $NaClO_4$ and showed absorption bands at 21.2 kK (472 nm) and 21.1 kK (475 nm) respectively. Other bands ∿28.2 kK (355 nm) were not resolved for both complexes because of high absorption in the UV region. UV spectra of the orange components showed band similar in shape to protonated TAP, with maxima at 41.8 kK (239 nm) and 41.5 kK (241 nm) respectively. A brown band was eluted with 0.5M $NaClO_4$ which showed an absorption at 28.5 kK (351 nm). This component did not appear to contain any TAP by its UV spectrum. In another attempt at chelation of 3-(N-Ac-gly)-TAP, only two major coloured bands were obtained - a red band which could be eluted from the ion-exchange column with pure water with visible absorptions at 28.2 kK (355 nm) and 19.8 kK (505 nm). An orange band was eluted with 0.2M $NaClO_4$ with only one resolved band in the visible region at 21.1 kK (474 nm), and a TAP-like band in the UV region at 41.8 kK (239 nm). The two orange components were not resolved in this case because a smaller column with less resolving power was used for the latter separation. Some brown material always remained firmly stuck at the top of the ion-exchange column which could not be eluted even with 1M $NaClO_4$ solution (Fig. 4.13).

By comparison with the isolation of the β-[Co(trien)(2-AMPy)]$^{3+}$ isomers, the two orange bands separated from the reaction mixture were identified as two isomers of the β-[Co(trien)(3-(N-Ac-gly)-TAP]$^{2+}$ ion:
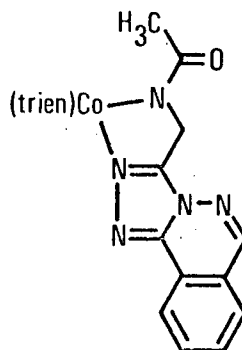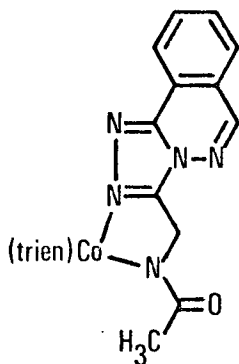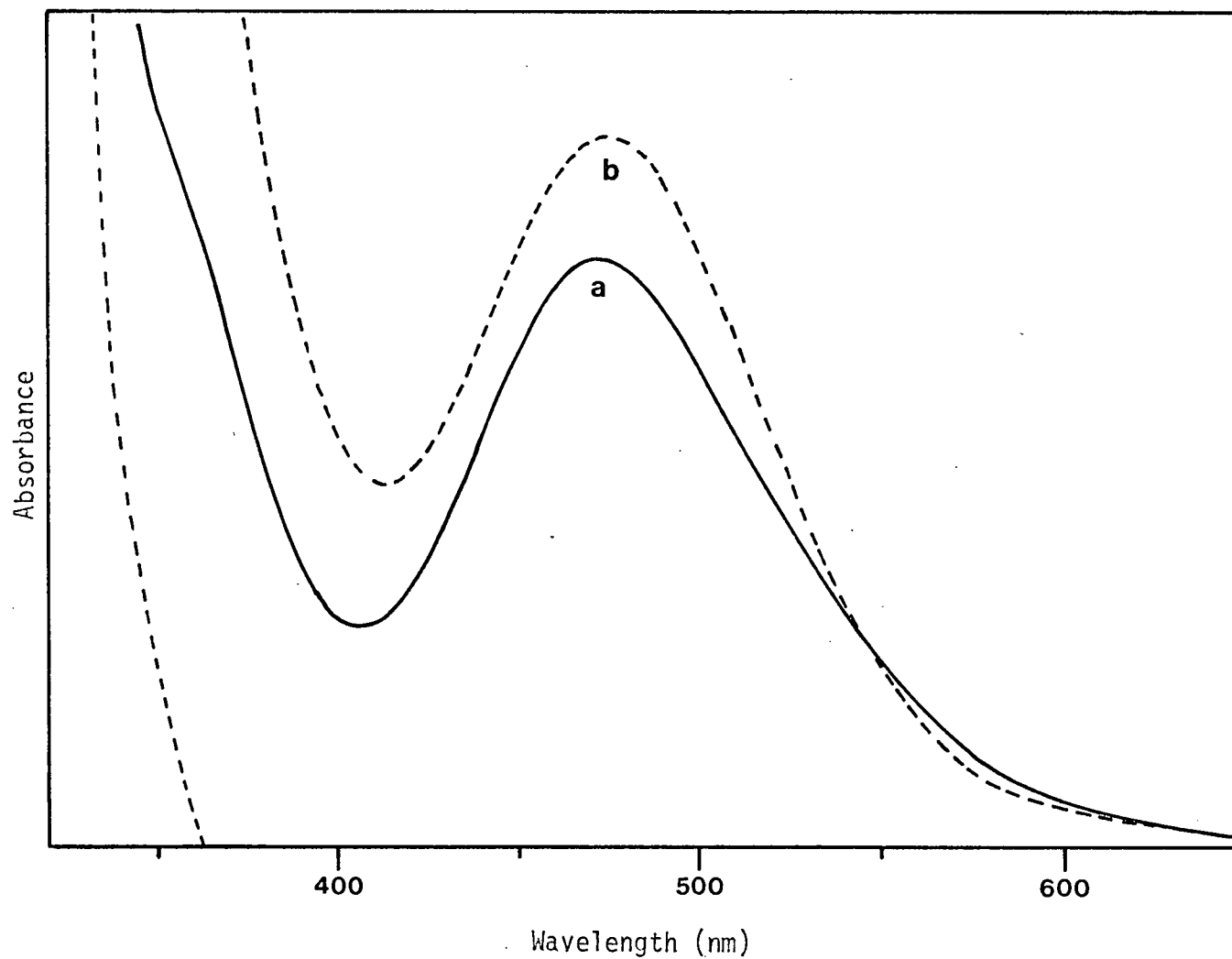
Figure 4.13   Isomers of [Co(trien)(3-(N-Ac-gly)-TAP)]$^{2+}$ Isolated by Ion-exchange
Chromatography.   (a) 1st Orange, (b) 2nd Orange

A difference between the cobalt(III) complexes of the two hetero-cyclic ligands is that the complexes with 2-AMPy are obtained as 3+ ions whereas those with 3-(N-Ac-gly)-TAP are obtained as 2+ ions. This is evident in their elution behaviour on an ion-exchange column. Sodium perchlorate solutions of concentration ca. 1.0M were needed to elute the $[Co(trien)(2-AMPy)]^{3+}$ isomers, whereas $[Co(trien)(3-(N-Ac-gly)-TAP)]^{2+}$ could be eluted with ca. 0.25M $NaClO_4$.

In $[Co(trien)(3-(N-Ac-gly)-TAP)]^{2+}$, chelation of 3-(N-Ac-gly)-TAP to the metal is expected to occur at N-2 of the triazole ring, and at the ionized amide nitrogen of the side-chain substituent. Coordination of an amide or peptide nitrogen atom to a metal ion after deprotonation is well established.[181] It seems likely that the propensity of a coordinated amide to lose a proton from the bound amido group is a function of the combined electron-withdrawing capacity of the metal ion and the C = O group.

With the conditions established for the chelation of 3-(N-Ac-gly)-TAP to a cobalt(III) complex, and the presence of the desired product demonstrated by its isolation from the reaction mixture, we next considered what would happen to the complex under acidic or basic conditions.

Hydrolysis of an amide under acidic conditions involves attack by water on the protonated amide:

$$
\begin{array}{ccccccc}
\overset{\text{O}}{\underset{\text{R-C-NHR'}}{\parallel}} & \xrightarrow{\text{H}^+} & \overset{\text{OH}}{\underset{\underset{\text{NHR'}}{|}}{\underset{\text{R-C+}}{|}}} & \xrightarrow{\text{H}_2\text{O}} & \overset{\text{OH}}{\underset{\underset{\text{NHR'}}{|}}{\underset{\text{R-C-OH}_2}{|\;+}}} & \longrightarrow & \overset{\text{O}}{\underset{\text{R-C-OH}}{\parallel}} + \text{R'NH}_2
\end{array}
$$

$$
\begin{array}{c}
\downarrow \\
\overset{\text{O}}{\underset{\text{R-C-O}^-\;\text{R'NH}_3^+}{\parallel}}
\end{array}
$$
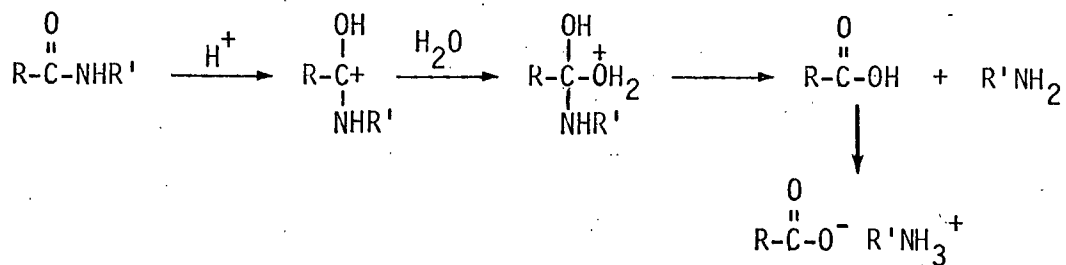
A similar mechanism may be applicable for metal complexes with a chelating TAP ligand containing an amide side-chain, e.g. the hydrolysis of [Co(trien)(3-(N-Ac-gly)-TAP)]$^{2+}$ (Scheme 4.5). The most basic site in an
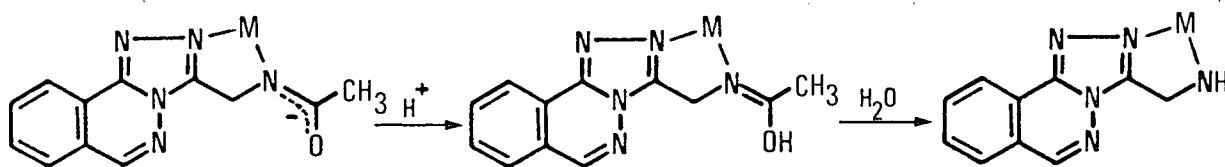
Scheme 4.5   Metal-assisted Hydrolysis of 3-(N-Ac-gly)-TAP

amide linkage is the carbonyl oxygen so that protonation occurs at that atom. This has been demonstrated in an X-ray crystal study of the protonated product of the [Co^{III}(gly-gly)$_2$]$^-$ anion (79) which showed that protonation occurred at the amide oxygen atom to form the iminol tautomer of glycylglycine, [Co^{III}(gly-glyH)$_2$]$^+$ (80).[182] Polarization of the iminol group in protonated [Co(trien)(3-(N-Ac-gly)-TAP)]$^{2+}$ by the metal ion should promote nucleophilic attack at the iminol carbon by water. In this respect, the metal ion is like a "super-proton" to enhance the hydrolysis reaction.
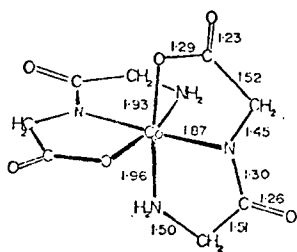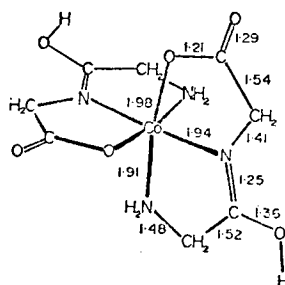
(79)   (80)

We anticipate that chelated 3-(N-Ac-gly)-TAP complex with coordinating imide nitrogen would be inactive toward base hydrolysis due to resonance stabilization of the coordinated imide group. A similar situation
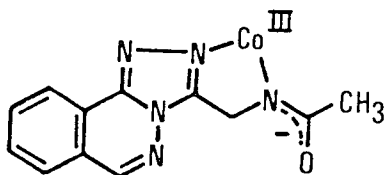
exists with Co(en)$_2$(glyNHR).[183] At high pH ($\sim$11) deprotonation of the amide nitrogen occurs, and the deprotonated species does not undergo base hydrolysis (Figure 4.2).

The peptide bond in β-[Co(trien)(Z-glyphe-H)]$^+$ (*81*) has been shown
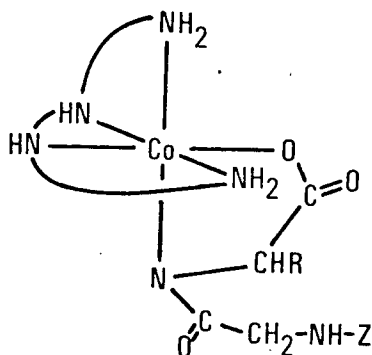
- (*81*)

to be stable under conditions (pH 7.5, 65°C) which rapidly hydrolyze the amide bond in β-[Co(trien)(glyphe)]$^{2+}$ with coordinated amino terminus and the amide carbonyl group (cf. Scheme 4.2).[155]

To investigate the metal-assisted hydrolysis of amide bonds under acidic conditions, [Co(trien)(3-(N-Ac-gly)-TAP)](ClO$_4$)$_2$ was dissolved in 0.05M HCl and incubated at 50°C. Under these conditions of acidity and temperature we expect hydrolysis of the amide bond in the side-chain

acetamidomethyl group. The reaction was checked by TLC on a sample of the solution treated with 1M NaCN which displaced the TAP ligand from the Co(III) coordination sphere. Alternatively, the Co(III) complex was reduced with solid $NaBH_4$ to the more labile Co(II) complex. Thin layer chromatograms of the ligands released by the metal were obtained after neutralization of the solution. There was no evidence of 3-aminomethyl-TAP from TLC after $[Co(trien)(3-(N-Ac-gly)-TAP)]^{2+}$ was heated for 4 hours.

In view of the apparent failure of Co(III) to facilitate the hydrolysis of side-chain amide groups in TAP derivatives we also considered hydrolysis by *in situ* formation of metal-TAP complexes.

An equimolar mixture of 3-(N-Ac-gly)-TAP and $CoCl_2.6H_2O$ solutions (0.0125M) in 1.0M HCl was incubated at 44°C. Another solution of 3-(N-Ac-gly)-TAP (0.0125M) in 1.0M HCl was also heated to 44° as a control experiment. After 3 hours at elevated temperature, the hydrolysis and control reactions were neutralized and analyzed by TLC on silica gel. The thin layer chromatograms showed that most of the 3-(N-Ac-gly)-TAP remained unhydrolyzed with only a little $3-NH_2CH_2$-TAP present. The identities of the TAP derivatives were confirmed by comparison with standards, and by reaction with ninhydrin. From visual inspection of the TLC's under UV light, we concluded that there was no significant difference between the hydrolysis mixture containing metal ions and the control solution.

It thus appears that under the hydrolysis reactions with $CoCl_2$ 3-(N-Ac-gly)-TAP is coordinated to the metal only at the N-2 site of the TAP moiety. At low pH, the hydrogen on the amide nitrogen of the side-chain is un-ionized and chelation does not occur, hence there is no metal-assisted hydrolysis of the amide bond. Thus, cobalt(II) ion promoted ionization of the amide hydrogens in peptides does not appear to occur below pH 10-11 (cf. pH 4-6 for copper(II) and pH 7-8 for nickel(II) ions).[184]

To ensure that chelation of 3-(N-Ac-gly)-TAP actually took place, an hydrolysis reaction was conducted with equimolar amounts of 3-(N-Ac-gly)-TAP and $CuSO_4$. The mixture was adjusted to pH 10 to ionize the side-chain amide hydrogen and then acidified to pH 0.8 for hydrolysis at 50°C. Comparison of the hydrolysis mixture with a control hydrolysis solution without Cu^{2+} after 5 hours showed that the solution contained predominantly unhydrolyzed 3-$CH_3CONHCH_2$-TAP. However, the concentration of the hydrolysis product 3-NH_2CH_2-TAP, was slightly higher in the presence of Cu^{2+} than in the control solution.

Attempts to study the hydrolysis of 3-(N-Ac-gly)-TAP under basic conditions with Co^{2+} and Cu^{2+} were frustrated by precipitation of the metal hydroxides on addition of base to neutral solutions of the metal salt and ligand.

In addition to our attempts with metal ions for assisting in the hydrolysis of amide bonds we also considered the method of Yamashita[146] in which ion-exchangers in the hydrogen form were used to effect the hydrolysis of peptides under mild conditions.

An aqueous solution of 3-(N-Ac-gly)-TAP was eluted through a chromatography column containing an excess of Dowex 50W-X8 (Bio-Rad, 200-400 mesh, 5.1 meq/dry g). The TAP compound bound strongly to the cation-exchange material and was removed from the column with saturated NaCl solution. UV spectra of the solution eluted from the column indicated the presence of protonated TAP [Figure 4.14(a)]. After neutralization of the solution, UV spectra showed the band structure characteristic of the TAP chromophore [Figure 4.13(b)] and thin-layer chromatography of the product showed only one product from ion-exchange chromatography which was identical with 3-(N-Ac-gly)-TAP standard. The product also showed no reaction with ninhydrin confirming the absence of the hydrolysis product, 3-NH_2CH_2-TAP.
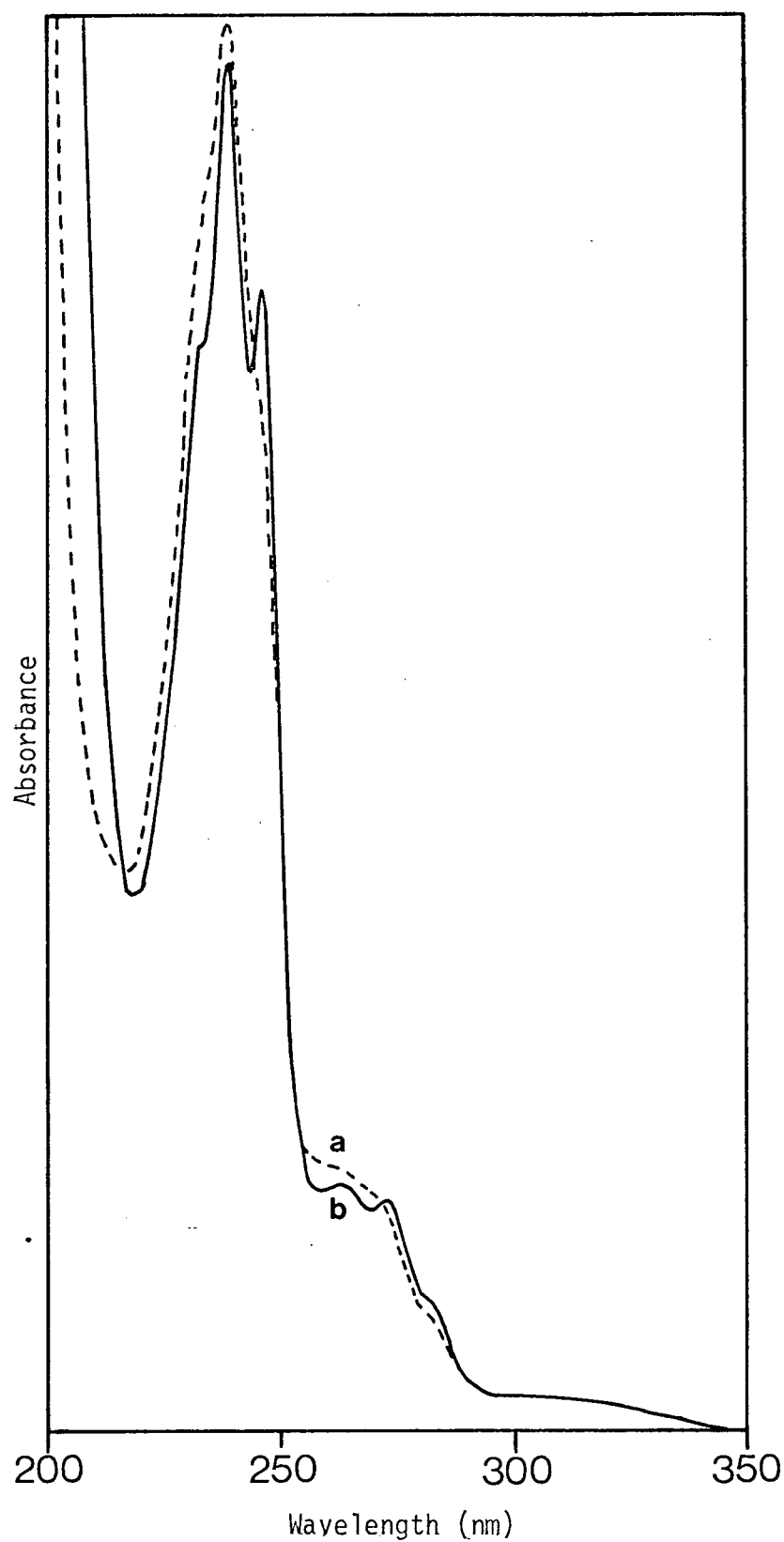
Figure 4.14 Ultraviolet Spectra of 3-(N-Ac-gly)-TAP Eluted from
Cation-exchange Resin. (a) Protonated, (b) Neutralized

From our investigations with metal ions we have shown that TAP derivatives coordinate strongly with metal ions (Co^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}) to form well defined complexes. Furthermore, Co^{3+} and Co^{2+} ions do not promote the acid-hydrolysis of amide bonds in side-chains of 3-substituted TAP compounds. Copper(II) ions show a slightly beneficial effect but not to any great extent under the conditions studied. We therefore conclude that metal-assisted acid-hydrolysis of peptide-TAP derivatives does not offer any significant advantages for selectively and mildly cleaving the C-terminal peptide bond. Relevant to our studies is the observation by Buckingham that the peptide bond in $[Co(en)_2(Z\text{-glygly})]^+$ is stable to hydrolysis in 1M $[H^+]$ over a period of weeks.[185]

Broader implications of our limited success with metal-assisted hydrolysis are that our method for modifying carboxylates with hydralazine is presently restricted to C-terminal amino acid analysis of peptides only. Extension to a sequencing method awaits development of an appropriate hydrolysis procedure.

CHAPTER 5

HYDRALAZINE DECOMPOSITION

5.1 INTRODUCTION

Hydralazine (*12*, 1-hydrazinophthalazine) is commercially available as a stable hydrochloride salt, the form in which it is usually used for most of its applications, since the free base is unstable both in the solid state and in solution.

Hydralazine is very unstable in basic solution with many factors contributing to this behavior:

a. Oxygen is necessary for the breakdown to occur.

b. pH is a critical factor in the reaction.

c. Type and concentration of ions in aqueous solution are important.[193]

Very little hydralazine decomposition occurs at neutral or acidic pH, whereas decomposition is rapid at basic pH, e.g., 30-50% decomposition in 1/2 hr. in pH 7.4 Sørensen buffer at 37°C.[40] Phosphate buffer causes more rapid disappearance of hydralazine than borate or glycine buffers,[193] but Na_2EDTA inhibits the decomposition in aqueous solution.[40] The rate of breakdown is independent of the concentration of hydralazine.

The major product obtained from degradation of hydralazine in aqueous solution is phthalazine, which is hydralazine minus its hydrazine side-chain.[40,193] Oxidation of hydralazine with oxygen in ethanol alkali or with copper sulfate at pH8 is also reported to furnish phthalazine.[194]

In apparent conflict with other work (*vide supra*), McIsaac and Kanda observed a major product which does not appear to be phthalazine.[36] Using 1-hydrazinophthalazine-1-C[14], they observed a new radioactive compound which had a much lower R_f than phthalazine on paper chromatography with n-BuOH-HOAc-H_2O(4 :1:5), and which did not give colour reactions with diazotized sulfanilic acid or *p*-dimethylaminobenzaldehyde, indicating the absence of free amine or hydrazine groups. To explain the negative results with the colour reactions, they speculated that the product might be diphthalazinylhydrazine (*82*). However, in the vast literature on hydralazine
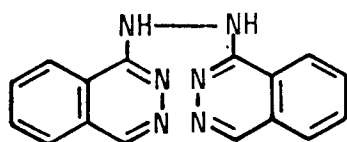
(*82*)

there is no further evidence to support this speculation.

The instability of free base hydralazine has plagued the work described in this thesis from the beginning. While hydralazine degradation in aqueous media has been amply described in the literature, nothing is reported on degradation in non-aqueous solution, or in the solid state. We therefore considered it to be a worthwhile digression to investigate the decomposition process since it would be beneficial for determining appropriate conditions for reactions involving hydralazine, and for interpreting UV and NMR spectra.

5.2 RESULTS AND DISCUSSION

The decomposition of hydralazine in the solid state is character-
ized visually by a change in colour from light lemon-yellow to dark orange.
This change occurs gradually at room temperature within minutes of the
solid being exposed to the atmosphere, and very much slower (days) *in vacuo*.
Storage of the free base below 0°C retards the decomposition but does not
stop it completely.

The decomposition rate of hydralazine in solution varies with the
solvent. To compare these rates qualitatively, the UV spectra of freshly-
prepared hydralazine in various solvents were monitored at various time
intervals. The changes in the spectra observed were band shifts, band
shapes and intensities, and the appearance of new bands.

The solvents used in this investigation were: water (pH 7),
methanol, DMSO, DMF, DMA, THF, dioxane, acetonitrile, methylene chloride,
and chloroform. Hydralazine decomposition was monitored up to 42 hours
after the solutions were prepared, and care was taken to ensure that
conditions were standardized for all the solutions. The same batch of
freshly-prepared hydralazine was used for all the degradation studies.

Some general conclusions can be drawn concerning the suitability
of certain solvents as media for reactions with hydralazine. Hydralazine
showed the greatest stability in $CHCl_3$ and CH_2Cl_2, and UV spectra of hydra-
lazine in these solvents were essentially unchanged within the time period
studied. In aqueous solution at pH 7, hydralazine showed slight decomposi-
tion, presumably due to the formation of phthalazine. The decomposition
was indicated in UV spectra by a reduction in relative intensity of the
263 nm band of hydralazine. A methanol solution showed slightly increased
absorption in the 240-250 nm region. This change was attributed to the

formation of 1,2(H)-phthalazone with supporting evidence from TLC. However, the product was not isolated for definitive confirmation of its identity. Hydralazine solutions in CH_3CN, DMSO, DMA, and DMF showed the growth of new bands at 286 nm and ~395 nm. These bands were most evident in DMF solution where the 286 nm band became the strongest band in the UV spectrum. In CH_3CN, DMSO, and DMA solutions, the 286 nm absorption was only slightly resolved above the background. Dioxane and THF solutions showed different UV band shapes from the other solutions. In the UV spectra of the solutions, an absorption at 286 nm appeared within one minute of preparation of the solution as a shoulder on a more intense 272 nm band and gradually decreased in intensity, and was replaced by a strong unresolved band ~278 nm. Dioxane solutions showed the presence of this 278 nm band from the onset and, in common with THF solutions, the 263 nm band of hydralazine decreased to a weak shoulder on the 273 nm band.

Another series of studies on hydralazine decomposition in various solvents using hydralazine HCl in the presence of excess Et_3N under an argon atmosphere gave slightly different results from the one where free base hydralazine was used with tertiary base absent. The UV spectra of the hydralazine solutions all showed the 286 nm band to varying degrees with the exception of a dioxane solution which, as before, showed a strong 278 nm band, and a THF solution which in contrast with previous results, showed no 286 nm band at all. The variability of these results serves to demonstrate that hydralazine degradation is dependent on factors other than the solvent. Thus, decomposition occurs even under an argon atmosphere, and oxygen has been reported to be essential for the degradation to occur.[193] It appears that dissolved oxygen in the solvent is sufficient. Presumably, impurities in the solvent may also play a role in the decomposition, e.g. diethylamine in DMF.

Some conclusions may be drawn regarding the stability of hydralazine in solution although different decomposition products may be formed in different solvents. The stability of hydralazine in various solvents appears to follow the order:

$$\begin{matrix} CH_2Cl_2 \\ CHCl_3 \end{matrix} > \begin{matrix} H_2O \\ CH_3OH \end{matrix} > CH_3CN > \begin{matrix} DMSO \\ DMA \end{matrix} > Dioxane > \begin{matrix} THF \\ DMF \end{matrix}$$
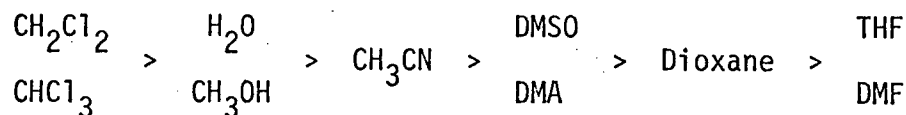
Methyl Cellosolve (2-methoxyethanol) was not included in any of the series of hydralazine decomposition studies, but hydralazine showed little tendency to decompose in this solvent, which may therefore be placed close to the chlorinated hydrocarbons in the stability order.

Of the solvents indicated above, dioxane, THF, and DMF are not recommended as media for reactions involving hydralazine. The remaining solvents are satisfactory provided that the hydralazine reaction is completed within 1 day, or that an excess of hydralazine is used in the reaction. Other solvents which are unsuitable as reaction media are morpholine and N-methyl-pyrrolidone.

The decomposition of hydralazine in aqueous and organic solvents is accelerated in the presence of a tertiary base. In addition, the degradation product responsible for the 286 nm band in the UV spectrum itself breaks down further to give a species absorbing at 278 nm in the UV spectrum. Thus, in 1,5-diazabicyclo[5.4.0]undec-5-ene (DBU) solution, the intensity of the 286 nm band grows to a maximum and then decreases which is concomitant with growth of the 278 nm band. The UV spectra showed isosbestic points at 277 nm and 291 nm. In the presence of a large excess of strong base, e.g., Et$_3$N, the species absorbing at 286 nm has a very short lifetime since it was

not observed, and UV spectra showed only the decomposition product absorbing at 278 nm. Clean isosbestic points were observed in the UV spectra.

From the multiplicity of changes seen in the UV spectra of hydralazine during solvent-induced decomposition, it is clear that the decomposition phenomenon is a complex one. Thin layer chromatography of the decomposition products from DMF solution on silica gel with $CHCl_3$/MeOH (10:1) showed at least five components, the major ones being a yellow compound at R_f0.76, and fluorescent components at R_f0.47 and 0.18.

A large-scale degradation of hydralazine was performed in DMF solution in order to identify the major decomposition products. The products were separated by preparative scale TLC on silica gel.

The major component of the decomposition was isolated as an orange amorphous solid whose UV spectrum showed strong bands at 286 nm and 393 nm. However, it was unstable in chloroform and methanol solution, and its UV spectrum changed even during the time it was being scanned. Notwithstanding these changes, the 286 nm band remained, and changes were mainly of intensity, a red shift of the higher wavelength band, and the appearance of two new bands at 255 nm and 265 nm. The UV spectrum of the major decomposition product isolated by preparative TLC, and purified by column chromatography on silica gel is shown in Figure 5.1.

A 100MHz Fourier-transform NMR spectrum of the major decomposition product freshly obtained by column chromatography is shown in Figure 5.2. The resonances between 0.8 and 2.0 ppm probably arise from degradation of the "286 nm" species, since aged solutions show new bands in this region and a growth of the bands evident in Figure 5.2.

Whilst it was difficult to obtain clean UV and NMR spectra, good mass spectra of the major decomposition product were consistently obtained.
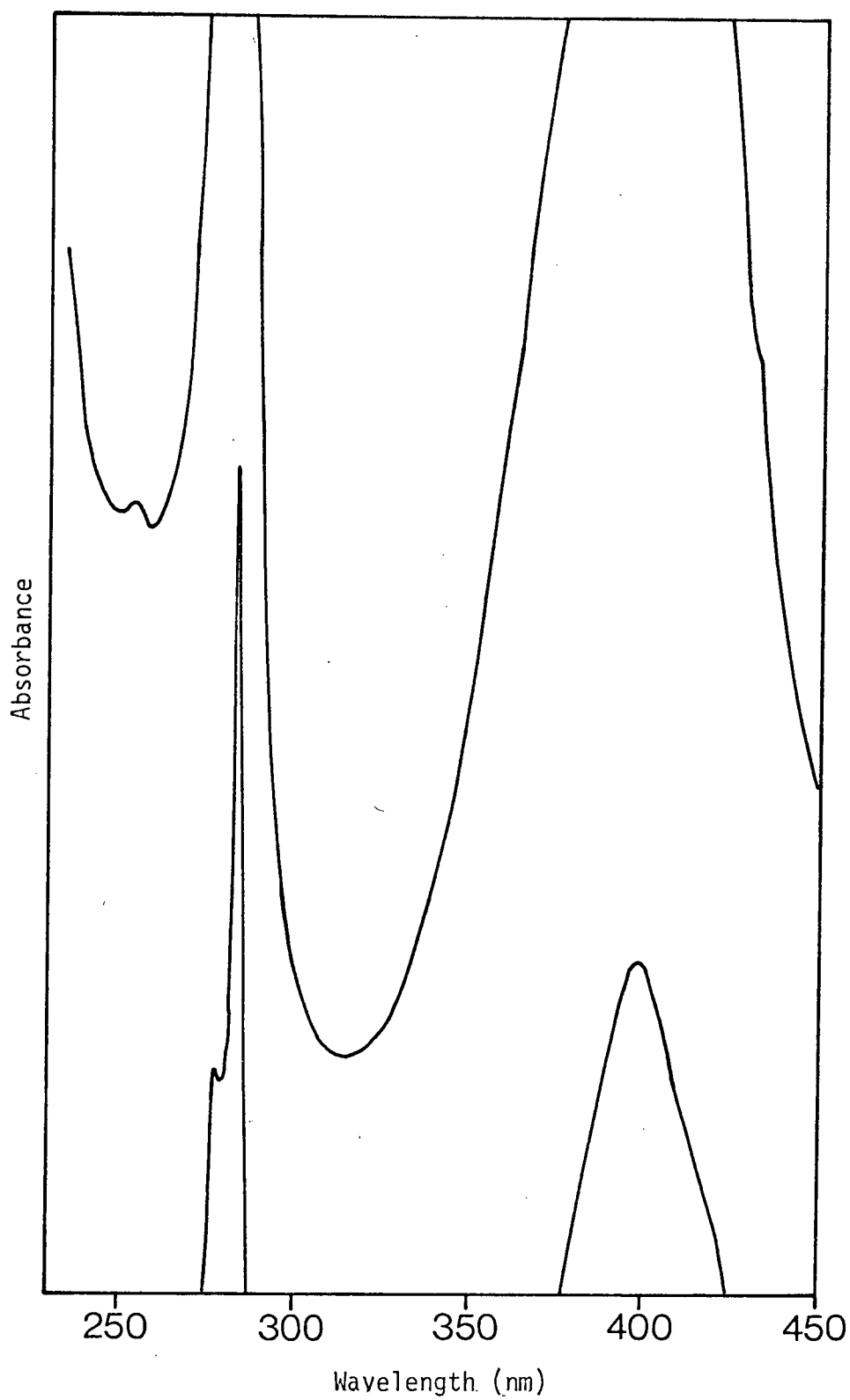
Figure 5.1 UV Spectrum of a CH_2Cl_2 Solution of the Major Product from Decomposition of Hydralazine in DMF
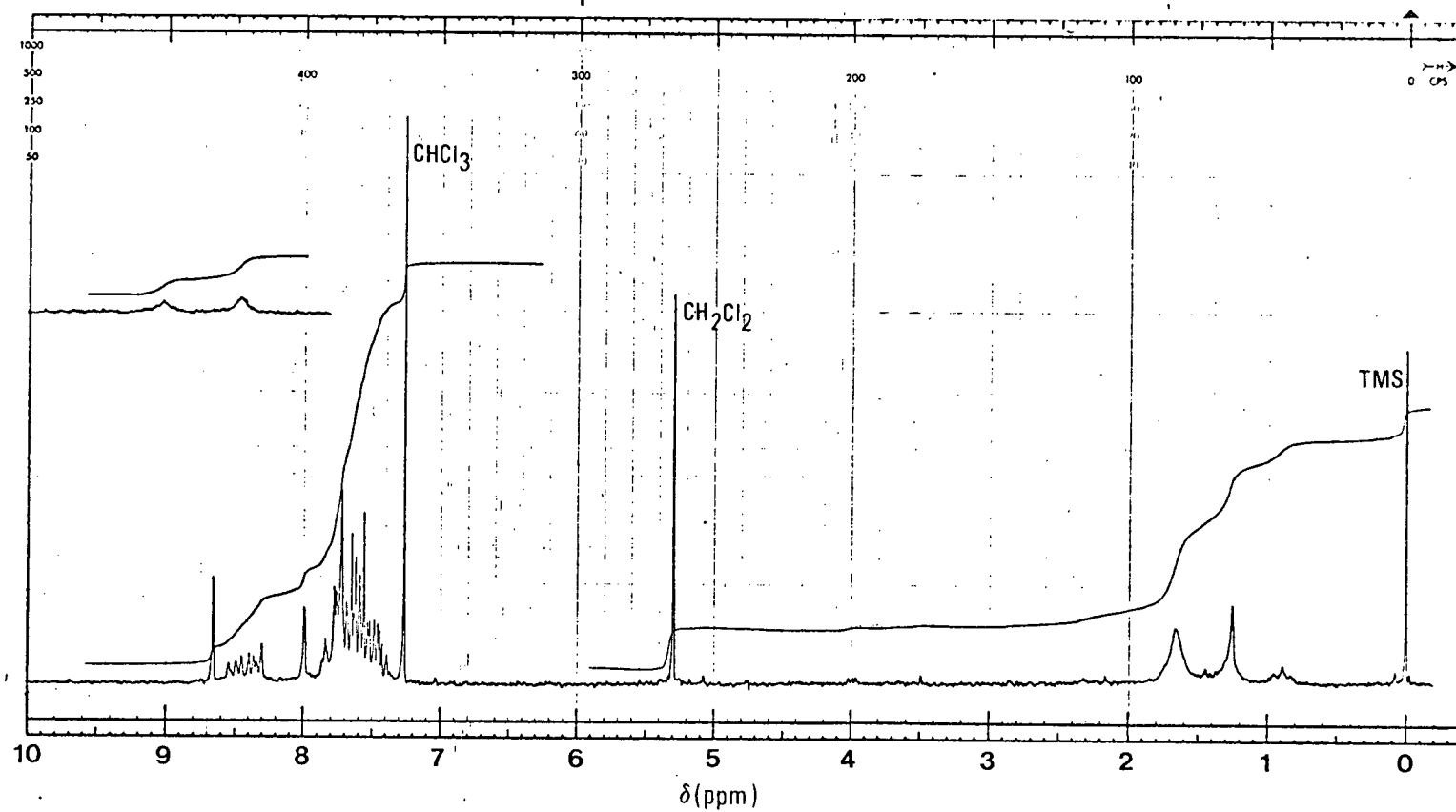
Figure 5.2   100 MHz Fourier-transform NMR Spectrum of the Major Product from Decomposition of Hydralazine in DMF

165

High-resolution mass spectra showed a parent m/e at 288.1118 indicating a composition of $C_{16}H_{12}N_6$ (calculated mass 288.1123). The low-resolution mass spectrum obtained with a probe temperature of 200°C is shown in Figure 5.3. The mass spectrum is characterized by intense fragment ions at m/e 273, 272, and 171 in the higher m/e region. At probe temperatures below 200°C, the m/e 171 ion is the base peak in the mass spectrum on the basis of high and low resolution mass spectra, the principal decomposition product of hydralazine was identified as diphthalazinylhydrazine (82). A fragmentation scheme consistent with this assignment is shown in Scheme 5.1. The fragmentation steps notated with an asterisk (*) were verified by the appearance of metastable peaks at the appropriate (m_d^2/m_p) values.

Diphthalazinylhydrazine is unstable in the solid state, and apparently undergoes air oxidation. Preparative scale TLC of the oxidized product on silica gel with $CHCl_3$/MeOH (10:1) gives two components about R_f's 0.12 and 0.22 which exhibit blue fluorescence under short wavelength UV light. These components in $CHCl_3$ solution show strong bands in their UV spectra at 280 and 267 nm, respectively. Variability of the band intensities in the UV spectra indicated that the components were not pure. The R_f 0.22 component showed fragment ions in the mass spectrum at m/e 149 (100), 167 (54), 185 (22), 200 (6), and 279 (48). The numbers in brackets correspond to the relative intensities of the ions. High-resolution mass spectrometry did not give unambiguous atomic compositions for these ions, but the parent ion at m/e 279 appears to have the composition $C_{14}H_{21}N_3O_3$ (observed 279.1569, calculated 279.1583). The mass spectrum of the R_f 0.12 component showed the same fragment ions, but with different relative intensities: m/e 149 (53), 167 (35), 185 (100), 200 (26), and 279 (37). No substantial effort was expended in attempting to obtain these secondary decomposition products in a pure state, or to determine their identities.
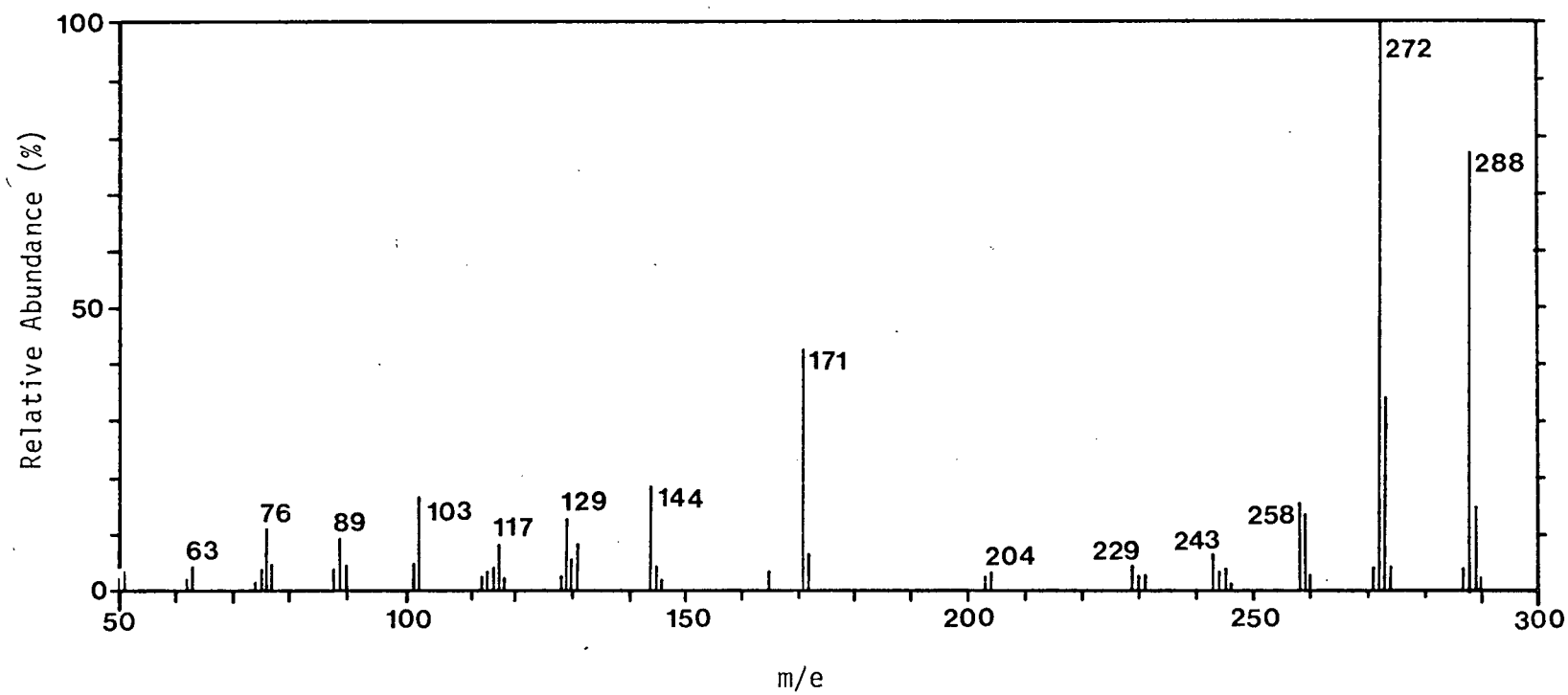
Figure 5.3   Mass Spectrum of the Major Product from Decomposition of Hydralazine in DMF
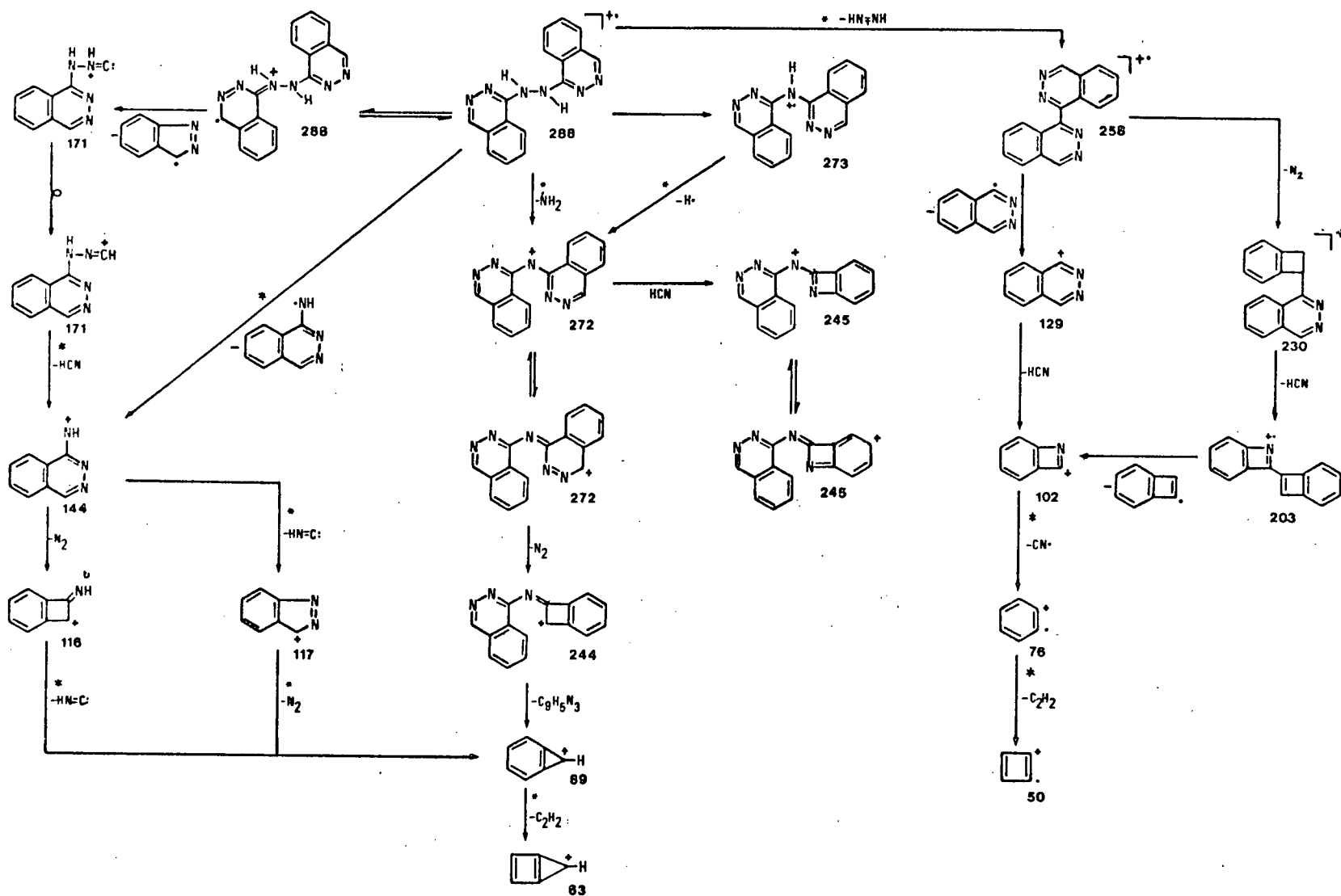
Scheme 5.1. Fragmentation Scheme for Diphthalazinylhydrazine

We have therefore shown that diphthalazinylhydrazine is a major decomposition product of 1-hydrazinophthalazine in non-aqueous solvents. It therefore appears that the speculation by McIsaac and Kanda[36] is correct after all. While we did not observe diphthalazinylhydrazine under the conditions they reported, we have been able to isolate and characterize it for the first time.

The formation of an N,N'-disubstituted hydrazine from a N-mono-substituted hydrazine is not without precedence. Isonicotinylhydrazine (*83*, Isoniazid) is oxidized at alkaline pH to give diisonicotinylhydrazine (*84*) as a major product.

CONHNH₂

[o] ⟶

CONHNHCO

(83)   *(84)*
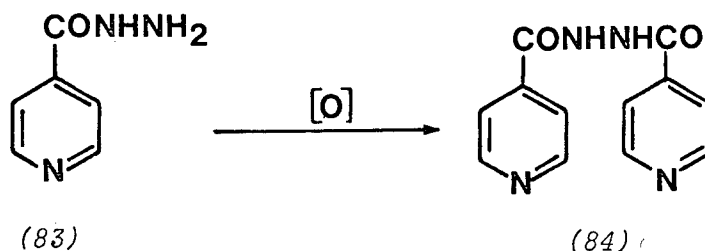
From our studies on the decomposition of hydralazine in solution we can recommend certain conditions to minimize the degradation. If free-base hydralazine is obtained by neutralization of the HCl salt, it should be freshly prepared immediately before use. CH_2Cl_2 and $CHCl_3$ should be used as solvent media whenever possible since hydralazine shows little tendency to decompose in these solvents. Methanol, water (pH 7), acetonitrile, dimethylacetamide and dimethylsulfoxide may also be used. Reactions should preferably be conducted under an inert atmosphere, since hydralazine is sensitive to oxygen, and the solutions should be de-oxygenated. An excess of strong base ought to be avoided if possible since this promotes decomposition of hydralazine. Hydralazine in excess of that required for reaction should be used to compensate for decomposed reagent if strong base cannot be avoided.

CHAPTER 6

EXPERIMENTAL

6.1 GENERAL METHODS

Electronic Spectroscopy

Cary recording spectrophotometers Model 17, 14 or 15 were used to obtain ultra-violet, visible and near-infrared spectra. Solution spectra were obtained with matched silica glass cells of 1 mm path length. Solid state mull spectra were run using nujol mulls pressed between silica windows. Light scattered by the mull was compensated for with a nujol-soaked filter paper in the reference beam of the spectrophotometer. The light intensity in the sample and reference beams were balanced by placing appropriate attenuators consisting of metal screens of varying meshes in the reference beam.

Diffuse reflectance spectra were recorded on a Bausch and Lomb Spectronic 600 spectrophotometer equipped with a visible reflectance attachment and a Sargent recorder, Model SR. Spectra were obtained over the wavelength range 740-350 nm. Magnesium carbonate was used as the reflectance standard.

Infrared Spectroscopy

Infrared spectra were recorded on a Perkin-Elmer Model 457 grating spectrophotometer covering the frequency range 4000-250 cm^{-1}. The cell windows used to contain the sample were KBr and NaCl. The cut-off points with these cells were approximately 450 cm^{-1} and 550 cm^{-1} respectively. Nujol and hexachlorobutadiene were used as mulling agents. IR spectra were calibrated with polystyrene film at 1601.4 cm^{-1} and 906.7 cm^{-1}.

Nuclear Magnetic Resonance

Nuclear magnetic resonance spectra were obtained at 60 MHz with a Varian T-60 or Varian EM-360A spectrometer, and at 100 MHz with a Varian HA-100 spectrometer for continuous-wave spectra and a Varian XL-100 or Nicolet Model NIC-80 spectrometer for Fourier-transform spectra. The chemical shifts are recorded in the δ(ppm) scale with tetramethylsilane (TMS) as an internal standard.

Mass Spectrometry

Mass spectra were recorded on an Atlas CH-4 spectrometer or an A.E.I. MS-902 spectrometer, high resolution measurements being obtained on the latter instrument.

Melting Point Determination

Melting points of solids were measured with a Thomas-Hoover capillary melting point apparatus and are uncorrected.

Elemental Analysis

Elemental analyses for carbon, hydrogen and nitrogen were performed by Mr. P. Borda of the Microanalytical Laboratory, U. B. C.

Chromatography

Column chromatography was performed using silica gel obtained from ICN Pharmaceuticals (silica gel Woelm for Adsorption, Act. I) or Baker (column chromatography grade).

Thin layer chromatography (TLC) was performed using Silica Gel GF precoated plates (Analtech-Uniplate, 250μ). Precoated plates 2000μ thick were used for preparative scale TLC. Drummond Microcap capillary tubes were used for spotting samples to analytical TLC plates. Compounds were detected by UV light (254 nm) or iodine absorption, or by spraying the plates with ninhydrin solution in ethanol followed by warming at 110°C. Unless specified otherwise, the solvent system used for developing TLC plates was $CHCl_3$-MeOH (10:1).

Ion-exchange chromatography was performed using Carboxymethyl-Sephadex CM-25 (Pharmacia) with a capacity of 4.5 meq/g.

6.2 CHEMICALS

All chemicals were reagent grade unless otherwise indicated. Spectral grade solvents were used in all solutions for ultra-violet and visible spectra.

Triethylamine

Triethylamine was purified by distillation from sodium hydroxide followed by double distillation with 2% 1-naphthyl isocyanate, and a single distillation from sodium turnings.

N,N-Dimethylformamide

N,N-Dimethylformamide was purified by distillation from anhydrous copper(II) sulfate at reduced pressure under a nitrogen atmosphere. The middle fraction boiling at 53°C at 20 nm pressure was collected and stored over molecular sieves 4A in serum-capped bottles.

Tetrahydrofuran

Tetrahydrofuran was dried by refluxing over lithium aluminum hydride for 15 hours and distilling. The fraction distilling at 64.9°C was collected. To remove peroxides, THF was eluted through a column of Alumina Brockman Activity I which had been dried at 110°C.

Hexamethylphosphoramide

Hexamethylphosphoramide (HMPA) was distilled from calcium hydride at water-aspirator pressure (b.p.~135°C) under an argon atmosphere. The distilled material was stored under argon.

Chloroform

Chloroform was refluxed over calcium hydride, fractionally distilled, and stored over molecular sieves 3A.

Ethanol [186]

Absolute ethanol (1 ℓ) was stirred with clean sodium turnings (14 g) until the sodium had reacted completely. Ethyl formate (40 g) was added and the mixture refluxed for 3 hours before being distilled. The first 25 ml of distillate was discarded. The dry ethanol was stored in a tightly stoppered bottle with Parafilm wrapped around the bottle cap.

N-Ethyl-5-phenylisoxazolium-3'-sulfonate (*17*, NEPIS, Woodward's Reagent K)[56]

NEPIS (Aldrich) was dissolved in excess 1N HCl, precipitated with acetone, filtered, washed with acetone, and dried *in vacuo* to give a fluffy white product.

Glycine Isopropyl Ester Hydrochloride,[187] $H_3\overset{+}{N}CH_2\overset{O}{\overset{\|}{C}}OC_3H_7$ Cl^-

Freshly distilled thionyl chloride (15 ml) was added slowly to a stirred suspension of glycine (15.1 g, 0.250 mol) in isopropyl alcohol (150 ml) which was maintained at 0°C. The suspension was heated to 80°C for 12 hours protected from moisture by Drierite. The clear solution thus obtained was concentrated to about 50 ml *in vacuo* and ether added until the solution became turbid. On cooling, the product crystallized from solution and was collected by suction filtration, and recrystallized twice from absolute ethanol/anhydrous ether. The twice-recrystallized product weighed 26.5 g (86%).

NMR (CDCl$_3$): 1.27 (doublet, J = 6 Hz, 6, 2CH$_3$), 3.92 (singlet, 2, CH$_2$), 5.05 (septet, 1, CH), 8.40 (broad singlet, 3, NH$_3$).

DL-Alanine Ethyl Ester Hydrochloride, $H_3\overset{+}{N}\underset{\underset{CH_3}{|}}{C}H\overset{O}{\overset{\|}{C}}OC_2H_5$ Cl^-

DL-Alanine (18.8 g, 0.211 mol) was suspended in absolute ethanol (350 ml) and dry hydrogen chloride was bubbled through the stirred suspension until no more gas appeared to be absorbed. The clear solution thus obtained was cooled to 0°C and re-saturated with hydrogen chloride. The solution was stirred at room temperature for 15 hours protected from the atmosphere with a Drierite drying tube. The solvent was removed under reduced pressure, and the resulting syrup was twice distilled with 200 ml

absolute ethanol to remove water as the alcohol-water azeotrope. On triturating with dry ether, the syrup crystallized to a white solid. Yield of crude product = 31.7 g (98%). The product was recrystallized from ethanol-petroleum ether and dried under reduced pressure over Drierite.

Glycylglycine Ethyl Ester Hydrochloride, $H_3\overset{+}{N}CH_2CONHCH_2\overset{O}{\overset{\|}{C}}OC_2H_5 \quad Cl^-$

Glycylglycine (9.98 g, 75.5 mmol) was suspended in dry ethanol (200 ml) and hydrogen chloride was bubbled through the stirred suspension. Within 10 minutes, almost all the glycylglycine had dissolved and a white precipitate appeared. Hydrogen chloride treatment was continued for 5 minutes before the reaction mixture was cooled to 0°C and resaturated with HCl for an additional 5 minutes. The mixture was stirred for 21 hours and the solid was collected by suction filtration. Another crop of product was obtained from the concentrated filtrate. The product was recrystallized from absolute ethanol in 82 % yield.

N-Butyloxycarbonyl-glycine,[188] $Me_3CO\overset{O}{\overset{\|}{C}}NHCH_2COOH$

A solution of butyloxycarbonyl azide (17 ml) in 150 ml dioxane was slowly added to a stirred solution of glycine (7.51 g, 100 mmol) in 150 ml water and 42 ml triethylamine. After 2 hours, suspended material was removed by filtration, and dioxane was distilled off from the solution under reduced pressure. The aqueous solution was acidified with 1N HCl and then extracted with ethyl acetate. The organic extract was dried with anhydrous magnesium sulfate and taken to dryness to give an orange syrup. On cooling, the syrup crystallized. The crude product recrystallized from ethyl acetate/petroleum ether as large translucent needles in 75% yield. Caution: BOC-N_3 is shock sensitive and has a thermal instability range of 100°-135°C and an autoignition temperature of 143°C.[189]

NMR (CDCl$_3$): 1.45 (singlet, 9, C(CH$_3$)$_3$), 3.87 (doublet, J = 6 Hz, 2, CH$_2$), 5.32, 6.48 (broad singlets, 1, NH), 11.38 (singlet, 1, COOH).

Mass spectrum, m/e (Rel. intensity): Parent at 175 not observed, Base at 120; 160 (3), 130 (9), 120 (100), 116 (3), 102 (5), 86 (1), 76 (17), 75 (6), 74 (8).

N-Trifluoroacetyl-glycylglycine,[190] CF$_3$CONHCH$_2$CONHCH$_2$COOH

S-Ethyl thioltrifluoroacetate (1.60 ml, 12.5 mmol) was stirred with glycylglycine (1.06 g, 8.00 mmol) dissolved in 1N NaOH (8 ml) for 24 hours. The reaction was quenched with conc. HCl (5 ml) whereupon a white precipitate appeared. The product was collected by suction filtration after being placed in a refrigerator for 2 hours. Additional product was obtained by extracting the filtrate with ethyl acetate. Weight of the crude product was 1.53 g (84%). Recrystallization from 95% ethanol gave small colourless crystals.

Azidotris(dimethylaminophosphonium) Hexafluorophosphate,[68] (37)
[(Me$_2$N)$_3$P-N$_3$]PF$_6$

Tris(dimethylaminophosphine) (10 g, 0.061 mol) was dissolved in ice-cold anhydrous ether (250 ml), and the temperature maintained at 0°C in an ice-water bath. Bromine (10 g, 0.063 mol) was added slowly and with efficient mixing (Caution: this is an extremely vigorous reaction, and appropriate safety precautions must be taken). After a few minutes, sodium hexafluorophosphate (10.4 g, 0.061 mol) in 250 ml water was added. The precipitate of [(Me$_2$N)$_3$P-Br]PF$_6$ was filtered, and washed with water and ether until all the excess bromine was removed. After the light yellow solid was dried overnight in a vacuum desiccator over P$_2$O$_5$, it was dissolved in 200 ml acetone (distilled from KMnO$_4$) and an excess of sodium azide (5 g) was added.

The solution was stirred overnight and NaBr and excess NaN_3 filtered off. After solvent was removed with a rotary evaporator, the product was obtained as a white solid which was recrystallized from acetone/ether in 83% yield.

Melting point > 250°C.

IR (nujol mull): $\nu(N_3)$ = 2173 cm^{-1} (lit. 2176 cm^{-1}).[68]

NMR (DMSO-d$_6$): 2.82 (doublet, J = 11 Hz, 6, C-$(CH_3)_2$) (lit. 2.79 ppm, J = 11 Hz).[68]

1-Ethoxycarbonyl-2-ethoxy-1,3-dihydroquinoline,[191] (27, EEDQ)
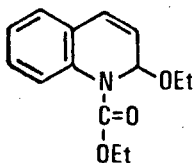
A solution of absolute ethanol (92 ml, 2.0 mol) and triethylamine (155 ml, 1.07 mol) was added dropwise to a stirred and well-cooled (-5°C) mixture of ethyl chloroformate (97 ml, 1 mol) and quinoline (tech. grade) (130 g, 1.0 mol) in benzene (300 ml). After stirring for 1 hour more, the mixture was washed with water and the aqueous layer extracted with chloroform (300 ml). The combined organic solution was evaporated to dryness under reduced pressure. On the addition of ether (∿50 ml) to the residue, an off-white solid separated and after standing in the cold, it was collected and washed with cold ether. Recrystallization from ether afforded large colourless crystals in 65% yield.

s-Triazolo[3,4-a]phthalazine[44]
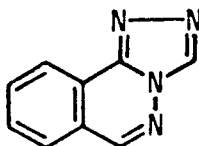
A mixture of hydralazine.HCl (5.668 g, 28.8 mmol), triethyl orthoformate (60 ml), and triethylamine (4 ml) was refluxed for 3 hours, and stirred at room temperature for 1 day. The solid was filtered off, washed with triethyl orthoformate, and recrystallized from water and from methanol. The product was obtained as long colorless needles. Anal. Calcd. for 3-H-TAP: C, 63.5; H, 3.6; N, 33.0. Found C, 63.5; H, 3.6; N, 33.0.

Melting point: 186.5 - 187.5°C.

UV (CH_2Cl_2), λ_{max} (nm): 234 sh, 239, 246, 264, 274, 284 sh.

NMR ($CDCl_3$): 7.92 (multiplet, 3, H-7, 8, 9), 8.65 (singlet-multiplet, 2, H-6, 10), 9.04 (singlet, 1, H-3).

Mass spectrum, m/e (Rel. Intensity): Parent and Base at 170; 170 (100),129 (2), 116 (4), 115 (33), 114 (9), 102 (2), 88 (15), 76 (4).

3-Aminomethyl-s-Triazolo[3,4-a]phthalazine . $HOCCF_3$
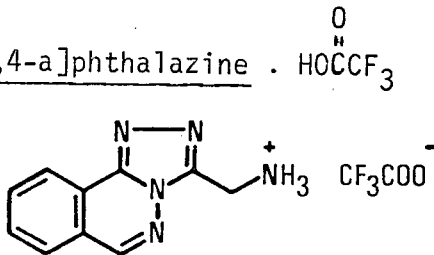
3-(N-BOC-gly)-TAP (100 mg) was dissolved in 50% (V/V) trifluoroacetic acid (2 ml) in CH_2Cl_2. The deprotection of the BOC-group was monitored by TLC on silica gel GF with $CHCl_3$-MeOH (10:1) developer. When deprotection was completed (approx. 10 mins), the solution was concentrated, and the product was precipitated by adding CH_2Cl_2. The yield was 95%.

UV (H_2O), λ_{max} (nm): 233 sh, 238, 244, 262, 271, 281.

Mass spectrum, m/e (Rel. Intensity): Parent and Base at 199; 199 (100), 198 (53), 183 (10), 171 (47), 149 (9), 145 (10), 129 (22), 117 (11), 115 (16), 102 (11).

3-Methyl-s-Triazolo[3,4-a]phthalazine Hydrochloride[45]
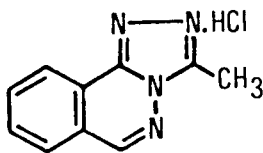
1-Hydrazinophthalazine hydrochloride (0.485 g, 2.47 mmol) in glacial acetic acid (50 ml) was refluxed for 4 hours. Excess acetic acid was removed from the light blue solution under reduced pressure, and the product dried *in vacuo* for 18 hours. Yield of 3-Me-TAP.HCl = 0.505 g (93%).

Melting point: 233 - 245°C dec.

NMR (DMSO-d_6): ∿8.15 (multiplet, 3, H-7, 8, 9), 8.59 (doublet, J ∿ 7 Hz, 1, H-10), 9.17 (singlet, 1, H-6), 9.71 (singlet, 1, H-3).

3-Methyl-s-Triazolo[3,4-a]phthalazine[44]
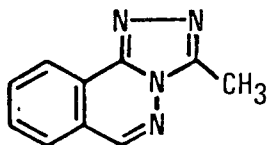
1-Hydrazinophthalazine (0.993 g, 5.05 mmol) in triethylorthoacetate (15 ml) was heated to reflux for 1 hour. The product separated from the gold-colored reaction mixture as yellowish needles and was recrystallized from water containing a trace of NaOH, as colorless needles in 96% yield.

Melting point: 167 - 169°C.

UV (MeOH), λ_{max} (nm): 236 sh, 240, 248, 265, 275, 285 sh.

NMR (CDCl$_3$): 2.78 (singlet, 3, CH$_3$), 7.84 (multiplet, 3, aromatic), 8.58 (possible doublet, 2, aromatic).

Mass spectrum, m/e (Rel. Intensity): Parent and Base at 184; 184 (100), 156 (7), 155 (8), 129 (11), 116 (11), 115 (82), 114 (20), 102 (8), 88 (26), 76 (9).

β-Alanine Methyl Ester Hydrochloride, $H_3\overset{+}{N}(CH_2)_2\overset{O}{\overset{\|}{C}}OCH_3$ Cl^-

Hydrogen chloride was bubbled through a suspension of β-alanine

(17.8 g, 0.200 mol) in dry methanol (300 ml) at 0°C until the solid had

dissolved completely and the solution was saturated with HCl. The solution

was stirred at room temperature for 18 hours and then rotary evaporated to

dryness. The product was recrystallized from ethanol-ether in 80% yield.

β-Alanine Iso-Propyl Ester Hydrochloride, $H_3\overset{+}{N}(CH_2)_2\overset{O}{\overset{\|}{C}}OCH(CH_3)_2$ Cl^-

Hydrogen chloride was bubbled through a suspension of β-alanine

(17.8 g, 0.200 mol) in iso-propanol (200 ml) at 0°C for 2 hours. The mixture

was then stirred for 1 day at room temperature when all solid dissolved.

The solvent was removed by rotary evaporation and the product recrystallized

from ethanol-ether in 85% yield.

1-Hydrazinophthalazine (Hydralazine)

1-Hydrazinophthalazine HCl (Hydralazine HCl, Apresoline HCl ;

Sigma, CIBA Pharm Co.) (washed with $CHCl_3$ and recrystallized from CH_3OH)

was dissolved in a minimum volume of hot water and neutralized with a

stoichiometric amount of 1N NaOH solution. The solution was immediately

extracted with chloroform under an inert atmosphere (Note: the extraction

procedure is not very efficient). The organic phase was dried with

anhydrous Na_2SO_4 and concentrated to dryness by rotary evaporation with as

little heating as possible. Hydralazine decomposition products may be

sometimes seen as a dark band of material in the flask. The impurities

precipitate from solution before the bulk of hydralazine when the solution

is concentrated and may thus be separated easily from the hydralazine.

The free-base hydralazine was dried under reduced pressure and was used

immediately , or stored at 5°C in vacuo or under an inert atmosphere.

6.3 COUPLING REACTIONS WITH THE ISOXAZOLIUM SALT METHOD

Synthesis of 3-(N-Ac-met)-TAP in Acetonitrile Solution

N-acetyl-DL-methionine (0.319 g, 1.67 mmol) was dissolved in warm acetonitrile (10 ml) containing triethylamine (0.175 g, 1.72 mmol), cooled to 0°C and stirred with a suspension of purified NEPIS (17) (0.424 g, 1.68 mmol) in ice-cold acetonitrile (10 ml). After all solid had dissolved (1 hour), hydralazine HCl (0.326 g, 1.66 mmol) was added together with triethylamine (0.166 g, 1.64 mmol), and the reaction mixture stirred at room temperature. After 2 days a light yellow solid was removed by filtration. UV spectra of the solid in methanol showed bands at 268, 278, and 318 nm. The solution was concentrated to an orange syrup which was dissolved in methylene chloride and extracted with water. Most of the colour is transferred to the aqueous phase. On re-extracting the organic layer with water, some product is lost into the aqueous phase. The CH_2Cl_2 solution was extracted with saturated NaCl solution, dried with anhydrous sodium sulfate, and concentrated to dryness. The crude product was obtained in 50-60% yield.

Melting point: 166 - 167°C.

UV (CH_2Cl_2), λ_{max} (nm): 237 sh, 243, 252, 264, 278.

Mass spectrum, m/e (Rel. Intensity): Parent at 315, Base Peaks at 240 and 197; 315 (11), 253 (11), 245 (5), 241 (16), 240 (100), 229 (6), 223 (5), 211 (17), 198 (17), 197 (100), 183 (9), 170 (25), 144 (5), 129 (11), 117 (5), 115 (9), 102 (7), 89 (8).

High resolution mass spectrum: Calcd. for $C_{15}H_{17}N_5OS$, 315.1154. Found 315.1152.

Synthesis of 3-(N-BOC-gly)-TAP in DMF Solution

A solution of N-BOC-glycine (0.300 g, 1.71 mmol) in dry, purified DMF (2 ml) containing triethylamine (0.24 ml, 1.7 mmol) was stirred with a suspension of purified NEPIS (*17*) (0.434 g, 1.71 mmol) in dry DMF (8 ml) at 0°C. After 1 hour, all the solid dissolved and hydralazine.HCl (0.337 g, 1.71 mmol) and triethylamine (0.24 ml, 1.7 mmol) was added to the reaction mixture. The solution was stirred for 2 days at room temperature, and then filtered. The white solid thus obtained was shown by nmr and mass spectrometry to be $Et_3N.HCl$. The lack of any UV absorptions indicated the absence of undissolved hydralazine.HCl. After DMF was removed from the filtrate under reduced pressure, the residue was dissolved in CH_2Cl_2, and extracted with 1N HCl and water. The CH_2Cl_2 solution was dried over anhydrous $MgSO_4$, reduced to dryness, and the light yellow residue washed with ether to remove most of the coloured impurities. Yield of crude 3-(N-BOC-gly)-TAP = 0.450 g (88%). Recrystallization from methanol-hexane gave fluffy white flakes of 3-(N-BOC-gly)-TAP which showed only one spot by TLC on silica gel GF with $CHCl_3/MeOH$ (10:1).

Melting point: 166 - 167°C.

UV (CH_2Cl_2), λ_{max} (nm): 238 sh, 243, 251.5, 267, 277, 288 sh.

NMR ($CDCl_3$): 1.47 (singlet, 9, $C(CH_3)_3$), 4.93 (doublet, J = 6 Hz, 2, CH_2), 5.51 (broad singlet, 1, NH), 7.9 (multiplet, 3, aromatic), 8.66 (singlet-multiplet, 2, aromatic).

Mass spectrum, m/e (Rel. Intensity): Parent at 299, Base at 243; 299 (11), 244 (22), 243 (100), 226 (20), 199 (23), 198 (39), 183 (22), 172 (10), 171 (16), 145 (9), 129 (10), 115 (9), 102 (5), 88 (5).

High resolution mass spectrum: Calcd. for $C_{15}H_{17}N_5O_2$, 299.1442. Found 299.1412.

6.4 COUPLING REACTIONS WITH THE EEDQ COUPLING REAGENT

Synthesis of 3-(N-Ac-gly)-TAP in Acetonitrile Solution

EEDQ (*27*) (51 mg, 0.21 mmol) was added to a mixture of hydralazine (32 mg, 0.20 mmol) and N-acetyl-glycine (27 mg, 0.23 mmol) in acetonitrile (10 ml), and stirred at room temperature for 4 1/2 hours. The reaction mixture was filtered to remove a yellow solid which showed UV absorption bands at 265 nm and 278 nm, indicative of decomposed hydralazine, and bands at 239 nm and 248 nm indicative of the TAP chromophore. Due to the apparently low concentration of TAP in solution and in the solid, no effort was made to work up the reaction mixture.

Synthesis of 3-(N-Ac-DL-ala)-TAP in Acetonitrile Solution

EEDQ (70 mg, 0.28 mmol) was stirred with a solution of hydralazine (45 mg, 0.28 mmol) and N-acetyl-DL-alanine (48 mg, .37 mmol) in acetonitrile (10 ml). After 18 hours at room temperature, the solution was filtered to remove precipitated solid. UV spectra of the solution showed a high concentration of TAP in addition to the quinoline by-product. The solution was reduced to dryness, the resultant residue dissolved in water, and extracted with CH_2Cl_2. UV spectra and TLC showed both quinoline and TAP in the organic phase. Solvent was removed from the CH_2Cl_2 solution under reduced pressure, and the solid obtained was washed with cyclohexane to remove all traces of quinoline. A small amount of yellow impurity was removed from the crude 3-(N-Ac-DL-ala)-TAP by preparative scale TLC on silica gel G with ethanol.

Melting Point: ~215° dec.

UV (CH_2Cl_2), λ_{max} (nm): 237 sh, 243, 251.5, 267, 287, 288 sh.

Mass spectrum, m/e (Rel. Intensity): Parent at 255, Base at 212; 255 (47), 213 (18), 212 (100), 198 (33), 197 (12), 184 (7), 172 (12), 171 (79), 145 (6), 144 (7), 129 (26), 117 (10), 115 (14), 102 (11), 89 (11), 76 (6), 69 (7).

Synthesis of 3-(N-BOC-L-ala)-TAP in Methylene Chloride Solution

EEDQ (0.136 g, 0.552 mmol) was stirred with a solution of hydrala-zine (0.088 g, 0.55 mmol) and N-BOC-L-alanine (0.103 g, 0.546 mmol) in CH_2Cl_2 (20 ml). Within 4 hours the reaction mixture showed good formation of TAP by UV spectroscopy, nevertheless, the reaction was continued for 23 hours at room temperature. The CH_2Cl_2 solution was extracted with dilute HCl until all quinoline was removed, then with 5% $NaHCO_3$ solution, and finally dried over anhydrous sodium sulfate. After solvent was removed, crude 3-(N-BOC-L-ala)-TAP was obtained as a yellow solid in ∿50% yield.

UV (CH_2Cl_2), λ_{max} (nm): 237 sh, 243, 252, 268, 278, 288 sh.

185

6.5 COUPLING REACTIONS WITH THE ACYLOXYPHOSPHONIUM SALT METHODS

6.5.1 The Kenner-Sheppard Reaction

A solution of N-acetyl-glycine (0.340 g, 2.91 mmol) in dry, distilled HMPA (2.9 ml) was added to a solution of recrystallized tosyl chloride (0.549 g, 2.88 mmol) in dry HMPA (4.64 g, 25.9 mmol) at 0°C. After 10 minutes, hydralazine.HCl (0.568 g, 2.89 mmol) and triethylamine (0.51 g, 5.0 mmol) were added, and the mixture was allowed to warm to room temperature. After 2 hours the reaction was checked by UV spectroscopy and TLC on silica gel G with n-BuOH developer. Thin layer chromatograms showed a large fluorescent spot indicative of the TAP product. The TAP bands in UV spectra are partially obscured by strong absorptions of the tosyl group. The reaction was allowed to proceed overnight before undissolved solid was filtered. The solid was predominantly hydralazine.HCl by its UV spectrum. Attempts to extract the 3-(N-Ac-gly)-TAP product by extraction of the HMPA solution with benzene, petroleum ether, and diethyl ether were largely unsuccessful. On extraction of the HMPA solution with CH_2Cl_2 some TAP was transferred into the CH_2Cl_2 layer together with all the colored components of the reaction mixture. However, the extraction of TAP into CH_2Cl_2 solution was inefficient, and no further effort was made to isolate the product.

6.5.2 The Azido- Tris(dimethylamino)phosphonium Hexafluorophosphate Method

$[(Me_2N)_3P-N_3]PF_6$ (*37*) (0.072 g, 0.21 mmol) was added over a period of 1 - 1 1/2 hours to a stirred solution of N-BOC-glycine (0.036 g, 0.21 mmol) and triethylamine (33 µl) in DMF (10 ml) at -15°C. After 1 hour, hydralazine HCl (0.040 g, 0.20 mmol) and triethylamine (60 µl) were added, and the coupling reaction continued under an argon atmosphere for an additional 4 hours at -15°C and 15 hours at room temperature. Thin layer chromatograms of the reaction mixture showed a low concentration of the fluorescent TAP product. There was no evidence of 3-(N-BOC-gly)-TAP in UV spectra because of the high UV cut-off of DMF. However, hydralazine decomposition products were evident by absorption bands at 278 nm and 286 nm.

A coupling reaction in Methyl Cellosolve was conducted under identical conditions as the reaction in DMF and similar results were obtained. However, UV spectra of the Methyl Cellosolve reaction showed very little decomposition of hydralazine after 20 hours of reaction.

6.5.3 The "Oxidation-Reduction Condensation" Method

A suspension of N-acetyl-glycine (0.039 g, 0.34 mmol) and 2,2'-dithiodipyridine (*42*, 2-DTP) (0.077 g, 0.32 mmol) in dioxane (2 ml) was stirred with a mixture of hydralazine.HCl (0.064 g, 0.32 mmol), triethyl-amine (0.039 g, 0.38 mmol), and triphenylphosphine (0.110 g, 0.419 mmol) in dioxane (5 ml) at 40°C for 20 hours. Precipitated solid was removed by solution filtration and was shown by its UV spectrum to contain predominantly decomposed hydralazine (λ_{max} (nm): 267, 278, 317). NMR and mass spectra indicated that the solid also contained 3-(N-Ac-gly)-TAP and $Et_3N.HCl$. TLC on Silica Gel GF developed with n-BuOH showed a low concentration of TAP product in the solution.

6.5.4 The Triphenylphosphite-Imidazole Method

In view of the large number (24) of reactions conducted with the Mitin[71] procedure under similar conditions, only one coupling reaction typical of those used in this study will be described.

Synthesis of 3-(N-Z-gly)-TAP

A mixture of N-Z-glycine (0.307 g, 1.47 mmol), hydralazine.HCl (0.290 g, 1.48 mmol), triphenylphosphite (0.478 g, 1.54 mmol), and triethyl-amine (0.21 ml) was dissolved in dry DMF (5 ml). A solution of imidazole (recryst. toluene) (0.202 g, 2.96 mmol) in DMF (7 ml) was added to the other reagents, and the mixture stirred at 40°C. TLC of the reaction mixture showed the presence of TAP even before the solution was heated. After heating for one day, the solution was filtered and reduced to dryness under reduced pressure. UV spectra of the solid filtered from the solution showed the presence of undissolved hydralazine.HCl and a phosphorus derivative. The syrup obtained from concentrating the solution was dissolved in CH_2Cl_2 and extracted with 1M Na_2CO_3 and 0.7N HCl respectively. The oily residue which was obtained after CH_2Cl_2 was removed by rotary evaporation crystal-lized on standing. The crude product was recrystallized from ethanol solution in 60% yield.

6.5.5 The Diphenylphosphite-Pyridine Method

A mixture of N-BOC-L-alanine (0.289 g, 1.53 mmol), hydralazine.HCl
(0.301 g, 1.53 mmol), and diphenyl phosphite (practical grade) (0.362 g,
1.55 mmol) was stirred with pyridine (10 ml) for 2 days. Undissolved solid
(hydralazine.HCl) was filtered off and the gold solution concentrated under
reduced pressure. A solid which crystallized from solution was shown by
UV spectroscopy to be predominantly hydralazine.HCl after washing with
acetone. The acetone wash contained mostly phosphorus derivatives. There
was no evidence for TAP product in the reaction mixture.

6.6 COUPLING REACTIONS WITH THE CARBODIIMIDE METHOD

It would be impractical to describe all the coupling reactions
conducted with carbodiimides since they totalled some fifty in number.
Instead, several experiments will be described to illustrate certain features
and characteristics of the procedures involved.

Synthesis of 3-(N-Z-glycyl)-TAP with DCC in DMA Solution

Triethylamine (1.10 ml, 7.89 mmol) was added to a mixture of
N-Z-glycine (1.076 g, 5.141 mmol), hydralazine.HCl (1.034 g, 5.257 mmol), and
dicyclohexylcarbodiimide (51) (2.137 g, 10.36 mmol) in dry dimethylacetamide
(50 ml) at 0°C. After 1 hour, additional hydralazine.HCl (1.015 g, 5.162
mmol) and triethylamine (1.10 ml, 7.89 mmol) were added. The reaction
mixture was stirred at 0°C for 4 hours before being allowed to warm to room
temperature. The reaction was continued for 1 day at room temperature, and
5 hours to 60°C. On cooling the solution, dicyclohexylurea (DCU) crystallized
out, and was removed by filtration. Rotary evaporation of the DMA solution
gave a syrup which solidified when the last traces of DMA were removed by

rotary evaporation of the syrup dissolved in methanol or CH_2Cl_2. The residual solid was dissolved in CH_2Cl_2, the insoluble DCU filtered off, and the solution extracted with 2% HCl, 5% $NaHCO_3$, and saturated NaCl solutions, respectively. When the CH_2Cl_2 solution was concentrated, more DCU crystallized from solution which was removed by filtration. The product was further purified by column chromatography on silica gel with $CHCl_3$-MeOH (50:1) as the developing solution. The crude 3-(N-Z-gly)-TAP thus obtained was leached with benzene to remove all remaining colored impurities. Anal. Calcd. for 3-(N-Z-gly)-TAP: C, 64.9; H, 4.5; N, 21.0. Found C, 65.0; H, 4.5; N, 21.0.

Melting point: 147 - 148°C dec.

UV (CH_2Cl_2), λ_{max} (nm): 236 sh, 242.5, 251, 266, 276, 287 sh.

NMR $(CDCl_3)$: 5.00 (doublet, J = 6 Hz, 2, CH_2), 5.15 (singlet, 2, CH_2(benzyl)), 6.08 (broad singlet, 1, NH), 7.32 (singlet, 5, C_6H_5), 7.95 (multiplet, 3, aromatic), 8.59 (singlet-multiplet, 2, aromatic).

Mass spectrum, m/e (Rel. Intensity): Parent at 333, Base at 198; 333 (63), 242 (15), 225 (17), 199 (22), 198 (100), 197 (47), 184 (95), 183 (32), 171 (28), 155 (6), 129 (17), 115 (14), 108 (11), 102 (10), 91 (71), 79 (18), 77 (19), 66 (44), 51 (12).

High resolution mass spectrum: Calcd. for $C_{18}H_{15}N_5O_2$, 333.1226. Found 333.1208.

Synthesis of 3-(N-Ac-gly)-TAP with EDC in Methanol Solution

Triethylamine (0.071 ml, 0.51 mmol) was added to a mixture of N-Ac-glycine (0.0899 g, 0.513 mmol), hydralazine.HCl (0.100 g, 0.510 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (*16*, EDC) in dry methanol (15 ml) at 0°C. All the solids dissolved to give a light yellow solution. Reaction was continued for 4 hours at 0°C, and 18 hours at room temperature. The solvent was removed from the solution by rotary evaporation to give a dry solid. The residue was shaken and sonicated with CH_2Cl_2 until all TAP product was leached into solution. The CH_2Cl_2 solution was streaked onto a silica gel TLC plate (2000 μ) and developed with $CHCl_3$-MeOH (10:1). Two well-separated fluorescent bands were obtained which were identified as $3-CH_3$-TAP (upper band) and 3-(N-Ac-gly)-TAP (lower band) by their nmr and mass spectra.

Synthesis of 3-(N-BOC-gly)-TAP with DCC in Methylene Chloride Solution

Dicyclohexylcarbodiimide (*51*, DCC) (0.373 g, 1.81 mmol) was stirred with a mixture of freshly-prepared hydralazine (0.255 g, 1.59 mmol) and N-BOC-glycine (0.280 g, 1.60 mmol) in dry CH_2Cl_2 (25 ml) at 0°C. The reaction was allowed to proceed for 1 hour at 0°C and 18 hours at room temperature before it was filtered to remove dicyclohexylurea. The solution was extracted with 1N HCl, 5% Na_2CO_3, and saturated NaCl solution, respectively. After solvent was removed by rotary evaporation, the crude light-yellow 3-(N-BOC-gly)-TAP thus obtained was recrystallized from methanol in 76% yield.

Synthesis of 3-(N-Ac-gly)-TAP with DCC in Methanol Solution

Triethylamine (0.85 ml, 6.1 mmol) was added to a mixture of N-acetyl-glycine (1.444 g, 1.233 mmol), hydralazine.HCl (1.176 g, 5.983 mmol) and dicyclohexylcarbodiimide (51, DCC) (2.602 g, 12.61 mmol) in dry methanol (45 ml) at 0°C. UV spectra of the solution after 15 minutes showed the presence of protonated hydralazine. Excess triethylamine (0.85 ml, 6.1 mmol) was therefore added. The reaction was conducted at 0°C for 2 hours, and at ambient temperature for 18 hours. During this time the color of the solution changed from yellow to reddish-orange. When the reaction mixture was refluxed for 1 hour, UV spectra showed $\sim$ 3x increase in the concentration of TAP. However, TLC indicated that refluxing also effected the formation of 3-CH$_3$-TAP side-product. The solution was concentrated to dryness and the residue suspended in CHCl$_3$. Undissolved dicyclohexylurea (DCU) was filtered off. The TAP product is only moderately soluble in CHCl$_3$. The chloroform solution was purified by column chromatography on silica gel developed initially with pure CHCl$_3$, then with a CHCl$_3$-MeOH mixture whose composition varied from a ratio of 50:1 to 20:1. Crude 3-(N-Ac-gly)-TAP was obtained as a light yellow solid. Crystallization from H$_2$O-EtOH afford white needles of pure 3-(N-Ac-gly)-TAP. Anal. Calcd. for 3-(N-Ac-gly)-TAP. 1/2 H$_2$O: C, 57.6; H, 4.8, N, 28.0. Found C, 57.4; H, 4.8; N, 28.0.

Melting point: 209 - 210°C dec.

UV (CH$_2$Cl$_2$), λ_{max} (nm): 236 sh, 242.5, 251, 267, 277, 287 sh.

NMR (CDCl$_3$): 2.11 (singlet, 1, CH$_3$), 5.07 (doublet, J = 5 Hz, 2, CH$_2$), 6.78 (broad singlet, 1, NH), 7.9 (multiplet, 3, aromatic), 8.67 (singlet-multiplet, 2, aromatic).

Mass spectrum, m/e (Rel. Intensity): Parent at 241, Base at 198; 241 (77), 199 (16), 198 (100), 183 (7), 171 (32), 129 (11), 117 (4), 115 (4), 102 (4).

High resolution mass spectrum: Calcd. for C$_{12}$H$_{11}$N$_5$O , 241.0964. Found 241.0965.

Synthesis of 3-(N-BOC-gly)-TAP in Acetonitrile Solution

A solution of N-BOC-glycine (0.258 g, 1.47 mmol) in acetonitrile (10 ml) containing triethylamine (0.21 ml, 1.5 mmol) was stirred with a suspension of purified NEPIS (*17*) (0.373 g, 1.47 mmol) in acetonitrile (10 ml) at 0°C. After 1 1/4 hours, hydralazine hydrochloride (0.307 g, 1.56 mmol) and triethylamine (0.22 ml, 1.6 mmol) were added and the mixture allowed to warm to room temperature. Undissolved hydralazine HCl was filtered after 18 hours, and the solvent removed from the solution with a rotary evaporator. The residue -as dissolved in CH_2Cl_2 and extracted with 5% $NaHCO_3$, 5% HCl, and H_2O, respectively. Extractions were repeated until the aqueous phases showed no UV absorbing material. However, during extractions with 5% HCl, the TAP product was protonated and lost into aqueous solution. Yield of crude product = 0.132 g (30%). The work-up procedure in which 5% HCl was replaced with 1% or 1N HCl gave higher yields of 3-(N-Ac-gly)-TAP.

Attempted Synthesis of 3-(N-BOC-gly)-TAP in CH_3CN-DMF Solution

A solution of N-BOC-glycine (0.265 g, 1.5. mmol in acetonitrile (5 ml) containing triethylamine (0.21 ml, 1.5 mmol) was stirred with a suspension of NEPIS (*17*) (0.383 g, 1.51 mmol) in acetonitrile (7 ml) at 0°C, and added after 1 hour to a suspension of hydralazine HCl (0.299 g, 1.52 mmol) in DMF (8 ml) containing triethylamine (0.21 ml, 1.5 mmol). Almost all solid dissolved after the mixture was stirred for 18 hours at room temperature. Solvent was removed from the solution under reduced pressure, and the sticky residue sonicated with 0.5N HCl (40 ml) to give an amorphous solid. However, after a period of hours, a gas was evolved and most of the solid dissolved. Little TAP product could be extracted from the aqueous solution with CH_2Cl_2 or ethyl acetate. After the pH of the aqueous solution was adjusted to 7.5, TAP was extracted into CH_2Cl_2. TLC of the CH_2Cl_2 solution showed predominantly 3-NH2CH2-TAP and only a small amount of 3-(N-BOC-gly)-TAP.

6.7 SOLID-PHASE STUDIES

Hydroxymethyl-Resin,[116] P_s-CH$_2$OH

Chloromethyl-poly(styrene-co-1% divinylbenzene) (1.34 mmol of Cl/g, 200-400 mesh, Bio-Beads S-X1; 29.3 g, 39.2 mmol) was suspended in Methyl Cellosolve (200 ml) and stirred with potassium acetate (10.0g, 102 mmol) at 135°C for 70 hours. The resin was collected and washed successively with water and methanol. The product was stirred with freshly prepared 0.51N NaOH solution (200 ml, 101 mmol) for 72 hours, then filtered, washed with water and methanol, and vacuum dried. The resin was used immediately for the preparation of methylchloroformyl resin.

IR (HCB mull): ν(O-H), 3575 and 3385 cm^{-1} (lit. 3610 and 3448 cm^{-1}).[107]

Methylchloroformyl-Resin,[116] P_s-CH$_2$O$\overset{\text{O}}{\overset{\|}{\text{C}}}$-Cl

Hydroxymethyl resin (27.9 g) was treated with 12.5% phosgene in benzene solution (200 ml). The slurry was diluted further with benzene (50 ml) and stirred for 6 hours, followed by filtration and washing with benzene and ether. The resin thus obtained was dried *in vacuo* and stored in a vacuum desiccator over Drierite. Volhard titration for chloride indicated a resin capacity of 1.36 mmol of chloride/g of resin.

IR (HCB mull): ν(C=O), 1772 cm^{-1} (lit. 1779 cm^{-1}).[107]

Resin-Alanine, P_s-CH$_2$O$\overset{\text{O}}{\overset{\|}{\text{C}}}$NHCH$\overset{\text{O}}{\overset{\|}{\text{C}}}$OEt
$$\qquad\qquad\qquad\qquad\qquad \underset{CH_3}{|}$$

DL-Alanine ethyl ester hydrochloride (1.37 g, 8.94 mmol) and triethylamine (2.0 ml, 36 mmol) were stirred for 23 hours at room temperature with chloroformylmethyl resin (3.09 g, 4.20 mmol) suspended in dry chloroform (50 ml). The resin was filtered, washed with chloroform, then resuspended in

dry chloroform (50 ml) and reacted with diethylamine (0.50 ml, 4.9 mmol) for 4 hours. The resin was washed with a chloroform-ether mixture (concentration gradient from 0 to 100% ether) and dried *in vacuo*. The resin-alanine ethyl ester was saponified with 0.5N KOH (50 ml) in methanol-acetone (1:1) for 21 hours, filtered, acidified with dilute HCl, and washed with a methanol-ether mixture with a concentration gradient of ether varying from 0 to 100%. The resin was dried *in vacuo* for 18 hours. The carboxylate content by titration with NaOH is 0.63 mmol/g resin.

Resin-glycine, P_s-CH$_2$OCNHCH$_2$COH
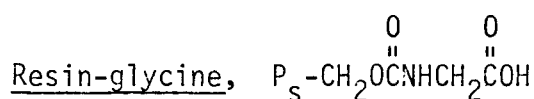
Glycine ethyl ester hydrochloride (1.60 g, 11.5 mmol) and triethylamine (5.0 ml, 36 mmol) were stirred for 21 hours at room temperature with chloroformylmethyl resin (8.49 g, 11.5 mmol) suspended in dry chloroform (100 ml). The resin was filtered, washed with chloroform, then resuspended in chloroform and stirred with diethylamine (1.0 ml, 9.7 mmol) for 5 hours to block unreacted acid chloride groups. The product was washed -ith a mixture of chloroform and methanol whose composition was varied from pure chloroform to pure methanol. The resin was saponified with 0.5N KOH (100 ml) in methanol-acetone (1:1) for 18 hours. After washing with a methanol-chloroform-ether mixture (mixture composition varied from 100% CH$_3$OH to 100% Et$_2$O), the resin was suspended in 75 ml of a methanol-acetone mixture (2:1), and stirred with 12N HCl (1 ml) for 1 hour.. The resin-glycine was washed with copious amounts of water, ethanol and ether, then dried *in vacuo*. The carboxylate content by titration with NaOH is 0.62 mmol/g resin.

Resin-glycylglycine, P_s-CHOCONHCH$_2$CONHCH$_2$COH

(with O above first CO and O above second CO)
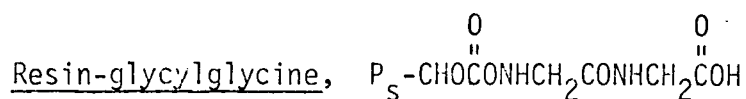
Glycylglycine ethyl ester hydrochloride (1.89 g, 9.63 mmol) and triethylamine (5.0 ml, 36 mmol) were stirred for 23 hours with chloroformyl-methyl resin (3.50 g, 4.75 mmol) suspended in dry chloroform (100 ml). The resin was filtered, washed with chloroform and ether, and dried *in vacuo*. The product was suspended in chloroform (75 ml) and reacted with diethylamine (0.50 ml, 4.9 mmol) for 5 hours to block unreacted acid chloride groups. After washing with chloroform and ether, the resin-glyglyOEt was dried by pumping on a vacuum line. The resin was saponified with 0.5N NaOH (50 ml) in methanol-acetone (1:1) for 17 hours, then washed with water, methanol, and ether prior to drying *in vacuo*. Analysis for carboxylic acid by titration with NaOH gave a value of 0.19 mmol/g. The low value probably reflects the glycylglycine substituent being present primarily as the sodium salt, and not necessarily from low substitution of dipeptide onto the resin.

Coupling of Resin-glycine with Hydralazine using Iso-butyl Chloroformate

Isobutyl chloroformate (5 g, 37 mmol) was stirred at -8°C with a suspension of resin-glycine (1.86 g, 0.689 mmol) in dry chloroform (25 ml) and triethylamine (5.0 ml). After one hour, the resin was filtered and washed with dry chloroform. A cold mixture of hydralazine hydrochloride (1.96 g, 10.0 mmol) in chloroform (100 ml) containing triethylamine (1.56 ml, 11.2 mmol) was added. The suspension was stirred for 1 hour at -8°C and for 19 hours at room temperature, filtered, and stirred with 75 ml water for 2 hours to remove any undissolved hydralazine hydrochloride. The resin was filtered, washed with water, acetone, chloroform, and ether, and dried under reduced pressure.

Cleavage from the Resin: 3-Aminomethyl-s-Triazolo[3,4-a]phthalazine from the Coupling Reaction with Isobutyl Chloroformate as Carboxyl Activating Agent for Resin-glycine

Hydrogen bromide was introduced for 3 1/2 hours into a suspension of resin-3-glycyl-TAP in trifluoroacetic acid (5 ml) and the suspension was allowed to stand for 15 hours. The resin was removed by filtration and washed with trifluoroacetic acid. Evaporation of the filtrate under reduced pressure gave a light orange syrup.

Purification of the residue by preparative scale TLC on silica gel with $CHCl_3/CH_3OH$ (10:1) gave a main fluorescent band at low R_f which showed UV spectra characteristic of TAP, and a yellow non-fluorescent band at high R_f which showed a prominent band at 278 nm in the UV spectrum reminiscent of decomposed hydralazine (cf. Chapter 5). The fluorescent band was extracted with methanol and purified two more times by preparative scale TLC. A yellow impurity was removed by washing the product with chloroform. Comparison of UV and mass spectra with those of authentic $3-NH_2CH_2$-TAP confirms the identity of the product.

UV (MeOH), λ_{max} (nm): 235 sh, 241, 248, 265, 274, 285 sh.

Mass spectrum, m/e (Rel. Intensity): Parent and Base at 199; 199 (100), 198 (59), 184 (15), 183 (15), 171 (48), 149 (13), 145 (15), 129 (24), 117 (11), 115 (14).

Coupling of Resin-glycine with Hydralazine using EEDQ

1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (27, EEDQ) (0.249 g, 1.01 mmol) was stirred at room temperature with resin-glycine (1.61 g, 1.0 mmol) suspended in dry tetrahydrofuran (25 ml). Triethylamine (0.40 ml) was added to a suspension of hydralazine.HCl (1.00 g, 5.09 mmol) in dry, peroxide-free THF (25 ml) under a nitrogen atmosphere and the suspension filtered.

After the resin-glycine was activated for 45 minutes, the hydralazine solution was added under a nitrogen atmosphere, and the mixture stirred for 21 hours. From the weight of hydralazine.HCl recovered, free-base hydralazine in THF solution was estimated to be 0.10 g (0.51 mmol). The resin was filtered, and washed with THF, methanol, and ether.

Cleavage from the Resin: 3-Aminomethyl-s-Triazolo[3,4-a]phthalazine from the Coupling Reaction with EEDQ as Carboxyl Activating Agent for Resin-glycine

Hydrogen bromide was bubbled for 4 hours into a suspension of resin-3-glycyl-TAP in glacial acetic acid and the suspension was left standing for 24 hours. The resin was removed by suction filtration and washed with acetic acid. Evaporation of the filtrate under reduced pressure gave a light orange syrup which showed relatively clean UV spectra in methanol solution characteristic of the TAP chromophore: λ_{max} (nm), 233 sh, 238, 245, 261, 271, 281 sh.

Coupling of Resin-glycine with Hydralazine using Dicyclohexylcarbodiimide (51)

Hydralazine hydrochloride (1.02 g, 5.19 mmol) was suspended in dry chloroform (50 ml) under a nitrogen atmosphere, and stirred with triethylamine (0.72 ml, 5.2 mmol) for 1 hour. The undissolved hydralazine.HCl was removed by filtration under N_2 with Schlenk glass-ware, and the hydralazine solution, after cooling to 0°C was added to resin-glycine (1.60 g, 1.01 mmol) with dicyclohexylcarbodiimide (0.286 g, 1.39 mmol). The mixture was stirred at 0°C for 2 hours, and at room temperature for 15 hours. UV spectra of the solution showed considerable hydralazine decomposition evidenced by a strong band at 278 nm after overnight reaction. Refluxing the reaction mixture for 2 hours showed no change in the UV spectra of the solution. The resin was filtered, washed with chloroform, methanol, and ether, and dried *in vacuo* for 4 hours.

Cleavage from the Resin: 3-Aminomethyl-s-Triazolo[3,4-a]phthalazine from the Coupling Reaction with DCC as Carboxyl Activating Agent for Resin-glycine

Hydrogen bromide was bubbled for 3 1/2 hours into a suspension of resin-3-glycyl-TAP (1.63 g) in glacial acetic acid (10 ml). The suspension was allowed to stand for 16 hours, filtered, and washed with acetic acid. Evaporation of the solution *in vacuo* gave an orange syrup, which on triturating with chloroform, crystallized to a white solid. UV spectra of the solid in methanol solution showed bands characteristics of the clean TAP chromophore.

Polyacrylamide Acid Chloride

Carboxypolyacrylamide (Bio-Gel CM-2; 100-200 mesh, 5 meq/g, Na^+ form) (50 g, 250 meq) was suspended in Dioxane/2N NaOH (3:1) (250 ml) for 2 hours, and in Dioxane/2N HCl (3:1) for 2 hours. The polymer was washed with water, acetone, and ether, and dried *in vacuo* for 19 hours. The polymer thus purified was stirred for 3 1/2 hours in freshly distilled thionyl chloride (containing 5% pyridine) (100 ml). The derivatized product was filtered, washed with dry benzene and ether, and dried *in vacuo* for 18 hours. The polyacrylamide acid chloride was stored over drierite.

Chloride Analysis of the Chloroformylmethyl Resin by the modified Volhard Method [192]

The chloroformylmethyl resin ($\sim$200 mg) was hydrolyzed in pyridine (3 ml) for 3 hours at 100°C. The solution was transferred quantitatively to a 125 ml Erlenmeyer flask with 50% aqueous acetic acid (30 ml), and acidified with conc. nitric acid (5 ml). The chloride was precipitated with 0.100N $AgNO_3$ (5.00 ml). The AgCl formed was coated with toluene (about a 1/4 inch

layer of toluene on the water surface) and the excess $AgNO_3$ back-titrated with standard 0.100N NH_4SCN solution, using a saturated ferric alum solution as an indicator. A red color, due to the formation of $Fe(SCN)_3$, indicated that the end point has been reached.

Analysis for chloride content in P_s-CH_2OCOCl was done in duplicate, and agreed within $\pm$ 0.01 mmol Cl/g resin. Hydrolysis of the resin was inefficient with 1N NaOH since the resin floated in this medium, and tended to rise up the side of the container. In addition, the resin swelled poorly in 1N NaOH solution.

Analysis of the Resin-Amino Acid (peptide) by Titration with Hydrochloric Acid

The resin ($\sim$500 mg) was suspended in 95% ethanol (25 ml) and standard 0.100N NaOH solution (25.00 ml) added. The mixture was heated to reflux for 10 minutes, and cooled to room temperature. The excess base was back-titrated with 0.100N HCl, using phenolphthalein as indicator. All analyses were done in duplicate, and usually agreed within $\pm$ 0.02 mmol/g resin. The titration for acid content is indirectly a measure of the amino acid substitution on the resin.

6.8 TRANSITION METAL COMPLEXES AND HYDROLYSIS STUDIES

<u>Sodium Triscarbonatocobaltate(III) Trihydrate</u>,[168] $Na[Co(CO_3)_3].3H_2O$

    A stirred slurry of sodium bicarbonate (42.0 g, 0.50 mol) in 50 ml of H_2O was cooled to 0°C and a solution of $CoCl_2.6H_2O$ (23.8 g, 0.10 mol) and 30% H_2O_2 (10 ml) in 50 ml of H_2O was added dropwise over a period of 20 mins. (<u>Note</u>: vigorous effervescence). The olive-green slurry was allowed to stand at 0°C for 1 hour with continuous stirring. The product was filtered and washed with three 10 ml portions of cold H_2O. The complex was usually used immediately for the preparation of cis-β-[Co(trien)OH(H_2O)](ClO_4)$_2$, however, it may be stored for future use if protected from moisture.

<u>*cis*-β-Carbonatotriethylenetetraminecobalt(III) Perchlorate Monohydrate</u>
[Co(trien)CO$_3$](ClO$_4$)$_2$.H$_2$O
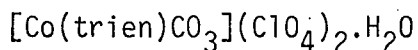
    Concentrated (60%) perchloric acid (15.2 ml) was slowly added to an ice-cold solution of triethylenetetramine (12.5 ml) in 75 ml of H_2O. The complete batch of freshly prepared $Na_3[Co(CO_3)_3].3H_2O$ was added and the mixture stirred for 30 minutes at 0°C, (<u>Note</u>: vigorous effervescence) and then warmed to 60°C for 30 minutes. A light purple solid was removed from the hot solution by suction filtration with a coarse, sintered-glass funnel. The solution was concentrated to ∿75 ml by rotary evaporation, and then placed in the cold for 1 hour to complete crystallization of the complex. The product was collected by suction filtration and washed with 95% methanol.

    The red solid was dissolved in hot water (35 ml), and sodium perchlorate (8 g) and methanol (15 ml) were added. The product recrystallized on cooling and was filtered and washed with 95% ethanol until the washing was colorless. One further recrystallization from water gave analytically pure material.

    Absorption spectrum, λ_{max} (nm): 506, 359.

Tetraaquobis(s-triazolo[3,4-a]phthalazine) nickel(II) Perchlorate

$[Ni(3-H-TAP)_2(H_2O)_4](ClO_4)_2$
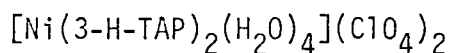
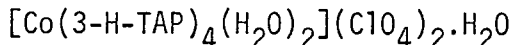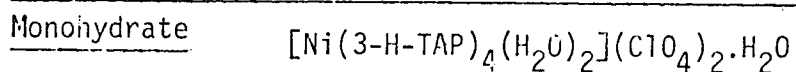On stirring nickel(II) perchlorate hexahydrate (1.101 g, 3.01 mmol) with 10 ml 2,2-dimethoxypropane a dense immiscible oily layer of the metal salt was formed. Within an hour, the color changed from green to yellow. After 1 1/2 hours, a solution 3-H-TAP (0.881 g, 5.18 mmol) in methanol was added to the mixture. A pale blue solid precipitated slowly as a fine powder. The reaction mixture was stirred overnight, filtered, and the solid washed with methanol (slightly soluble). The product was dried *in vacuo* at room temperature for 18 hours over Drierite. Anal. Calcd. for $[Ni(3-H-TAP)_2(H_2O)_4]$-$(ClO_4)_2$: C, 32.3; H, 3.0; N, 16.7. Found C, 32.2; H, 3.0; N, 16.7.

Diaquotetrakis(s-triazolo[3,4-a]phthalazine) cobalt(II) Perchlorate Mono-hydrate $[Co(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$

Cobalt(II) perchlorate hexahydrate (0.391 g, 1.07 mmol) was stirred with 20 ml 2,2-dimethoxypropane under a nitrogen atmosphere for 2 hours. s-Triazolo[3,4-a]phthalazine (0.726 g, 4.27 mmol) was dissolved in 20 ml warm methanol and added to the solution of cobalt perchlorate. A tangerine precipitate appeared slowly which was filtered after two hours, washed with dry methanol and ether, and dried under vacuum at room temperature for 18 hours. The complex did not show any definite melting point, but turned violet ca. 224°C. Anal. Calcd. for $[Co(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$: C, 43.6; H, 3.1; N, 22.6. Found: C, 43.7; H, 3.0; N, 22.6.

Diaquotetrakis(s-triazolo[3,4-a]phthalazine)nickel(II) Perchlorate
Monohydrate $[Ni(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$

Nickel(II) perchlorate hexahydrate (0.166 g, 0.455 mmol) was stirred in 5 ml 2,2-dimethoxypropane for one hour, during which time the suspended solid changed from a pale green color to orange. Methanol (1.5 ml) was added to dissolve all the solid, and the deep orange solution was stirred for a further period of one hour. s-Triazolo[3,4-a]phthalazine (0.308 g, 1.81 mmol) was dissolved in 15 ml warm methanol and added to the solution of nickel perchlorate. The color of the solution lightened until it was almost colorless, and a pale blue solid gradually precipitated from solution. After two hours, the solid was filtered, washed with dry methanol (the complex is moderately soluble), and ether, and dried *in vacuo* at room temperature for 18 hours. Anal. Calcd. for $[Ni(3-H-TAP)_4(H_2O)_2](ClO_4)_2 \cdot H_2O$: C, 43.6; H, 3.1; N, 22.6. Found C, 43.8; H, 3.0; N, 22.5.

## Diaquotetrakis(s-triazolo[3,4-a]phthalazine)copper(II) Perchlorate
$[Cu(3-H-TAP)_4(H_2O)_2](ClO_4)_2$
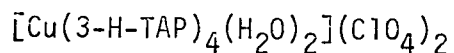
Copper(II) perchlorate hexahydrate (0.245 g, 0.662 mmol) was stirred with 5 ml 2,2-dimethoxypropane for two hours. s-Triazolo[3,4-a]-phthalazine (0.449 g, 2.64 mmol) was dissolved in 15 ml warm methanol and added to the blue oil of copper perchlorate. A light blue precipitate was formed which immediately changed to purple. The precipitate was filtered, and on washing with methanol (the complex is slightly soluble), the blue color returned. On drying *in vacuo* at room temperature for 48 hours, the product was obtained as a purple amorphous solid. On exposure to moist air or solvents, the colour changed to light blue. The interconversion between the purple and blue forms appear to be readily reversible. Anal. Calcd. for $[Cu(3-H-TAP)_4(H_2O)_2](ClO_4)_2$ (purple form): C, 44.2; H, 2.9; N, 22.9. Found: C, 44.0; H, 2.8; N, 22.9.

Hexakis(s-triazolo[3,4-a]phthalazine)cobalt(III) Perchlorate Monohydrate
[Co(3-H-TAP)$_6$](ClO$_4$)$_3$·H$_2$O
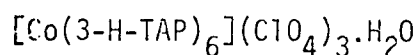
Sodium tris(carbonato)cobaltate(III) (0.749 g, 1.55 mmol) and s-triazolo[3,4-a]phthalazine (1.582 g, 9.30 mmol) were suspended in 75 ml 95% ethanol and 10 ml 2M HClO$_4$. The suspension was heated to reflux for 3 hours, during which time the color of the solid changed from dark green to a light brown. Reaction was continued for an hour at room temperature. The solid was filtered, washed with 95% ethanol and ether, and dried *in vacuo* at room temperature for 18 hours. Anal. Calcd. for [Co(3-H-TAP)$_6$]-(ClO$_4$)$_3$·H$_2$O : C, 46.5; H, 2.7; N, 24.1. Found: C, 46.5; H, 2.9; N, 24.3.

cis-β-2-aminomethylpyridine(triethylenetetramine)cobalt(III) Perchlorate
[Co(trien)(2-AMPy)](ClO$_4$)$_3$
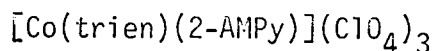
Two equivalents of 2M HClO$_4$ (2.8 ml) were added to β-[Co(trien)CO$_3$]-ClO$_4$·H$_2$O (1.045 g, 2.731 mmol) and the solution warmed at 70°C until CO$_2$ ceased to be liberated (10 min). On cooling, the pH of the solution was adjusted to about 4 with 1M NaOH, and 2-aminomethylpyridine (2-AMPy) (0.296 g, 2.74 mmol) added. After heating the solution to 70°C for 15 minutes, the solution was quenched to pH 4 with 2M HClO$_4$, and a purple precipitate filtered off. After the solution was concentrated by rotary evaporation, the product was loaded onto the top of a chromatography column containing Sephadex CM-25 cation exchange resin, and eluted with sodium perchlorate solution (0.1-2.0M). If a sufficiently long column is used, two isomers of the product can be obtained as separate orange bands. Sodium perchlorate impurity was removed by concentrating the eluents containing the complexes and crystallizing the NaClO$_4$ from solution while replacing the water with methanol. The solid thus obtained always contained some NaClO$_4$ impurity, however.

Absorption spectrum, λ_{max} (nm): 1st isomer eluted, 342 and 471.
2nd isomer eluted, 339 and 467.

cis-β-(3-Acetimidomethyl-s-triazolo[3,4-a]phthalazine)triethylenetetramine-
cobalt(III) Perchlorate   [Co(trien)(3-(N-Ac-gly)-TAP)](ClO$_4$)$_2$

β-[Co(trien)CO$_3$]ClO$_4$·H$_2$O (0.126 g, 0.329 mmol) was converted to
β-[Co(trien)(OH$_2$)$_2$]$^{3+}$ with two equivalents of 2.0M HClO$_4$ (0.33 ml). After the
evolution of CO$_2$ ceased, the solution was heated to 60°C for 10 minutes. On
cooling, the pH of the solution was adjusted to about 4 by addition of 1M NaOH,
and 3-(N-Ac-gly)-TAP (0.080 g, 0.33 mmol) was added. The pH of the solution
was raised to 8.0 with NaOH solution. After heating the solution to 60°C,
methanol was added to dissolve 3-(N-Ac-gly)-TAP completely, and the reaction
continued for 1 hour at 60°C and 18 hours at room temperature. 3-(N-Ac-gly)-
TAP tended to precipitate from solution, so sufficient methanol was added to
prevent this from occurring. The solution was concentrated by rotary evapora-
tion, and the components of the solution were separated by ion-exchange
chromatography on Sephadex CM-25 using sodium perchlorate (0.1M - 0.5M) for
elution. Two orange bands which appear to be isomers of [Co(trien)(3-(N-Ac-
gly)-TAP)]$^{2+}$ were eluted with 0.2 - 0.3M NaClO$_4$. The products were contami-
nated with NaClO$_4$, and purified by fractional recrystallization from methanol
solution. However, it was difficult to remove all traces of NaClO$_4$ from the
complexes.

Absorption spectrum, λ$_{max}$ (nm): 1st isomer eluted ∿355 - 360 sh,
and 472. 2nd isomer eluted ∿355 sh and 475.

Hydrolysis Study on 3-(N-Ac-gly)-TAP in the Presence of Co^{2+}

A solution containing equimolar amounts of 3-(N-Ac-gly)-TAP and
CoCl$_2$·6H$_2$O (0.0125 M) in 1.00 M HCl, and a control solution containing only
3-(N-Ac-gly)-TAP at the same concentration (0.0125 M) in 1.00 M HCl were
incubated at 44°C. The progress of the hydrolysis reactions was monitored
by TLC on silica gel GF after neutralization of a sample of the solution

with dilute NaOH. The concentrations of the spots on the TLC plates were estimated visually. After 3 hours the degree of hydrolysis was small, and there was no significant difference in the concentration of hydrolyzed product containing Co^{2+} ions from the control solution.

Hydrolysis study on 3-(N-Ac-gly)-TAP in the Presence of Cu^{2+}

An aqueous solution containing equimolar amounts of 3-(N-Ac-gly)-TAP and $CuSO_4 \cdot 5H_2O$ was adjusted to pH 10 with concentrated NaOH (Cu^{2+} precipitates as $Cu(OH)_2$), and then to pH 0.8 with 5N HCl. A control solution containing only 3-(N-Ac-gly)-TAP at the same concentration (0.0125 M) was treated similarly with base and acid. Both solutions were incubated at 50°C. The rate of hydrolysis of the acetamidomethyl group was monitored by TLC with silica gel on a neutralized sample. After 5 hours, TLC analysis showed a relatively low concentration of hydrolyzed product, which however, was slightly higher in the solution containing Cu^{2+} ions.

Attempted Hydrolysis of 3-(N-Ac-gly)-TAP with a Cation-exchange Resin

An aqueous solution of 3-(N-Ac-gly)-TAP was eluted through a chromatography column containing cation-exchange resin (Bio-Rad, Dowex 50W-X8; 200-400 mesh, 5.1 meq/g, H^+ form) (2.335 g, 11.91 meq) with water. The eluent was monitored by UV spectroscopy and TLC on silica gel GF. No UV-absorbing material passed through the column. The bound TAP was eluted from the column with saturated NaCl solution. UV spectra were consistent with protonated 3-(N-Ac-gly)-TAP. There was no evidence of hydrolyzed product in thin layer chromatograms. The eluent gave negative results to the ninhydrin test for amines after neutralization of the solution.

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