Radical Reactions for Chemoselective Modification of Peptides

A Thesis

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Degree of

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by

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Contents

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Statement

To the best of my knowledge, this thesis contains no material previously submitted for a degree or diploma and contains no material previously published except where due reference is made.

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Caroline Ward

Abstract

With a view to developing procedures for chemoselective modification of peptides and proteins, generation of carbon-centred radicals in amino acid derivatives, amino acids, and peptides has been investigated. Trapping and detection of these radicals has also been studied.

In the photolytic reaction of triglycine with di-*tert*-butyl peroxide, α-centred radicals were trapped as either dimers of triglycine or tripeptides resulting from conversion of one glycine residue to alanine. Alanine residues were formed at either the *C*-terminal or non-terminal residue of the tripeptide. The dimers were either symmetric, crosslinked between *C*-terminal residues, or non-symmetric, linked between *C*-terminal and non-terminal residues. Product studies on this reaction demonstrated the decreasing relative ease of formation of *C*-terminal, non-terminal, and *N*-terminal α-centred radicals in peptides.

The α-centred glycyl radical generated *via* reaction of *N*-benzoylglycine methyl ester with di-*tert*-butyl peroxide, was trapped by the radical scavenger 1,1,3,3-tetramethylisoindolin-2-yloxyl.

A series of attempts to generate a β-carbon-centred radical from *N*-benzoyl-*S*-methylcysteine methyl ester and *N*-benzoyl-*S*-methylcysteine sulfoxide methyl ester is discussed. Evidence for efficient generation of a β-centred radical was derived from product studies of photolytic reactions of the *S*-methylcysteine sulfoxide derivative with di-*tert*-butyl peroxide in either benzene, benzene/carbon tetrachloride, or α,α,α-trichlorotoluene. The *S*-methylcysteine sulfoxide derivative was converted to the corresponding alanine, phenylalanine, or β-chloroalanine derivatives in these reactions.

Generation of carbon-centred radicals through reaction of sulfides and sulfoxides with hydrogen peroxide, initiated by ferrous ions or photolysis, is reported. Radicals generated from a variety of sulfides and sulfoxides have been

trapped with sodium 3,5-dibromo-4-nitrosobenzene sulfonate. The resultant spin adducts were detected by electron spin resonance (e.s.r.) spectroscopy and structurally assigned by examination of the e.s.r. spectra. E.s.r. signals arising from spin adducts of radicals formed by carbon-sulfur bond homolysis were observed. Studies of compounds containing both amine and sulfide functionality indicated that the amino group must be protonated to ensure that reaction occurs at the sulfide moiety, otherwise alternative reactions involving the deprotonated amino group may occur.

Evidence for selective generation of side-chain carbon-centred radicals was obtained when the methodology described above was applied to sulfur containing amino acids and peptides. Reactions of methionine and methionine sulfoxide gave rise to e.s.r. spectra attributable to generation of a γ-centred amino acid radical. Similarly, an e.s.r. spectrum attributable to formation of a β-centred radical was observed on reaction of acidified *S*-methylcysteine. Reactions of glycylmethionylglycine, methionylleucylphenylalanine, and acidified *S*-methyl-glutathione gave rise to e.s.r. spectra attributable to generation of side-chain carbon-centred radicals *via* selective reaction of the sulfur containing side-chains.

Introduction

Proteins play important roles in all biological systems.[1] Almost all catalysts in biological systems are proteins, namely enzymes, and hence, proteins determine the paths of chemical transformations in cells. Proteins control functions such as chemical transport and storage, immune system protection, integration of metabolism, and cell growth and differentiation. Proteins are made up of a set of twenty amino acids and the versatility of proteins results from their folding into many distinctive three-dimensional structures, determined by the amino acid sequences in the peptide chains.

Chemical modification of peptides and proteins is an area of particular interest. The properties of a protein can be significantly altered by modification of its amino acid residues. Modification of an enzyme, for example, can change its mechanism of action or its catalytic activity, particularly if an amino acid in its active site is altered.[2] Attachment of molecules to proteins is extremely useful as protein labelling is used for mechanistic studies of biochemical reactions[3] and dyes have been covalently attached to wool protein by alkylation of nucleophilic amino acid residues with appropriate chromophores.[4]

The conformation of a protein is the critical property which determines its biological function. Crosslinking the peptide strand or strands of a protein imposes restriction of its conformational freedom and is one of few methods available for the introduction of such constraints.[5] Conformationally restricted peptides have been used to determine the properties of three-dimensional structures in peptides and proteins[6] and to investigate the structural features important to the interactions of a biologically active ligand with its receptor.[7]

The peptide chains of the protein keratin,[8] the major component of wool, are linked by disulfide bonds of cystine residues. Some of the unique physical properties of wool are attributed to the presence of these disulfide crosslinks.

Reagents used in wool processing, however, can destroy the links. Therefore development of methods for the introduction of new crosslinks to wool protein, stable to the processing conditions, is important.

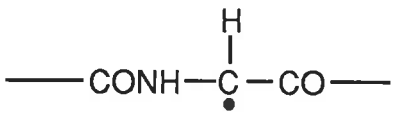
Selective chemical modification of peptides and proteins has been such a difficult task that required peptides have often been obtained by sequential synthesis from individual amino acids and required proteins by gene technology. Sequential synthesis is not an option when modification of a large mass of protein is required and problems arise in the incorporation of polyfunctional or unusual amino acids into a peptide. In such cases, procedures for selective modification of peptides are particularly useful.

Effective protein modification needs to be chemoselective. For example, when crosslinking wool protein, any new crosslink requires the chemical stability to withstand processing conditions. Hence, the protein needs to be crosslinked at selected amino acid residues with the desired stable crosslink being produced. The catalytic power of enzymes comes from their ability to bind substrates in precise orientations in their active sites[1] and alteration of the activity of an enzyme may be controlled by selective reaction on amino acid residues in its active site.[2] In mechanistic studies, selective labelling of a protein would reduce the chance of the label interfering with the chemistry being examined.

Most current methods of protein modification involve reactions of the thiol, amino, and hydroxyl groups on the constituent amino acids' side-chains, to form heteroatom-carbon or heteroatom-heteroatom bonds. Molecules can also be bound to proteins *via* weak forces such as hydrogen bonding, van der Waals forces, and ionic attraction. The Si-Ro-Set™ process[9] and its modified modern equivalents,[10] used in wool setting, involve rearrangement of the hydrogen and disulfide bonds between keratin chains by steaming the wool in the presence of thiolate or sulfite anions. Modification of peptides and proteins through formation of more stable, stronger carbon-carbon bonds would be preferable.

Such bonds can be produced by reactions of carbanions and carbocations but selective generation of these in proteins is very difficult due to the variety of functionality present. In addition, most ionic reactions involve dissolution of the protein in harsh acidic or basic reagents, and protein damage can result. Alternatively, carbon-carbon bonds can be formed by the coupling of two carbon-centred radicals and by addition of carbon-centred radicals to alkenes and other unsaturated functionality. The use of free-radical reactions to modify proteins would be advantageous in that they can be initiated by mild heat or ultraviolet light. Reagents can be evenly distributed throughout the protein before initiation and there is less chance of damage occurring under these moderate conditions.

Accordingly, the aim of the work presented in this Thesis was the development of chemoselective methods for generating carbon-centred radicals in peptides, with a view to using this chemistry for selective modification of peptides and proteins.

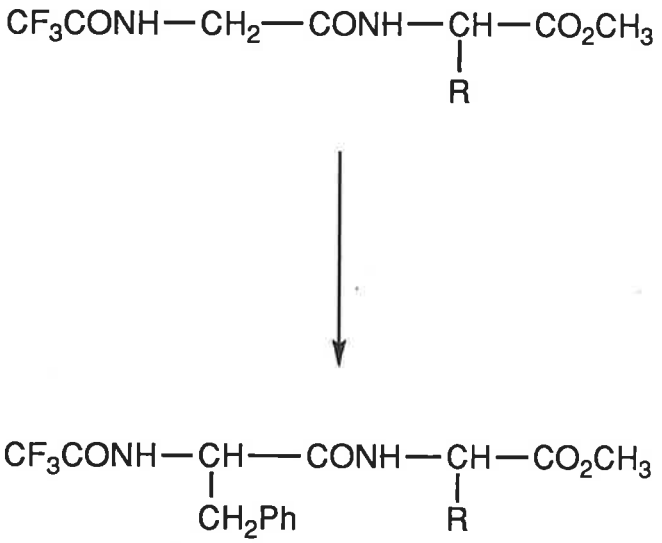


(1)

Free-radical reactions of peptides and proteins have already attracted considerable attention. Two main types of radicals have been detected by electron spin resonance (e.s.r.) spectroscopy during the irradiation of proteins.[11] The broad and anisotropic e.s.r. spectra of one type have been attributed to sulfur-centred radicals, because a similar spectrum was observed when cystine dihydrochloride was irradiated.[12] The doublet resonance of the other radical type is characteristic of resonances found for radicals generated from *N*-acetylglycine and glycylglycine,[13] and was thus assigned to α-carbon-centred radicals (1), formed by selective hydrogen abstraction from glycine residues. The assignment of the doublet to glycyl radicals was validated by e.s.r. studies which showed that the

ratio of the doublet resonance to the sulfur resonance was dependent on the ratio of glycine to cysteine and cystine residues in the protein.[14]

Selectivity for reaction of glycine residues has also been shown[15-19] through photoalkylation experiments on protected dipeptides and proteins. The degree of selectivity for alkylation of glycine residues was dependent on their location in the peptide, and the molecular weight and conformation of the peptide.[17,18] The glycine residues of the dipeptide derivatives (2), (3), (4), and (5) were converted to phenylalanine residues in yields of 25-60%, on exposure to visible light in the presence of an α-diketone as a photoinitiator, di-*tert*-butyl peroxide, and toluene (Scheme 1).[16] Photolysis of the proteins lysozyme, collagen, and ribonuclease,



(2) R = CH_3

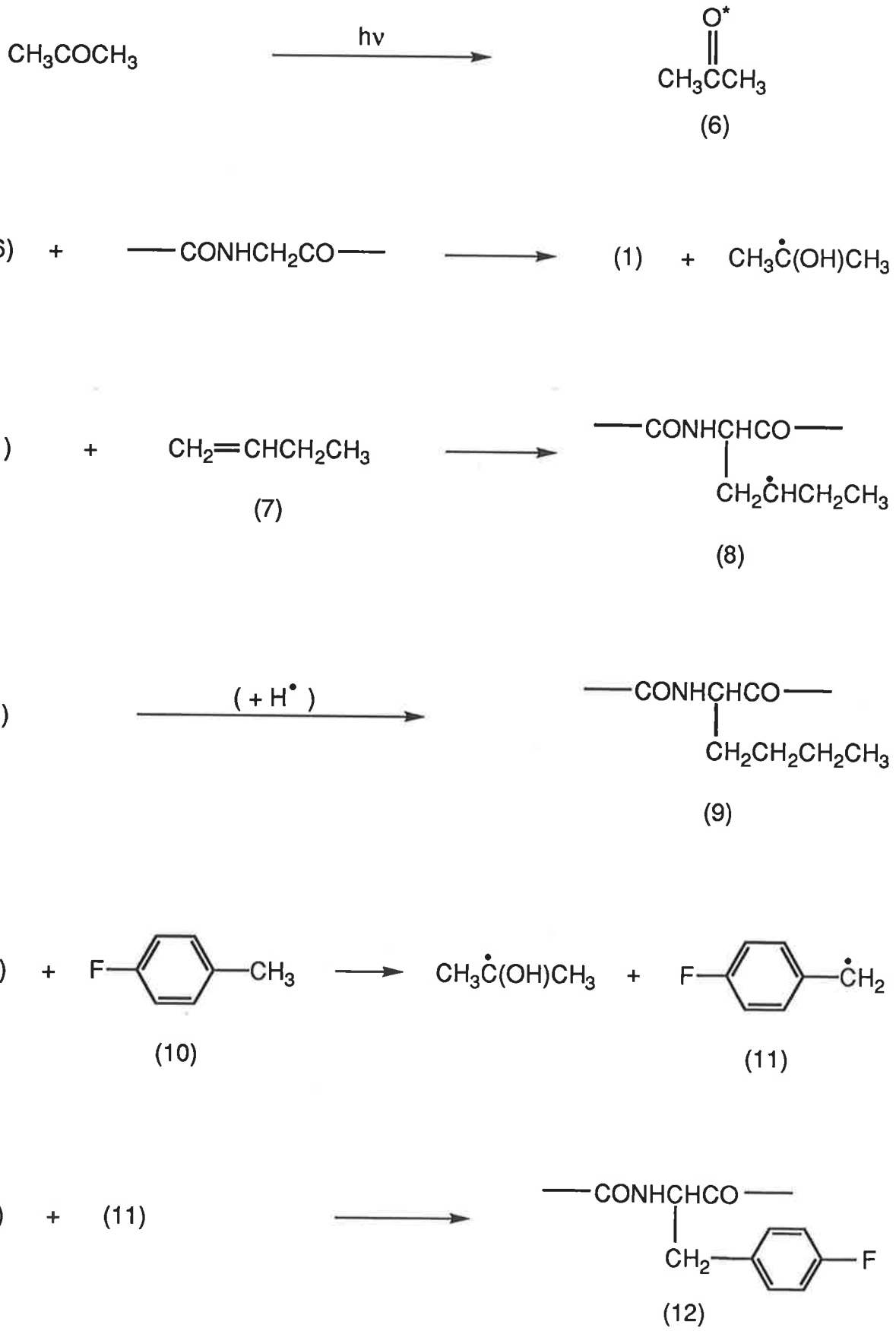
(3) R = $CH(CH_3)_2$

(4) R = CH_2Ph

(5) R = $CH_2(C_6H_4\text{-}p\text{-}OCH_3)$

Scheme 1

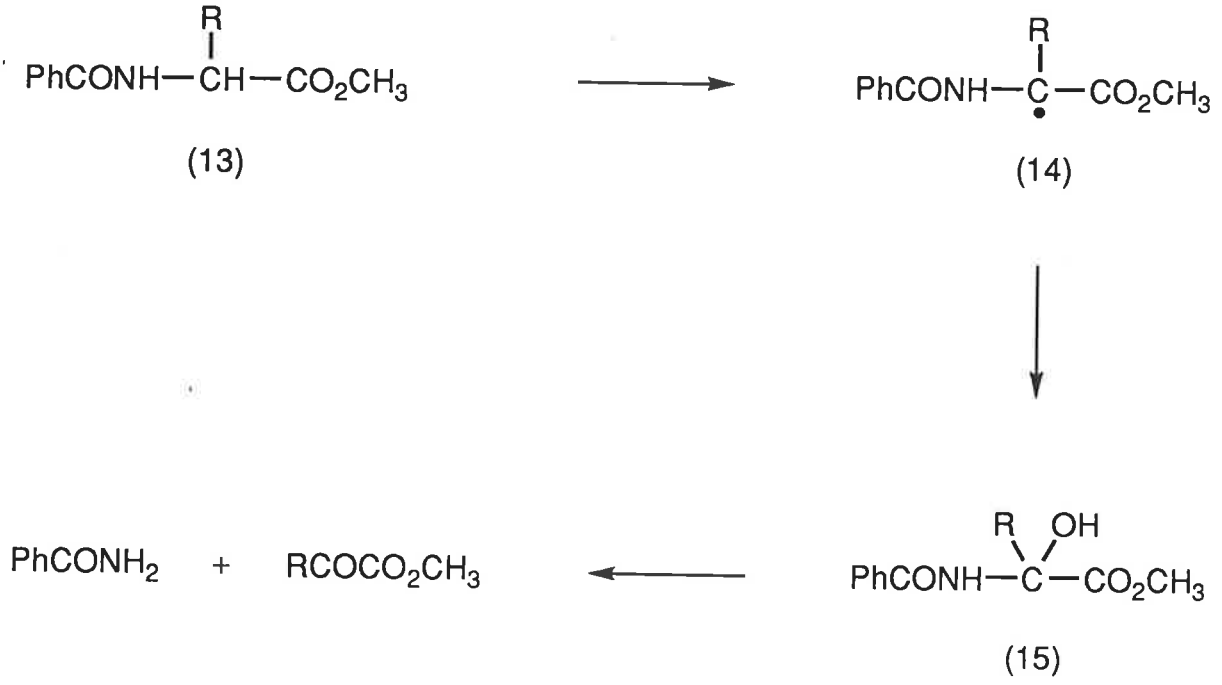
with acetone and either but-1-ene (7) or *p*-fluorotoluene (10) resulted in conversion of glycine residues to either derivatives of norleucine (9) or *p*-fluorophenylalanine (12).[17] The proposed free-radical mechanisms for the alkylations[15,18] are outlined in Scheme 2. Photosensitized acetone (6) abstracts an α-hydrogen from a glycine residue. The resultant glycyl radical (1) adds to but-1-ene (7) to form the radical (8), or couples with *p*-fluorobenzyl radical (11),


CH3COCH3
hν
O*
CH3CCH3
(6)
(6) + —CONHCH2CO— → (1) + CH3Ċ(OH)CH3
(1) + CH2=CHCH2CH3
(7)
—CONHCHCO—
CH2ĊHCH2CH3
(8)
(8)
(+ H•)
—CONHCHCO—
CH2CH2CH2CH3
(9)
(6) + F— —CH3
(10)
CH3Ċ(OH)CH3 + F— —ĊH2
(11)
(1) + (11)
—CONHCHCO—
CH2— —F
(12)

Scheme 2

formed by hydrogen abstraction from *p*-fluorotoluene (10), to form a *p*-fluorophenylalanine residue (12). The radical (8) abstracts a hydrogen atom to form a norleucine residue (9).

Selectivity for reaction of glycine residues was also observed in a study of the reaction of amino acid derivatives with nickel peroxide.[20] The relative rates of reaction of the amino acid derivatives (13a-c) were found to be 10.0:1.0:0.14. Preferential reaction of the glycine residues of the *N*-benzoyldipeptide methyl esters of alanylglycine, valylglycine, leucylglycine, and glycylvaline has also been observed.[21] Nickel peroxide is a free radical oxidant which acts as both a hydrogen-atom abstractor and a source of hydroxyl radicals.[22,23] The mechanism of its reaction with the amino acid derivatives (13a-c) is thought to involve hydrogen-atom abstraction followed by combination of the resultant α-centred radicals (14a-c) with hydroxyl radical to form the α-hydroxy amino acid derivatives (15a-c) (Scheme 3). Elimination of benzamide from the alcohols (15a-c) gives methyl glyoxylates. This mechanism is analogous to that proposed

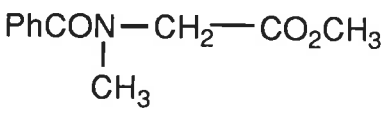


(a) R = H
(b) R = CH_3
(c) R = $CH(CH_3)_2$

Scheme 3

for the cleavage of *C*-terminal glycine residues in the bioactivation of peptide hormones, catalysed by peptidylglycine α-amidating monooxygenase (PAM).[24] The selective reaction of the glycine derivative (13a) with nickel peroxide suggests that the substrates of PAM are synthesized with a *C*-terminal glycine residue because it is so readily removed by oxidation.

The preferential reaction of glycine derivatives is contrary to the expected relative stability of secondary and tertiary radicals.[25] Expectations are that radicals should form more easily at the tertiary α-centres of other amino acid derivatives due to the greater relief of steric compression on conversion from sp^3 to sp^2 hybridized molecular orbitals. In addition, there is greater hyperconjugative stabilization of tertiary radicals. As a result, the selectivity for hydrogen-atom abstraction from glycine derivatives has been the subject of several investigations in recent years. One study exploited the photolytic reaction of di-*tert*-butyl peroxide with aliphatic amino acid derivatives.[26] The mechanism of photolytic reactions of di-*tert*-butyl peroxide with organic substrates is outlined in Scheme 4.[27] *tert*-Butoxy radical, produced by photolytic cleavage of di-*tert*-butyl peroxide, undergoes either β-scission to give acetone and methyl radical or hydrogen-atom abstraction from the substrate to give a carbon-centred radical and *tert*-butanol. The substrate radical either couples with methyl radical or dimerizes. The relative rates of reaction of the derivatives of glycine (13a), alanine (13b), valine (13c), and sarcosine (16), with di-*tert*-butyl peroxide indicated the greater stability of the radical (14a), produced by the abstraction of an α-hydrogen from the glycine derivative (13a), compared with the radicals (14b), (14c), and (17), derived from the amino acid derivatives (13b), (13c), and (16), respectively.



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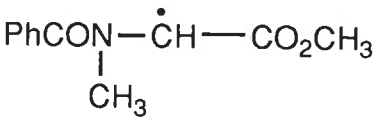
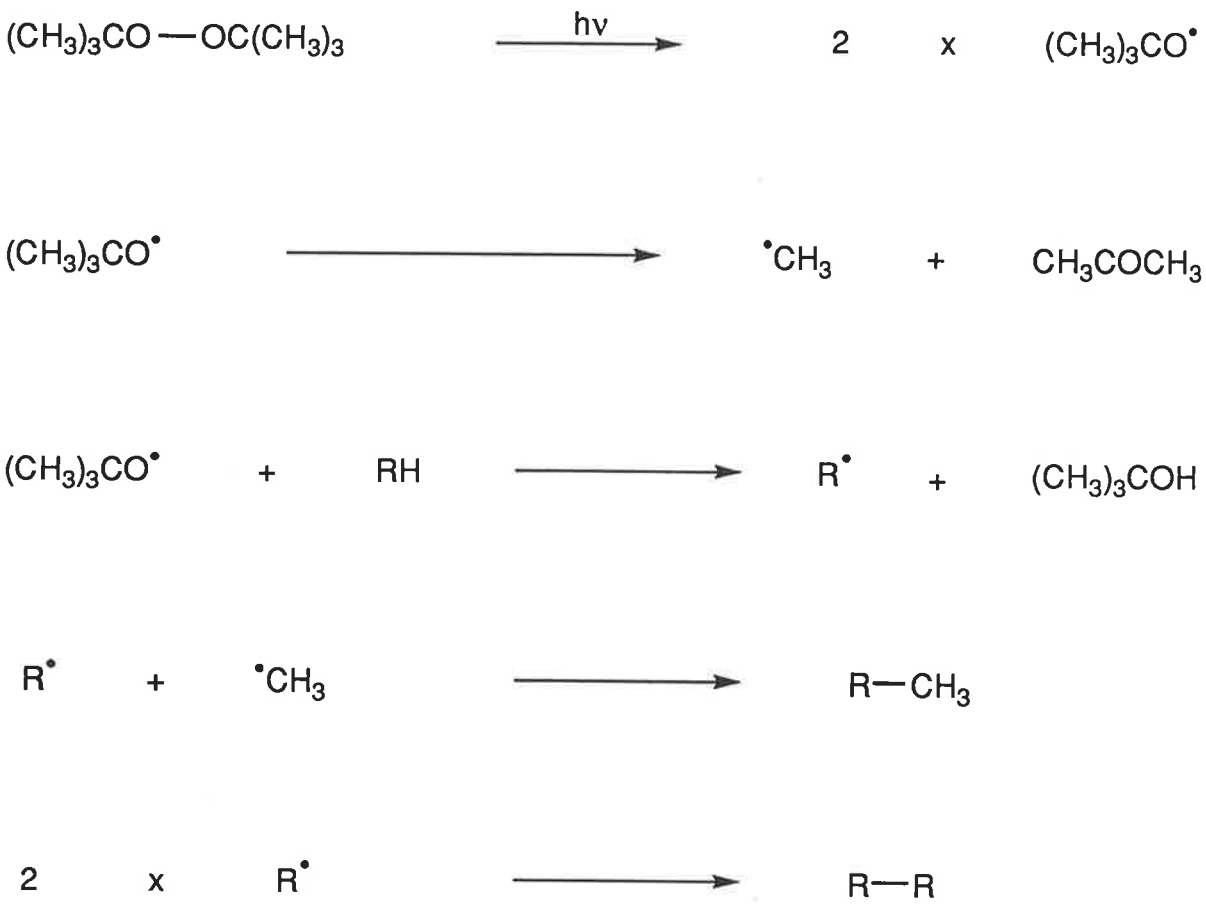
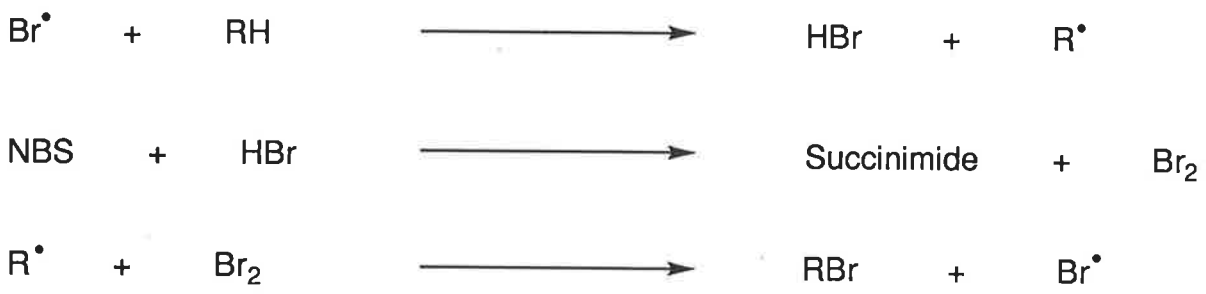


(17)



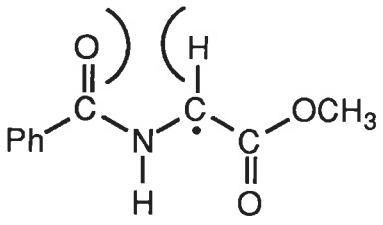
Scheme 4

Similar results were obtained in a study of the bromination of amino acid derivatives with *N*-bromosuccinimide (NBS).[26,28] The radical bromination cycle is shown in Scheme 5 and is believed to involve hydrogen-atom abstraction by bromine atom.[29] The amino acid derivatives (13a-c) and (16) react with NBS by α-hydrogen-atom abstraction and the relative rates of reaction indicated the greater stability of the glycyl radical (14a) compared with the α-centred radicals (14b), (14c), and (17). In addition, selective reaction of glycine residues has been observed in reactions of NBS with dipeptide derivatives containing glycine and either alanine, valine, phenylalanine, leucine, or aspartic acid.[30,31]

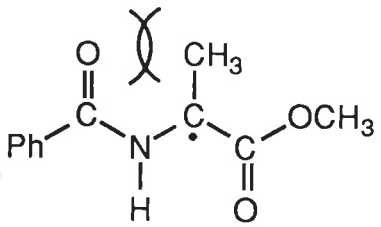


Scheme 5

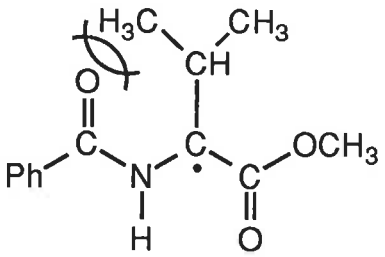
α-Carbon-centred radicals derived from amino acid derivatives are stabilized by the combined resonance effects of an electron-releasing amido substituent and an electron-withdrawing carboxy substituent and are termed captodative,[32] merostabilized,[33] or "push-pull" stabilized.[34] Their stability is dependent on the degree of delocalization of the unpaired spin density over these neighbouring groups. Overlap of the semi-occupied p-orbital with the π-orbitals of the amido and methoxycarbonyl substituents is required for spin delocalization and there is maximum overlap in planar conformations of these systems. The differences in stability of the α-centred radicals (14a-c) and (17) have been attributed[26,28] to the relative ease with which they adopt these planar conformations. The non-bonding interactions in the α-centred radicals (14a-c) and (17), shown below, can distort the radicals out of planarity. The larger the α- or *N*-substituent, the greater the distortion. This is consistent with the observed relative rates of formation of the α-centred radicals (14a-c) and (17).



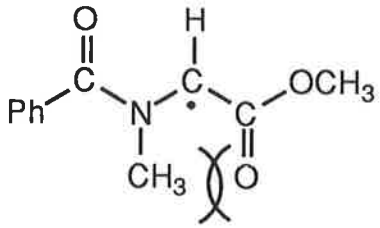
(14a)



(14b)

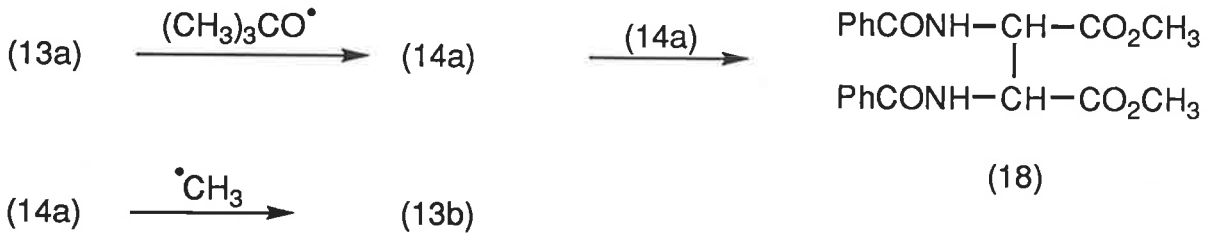


(14c)



(17)

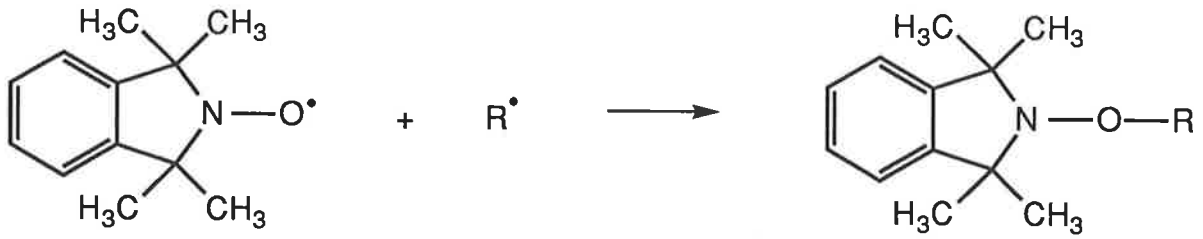
In the photolytic reaction of *N*-benzoylglycine methyl ester (13a) with di-*tert*-butyl peroxide, the radical (14a), produced by α-hydrogen-atom abstraction from the glycine derivative (13a) by *tert*-butoxy radical, either reacts to produce the dimer (18) or couples with methyl radical to give the alanine derivative (13b) (Scheme 6).[35] In work described in this Thesis, the trapping of the glycyl radical (14a) was investigated with a view to trapping carbon-centred radicals in proteins. The ability to trap carbon-centred radicals in proteins would be advantageous in the formation of new carbon-carbon bonds in a protein as it would give an indication of the number and position of such radicals.



Scheme 6

Free radicals can be trapped as stable neutral spin adducts by coupling with other radicals. Di-*tert*-alkyl nitroxides react with alkyl radicals at close to diffusion controlled rates ($k = 10^7$-$10^9\ M^{-1}s^{-1}$) to give stable alkoxyamines, as shown in Scheme 7 for 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO) (19), but they do not trap oxygen-centred radicals efficiently.[36] It was thus envisaged that a trap of this type could trap the glycyl radical (14a) in the presence of *tert*-butoxy radical. TMIO (19) has been successfully utilized in studies of the mechanisms of

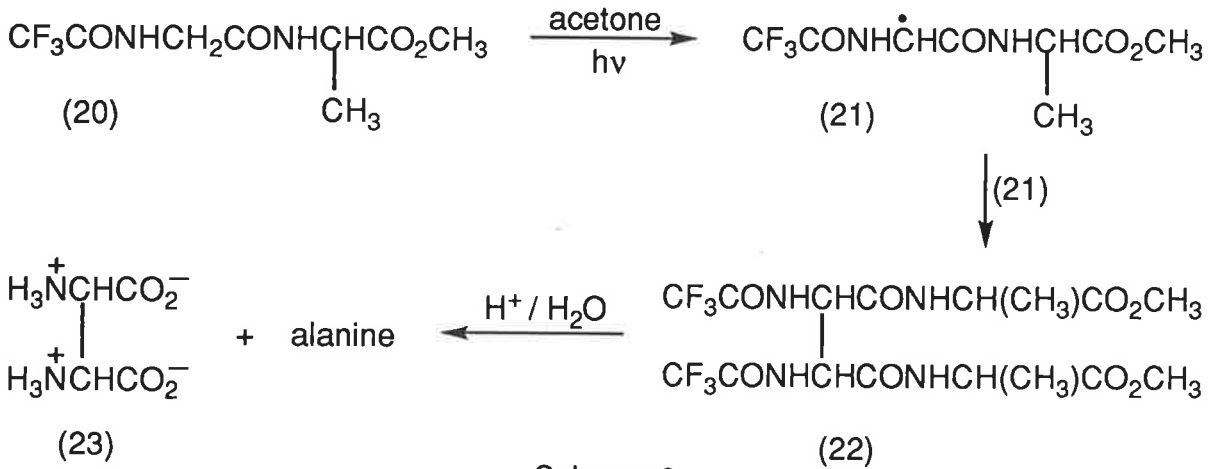
initiation of radical polymerizations[36-38] and its use was favoured over other nitroxide spin traps because it provides adducts which are usually crystalline, stable, and easily characterized.[38] In addition, the chromophore of TMIO (19) allows simple detection of its adducts by thin layer and high performance liquid chromatography. Attempts to trap the α-centred glycyl radical (14a) with TMIO (19) are discussed in Chapter One.



(19)

Scheme 7

In addition to the dimerization of *N*-benzoylglycine methyl ester (13a), crosslinking of glycine residues has been observed in the photolysis of the glycylalanine derivative (20) in acetone. The products obtained, after acid hydrolysis, were 1,2-diaminosuccinic acid (23) and alanine.[15] The formation of the crosslinked species (23) indicated that two of the α-centred glycyl radicals (21), formed by hydrogen-atom abstraction from the glycylalanine derivative (20) by photosensitized acetone (6), had coupled to give the crosslinked dipeptide derivative (22) (Scheme 8).



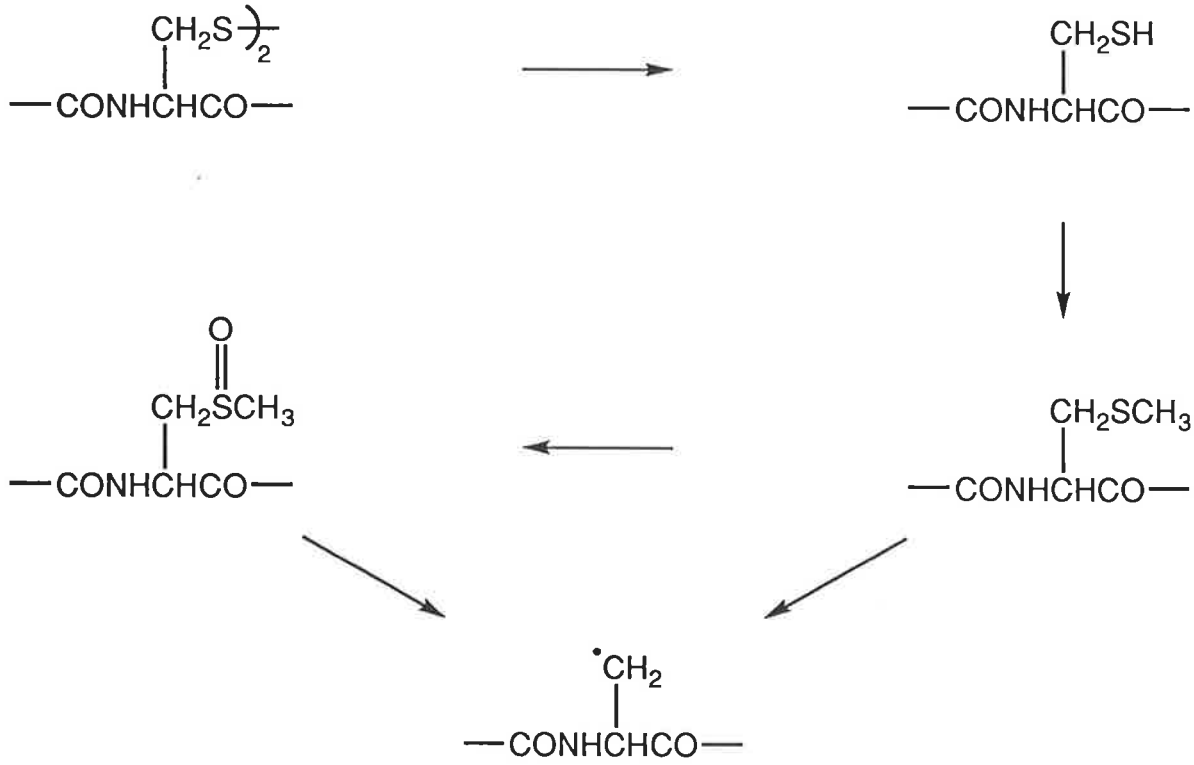
Scheme 8

At the outset of this work, some evidence of free-radical induced crosslinking of glycine residues in an unprotected peptide had also been observed in the reaction of triglycine (24) with di-*tert*-butyl peroxide.[39] A trace amount of the glycine dimer (23) was detected by high performance liquid chromatography, after acid hydrolysis of the product peptide mixture. Evidently, dimerization of triglycine (24) had occurred by coupling of two α-centred glycyl radicals, formed by hydrogen-atom abstraction from the peptide (24) by *tert*-butoxy radical. In the present work, the efficiency and synthetic utility of this dimerization was investigated with a view to extending the methodology to the formation of new carbon-carbon bonds in larger peptides and proteins. The results of this investigation are discussed in Chapter One.

$H_3\overset{+}{N}CH_2CONHCH_2CONHCH_2CO_2^-$

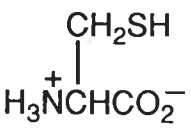
(24)

An alternative to the generation of carbon-centred radicals on peptide backbones is their generation on the more accessible side-chains. Different methodology is required to obtain side-chain radicals as the captodative nature of the α-centred radicals is unique to that position. In the present work, it was envisaged that the reactive thiol moiety of cysteine residues could be manipulated to give rise to β-centred radicals *via* homolytic cleavage of the carbon-sulfur bond. The proposed method for generating a β-centred radical from a cysteine residue in a protein is outlined in Scheme 9. The proposal involves reduction of any cysteine residues present as disulfides, selective methylation of the thiol moiety, and displacement of a β-centred radical from the resultant sulfide or its sulfoxide.



Scheme 9

Cysteine (25) and methionine (26) are the only sulfur-containing amino acids found in naturally occurring proteins. Cysteine (25), often found as its disulfide cystine (27), is the most abundant. Cystine residues can readily be converted to cysteine residues *via* nucleophilic thiol interchange reactions on treatment of a protein with a thiol such as 2-mercaptoethanol.[40] Free-radical reactions of thiols usually involve abstraction of the thiol hydrogen to give sulfur-centred radicals.[41] Methylation of the thiol group of cysteine (25), to form *S*-methylcysteine (28), eliminates this possibility. Selective alkylation of cysteine



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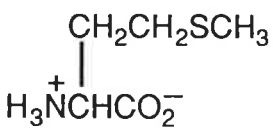

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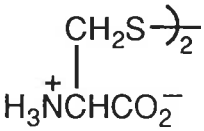

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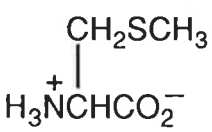
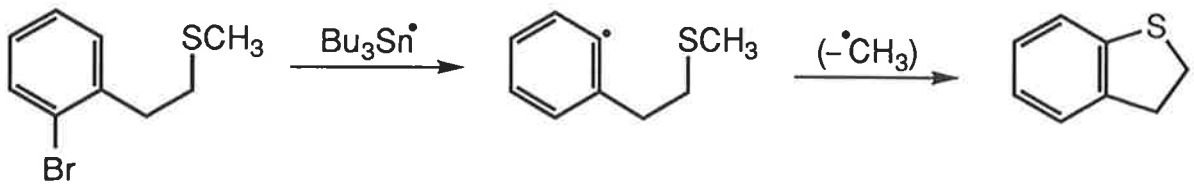


(28)

(25) in a peptide possessing several potentially competing nucleophilic groups such as hydroxy, amino, and carboxyl groups has been achieved by treatment with alkylating agents, including alkyl halides, in saturated methanolic ammonia solution.[42] In addition, the methylating reagent trimethylphosphate (TMP) has been found to be particularly selective for cysteine (25).[43] Out of twenty-one amino acids treated individually with TMP at pH 5-8, cysteine (25) was the only one that reacted. The selective *S*-methylation of the cysteine residue in a small peptide using TMP is discussed in Chapter Three. It was envisaged that both *S*-methylcysteine (28) and its homologue methionine (26) could be manipulated to give rise to carbon-centred radicals.

A sulfur-containing amino acid was chosen for elaboration because displacement of carbon-centred radicals from sulfides and sulfoxides is known to occur. Intramolecular homolytic substitution of carbon-centred radicals on sulfur has been used in the synthesis of sulfides and sulfoxides. Treatment of the sulfide (29) and its corresponding sulfoxide with tributyltin hydride gave cyclized products, as shown in Scheme 10 for the sulfide (29).[44,45]

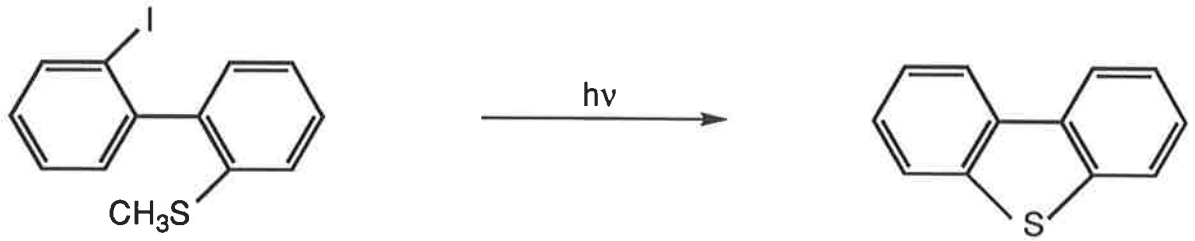


(29) (30)

Scheme 10

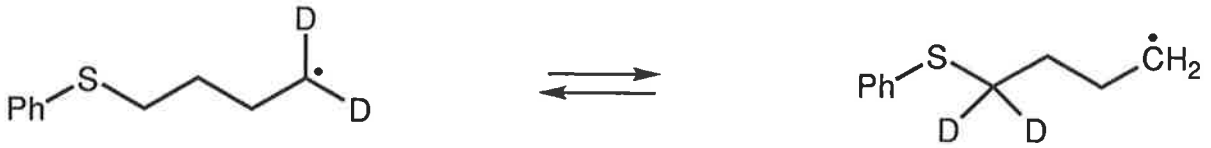
The proposed mechanism involved formation of the aryl radical (30) through abstraction of bromine by tributyltin radical. Homolytic substitution of this radical (30) at sulfur resulted in cyclization through displacement of the methyl radical which abstracted a hydrogen-atom from tributyltin hydride to give methane. The intramolecular nature of the substitution reaction, which was

believed to involve concerted bond formation and bond fission,[44,46] caused methyl radical to be displaced in preference to a more stable methylene radical. In a similar reaction, dibenzothiophene (32) was synthesized by photolysis of the iodide (31) in cyclohexane (Scheme 11).[47]



(31) (32)

Scheme 11



(33) (34)

Scheme 12

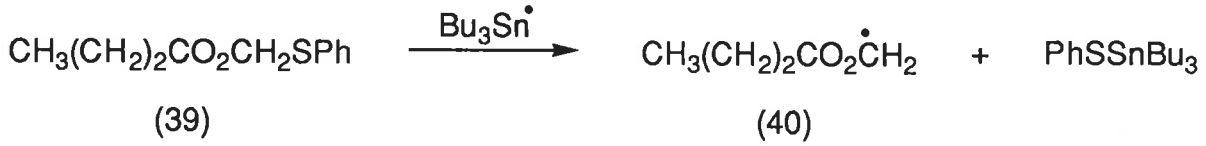
$$(PhCH_2)_2S + m\text{-}ClC_6H_4\dot{C}H_2 \longrightarrow m\text{-}ClC_6H_4CH_2SCH_2Ph + Ph\dot{C}H_2$$

(35) (36) (37) (38)

Scheme 13

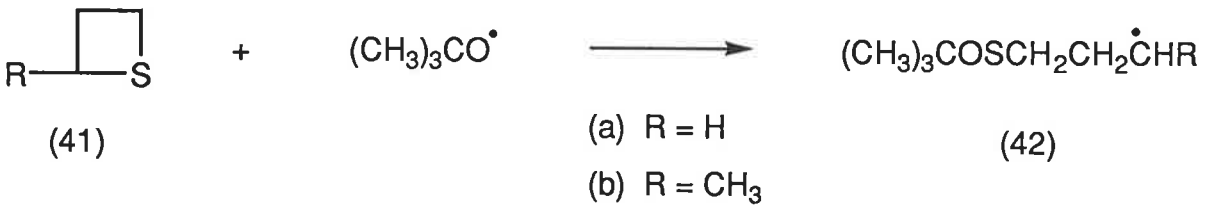
In mechanistic studies of homolytic substitution on sulfides, the radicals (33) and (34) rearranged *via* cleavage of carbon-sulfur bonds (Scheme 12).[48] In one of the few reported intermolecular reactions of this type, the thioether (37) was produced *via* displacement of the resonance stabilized benzyl radical (38) in the reaction of the *m*-chlorobenzyl radical (36) with dibenzylthioether (35) (Scheme 13).[49]

Carbon-centred radicals have also been generated by substitution of stannyl and oxygen-centred radicals on sulfides. Phenylthiomethyl butanoate (39) was reduced to methyl butanoate by tributyltin hydride *via* stannyl radical attack on sulfur and displacement of the radical (40) (Scheme 14).[50] Photolysis of thietan (41a) and 2-methylthietan (41b), with di-*tert*-butyl, diethyl, or bis-(trimethylsilyl) peroxides resulted in ring opening *via* homolytic substitution on sulfur by oxygen-centred radicals. In the reactions with di-*tert*-butyl peroxide, the radicals (42a) and (42b) were produced (Scheme 15).[51]



(39) (40)

Scheme 14



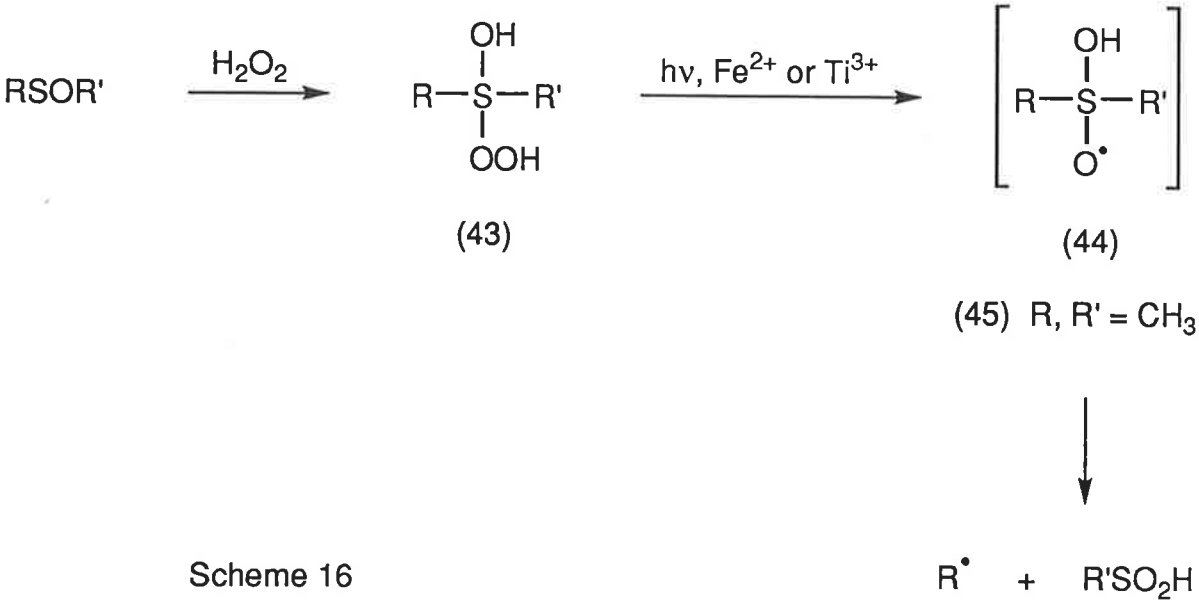
(41) (42)

(a) R = H
(b) R = CH_3

Scheme 15

In addition, alkyl radicals have been generated by treatment of sulfoxides with hydrogen peroxide, followed by photolysis or addition of ferrous or titanous ions.[52-54] The proposed mechanism[54] for this process involves addition of hydrogen peroxide to the sulfoxide to form a complex (43). On photolysis or addition of ferrous or titanous ions, the peroxide bond of this complex (43) is cleaved giving rise to the intermediate radical (44) which undergoes β-scission to displace an alkyl radical and leave an alkylsulfonic acid (Scheme 16). The ratio of the two radicals which can be displaced through cleavage of the two carbon-sulfur

bonds is dependent on their relative stability. The combination of dimethyl sulfoxide, hydrogen peroxide, and ferrous ions gives rise to methyl radicals and has been used to methylate various substrates.[54,55] The intermediate (45) is believed to be involved in methylation. The combination of hydrogen



Scheme 16

peroxide and ferrous ions, known as Fenton's reagent, gives rise to hydroxyl radicals (Equation 1) and hydroperoxy radicals (Equation 2) *via* electron transfer between ions and the peroxide.[56] Titanous ions are an alternative to ferrous ions in this process. The mechanism for displacement of radicals from sulfoxides outlined above is favoured over direct substitution on sulfoxides by hydroxyl radical (Equation 3) or hydroperoxy radical because no alkyl radicals or sulfonic

Equation 1 : $Fe^{2+} + HOOH \longrightarrow Fe^{3+} + HO^{-} + HO^{\bullet}$

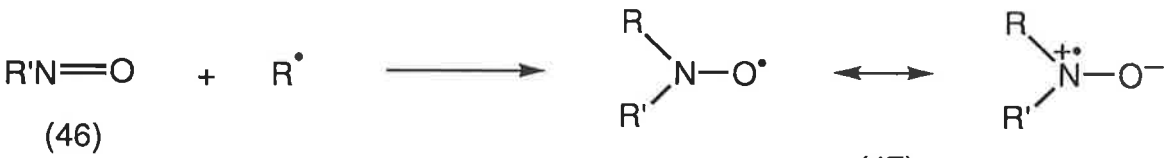
Equation 2 : $Fe^{3+} + HOOH \longrightarrow Fe^{2+} + H^{+} + HOO^{\bullet}$

Equation 3 : $RSOR' + HO^{\bullet} \longrightarrow$ (44)

acids were detected when hydroxyl and hydroperoxy radicals were replaced by *tert*-butoxy radicals generated from *tert*-butyl perbenzoate, *tert*-butyl peracetate, or *tert*-butylperoxalate.[54] These reagents do not form complexes with sulfoxides, whereas *tert*-butyl hydroperoxide, which can form a complex, gave minor amounts of methyl radicals on photolysis in dimethyl sulfoxide. In a conflicting study, however, production of alkyl radicals *via* carbon-sulfur bond cleavage was observed on treatment of sulfoxides with *tert*-butoxy radicals generated from di-*tert*-butyl peroxide.[57]

As treatment of a sulfide with hydrogen peroxide quickly results in the *in situ* formation of a complex (43), alkyl radicals can be generated from dialkylsulfides or sulfoxides using the methodology outlined in Scheme 16. In the precise conversion of sulfides to sulfoxides, however, it is difficult to control overoxidation using hydrogen peroxide and sulfones may be produced. Overoxidation is best avoided by oxidation of sulfides with sodium periodate.[58]

As in some of the examples discussed above,[51-53,57] free radicals can be detected and identified through the utilization of electron spin resonance (e.s.r.) spectroscopy, a powerful tool for the study of radicals.[59] Only molecules with unpaired electrons are detected and structural information on these is provided by splitting patterns in their e.s.r. spectra. In e.s.r. studies, short-lived radicals are often trapped to form relatively long-lived stable spin adducts. Nitroso compounds (46) are commonly used as radical traps.[60] Radicals produced in the presence of such traps add to the nitrogen to form resonance stabilized nitroxide radicals (47) (Scheme 17). The e.s.r spectra of these nitroxides (47) show triplet



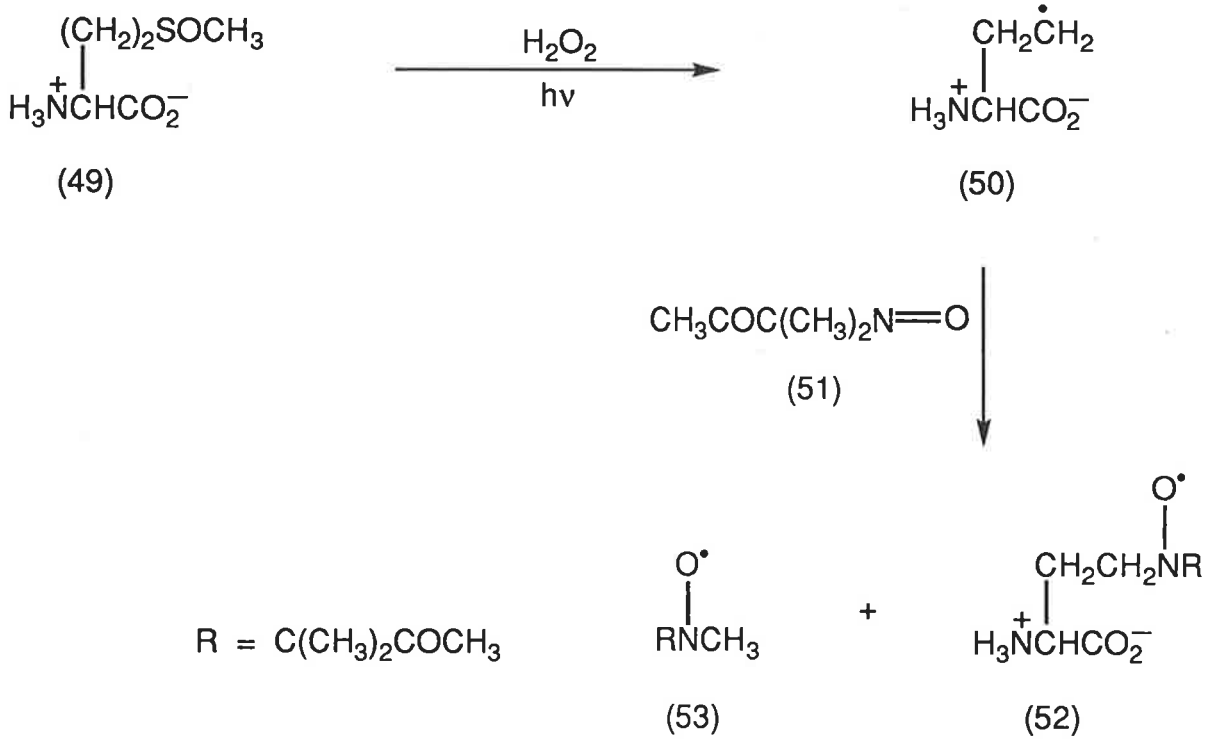
(46) (47)

(48) R' = $C(CH_3)_3$

Scheme 17

(1:1:1) hyperfine splitting, usually 15-17 gauss, caused by the nitrogen nucleus. In addition, splittings originate from magnetic nuclei of the trapped radical and provide information on its structure. Nitroso-*tert*-butane (NTB) (48) has been widely used in e.s.r. studies[60] including the identification of radicals produced from sulfides and sulfoxides.[52] Short-lived alkyl radicals produced on photolysis of mixtures of sulfides or sulfoxides with hydrogen peroxide have been trapped by NTB (48) to form alkyl-*tert*-butylnitroxides which gave stable, interpretable e.s.r. spectra.

Of particular relevance to the present work was the production of the γ-centred radical (50) from methionine (26) or its sulfoxide (49) *via* the methodology outlined in Scheme 16. In one study,[52] an aqueous solution of the sulfoxide (49) was photolysed with hydrogen peroxide in the presence of the trap, 2-methyl-2-nitrosobutan-3-one (51). The triplet of triplets of triplets (15.5, 12.0, 0.5 gauss) in the resultant e.s.r. spectrum was attributed to the adduct (52), formed by reaction of the γ-centred radical (50) with the trap (51) (Scheme 18). The smaller



Scheme 18

triplet splittings arise from coupling between the lone electron and the γ- and β-hydrogens. A second signal, a triplet of quartets (16.1, 13.7 gauss), was attributed to the methyl radical adduct (53). Homolytic cleavage of either carbon-sulfur bond gives rise to the radical (50) and methyl radical.

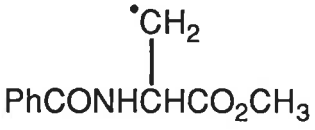
In similar studies using NTB (48) as the trap, a triplet of doublets of doublets of triplets was the dominant e.s.r. signal derived from methionine (26).[61] Distinct splitting by each of the γ-hydrogens gave rise to a more complicated signal than that described above for the adduct (52) of the radical (50). Similarly, methionylmethionine gave two triplets of doublets of doublets of triplets, indicating that γ-centred radicals had been generated from both residues.[62]

The e.s.r. spectrum of an acidic mixture of methionine (26) and Fenton's reagent in a continuous flow system, showed a triplet of triplets and a quartet for the protonated γ-centred radical (50) and methyl radical, respectively.[63] In a similar study, a triplet of triplets was observed when methionine (26) or its sulfoxide (49) were mixed with titanous chloride and hydrogen peroxide using a continuous flow method.[53]

In addition to e.s.r. studies, one product study involving carbon-sulfur bond homolysis in an amino acid was found in the literature. In research into flavour deterioration caused by food irradiation, the γ-irradiation of an aqueous solution of *S*-(*cis*-1-propenyl)-L-cysteine sulfoxide, a lachrymatory precursor ~~of~~ from onion, gave rise to alanine,[64] presumably *via* cleavage of a carbon-sulfur bond to give a β-centred radical.

Based on the precedents described above, the generation of carbon-centred side-chain radicals in proteins by the method outlined in Scheme 9 was investigated by studying reactions of cysteine (25) and methionine (26) and their derivatives. The selective methylation of cysteine (25) in peptides, the controlled oxidation of *S*-methylcysteine (28) and its derivatives, and the relative efficiency of radical generation from sulfides and sulfoxides were investigated. Generation

of the β-centred radical (54) *via* homolytic substitution on sulfur was investigated by studying reactions of *N*-benzoyl-*S*-methylcysteine methyl ester (55) and *N*-benzoyl-*S*-methylcysteine sulfoxide methyl ester (56) with alkyl, aryl, stannyl, and alkoxy radicals, and Fenton's reagent. The results of product studies in this area are discussed in Chapter Two.



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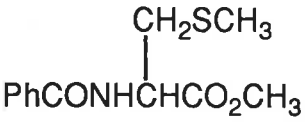

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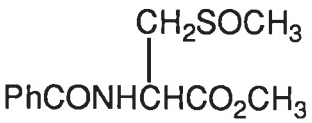
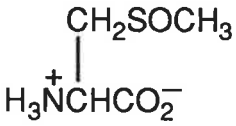


(56)

E.s.r. studies on reactions of hydrogen peroxide with *S*-methylcysteine (28), *S*-methylcysteine sulfoxide (57), methionine (26), methionine sulfoxide (49) and the commercially available tripeptides glycylmethionylglycine (58), methionylleucylphenylalanine (59), and *S*-methylglutathione (60), initiated by ferrous ions or photolysis, were also undertaken and the results are discussed in Chapter Three.



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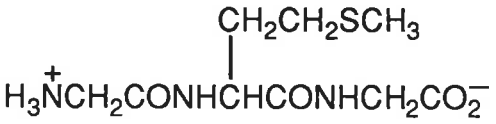

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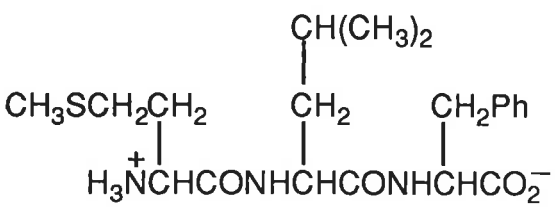

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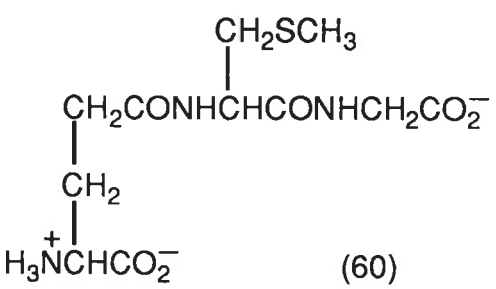
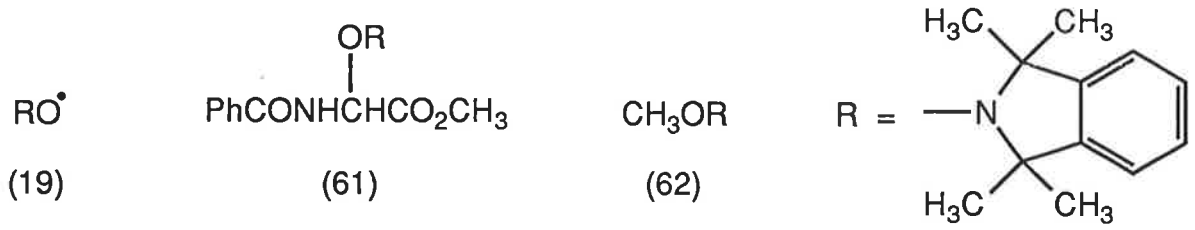


(60)

Results and Discussion Chapter One

Reactions of Glycyl Radicals

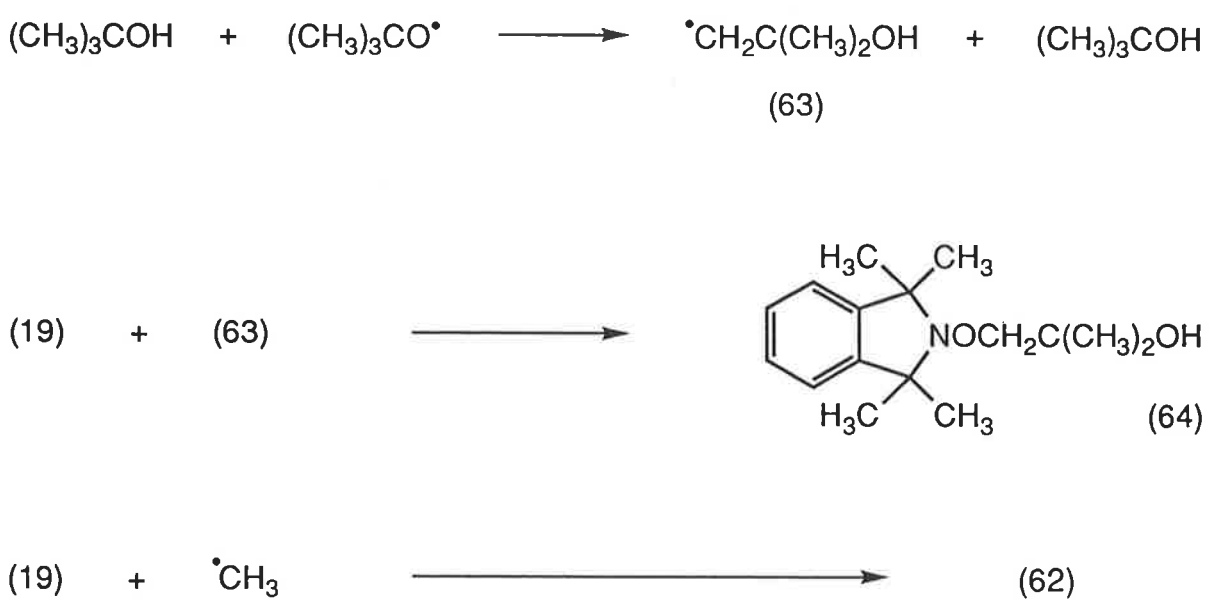
The α-centred radical (14a) has been generated and trapped as the alanine derivative (13b) and the diastereomers of the dimer (18) by photolysis of a mixture of the glycine derivative (13a) and di-*tert*-butyl peroxide in either benzene or *tert*-butanol (Scheme 6).[35] These reactions were repeated in the presence of the radical trap 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO) (19), in attempts to trap the radical (14a) and form the adduct (61), with a view to trapping carbon-centred radicals in proteins.



A solution of the glycine derivative (13a), TMIO (19), and di-*tert*-butylperoxide in benzene was irradiated with ultraviolet light under an inert atmosphere for twenty-four hours. Chromatography of the crude product on silica gave the adduct (61) in 14% yield (42% based on recovered starting material) and the starting glycine derivative (13a) in 66% yield. The ^{1}H n.m.r. spectrum of the adduct (61) showed a doublet (J = 9.5 Hz) at δ 6.22 for the α-proton. The fast atom bombardment (f.a.b.) mass spectrum showed a protonated molecular ion at *m/z* 383. The product (61) was insufficiently stable for complete characterization. The ^{1}H n.m.r. spectrum of one impure fraction obtained from the chromatography showed a singlet at δ 3.78 as well as the resonances expected for the aromatic and methyl protons of an adduct of TMIO (19). These signals are likely to have arisen from 2-methoxy-1,1,3,3-tetramethyl-isoindoline (62), produced by trapping of methyl radical, formed *via* β-scission of *tert*-butoxy radical (Scheme 4), by TMIO (19).[38] The ^{1}H n.m.r spectrum of the crude reaction

mixture showed three singlets at δ 3.76, 3.84, and 3.81 for methyl ester protons in an approximate integral ratio of 9:2:3 and these corresponded to the compounds (13a), (61), and (62), respectively. The yield of (61) indicated that the efficiency of trapping of the radical (14a) by TMIO (19) was about 40%, assuming that the only reaction of the glycine derivative (13a) was α-hydrogen abstraction. Competition for the trap (19) between the radical (14a) and methyl radical would have affected this efficiency.

As the goal of this research was to trap glycyl radicals formed in peptides, it was preferable to trap the radical (14a) in water miscible solvents such as *tert*-butanol. Accordingly, the above reaction was repeated in *tert*-butanol over eighteen hours. Thin layer chromatography (t.l.c.) of the reaction mixture indicated that little of the trap (19) remained but the ^{1}H n.m.r. spectrum showed two small peaks near δ 6.25 which indicated that at most 5% of the adduct (61) had been produced. Chromatography of the reaction mixture on silica gave the methoxyamine (62) in 17% yield, 2-(2-hydroxy-2-methylpropoxy)-1,1,3,3-tetramethylisoindoline (64) in 15% yield, and the starting glycine derivative (13a) in 44% yield. The ^{1}H n.m.r. spectrum of the methoxyamine (62), consistent with that reported,[38] showed a singlet at δ 3.78 attributable to the methoxy protons. Satisfactory microanalytical data was obtained for the alcohol (64) and the mass spectrum showed a molecular ion at *m*/z 263. The infra-red spectrum showed a broad signal at 3384 cm^{-1} for the hydroxyl group. The ^{1}H n.m.r. spectrum showed singlets at δ 1.31 and 3.83, for two methyl groups and the methylene group, respectively, along with two multiplets centred at δ 7.09 and 7.24 for the aromatic protons. Broad resonances at δ 1.46 and δ 2.50 corresponded to the methyl protons of the tetramethylisoindoline moiety and the hydroxyl proton, respectively. The ^{13}C n.m.r. spectrum showed peaks at δ 26.48, 25.22 and 30.12, for the six methyl groups, peaks at δ 67.50, 70.67 and 144.88, for the five quaternary carbons, peaks at δ 121.39 and 127.25, for the remaining four aromatic carbons,

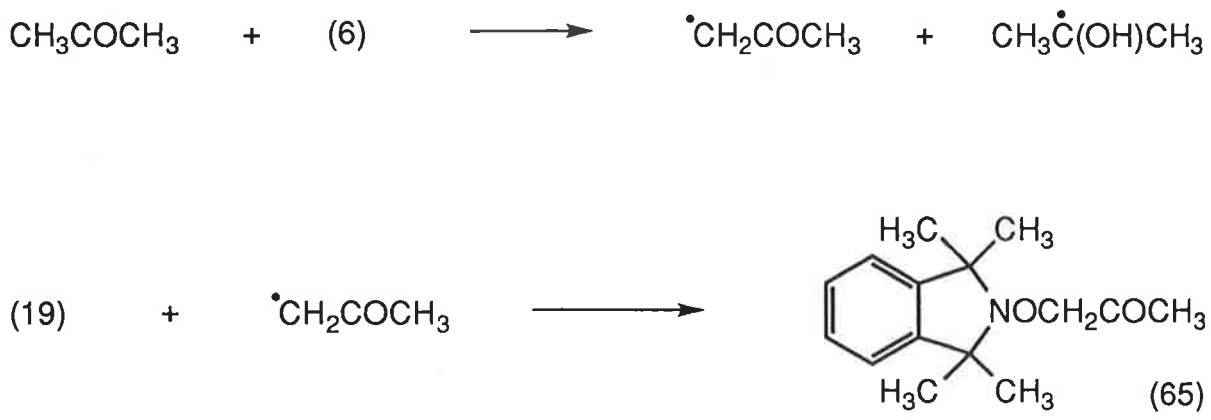


Scheme 19

and a peak at δ 84.89, for the methylene carbon. Formation of the products (62) and (64) can be attributed to TMIO (19) trapping the radical (63) and methyl radical (Scheme 19), produced by hydrogen-atom abstraction from *tert*-butanol by *tert*-butoxy radical and β-scission of *tert*-butoxy radical, respectively. The α-centred glycyl radical (14a) was either formed only in minor amounts or not trapped due to the consumption of the trap (19) by other radicals. When the above reaction was repeated in the absence of the glycine derivative (13a), chromatography of the reaction mixture on silica gave the methoxyamine (62) in 22% yield and the alcohol (64) in 40% yield.

As it is probable that the radical (63) would be formed in most radical reactions in *tert*-butanol, this solvent is unsuitable for trapping of radicals in peptides, and trapping of the radical (14a) in the alternative polar solvent, acetone, was investigated. As reactions involving *tert*-butoxy radicals and TMIO (19) in acetone were known to produce the adduct (65) of TMIO (19) and acetonyl radical,[37,65,66] formed by hydrogen-atom abstraction from the solvent, use of di-*tert*-butyl peroxide in acetone was avoided. As outlined in the Introduction,

generation of α-centred glycyl radicals in peptides and protected dipeptides *via* hydrogen-atom abstraction by photosensitized acetone (as in Scheme 2) has been reported.[15,17,18] Accordingly, a solution of the glycine derivative (13a) and TMIO (19) in acetone was photolysed, for fourteen days as periodic t.l.c. analysis showed that reaction was slow. Chromatography of the crude product on silica recovered the starting materials (19) (65%) and (13a) (69%). Another fraction obtained was dominated by resonances of the adduct (65) of acetonyl radical and the trap (19). The ^{1}H n.m.r. resonances of the ketone (65), consistent with those reported,[37] were singlets at δ 2.27 and 4.57 for the acetyl and methylene protons, respectively, along with two multiplets centred at δ 7.12 and 7.25, and a broad singlet at δ 1.46, for the aromatic and methyl protons of the tetramethylisoindoline moiety, respectively. The mass spectrum of this fraction showed a small molecular ion for the adduct (65) at *m/z* 247 with a base peak at *m/z* 232 corresponding to loss of a methyl group from the molecular ion. Accurate mass measurement of the peak at *m/z* 247 confirmed the identity of this compound. Formation of the adduct (65) can be attributed to trapping of acetonyl radical, generated through hydrogen-atom abstraction from acetone by photosensitized acetone (6), by TMIO (19) (Scheme 20). Integration of peaks in the ^{1}H n.m.r. spectrum of the reaction mixture indicated that the adduct (65) constituted approximately 15% of the crude mixture. This spectrum did not show a doublet near δ 6.2 for the adduct (61).



Scheme 20

After chromatography of the crude product on silica, however, the ^{1}H n.m.r. spectrum of an impure fraction showed a doublet at δ 6.24 (J = 9.5 Hz) which may have arisen from the α-proton of the adduct (61). The mass spectrum of this fraction showed its highest value peak at *m/z* 351 which corresponds to loss of a methoxy group from the molecular ion of the adduct (61). Evidently, the resonances of the adduct (61), formed in less than 1% yield, were negligible in the crude ^{1}H n.m.r. spectrum.

The possibility that the adduct (61) was unstable under the photolytic conditions used in the above reactions was excluded by photolysing it in toluene under nitrogen for five days. The mixture was monitored by t.l.c. which indicated that the adduct (61) remained the major compound present. The ^{1}H n.m.r. spectrum of the final reaction mixture showed a doublet at δ 6.17 (J = 9.5 Hz) for the α-proton of the starting material (61) and no resonances from the glycine derivative (13a), a likely product of decomposition of the adduct (61). There was only one sizeable methyl ester singlet at δ 3.80 which corresponded to the adduct (61).

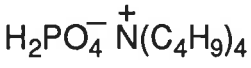
Adducts of the radical trap TMIO (19) have been shown to exhibit reversible homolysis at elevated temperatures.[67] The thermal stability of the adduct (61) was investigated by heating a solution of the adduct (61) in toluene at reflux for thirty hours. The reaction was followed by t.l.c. which indicated that the adduct (61) decomposed to form several compounds. The ^{1}H n.m.r. spectrum showed no signals for the possible decomposition product (13a) or its dimer (18). There were five peaks from δ 3.5-4.0, likely to be methyl ester singlets, but it was not obvious as to which compounds these corresponded. As the adduct (61) has a structure analogous to an acetal of a glyoxylate, it was possible that it decomposed to give benzamide and an alcohol. T.l.c. indicated, however, that no benzamide was produced. Evidently, the adduct (61) was photolytically stable but thermally unstable.

Generation of radicals from the polar solvents used above indicated that use of TMIO (19) to trap radicals in peptides and proteins was limited. Although the α-centred radical (14a) was trapped by TMIO (19) in benzene, this reaction was not as efficient as trapping of the radical (14a) as the dimer (18) or the alanine derivative (13b) (Scheme 6).[35] It was thus decided to investigate formation of dimers of peptides as a method of establishing the number and position of α-carbon-centred radicals generated in a peptide. As mentioned in the Introduction, the extension of the methodology with which the radical (14a) was generated and dimerized to the synthesis of crosslinked peptides has been briefly investigated by photolysis of a mixture of triglycine (24) and di-*tert*-butyl peroxide in water/*tert*-butanol (2:3) for twenty-one hours.[39] As analysis of the reaction mixture indicated that a small amount of dimerization of triglycine (24) occurred, this reaction was investigated using the conditions described above as a basis.

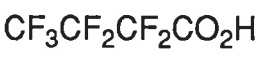
Initial work focussed on the development of a high performance liquid chromatography (h.p.l.c.) assay for use in analysis of mixtures obtained from reactions of triglycine (24). Water, containing 0.05-0.1% of an ion-pairing reagent,[68] running through a reverse phase silica (C18) column was used to chromatograph the polar peptide mixtures. The ultraviolet absorption spectrum of triglycine (24) shows a maximum absorption at $\lambda \sim 185$ nm[69] but light of wavelength 200-220 nm was used for the detection of peptides in reaction mixtures because water absorbs light of wavelength less than 190 nm.

The retention time of triglycine (24) in aqueous trifluoroacetic acid (TFA) , a commonly used ion-pairing reagent that interacts with amino groups of peptides,[70] was too short to enable use of this solvent in analysis of reaction mixtures. Acetylation of the amino group of triglycine (24), by treatment with acetic anhydride in acetic acid, increased its retention time in aqueous TFA. Use of tetrabutylammonium phosphate (TBAP) (66), which pairs with carboxyl groups, as the ion-pairing reagent,[71] further increased the retention time of *N*-acetyltriglycine. During the course of this work, it was found that triglycine

(24) had a suitably long retention time in aqueous heptafluorobutyric acid (HFBA) (67), another reagent which forms ion-pairs with amino groups,[72] to enable use of this solvent in analysis of non-acetylated reaction mixtures.



(66)



(67)

It was observed that triglycine (24) was retained longer than *N*-acetyltriglycine when aqueous HFBA (67) was the h.p.l.c. solvent. Evidently, the ion-pair of the heptafluorobutyrate group and triglycine (24) is more hydrophobic than *N*-acetyltriglycine and the ion-paired triglycine is thus retained longer by the reverse-phase silica. In aqueous TFA, triglycine (24) was retained less than *N*-acetyltriglycine because the ion-pair of the trifluoroacetate group and triglycine (24) is less hydrophobic than *N*-acetyltriglycine. These observations are indicative of the strength of the ion-pair formed by heptafluorobutyric acid and peptides. As expected, *N*-acetyltriglycine was retained similarly in aqueous TFA or HFBA (67) and this retention was less than that in aqueous TBAP (66).

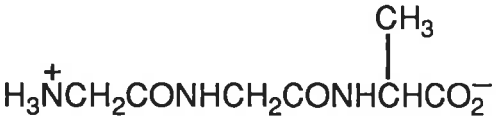
The development of the h.p.l.c. assay took place during initial studies of reactions of triglycine (24) and hence different reactions are discussed in terms of different assays. Attempted separation of one reaction mixture using a preparative h.p.l.c. column was unsuccessful and this lead to the use of an analytical h.p.l.c. column for separation of mixtures. Some reaction mixtures were acetylated, by treatment with acetic anhydride in acetic acid, and analysed using aqueous TBAP (66).

^{1}H N.m.r. spectra of reaction mixtures and separated components were obtained using deuterium oxide as the solvent but resonances for α-protons of α-substituted amino acid residues appeared near the residual HOD (δ 4.80) peak.

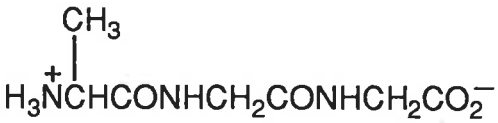
After experimentation with several solvents, such as d_6-dimethylsulfoxide and deuterated trifluoroacetic acid, it was found that reasonable spectra could be obtained in d_4-acetic acid, even though this solvent did not completely dissolve the peptides. Therefore, most spectra were obtained in deuterium oxide but for some isolated products, the existence and nature of peaks near δ 4.80 was confirmed by obtaining spectra using d_4-acetic acid and/or d_4-acetic acid/deuterium oxide mixtures as solvent. Several different compounds were experimented with as matrices for use in obtaining positive ion f.a.b. mass spectra of the products. Best results were achieved using glycerol as the matrix, with mass-analysed ion kinetic energy (MIKE) spectra obtained through selection of protonated molecular ions. MIKE spectra are obtained by mass analysis of ions before they enter the electric sector of a reversed-sector mass spectrometer.[73] A daughter ion mass spectrum is obtained from the selected ion.

Initially, a mixture of di-*tert*-butyl peroxide and triglycine (24) was photolysed with light of wavelength 350 nm in water/*tert*-butanol (2:3) for six days, with periodic additions of more di-*tert*-butyl peroxide. The ^{1}H n.m.r. spectrum of the resultant reaction mixture was dominated by resonances for triglycine (24) with low intensity peaks for minor amounts of products. The ^{1}H n.m.r. spectra of reaction samples taken periodically during a twenty-four hour repeat of the above reaction, indicated that although the starting material (24) remained the major compound, increasing amounts of products formed with time. The f.a.b. mass spectrum of an eight hour sample showed a peak at *m*/z 377, possibly for a protonated molecular ion of a dimer of triglycine (24). H.p.l.c. analysis (aqueous TBAP (66)) of acetylated reaction samples showed that all were dominated by *N*-acetyltriglycine, as verified by its coinjection with the samples, with the twenty-four hour sample containing the greatest amount of the minor products. When it was coinjected with the acetylated twenty-four hour reaction mixture, *N*-acetylglycylglycylalanine, synthesized by treatment of glycylglycylalanine (68) with acetic anhydride in acetic acid, coeluted with a peak

in the mixture, indicating that glycylglycylalanine (68) may have been produced in the reaction. Coinjections (aqueous HFBA (67)) of the mixture obtained from a forty-eight hour repeat of this reaction, with glycylglycylalanine (68) and alanylglycylglycine (69), indicated that the peptide (68) was probably one of the products but alanylglycylglycine (69) was not produced in detectable amounts.

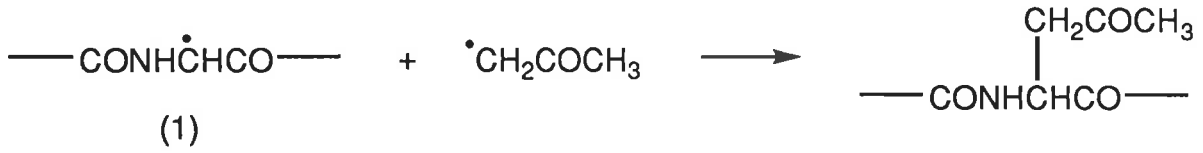


(68)



(69)

The reaction was repeated with photolysis (λ = 350 nm) for forty-six hours and preparative h.p.l.c. (aqueous HFBA (67)) of the reaction mixture afforded small amounts of several compounds, the most abundant being triglycine (24). One of the products was glycylglycylalanine (68), identified by comparison of its h.p.l.c. and ^{1}H n.m.r. spectral properties with those of an authentic sample. ^{1}H N.m.r. signals possibly arising from α-protons of substituted residues in the other products were either concealed or unclear due to the residual HOD peak at δ 4.80 in the deuterium oxide solvent. The formation of one compound was attributed to incorporation of an acetonyl group at the α-position of a residue of triglycine (24). The ^{1}H n.m.r. spectrum of this compound showed singlets of relative integral 3:2:2 at δ 2.21, 3.88, and 4.00, for the acetyl protons and the α-protons of two glycine residues, respectively. In addition, a two proton doublet at δ 3.16 (J = 5.5 Hz), and a multiplet centred at δ 4.73 corresponded to the methylene protons of the acetonyl moiety and the α-proton of the substituted glycine residue, respectively. Formation of this compound was attributed to coupling of an α-centred radical of triglycine (24) with acetonyl radical (Scheme 21), produced by hydrogen-atom abstraction from acetone which is formed on β-scission of *tert*-butoxy radical.



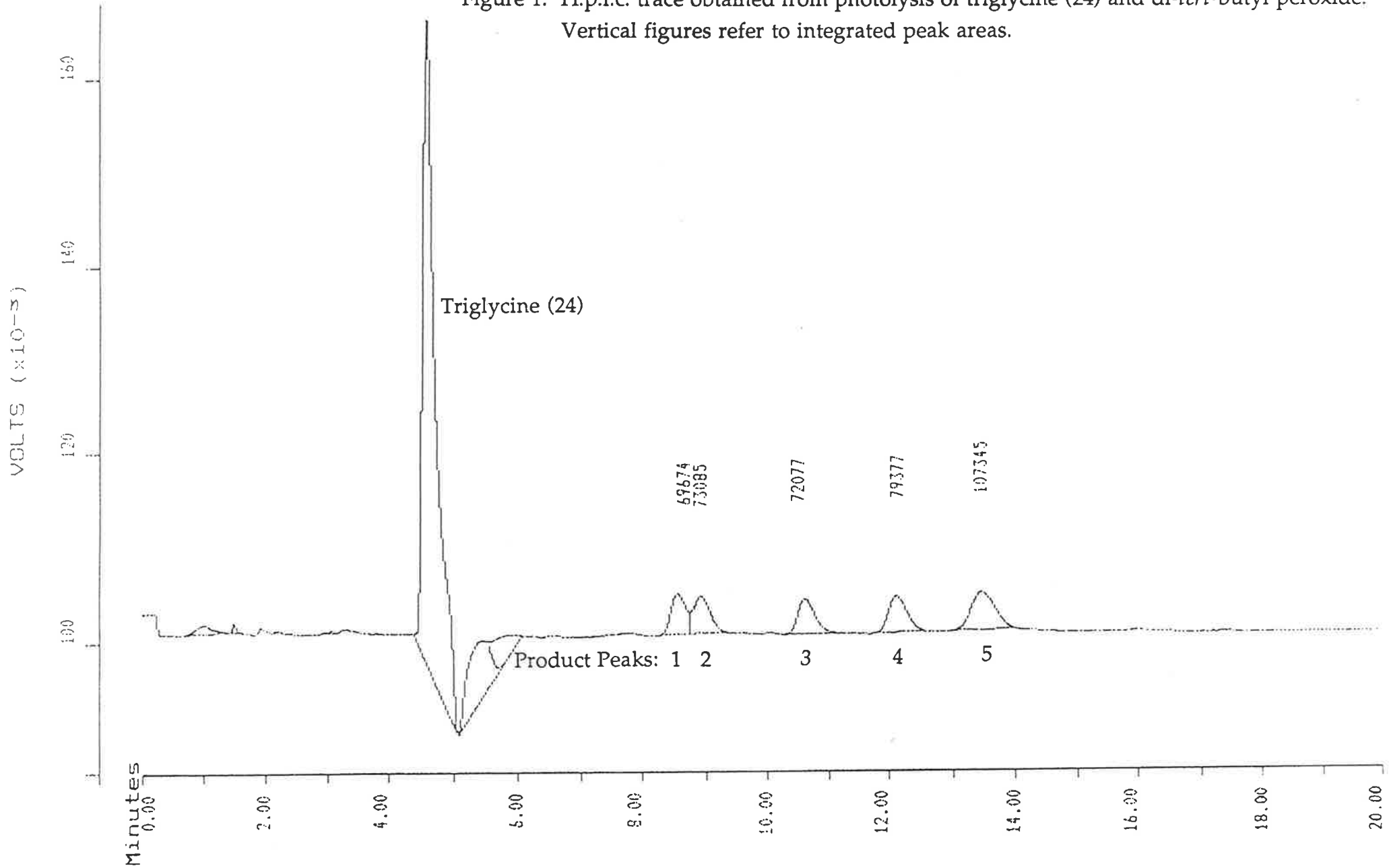
Scheme 21

The ^{1}H n.m.r. spectrum of another h.p.l.c. fraction showed singlets at δ 3.84, 3.85, and 3.99, and an apparent doublet at δ 3.97, along with some small peaks in the same region (δ 3.8-4.1). These resonances corresponded to α-protons of glycine residues and were consistent with a diastereomeric pair of dimers of triglycine (24), as discussed in detail later in this Chapter. A small singlet at δ 5.02, possibly arose from substituted glycine residues of one of these dimers. The ^{1}H n.m.r. spectrum of another fraction showed two singlets at δ 3.86 and 3.96 integrating in a 1:1 ratio, attributable to two glycine residues, possibly in a dimer of triglycine (24).

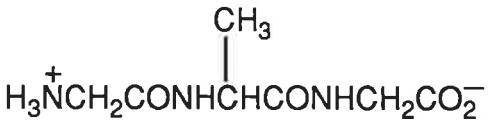
Periodic h.p.l.c. analysis of a repeat of the reaction photolysed with light of a different wavelength, 300 nm, showed that the triglycine (24) was consumed faster than in previous reactions. The reaction mixture became increasingly complex with time and after twenty hours, little triglycine (24) remained. The h.p.l.c. trace of a six hour sample from this reaction indicated that separation of mixtures from reactions photolysed for more than a few hours would be difficult.

As there was evidence suggesting that α-centred radicals of triglycine (24) were being produced and either dimerizing or coupling with relatively small methyl or acetonyl radicals, it was decided to repeat the reaction for a short time using light of wavelength 300 nm and analyse the primary products. Triglycine (24) and di-*tert*-butyl peroxide were dissolved in water/*tert*-butanol (2.5:3) and irradiated with ultraviolet light (300 nm) under an inert atmosphere for 1.75 hours. Analytical h.p.l.c. (aqueous HFBA (67)) of the reaction mixture showed that at least five products had formed but a large amount of the starting material (24) remained as shown in the h.p.l.c. trace (Figure 1). A small amount of the

Figure 1: H.p.l.c. trace obtained from photolysis of triglycine (24) and di-*tert*-butyl peroxide. Vertical figures refer to integrated peak areas.



reaction mixture was irradiated for a further 1.25 hours at which time h.p.l.c. analysis showed that all five product peaks had increased in size relative to triglycine (24) but none had increased significantly relative to the others. This confirmed that all of these products, referred to as the first to fifth peaks in order of increasing retention times, were primary. The relative proportions of the peaks in the h.p.l.c. trace of the 1.75 hour reaction mixture were the same at detection wavelengths of 205, 210, 220 and 230 nm, indicating that the products were absorbing light through similar functionality, presumably amide bonds.



(70)

Coinjections onto the h.p.l.c. column of the 1.75 hour reaction mixture with authentic samples of triglycine (24), glycylglycylalanine (68), glycylalanylglycine (70), and alanylglycylglycine (69) provided information about some of the products. As expected, triglycine (24) coeluted with the largest peak. Glycylglycylalanine (68) coeluted with the third peak and glycylalanylglycine (70) coeluted with the first peak, indicating that the alanyl peptides (68) and (70) may have been produced in the reaction. It was shown that alanylglycylglycine (69) was not a product as it eluted between the largest peak and the first product peak. In addition, in both aqueous HFBA (67) and TBAP (66) running solvent *N*-acetylglycylglycylalanine and *N*-acetylglycylalanylglycine coeluted with peaks in the acetylated reaction mixture.

The ^{1}H n.m.r. spectrum of this reaction mixture was dominated by resonances of triglycine (24) but contained smaller peaks from δ 1.0-2.5 and δ 3.5-5.0. Samples of the reaction mixture were enriched with authentic samples of glycylglycylalanine (68), glycylalanylglycine (70), and alanylglycylglycine (69),

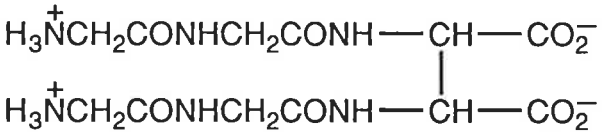
and the resultant ^1H n.m.r. spectra indicated that the peptides (68) and (70) had been produced in an approximate ratio of 5:1 and that alanylglycylglycine (69) was not a product. Doublets (J = 7.5 Hz) at δ 1.37 and δ 1.45 enlarged with respect to other peaks upon enrichment of the reaction mixture with glycylglycylalanine (68) and glycylalanylglycine (70), respectively. In the spectrum of the reaction mixture the approximate ratio of these two doublets was 5:1 (based on peak heights). As the first and third product peaks were of approximately equal ultraviolet absorbance (Figure 1), this unequal ratio indicated that the first product peak consisted of glycylalanylglycine (70) and another product. The ^1H n.m.r. spectrum of the sample enriched with alanylglycylglycine (69) showed a doublet at δ 1.63 (J = 7.5 Hz) which was not in the original spectrum.

The primary products were isolated by h.p.l.c. of the 1.75 hour reaction mixture, using an analytical size column and aqueous HFBA (67). Numerous small portions of the reaction mixture were chromatographed and the isolated products were repurified several times. As a result, limited amounts of the pure products were obtained and these were identified using ^1H n.m.r. spectroscopy and positive ion f.a.b. mass spectrometry. The primary n.m.r. solvent was deuterium oxide but ^1H n.m.r. spectra of the products in the first, second, fourth and fifth peaks were also obtained in d_4-acetic acid and/or d_4-acetic acid/deuterium oxide to confirm the existence and nature of resonances at chemical shifts near that of the residual HOD (δ 4.80). The chemical shifts quoted are taken from the deuterium oxide spectra.

The ^1H n.m.r. spectrum of the largest peak in the h.p.l.c. trace (Figure 1), isolated to verify that it was triglycine (24), showed three singlets at δ 3.97, δ 4.09, and δ 4.13 in the ratio 1:1:1, and was similar to the spectrum of an authentic sample of triglycine (24). The f.a.b. mass spectrum showed a protonated molecular ion at *m/z* 190. The MIKE spectrum on this peak showed major peaks at *m/z* 172 and 115, for loss of water and loss of the C-terminal glycine residue, respectively. Minor peaks at *m/z* 146 and 133 were attributed to loss of carbon

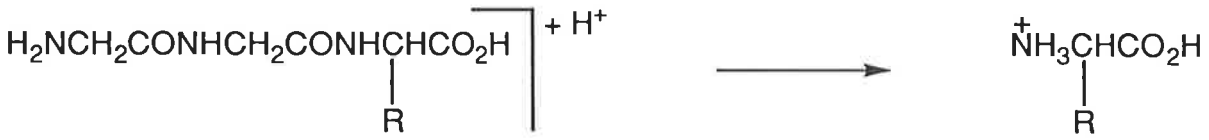
dioxide and loss of the *N*-terminal glycine residue, respectively. A small peak at *m/z* 76 arose from loss of glycylglycine from the *N*-terminus.

The first product peak consisted of one diastereomer (71A) of the *C*-terminal symmetric dimer (71) and glycylalanylglycine (70). The ^{1}H n.m.r. spectrum of this mixture showed a singlet at δ 3.95, two doublets at δ 4.06 (J = 17.0 Hz) and δ 4.10 (J = 17.0 Hz) (AB quartet), and a singlet at δ 5.16, for two methylene groups, the other four methylene protons, and for the two methine protons, respectively, of the dimer (71A). The occurrence of three resonances for six proton groups indicated that this dimer (71A) was symmetric. The AB quartet indicated that the



(71)

protons involved were diastereotopic. Other resonances were a doublet at δ 1.45 (J = 7.5 Hz) and a quartet at δ 4.45 (J = 7.5 Hz) attributable to the protons of the alanine residue, and two singlets at δ 3.89 and δ 4.04 for the protons of the glycine residues, of glycylalanylglycine (70). Comparison of integrals in the n.m.r. spectrum, indicated that the ratio of the products (71A) and (70) was slightly more than 1:1. As discussed later in this Chapter, this ratio is not the actual ratio of production, presumably because of selective purification. The complex f.a.b. mass spectrum of the mixture contained peaks at *m/z* 377 and 204 which corresponded to protonated molecular ions of the dimer (71A) and glycylalanylglycine (70), respectively. The MIKE spectrum on 377 showed major peaks at *m/z* 359 (loss of water), 320, 313, 285, 263, and 245. The peak at *m/z* 320 was attributed to loss of an *N*-terminal glycine residue from the molecular ion. The peaks at *m/z* 263 and 245 were attributed to loss of glycylglycine from the *N*-terminus (Scheme 22)



Scheme 22

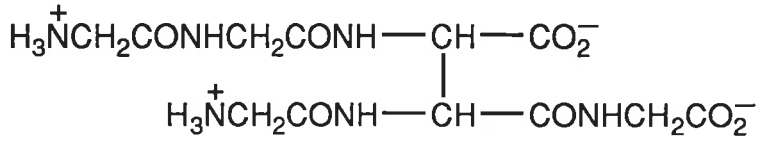
and the additional loss of a water molecule, respectively. The only symmetric dimer of triglycine (24) capable of fragmenting in this manner is crosslinked between the *C*-terminal residues.

The second product peak consisted of the other diastereomer (71B) of the *C*-terminal symmetric dimer (71). The ^{1}H n.m.r. spectrum of this dimer (71B) showed two singlets at δ 3.95 and δ 4.08, attributable to the eight methylene protons, and a singlet at δ 4.70 for the two methine protons. The simplicity of the spectrum indicated that this dimer (71B) is symmetric. The complex f.a.b. mass spectrum showed a protonated molecular ion at *m/z* 377, the MIKE spectrum on which showed major peaks at *m/z* 359 (loss of water), 313, 285, 263. The peak at *m/z* 263, attributable to loss of glycylglycine from the *N*-terminus (Scheme 22), again indicated that this dimer (71B) is crosslinked between the *C*-terminal residues.

The third product peak consisted of glycylglycylalanine (68). The ^{1}H n.m.r. spectrum showed a doublet at δ 1.44 (J = 7.5 Hz) and a quartet at δ 4.39 (J = 7.5 Hz) attributable to the protons of the alanine residue, and two singlets at δ 3.93 and δ 4.06 for the protons of the glycine residues. The f.a.b. mass spectrum showed a protonated molecular ion at *m/z* 204. The MIKE spectrum on 204 showed major peaks at *m/z* 186, 160, 147, 115, and 90 attributed to loss of water, loss of carbon dioxide, loss of *N*-terminal glycine, loss of *C*-terminal alanine, and loss of glycylglycine, respectively.

The compound in the fourth product peak was assigned as one diastereomer (72A) of the non-symmetric dimer (72) crosslinked between the *C*-terminal and

non-terminal glycine residues. The ^{1}H n.m.r. spectrum showed doublets at δ 3.75 and δ 3.85 (AB quartet) (J = 17.0 Hz) and singlets at δ 3.95, δ 3.97, and δ 4.08 for the methylene protons, and two doublets at δ 4.83 (J = 4.5 Hz) and δ 5.08 (J = 4.5 Hz) for the two methine protons. The complexity of this spectrum indicated that this dimer (72A) is not symmetric and the AB quartet indicated the diastereotopic nature of the protons involved. The complex f.a.b. mass spectrum contained a peak at *m*/*z* 377, corresponding to the protonated molecular ion of the dimer (72A). The MIKE spectrum on 377 showed major peaks at *m*/*z* 359 (loss of water), 313, 303, 285, 263, and 245. The peak at *m*/*z* 303 was attributed to loss of a *C*-terminal glycine residue from the molecular ion. The peaks at *m*/*z* 263 and 245 corresponded to loss of glycylglycine from the *N*-terminus (Scheme 22) and the additional loss of a water molecule, respectively. The loss of *N*-terminal glycylglycine showed that this dimer (72A) is not crosslinked between the *N*-terminal glycine residue and the non-terminal glycine residue.



(72)

The compound in the fifth product peak was assigned as the other diastereomer (72B) of the non-symmetric dimer (72). The ^{1}H n.m.r. spectrum showed a singlet at δ 3.97 and doublets at δ 3.84 (J = 17.5 Hz) and 3.94 (17.5) (AB quartet), δ 3.88 (16.0) and 3.95 (16.0) (AB quartet), and δ 4.07 (17.5) and 4.11 (17.5) (AB quartet), for the methylene protons, and two doublets at δ 4.82 (J = 4.5 Hz) and δ 5.19 (J = 4.5 Hz) for the two methine protons. The complexity of these resonances indicated that this dimer (72B) is not symmetric and there were several diastereotopic protons. The complex f.a.b. mass spectrum showed a protonated molecular ion at *m*/*z* 377, the MIKE spectrum on which showed major peaks at *m*/*z* 359 (loss of water), 313, 285, 263, and 245. The peaks at *m*/*z*

263 and 245 were attributed to loss of glycylglycine from the *N*-terminus (Scheme 22) and the additional loss of a water molecule, respectively. The loss of *N*-terminal glycylglycine showed that this dimer (72B) is not crosslinked between the *N*-terminal glycine residue and the non-terminal glycine residue.

Relative yields of the products (68), (70), (71A,B), and (72A,B), shown in Table 1, were calculated using their integrals on the h.p.l.c. trace (Figure 1) and the 5:1 production ratio of glycylglycylalanine (68) to glycylalanylglycine (70) established previously. Calculations are based on the assumption that amide bonds were the only functionality which absorbed ultraviolet light, as indicated previously. Under these circumstances, the dimers (71A,B) and (72A,B) absorb twice as much light as the monomers (68) and (70), assuming equal molar response ratios for each amide bond.

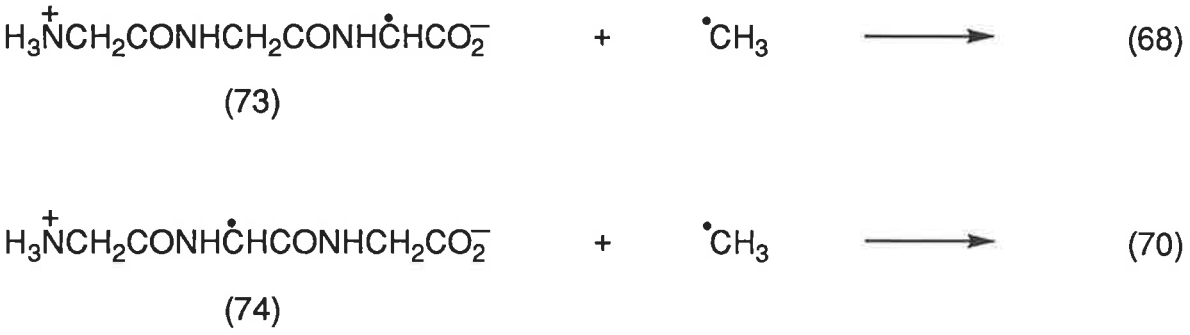
Table 1: Relative yields of the products (68), (70), (71A,B), and (72A,B).

Product	*Relative Yield*
Glycylglycylalanine (68)	1.0*
Glycylalanylglycine (70)	0.2
Dimer (71A)	0.4
Dimer (71B)	0.5
Dimer (72A)	0.5
Dimer (72B)	0.7

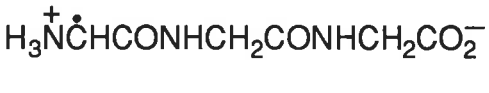
*Assigned as unity.

Formation of the alanyl peptides (68) and (70) can be attributed to α-hydrogen-atom abstraction from the *C*-terminal and non-terminal residues of triglycine (24) by *tert*-butoxy radical, giving rise to the radicals (73) and (74). Evidently, the radicals (73) and (74) coupled with methyl radical, produced by

β-scission of *tert*-butoxy radical, to give the alanyl peptides (68) and (70), respectively (Scheme 23). The absence of alanylglycylglycine (69) amongst the products indicated that the *N*-terminal α-centred radical (75) was not formed.



Scheme 23



(75)

The 5:1 ratio of the alanyl peptides (68) and (70) indicated that the *C*-terminal radical (73) was formed in preference to the non-terminal radical (74). This may be attributed to both steric crowding of the non-terminal α-hydrogens and polar effects. The inductively electron withdrawing carboxy group has a destabilizing effect on the formation of glycyl radicals (1).[74] As *tert*-butoxy radical abstracts an α-hydrogen, the developing positive charge at the α-carbon would be more disfavoured by the aminocarboxy group than the carboxylate group and formation of the *C*-terminal radical (73) is thus favoured over the non-terminal radical (74).

As shown below, glycyl radicals (1) are stabilized through delocalization of the unpaired electron *via* the adjacent nitrogen. The ammonium group of triglycine (24) is expected to disfavour formation of the *N*-terminal radical (75)

because the *N*-terminal nitrogen is protonated, and thus the nitrogen electrons are not available to stabilize the radical (75). Formation of the radical (75) would also be disfavoured by the development of adjacent electron deficient centres, namely the protonated nitrogen and the α-carbon, in the reaction transition state.

(1)

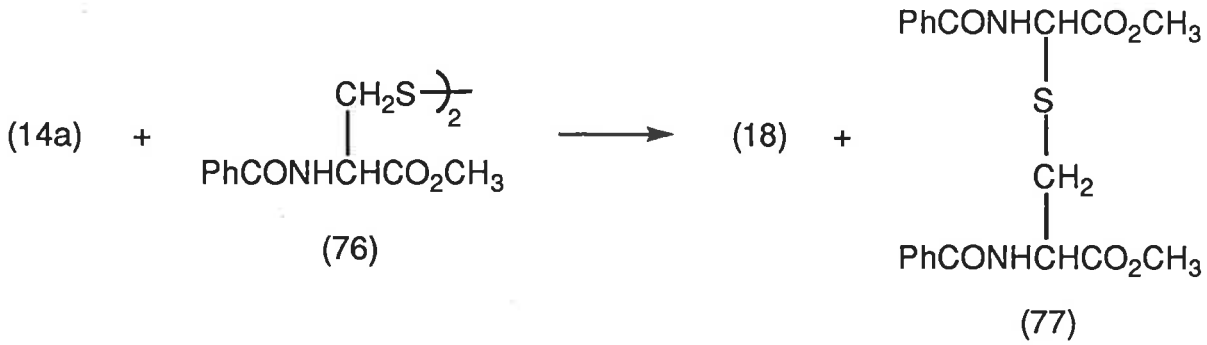
Formation of the dimer (71), attributable to coupling of two *C*-terminal radicals (73) (Scheme 24), as the only symmetric dimer is consistent with preferential formation of the radical (73). The absence of the non-terminal and *N*-terminal symmetric dimers amongst the products is consistent with formation of the radicals (74) and (75) being less favourable. The structures of the non-symmetric dimers (72A,B) were assigned as crosslinked between the *C*-terminal and non-terminal residues because there was evidence for formation of the non-terminal radical (74) but none for formation of the *N*-terminal radical (75). Formation of the dimer (72) can be attributed to coupling of a *C*-terminal (73) and a non-terminal radical (74) (Scheme 24).

Scheme 24

As coupling of the α-centred radicals (73) and (74) with methyl radical competed with formation of the dimers (71) and (72), the synthetic utility of coupling of α-centred glycyl radicals (1) for carbon-carbon bond formation between peptides is limited. The chance of two glycyl radicals (1) coupling would

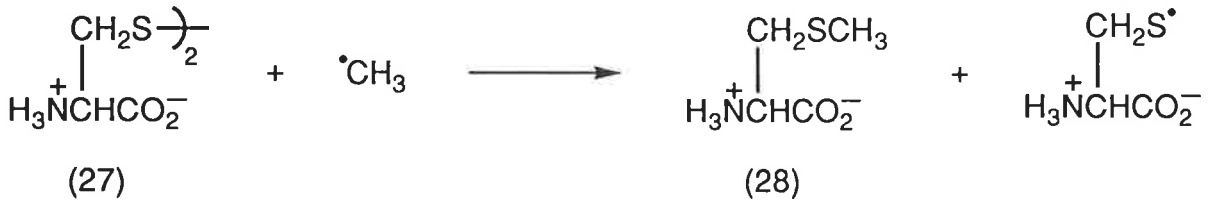
decrease with increasing size and complexity of peptides, and reactions with other radicals or peptide functionality would be expected to become prevalent.

It has been reported[75] that reaction of the glycyl radical (14a) with a disulfide competed with dimerization. The glycyl radical (14a), generated by treatment of the corresponding α-bromoglycine derivative with tributyltin radical, either substituted at sulfur in the cystine derivative (76), to give the methylene-thio crosslinked derivative (77) (Scheme 25), or coupled to give the protected glycine dimer (18). To ascertain whether reaction of glycyl radicals (1) with cystine



Scheme 25

residues in peptides would interfere with coupling of glycyl radicals (1), a mixture of triglycine (24), cystine (27), and di-*tert*-butyl peroxide was photolysed for two days. T.l.c. analysis of the reaction mixture showed that no cystine (27) remained but triglycine (24) and one product were present. H.p.l.c. of the mixture afforded *S*-methylcysteine (28), identified by comparison of its ^{1}H n.m.r. spectrum and t.l.c. properties with those of an authentic sample. Formation of *S*-methylcysteine (28) can be attributed to substitution of methyl radical, produced by β-scission of *tert*-butoxy radical, on sulfur in cystine (27) (Scheme 26). This selective reaction of methyl radical with cystine (27) indicated that reactive disulfide moieties, common in peptides, may react preferentially with reagents used to generate glycyl radicals (1), thus interfering with the generation and coupling of glycyl radicals (1).



(27) (28)

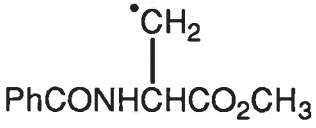
Scheme 26

In summary, the photolytic reaction of triglycine (24) with di-*tert*-butyl peroxide gave rise to α-centred radicals (1) which either coupled with methyl radicals or dimerized to give crosslinked triglycine (24). Product studies on this reaction demonstrated the decreasing relative ease of formation of *C*-terminal, non-terminal, and *N*-terminal α-centred radicals in peptides. Indications were that such carbon-carbon bond forming reactions of glycyl radicals (1) could be used for modification of small or uncomplicated peptides. For larger, more complicated peptides it was evident that radical reactions of other functionality, such as the reactive disulfide moiety of cystine (27), could interfere with generation of glycyl radicals (1). To take advantage of such reactivity, generation of β-carbon centred radicals *via* manipulation of cystine and cysteine residues was investigated, using the method outlined in the Introduction (Scheme 9). The results are discussed in Chapter Two.

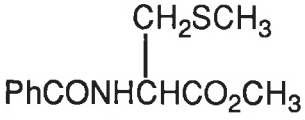
Results and Discussion Chapter Two

Reactions *Via* β-Centred Radicals from Cysteine Derivatives

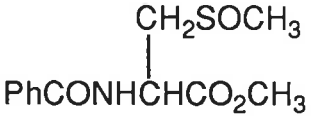
As outlined in the Introduction, generation of carbon-centred radicals on peptide side-chains was investigated through reactions of *N*-benzoyl-*S*-methyl-cysteine methyl ester (55) and *N*-benzoyl-*S*-methylcysteine sulfoxide methyl ester (56), with alkyl, aryl, stannyl, and alkoxy radicals, and Fenton's reagent, with a view to generating the β-centred radical (54). The results are discussed in this Chapter.



(54)

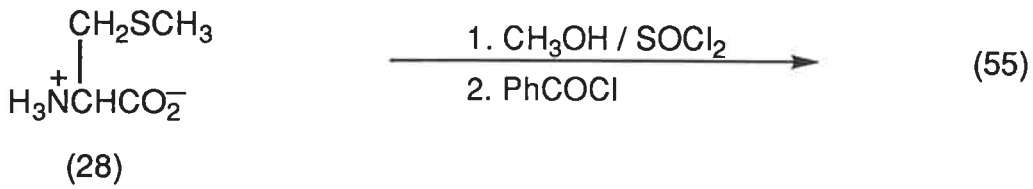


(55)



(56)

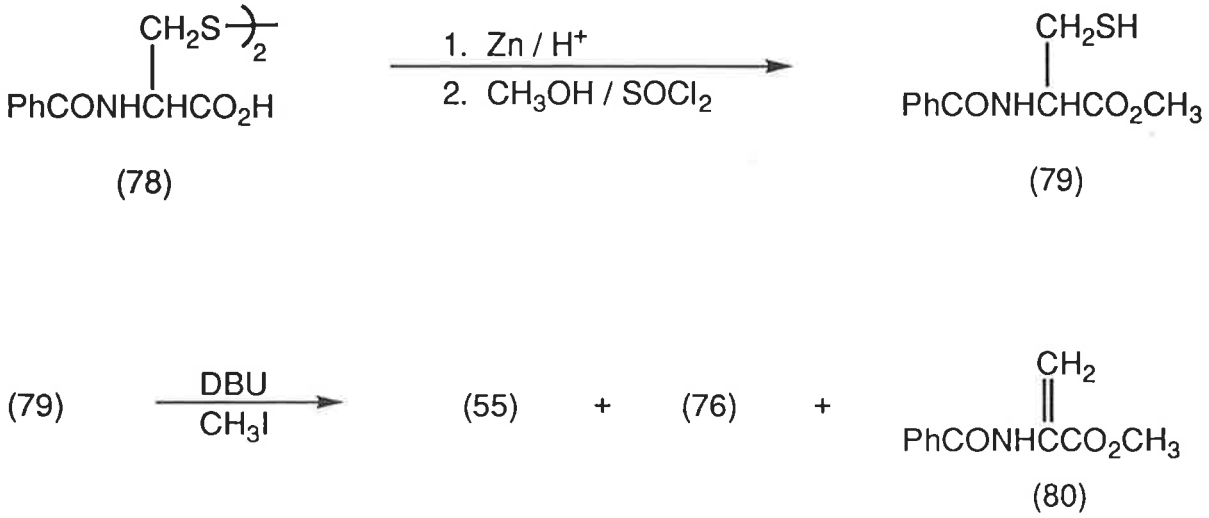
N-Benzoyl-*S*-methylcysteine methyl ester (55) was synthesized as shown in Scheme 27. *S*-Methylcysteine (28) was added to methanol which had been pretreated with thionyl chloride. The resultant methyl ester was treated with benzoyl chloride to give the *S*-methylcysteine derivative (55) which recrystallized from ethyl acetate/petroleum ether in 87% yield. Satisfactory microanalytical data was obtained for the sulfide (55) and the mass spectrum showed a molecular ion at *m/z* 253. The ^{1}H n.m.r. spectrum showed two doublets of doublets at δ 3.01 (J = 6.0, 14.0 Hz) and 3.10 (J = 5.0, 14.0 Hz) for the β-protons, a singlet at δ 2.10 for the *S*-methyl protons, and a multiplet centred at δ 5.00 for the α-proton.



(28) (55)

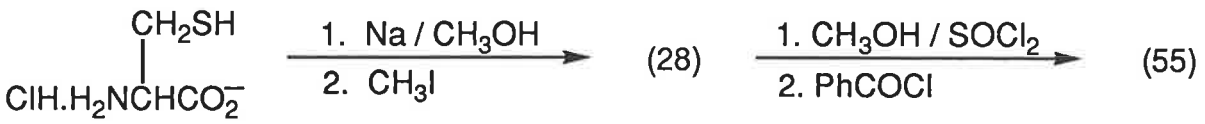
Scheme 27

In an earlier synthesis of the sulfide (55), before *S*-methylcysteine (28) was purchased, *N,N'*-dibenzoylcystine (78) was reduced by treatment with zinc powder in acetic acid/water/ethanol, as reported.[76] The resultant thiol was added to methanol that had been pretreated with thionyl chloride and gave the crude cysteine derivative (79) (Scheme 28). In a reaction based on a general procedure for alkylation of thiols,[77] the crude thiol (79) was treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and methyl iodide in benzene. Chromatography of the resultant mixture gave the *S*-methylcysteine derivative (55), with a ^{1}H n.m.r. spectrum consistent with that of the sample described above, *N*-benzoyldehydroalanine methyl ester (80), with a ^{1}H n.m.r. spectrum consistent with that reported,[78] and *N,N'*-dibenzoylcystine dimethyl diester (76), with a ^{1}H n.m.r. spectrum and t.l.c. properties consistent with those of an authentic sample. The products (55), (80), and (76) were obtained in respective yields of 30%, 38%, and 18% from the starting material (78). Formation of the dehydroalanine derivative (80) can be attributed to removal of the acidic α-hydrogen from the sulfide (55) or the thiol (79) by DBU and displacement of methanethiolate ion or thiolate ion from the respective resultant anions. Presumably, oxidation of the thiol (79) produced the cystine derivative (76).



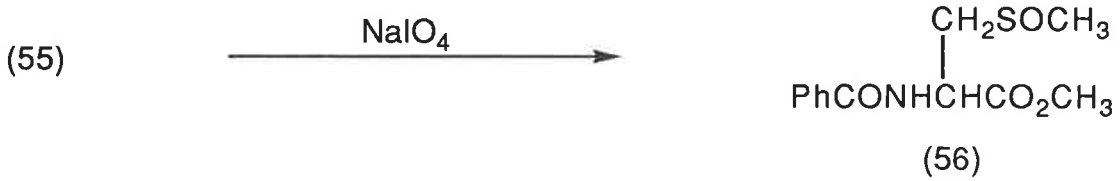
Scheme 28

In another earlier synthesis of the sulfide (55), *S*-methylation of cysteine (25) was carried out before derivatization to reduce the chances of oxidation and alkene formation. Anions formed by α-hydrogen removal from the cysteine derivatives (79) and (55) are stabilized by adjacent inductively electron withdrawing amido groups which are absent in the free amino acid (25). A suspension of the hydrochloride salt of cysteine (25) in methanol was treated with sodium followed by methyl iodide, as reported.[79] The resultant crude *S*-methylcysteine (28) was esterified and benzoylated to give the *S*-methylcysteine derivative (55) in 29% yield (Scheme 29). The relatively low yield was attributed to difficulties encountered in the methylation reaction and the isolation of the intermediate (28).



Scheme 29

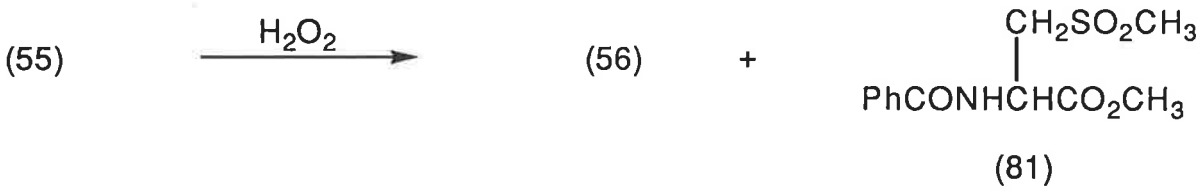
N-Benzoyl-*S*-methylcysteine sulfoxide methyl ester (56) was synthesized as shown in Scheme 30. Treatment of the *S*-methylcysteine derivative (55) with sodium metaperiodate in water/methanol gave a 1:1 mixture of the diastereomers of *N*-benzoyl-*S*-methylcysteine sulfoxide methyl ester (56). Recrystallization from ethyl acetate/light petroleum gave the sulfoxide (56) in 82% yield. Satisfactory microanalytical data was obtained for the sulfoxide (56)



Scheme 30

and the f.a.b. mass spectrum showed a protonated molecular ion at *m/z* 270. The ^{1}H n.m.r. spectrum showed singlets at δ 2.68 and 2.69, and doublets of doublets at δ 3.27 (J = 4.0, 13.5 Hz), δ 3.38 (J = 6.0, 13.5 Hz), δ 3.47 (J = 7.5, 13.5 Hz), and δ 3.52 (J = 5.5, 13.5 Hz), for the methylsulfoxy protons and the β-protons, respectively, of the two diastereomers.

In another synthesis of the sulfoxide (56), oxidation of the sulfide (55) with hydrogen peroxide gave the sulfoxide (56) and its corresponding sulfone (81) in a ratio of approximately 3:1 (Scheme 31). The f.a.b. mass spectrum of the mixture showed protonated molecular ions at *m/z* 270 and 286 for the products (56) and (81), respectively. The ^{1}H n.m.r. spectrum showed singlets at δ 2.68 and 2.69, and δ 2.97, for the methylsulfoxy protons of the two diastereomers of the sulfoxide (56), and the methylsulfonyl protons of the sulfone (81), respectively.



Scheme 31

The proposed method for generating β-centred radicals from cystine or cysteine residues in peptides outlined in the Introduction (Scheme 9), requires conversion of disulfides to thiols to sulfides and sulfoxides. The reactions described above indicated that this can be achieved by use of suitable reducing and methylating reagents and sodium periodate.

Homolytic substitution of radicals on sulfur in the sulfoxide (56) was initially investigated by using the combination of tributyltin hydride and alkyl or aryl halides to generate alkyl or aryl radicals. A mixture of the sulfoxide (56), bromobenzene, and the radical initiator azobisisobutyronitrile (AIBN) in benzene

was heated at reflux while tributyltin hydride was added dropwise. Slow addition of the tin hydride allowed time for the phenyl radical, produced by abstraction of bromine from bromobenzene by tributyltin radical, to react with the sulfoxide (56) before being reduced by tributyltin hydride. By comparison with t.l.c. properties of an authentic sample of the dehydroalanine derivative (80), t.l.c. analysis of the reaction mixture indicated that the only reaction was partial conversion of the sulfoxide (56) to the alkene (80). The reaction mixture was stirred with aqueous potassium fluoride in order to remove stannyl compounds and the ^{1}H n.m.r. spectrum of the resultant mixture indicated that no starting material (56) remained and showed signals at δ 6.00 and δ 6.81, characteristic of the olefinic protons of the dehydroalanine derivative (80).[78] Formation of the alkene (80) can be attributed to elimination of the methylsulfoxy group from the sulfoxide (56) and is evidently promoted by exposure of the sulfoxide (56) to heat and base. The spectrum also indicated that there was a significant amount of bromobenzene in the reaction mixture, suggesting that the production of phenyl radicals from bromobenzene was not particularly efficient. Consequently, the reaction was repeated using iodobenzene, from which abstraction of iodine is more facile than bromine abstraction from bromobenzene,[80] and initiated by photolysis, to avoid heat promoted formation of the alkene (80). The ^{1}H n.m.r. spectrum of this reaction mixture, which was not stirred with aqueous potassium fluoride, indicated that the starting material (56) remained unreacted and that there was a significant amount of iodobenzene remaining, suggesting that the production of phenyl radicals was inefficient. This reaction was repeated with methyl iodide instead of iodobenzene in an attempt to substitute methyl radical on sulfur but t.l.c. of the reaction mixture indicated that no reaction had occurred.

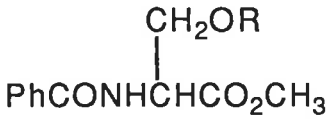
The lack of evidence for the formation of the β-centred radical (54) and the possible inefficient formation of phenyl radicals lead to use of benzoyl peroxide as the source of phenyl radicals. The peroxide bond of benzoyl peroxide cleaves on thermolysis and the resultant benzoyloxy radicals undergo β-scission to give

phenyl radical and carbon dioxide. A solution of the sulfoxide (56) and benzoyl peroxide in benzene was heated at reflux for five hours. The only compounds identified by examination of the ^{1}H n.m.r. spectrum of the complex reaction mixture were the starting material (56) and its elimination product (80). A small doublet at δ 1.50 (J = 7.0 Hz) corresponded to the β-protons of the alanine derivative (13b), as determined by comparison with the n.m.r. spectrum of an authentic sample. The alanine derivative (13b) may have been formed through hydrogen-atom abstraction by the β-centred radical (54). As there was a relatively small amount of this derivative (13b), if any, in the complex reaction mixture, this reaction was not pursued.

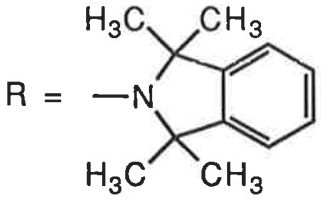
Direct substitution of stannyl radicals at sulfur in the cysteine derivatives (56) and (55) was investigated with the expectation that, if produced, the radical (54) would be reduced by tributyltin hydride to give the alanine derivative (13b). A mixture of the sulfide (55), tributyltin hydride and AIBN in benzene was heated at reflux. The ^{1}H n.m.r. spectrum of the reaction mixture indicated that hydrolysis of the ester moiety of the sulfide (55) may have occurred. The spectrum showed resonances for α- and β-protons of the sulfide (55) and a similar compound but showed only one methyl ester singlet. When the reaction was repeated with the sulfoxide (56), t.l.c. analysis of the reaction mixture indicated that most of the starting material (56) remained and that one of the minor compounds present was the dehydroalanine derivative (80). T.l.c. analysis of both reaction mixtures indicated that neither contained the alanine derivative (13b). Using an alternative method for generating tributyltin radicals, involving hydrogen-atom abstraction from tributyltin hydride by *tert*-butoxy radicals,[80] a solution of the sulfoxide (56), tributyltin hydride, and di-*tert*-butyl peroxide in benzene was photolysed. T.l.c. analysis of the reaction mixture indicated that no reaction had occurred.

As limited success was achieved in generating the β-centred radical (54) *via* radical substitution on sulfur, the alternative methodology of reaction of the

sulfoxide (56) with Fenton's reagent, as outlined for dialkylsulfoxides in the Introduction (Scheme 16), was investigated. Assuming that the hydrogen peroxide adduct of the sulfoxide (56) formed, it was envisaged that electron transfer from ferrous ion would cleave its peroxide bond. On β-scission, the resultant oxygen-centred radical was expected to displace the primary radical (54) in preference to methyl radical. As methyl radicals, produced by reaction of dimethylsulfoxide with Fenton's reagent, had been trapped by 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO) (19),[38] reactions were carried out in the presence of TMIO (19) in an attempt to trap the radical (54) thereby giving the adduct (82).



(82)

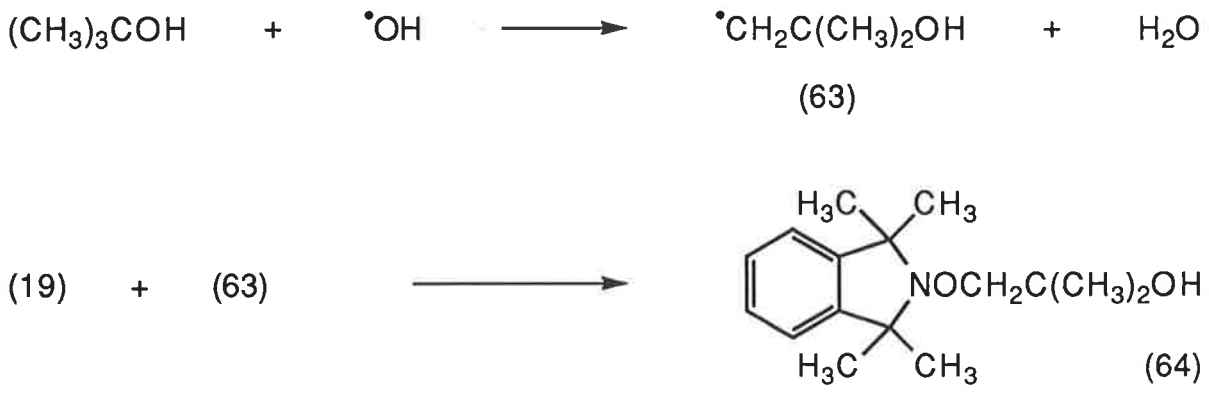


Accordingly, a mixture of a solution of the sulfoxide (56) and TMIO (19) in benzene and aqueous hydrogen peroxide was treated with an aqueous ferrous sulfate solution. The f.a.b. mass spectrum of the complex reaction mixture showed a peak at *m/z* 397, corresponding to the protonated molecular ion of the desired adduct (82). The mixture was chromatographed on silica and the f.a.b. mass spectrum of one fraction showed a peak at *m/z* 397 but no resonances expected for the adduct (82) were found in the ^{1}H n.m.r. spectrum. Both of the starting materials (56) and (19) were found in the fractions and the only product identified was the dehydroalanine derivative (80), the characteristic[78] olefinic signals of which were seen at δ 6.00 and 6.81 in the ^{1}H n.m.r. spectrum of one fraction.

In an attempt to simplify this reaction, it was repeated in a homogeneous solvent system, namely methanol/water. The ^{1}H n.m.r. spectrum of the reaction

mixture showed that the major compound was the starting material (56) and that the alkene (80) was a minor product. T.l.c. analysis of the mixture indicated that the TMIO (19) had been consumed but the structures of any resultant compounds could not be elucidated from the n.m.r. spectrum. It was possible that hydroxyl radical, formed by reaction of hydrogen peroxide and ferrous sulfate, abstracted a methyl hydrogen from methanol. The resultant radical, stabilized by resonance through its adjacent oxygen atom, would have been trapped by TMIO (19).

Consequently, this reaction was repeated in *tert*-butanol/water. As the hydroxyl group in *tert*-butanol is adjacent to a tertiary carbon, a radical stabilized through an adjacent oxygen cannot be produced. T.l.c. analysis of this reaction mixture indicated that the spin trap (19) had been converted to the adduct (64), a sample of which was obtained from a reaction involving TMIO (19) in *tert*-butanol discussed in Chapter One (page 23). The ^{1}H n.m.r. spectrum of the reaction mixture showed singlets at δ 1.31 and 1.46, 2.45 (broad), and 3.83 for the methyl, hydroxyl, and methyl ester protons of the adduct (64), respectively. In addition, singlets at δ 2.69 and δ 2.97 in a 2:1 integral ratio, corresponded to the methylsulfoxy protons of the sulfoxide (56) and its oxidation product, the sulfone (81), respectively. Formation of the alcohol (64) can be attributed to coupling of TMIO (19) with the alkyl radical (63), presumably formed by hydrogen-atom abstraction from *tert*-butanol by hydroxyl radical (Scheme 32).



Scheme 32

The reactions involving Fenton's reagent produced coloured, complex reaction mixtures and it was apparent that this was partially due to the ferrous ions. As an alternative, ultraviolet light was used to cleave peroxide bonds. The heterogeneous solvent system of benzene/water was used to avoid the problems associated with the alcohol solvents. Photolysis of a mixture of aqueous hydrogen peroxide and a solution of the sulfoxide (56) and TMIO (19) in benzene was followed by chromatography of the complex reaction mixture on silica. The sulfoxide (56), TMIO (19), and the alanine derivative (13b) were the only compounds identified in the partially purified fractions obtained. The ^{1}H n.m.r. spectrum of the fraction containing the product (13b) showed a doublet at δ 1.53 (J = 7.0 Hz), a singlet at δ 3.80, and a mulitplet centred at δ 4.80. The ^{1}H n.m.r. spectrum of an authentic sample of this compound (13b) showed that these resonances corresponded to its β-methyl, methyl ester, and α-protons, respectively. Formation of the alanine derivative (13b) can be attributed to hydrogen-atom abstraction by the β-centred radical (54). No evidence for the production of the adduct (82) was found even though it appeared that the radical (54) was generated. It was possible that TMIO (19) and the radical (54) were not combining efficiently or that the adduct (82) was unstable.

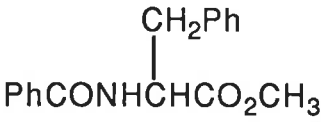
Generation of the radical (54) in reactions of the sulfoxide (56) with hydrogen peroxide in benzene/water, whilst evident, was apparently inefficient. This was possibly a result of inefficient formation of the hydrogen peroxide adduct of the sulfoxide (56) due to the heterogeneous nature of the solvent system. Reactions of a peroxide soluble in organic solvents with the cysteine derivatives (55) and (56) were therefore investigated. A solution of the sulfide (55) and di-*tert*-butyl peroxide in benzene was photolysed (350 nm) for twenty-four hours. The ^{1}H n.m.r. spectrum of the reaction mixture showed that little of the sulfide (55) had reacted but a small doublet at δ 1.52 (J = 7.0 Hz) may have corresponded to the β-protons of the alanine derivative (13b). This compound (13b) can be formed by hydrogen-atom abstraction by the radical (54), which may

have been displaced *via* substitution of *tert*-butoxy radical on sulfur in the sulfide (55). Similar results were obtained when the reaction was repeated using 300 nanometre light.

As the sulfide (55) showed low reactivity, a mixture of the sulfoxide (56) and di-*tert*-butyl peroxide in benzene was photolysed for three days. Periodic t.l.c. analysis of the mixture showed that increasing amounts of products were forming. The ^{1}H n.m.r. spectrum of the reaction mixture showed that it contained the alanine derivative (13b) amongst a mixture of several compounds. This was confirmed when addition of an authentic sample of the alanine derivative (13b) to the n.m.r. sample caused an increase in size of the doublet at δ 1.51 (J = 7.0 Hz), the multiplet centred at δ 4.81, and the singlet at δ 3.78 relative to the other resonances in the spectrum. Comparisons of the integrals of the α- and β-protons of the alanine derivative (13b) and the methylsulfoxy protons of the starting material (56) indicated an approximate 2:1 ratio of the compounds (13b) and (56). Assuming that the methyl ester peaks (δ 3.7-3.9) represented all of the amino acid derived compounds present in the mixture, including the sulfoxide (56), the proportion of the total integral of the methyl ester peaks arising from the methyl ester peak of the alanine derivative (13b) indicated that this product (13b) was approximately 20% of the amino acid derived material. The integral of the methyl ester peak of the product (13b) was determined from the integrals of the signals for the α- and β-protons. In the following reactions, most yields and ratios of products are estimated using integrals in a similar way to that described above.

This reaction was repeated and followed closely by t.l.c., with an extra portion of di-*tert*-butyl peroxide added after five hours in an attempt to obtain further reaction. One third of the reaction solution was removed after twenty-one hours and this complex mixture was chromatographed on silica. The alanine derivative (13b) and *N*-benzoylphenylalanine methyl ester (83) were identified as products by comparison of ^{1}H n.m.r. spectra and t.l.c. properties of

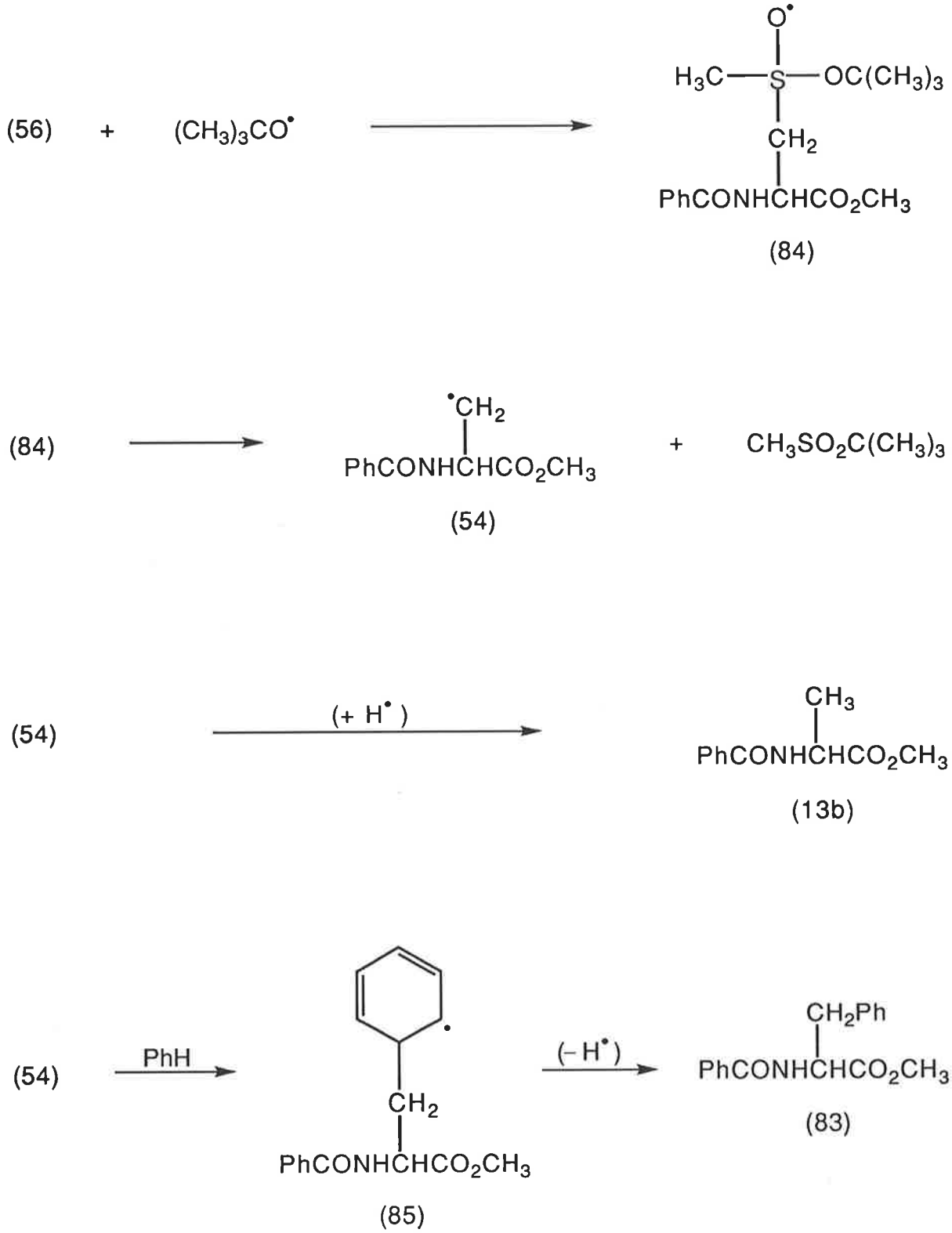
the partially purified fractions obtained with those of authentic samples. The ^1H n.m.r. spectrum of one fraction showed two doublets of doublets at δ 3.24 (J = 5.5, 14.0 Hz) and δ 3.31 (J = 6.0, 14.0 Hz) for the β-protons of the phenylalanine derivative (83), along with peaks for phenyl protons from δ 7.12-7.33. Integrals of peaks in the ^1H n.m.r. spectrum of the crude reaction mixture indicated that the alanine derivative (13b) made up approximately 30% of the amino acid derived material and the approximate ratio of the products (13b) and (83) and the starting material (56) was 2:1:1. It could now be seen that the phenylalanine derivative (83) was a product in the previous reaction, in which the ratio of the alanine derivative (13b) to the phenylalanine derivative (83) was also 2:1.



(83)

Di-*tert*-butyl peroxide was added to the remainder of the reaction mixture and photolysis was continued for a further twenty-seven hours with another addition of di-*tert*-butyl peroxide after seven hours. The ^1H n.m.r. spectrum of the complex reaction mixture showed that although there was little of the starting material (56) remaining, the alanine derivative (13b) remained approximately 30% of the total product. The alanine and phenylalanine derivatives (13b) and (83) were the major products, present in an approximate ratio of 2:1 as estimated by comparison of peak heights of the respective β-proton signals. Evidently, additional photolysis and di-*tert*-butyl peroxide did not significantly increase product yields.

Formation of the products (13b) and (83) can be attributed to addition of *tert*-butoxy radical at sulfur in the sulfoxide (56) followed by β-scission of the resultant oxygen-centred radical (84) to give the β-centred radical (54) (Scheme 33). Hydrogen-atom abstraction by the radical (54) gives rise to the alanine


(56)
+
$(CH_3)_3CO^{\bullet}$
H_3C
S
$OC(CH_3)_3$
O
CH_2
$PhCONHCHCO_2CH_3$
(84)
(84)
$^{\bullet}CH_2$
$PhCONHCHCO_2CH_3$
(54)
+
$CH_3SO_2C(CH_3)_3$
(54)
(+ $H^{\bullet}$)
CH_3
$PhCONHCHCO_2CH_3$
(13b)
(54)
PhH
CH_2
$PhCONHCHCO_2CH_3$
(85)
(- $H^{\bullet}$)
CH_2Ph
$PhCONHCHCO_2CH_3$
(83)

Scheme 33

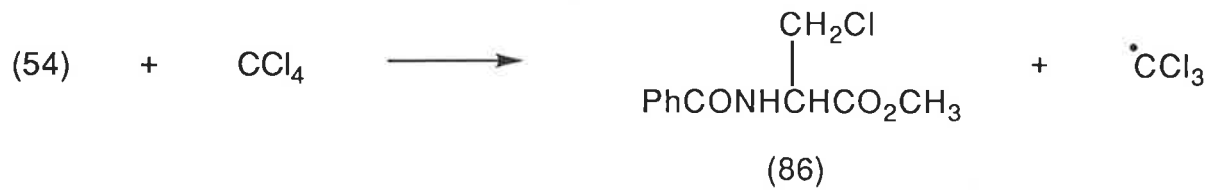
derivative (13b). Addition of the radical (54) to benzene followed by hydrogen-atom transfer from the intermediate radical (85) gives rise to the phenylalanine derivative (83). This apparent substitution of *tert*-butoxy radical on sulfur in the sulfoxide (56) is contrary to the report[54] which states that no radicals were detected when sulfoxides were exposed to *tert*-butoxy radicals. Generation of the radical (54) is, however, consistent with a report[57] which states that radicals formed by carbon-sulfur bond cleavage were detected on photolysis of sulfoxides with di-*tert*-butyl peroxide. Evidently, the sulfur in the sulfoxide (56) is far more susceptible to radical substitution than that in the sulfide (55).

To confirm that the radical (54) was not formed by spontaneous photolytic carbon-sulfur bond cleavage, a solution of the sulfoxide (56) in 2% *iso*-propanol in benzene was photolysed for fifty-two hours. It was thought that the radical (54), if produced, would abstract a hydrogen atom from *iso*-propanol to give the alanine derivative (13b). Catalytic amounts of the radical initiators benzoyl peroxide, AIBN, and acetone were added to the solution during photolysis as t.l.c. analysis indicated that no reaction was occurring. The ^{1}H n.m.r. spectrum of the reaction mixture showed that at least 90% of the starting material (56) remained. A small singlet at δ 2.98 indicated that a minor amount of the sulfone (81), produced by oxidation of the sulfoxide (56), may have formed. In addition, no reaction was observed during four days of photolysis of a solution of the sulfoxide (56) in *iso*-propanol only. These studies confirmed that *tert*-butoxy radical was required to displace the radical (54) from the sulfoxide (56).

The above reactions of the sulfoxide (56) with di-*tert*-butyl peroxide were conducted using light of wavelength 350 nanometres. A mixture of the sulfoxide (56) and di-*tert*-butyl peroxide was irradiated with 300 nanometre light for twenty-four hours. The ^{1}H n.m.r. spectrum of the reaction mixture showed that little of the starting material (56) remained, the alanine derivative (13b) was produced in approximately 30% yield, and the approximate ratio of the products (13b) and (83) was 2:1. The above reactions of the sulfoxide (56) with di-*tert*-butyl

peroxide were carried out with periodic additions, each of approximately five equivalents, of the peroxide. To determine whether larger portions of this reagent would further optimise the reaction, a solution of the sulfoxide (56) and ten equivalents of di-*tert*-butyl peroxide in benzene was photolysed for twenty-three hours with another ten equivalents of the peroxide added after seven hours. The ^1H n.m.r. spectrum of the reaction mixture indicated that the ratio of the compounds (13b), (83), and (56) was 2:1:1, as estimated by comparison of the respective methyl ester peak heights. The alanine derivative (13b) made up approximately 30% of the amino acid derived material. These results indicated that changes in wavelength of incident light and amount of di-*tert*-butyl peroxide did not significantly affect the progress of the reaction. Isolated product yields were not obtained for the reactions of the sulfoxide (56) with di-*tert*-butyl peroxide because the reaction mixtures were complex and some of the products had similar chromatographic properties.

To obtain further evidence for generation of the β-centred radical (54) from the sulfoxide (56) and gauge the synthetic potential of the reaction, carbon tetrachloride was introduced to the reaction. It was envisaged that the radical (54) would abstract a chlorine atom from carbon tetrachloride to give *N*-benzoyl-β-chloroalanine methyl ester (86) (Scheme 34). Poor solubility of the sulfoxide (56) in carbon tetrachloride lead to photolysis of a solution of the sulfoxide (56) and di-*tert*-butyl peroxide in benzene/carbon tetrachloride (11:1) for thirty hours, at which time very little of the starting material (56) remained. Chromatography of the complex reaction mixture on silica gave partially purified fractions containing the β-chloroalanine derivative (86) and the phenylalanine derivative



Scheme 34

(83), which were inseparable on silica, the alanine derivative (13b), and the dehydroalanine derivative (80). The ^{1}H n.m.r. spectrum of one fraction showed two doublets of doublets at δ 4.04 (J = 3.5, 11.5 Hz) and δ 4.09 (J = 3.0, 11.5 Hz), an apparent doublet of triplets at δ 5.20 (J = 7.0, 3.0 Hz), and a singlet at δ 3.86, which matched the resonances for the β-protons, the α-proton, and the methyl ester protons, respectively, in the n.m.r. spectrum of an authentic sample of the chloride (86). Signals at δ 6.01 and 6.81, characteristic of the olefinic protons of the alkene (80),[78] were not present in the ^{1}H n.m.r. spectrum of the crude reaction mixture and formation of the alkene (80) can be attributed to elimination of hydrogen chloride from the chloride (86) during chromatography. The ^{1}H n.m.r. spectrum of the reaction mixture showed that the major products, the β-chloroalanine derivative (86) and the alanine derivative (13b), formed in about 30% and 15% yield, respectively. The phenylalanine derivative (83) was a minor product. Evidently, abstraction of a chlorine atom from carbon tetrachloride by the radical (54), produced *via* radical substitution on the sulfoxide (56), was not efficient enough to exclude formation of the derivatives of alanine (13b) and phenylalanine (83).

In an attempt to optimise this synthesis of the β-chloroalanine derivative (86), a solution of the sulfoxide (56), di-*tert*-butyl peroxide, and chlorotriphenylmethane in benzene was photolysed for fifty hours. It was thought that chlorotriphenylmethane would be a good source of the chlorine atom as the triphenylmethyl radical, produced by chlorine-atom abstraction from chlorotriphenylmethane, is strongly stabilized through resonance. ^{1}H N.m.r. spectra of partially purified fractions obtained from chromatography of the reaction mixture on silica showed that the chloride (86), the alanine derivative (13b), and the phenylalanine derivative (83) were products. Chlorotriphenylmethane (60%) was recovered from the reaction mixture, indicating that chlorine-atom abstraction from chlorotriphenylmethane by the radical (54) was not particularly efficient.

The relatively low reactivity of chlorotriphenylmethane prompted the use of α,α,α-trichlorotoluene as the reaction solvent, thus providing a large excess of a chlorine atom source and eliminating production of the phenylalanine derivative (83). The dichlorobenzyl radical, produced by chlorine-atom abstraction from α,α,α-trichlorotoluene, is strongly stabilized through resonance. A solution of the sulfoxide (56) and di-*tert*-butyl peroxide in α,α,α-trichlorotoluene was photolysed for thirty hours. The ^{1}H n.m.r. spectrum of the reaction mixture indicated that no starting material (56) remained and that the ratio of the major product, the β-chloroalanine derivative (86), to the minor product, the alanine derivative (13b), was approximately 6:1. Integrals of peaks characteristic of the chloride (86) indicated that the approximate yield of this product (86) was 50%.

To establish yields for this reaction, it was repeated with addition of the external standard *tert*-butylbenzamide on completion. Photolysis of a solution of the sulfoxide (56) and di-*tert*-butyl peroxide in α,α,α-trichlorotoluene for thirty-four hours was followed by addition of one-fifth of an equivalent of the reference compound, *tert*-butylbenzamide. The ^{1}H n.m.r. spectrum of the resultant mixture showed that the β-chloroalanine derivative (86) and the alanine derivative (13b) were produced in approximately 45% and 4% yield, respectively. The yield of the chloride (86) based on remaining starting material (56) was 70%. Percentage yields were calculated by comparing integrals of resonances characteristic of the products (86) and (13b) and the starting material (56) with that of the *tert*-butyl singlet of the external reference compound. The yield of the chloride (86) demonstrated that the β-centred radical (54) can be efficiently generated from the sulfoxide (56) *via* substitution of *tert*-butoxy radical on sulfur. The reaction is potentially useful in the synthesis of β-substituted amino acid derivatives.

Reactions discussed in this Chapter indicate the feasibility of the proposal for generation of β-centred radicals from cystine or cysteine residues in peptides

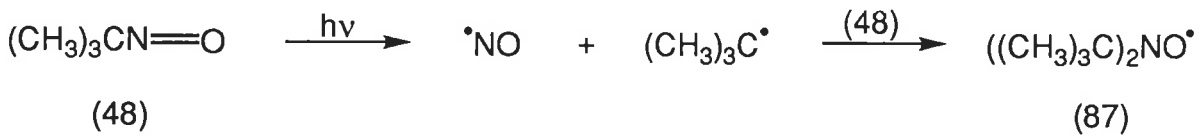
outlined in the Introduction (Scheme 9). Reactions of the amino acid derivatives (78), (79), (55), and (56) indicated that reduction of cystine residues in peptides, methylation of cysteine residues, oxidation of *S*-methylcysteine residues, and displacement of β-centred radicals from *S*-methylcysteine sulfoxide residues is achievable. Generation of radicals from methionine residues in peptides, in which case the reduction and methylation steps in Scheme 9 would be unnecessary, is also feasible. E.s.r. studies to obtain direct evidence for generation of carbon-centred side-chain radicals from *S*-methylcysteine (28) and methionine (26) and their derivatives are discussed in Chapter Three.

Results and Discussion Chapter Three

E.s.r. Studies of Radicals from Amino Acid Sulfides and Sulfoxides

E.s.r. studies to obtain direct evidence for generation of side-chain carbon-centred radicals from *S*-methylcysteine (28) and methionine (26) and their derivatives were carried out with a view to generating such radicals in peptides *via* the method outlined in the Introduction (Scheme 9). Direct detection of radicals by e.s.r. spectroscopy using two different radical traps is discussed. As mentioned in the Introduction, radicals produced by photolysis of mixtures of sulfides or sulfoxides and hydrogen peroxide have been trapped by nitroso-*tert*-butane (NTB) (48) to give stable adducts[52] and this method was used in initial studies.

A mixture of a solution of *N*-benzoyl-*S*-methylcysteine sulfoxide methyl ester (56) and NTB (48) in benzene and aqueous hydrogen peroxide was irradiated with ultraviolet light. The e.s.r. spectrum of the mixture showed a triplet (1:1:1, 15 gauss (G)). The absence of any splitting of the signal other than the characteristic nitrogen triplet of a nitroxide indicated that a tertiary radical had been trapped. The same triplet was observed in a control experiment, in which a solution of NTB (48) in benzene was photolysed, and was attributed to di-*tert*-butyl nitroxide (87). This nitroxide (87) has a reported coupling constant of fifteen gauss in benzene[81] and is known to form by photolytic cleavage of the weak carbon-nitrogen bond of NTB (48) and trapping of the resultant *tert*-butyl radical by NTB (48) (Scheme 35).[60]



Scheme 35

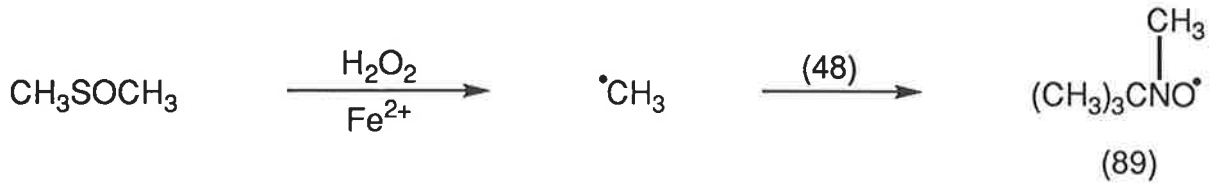
To avoid formation of this nitroxide (87), ferrous ion was used instead of photolysis to initiate the reaction. A mixture of a solution of the sulfoxide (56) and NTB (48) in benzene and aqueous hydrogen peroxide was treated with ferrous sulfate and the resultant green colouration indicated that electron transfer from ferrous ion to hydrogen peroxide, producing hydroxyl radical and green ferric ion (Equation 1, page 17), had occurred. The e.s.r. spectrum of the mixture showed two overlapping triplets (1:1:1, 15G and 27G) which were attributed to di-*tert*-butyl nitroxide (87) and *tert*-butylhydroxy nitroxide (88), formed by trapping of *tert*-butyl radical and hydroxyl radical by NTB (48), respectively (Schemes 35 and 36). Twenty-seven gauss is the reported coupling constant for deprotonated *tert*-butylhydroxy nitroxide (88) in aqueous solution.[82] Evidently, the carbon-nitrogen bond of NTB (48) cleaved to give *tert*-butyl radical. This cleavage may have been spontaneous or caused by electron transfer from ferrous ion to NTB (48).

(88)

Scheme 36

As it seemed probable that the heterogeneity of the above reaction mixtures was hindering reaction of hydrogen peroxide with the sulfoxide (56), the methodology was tested in a homogeneous solvent system. Dimethyl sulfoxide, aqueous hydrogen peroxide, and ferrous sulfate, a mixture reported to generate methyl radical by the mechanism outlined in Scheme 16,[54] were combined with NTB (48). The e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, 17G) of quartets (1:3:3:1, 14G) for the adduct (89), formed by trapping of methyl radical by NTB (48) (Scheme 37). A triplet (1:1:1, 17G) for di-*tert*-butyl nitroxide (87) was also observed. The nitroxide (87) has reported coupling constants of seventeen and sixteen gauss in water and dimethyl sulfoxide, respectively.[81] The signal for the adduct (89), consistent with that reported,[82] arises through

interaction of the unpaired electron with the nitrogen (triplet) and methyl hydrogens (quartet). This result indicated that the lack of evidence for radical generation from the sulfoxide (56) was probably due to the heterogeneity of the solvent system.



Scheme 37

The successful use of a homogeneous aqueous solvent system prompted an investigation into generation of radicals from water soluble amino acid sulfoxides. The only such sulfoxide found to be commercially available, methionine sulfoxide (49), was chosen for initial studies. The e.s.r. spectrum of the mixture obtained from treatment of an aqueous solution of methionine sulfoxide (49) and NTB (48) with hydrogen peroxide and ferrous sulfate showed a triplet (1:1:1, 16G) of doublets (2G) (Figure 2). This signal was assigned to the adduct of NTB (48) and the α-centred radical formed by decarboxylation of methionine sulfoxide (49), with the doublet arising through interaction of the unpaired electron with the α-hydrogen. Reaction of *N*-acetylglycine with titanous ion and hydrogen peroxide has been reported to give a signal corresponding to the radical formed by decarboxylation.[83] Decarboxylation of acetic acid and malonic acid under similar conditions has also been reported,[84] and the proposed mechanism involved carboxyl hydrogen-atom abstraction by hydroxyl radical (Equation 4), followed by decarboxylation of the resultant carboxyl radical (Equation 5). Decarboxylation *via* the carboxylic acid form of methionine sulfoxide (49) is proposed because thermodynamic calculations, based on reported methods,[85] and using values for formic acid,[85,86] indicate that carboxyl hydrogen-atom abstraction by hydroxyl radical (Equation 4) is exothermic

Equation 4 : $RCO_2H + {}^{\bullet}OH \longrightarrow RCO_2^{\bullet} + H_2O$

Equation 5 : $RCO_2^{\bullet} \longrightarrow R^{\bullet} + CO_2$

Equation 6 : $RCO_2^{-} + {}^{\bullet}OH \longrightarrow RCO_2^{\bullet} + {}^{-}OH$

whereas electron transfer from carboxylate to hydroxyl radical (Equation 6) is endothermic. Presumably, decarboxylation of methionine sulfoxide (49) occurred at a faster rate than the desired reaction at the sulfoxide moiety. Similarly, the absence of the triplet for di-*tert*-butyl nitroxide (87) indicated that displacement of *tert*-butyl radical from NTB (48) was slower than decarboxylation of the amino acid (49).

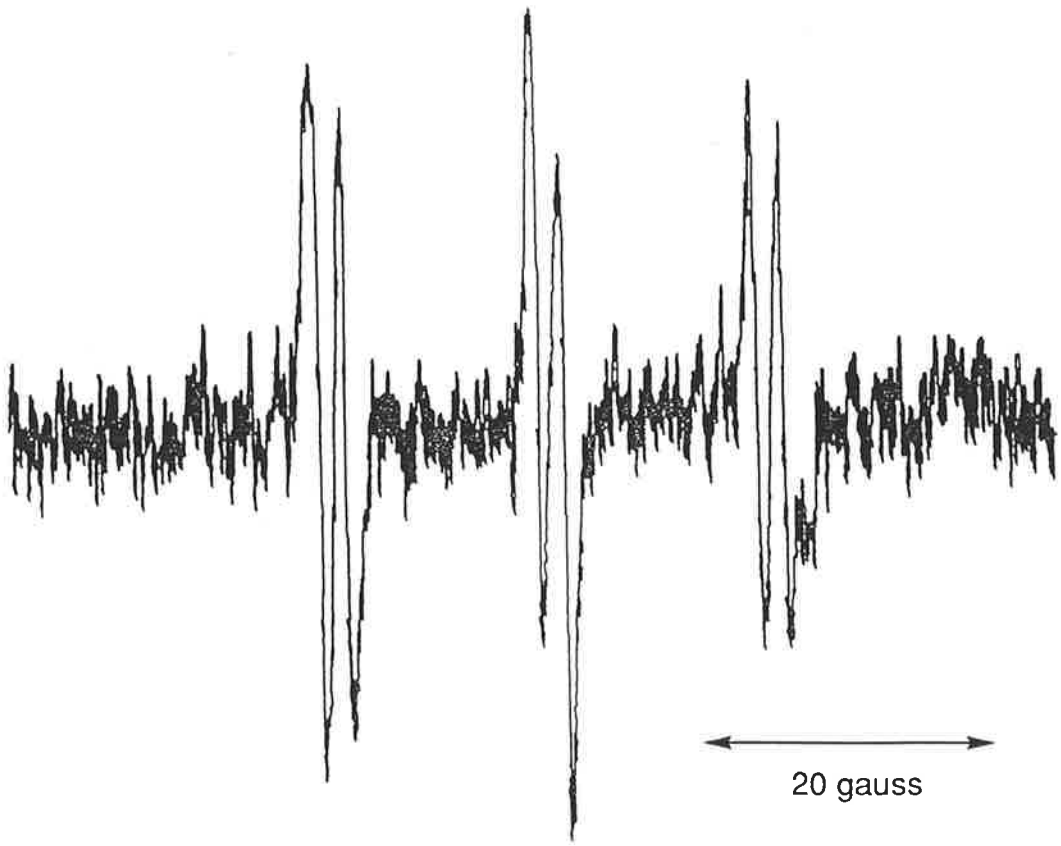


Figure 2: E.s.r. spectrum obtained from reaction of methionine sulfoxide (49), NTB (48), hydrogen peroxide, and ferrous sulfate.

The experiment described above was repeated using photolysis to initiate reaction instead of electron transfer from ferrous ion. Photolysis of hydrogen peroxide to give hydroxyl radical is relatively inefficient and it was envisaged that decreasing the concentration of hydroxyl radical by this method would abate decarboxylation. The resultant e.s.r. spectrum showed a triplet (1:1:1, 17G) and a triplet (1:1:1, 17G) of triplets (1:2:1, 12G), overlapping (Figure 3), attributable to

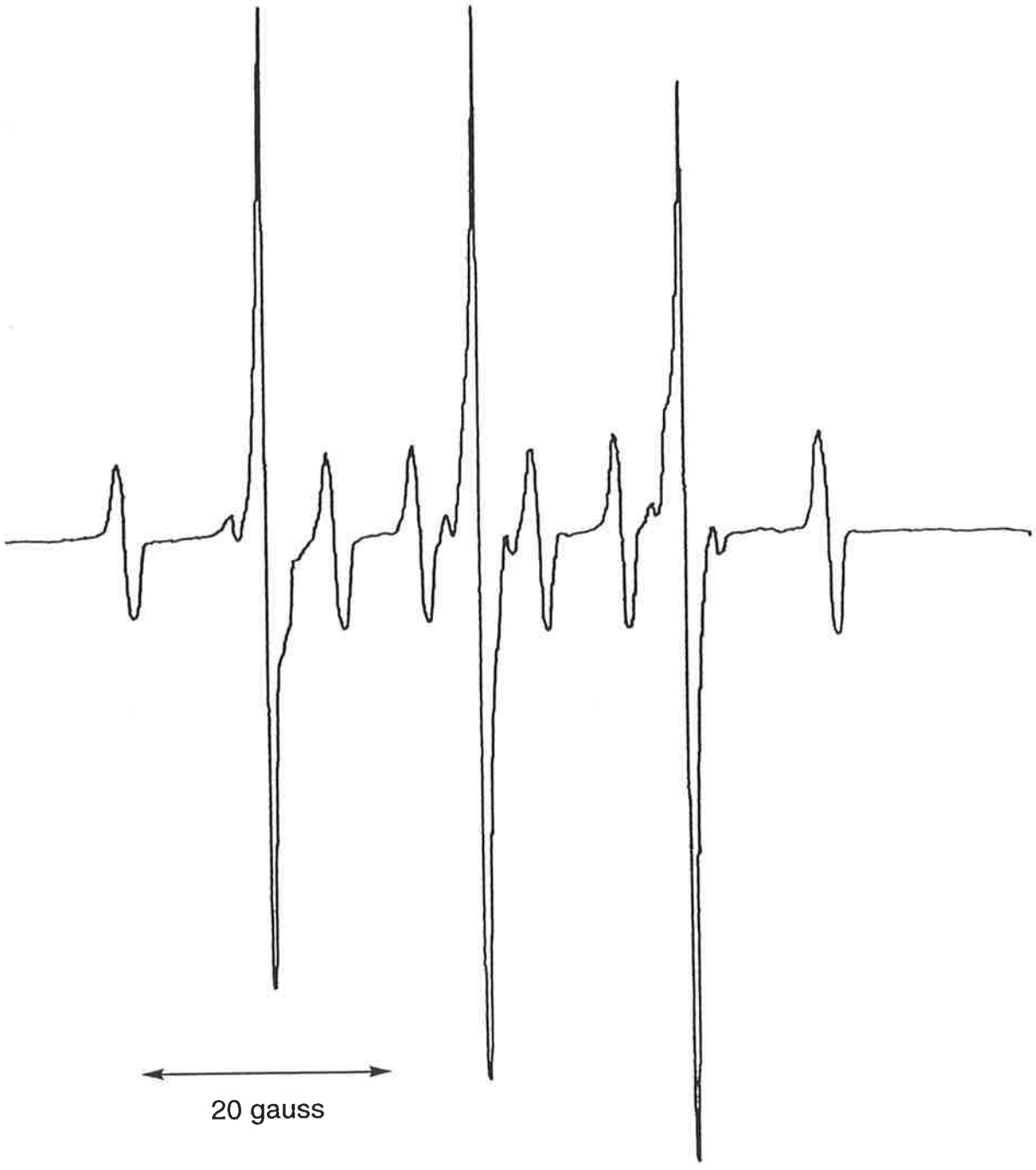


Figure 3: E.s.r. spectrum obtained from photolysis of methionine sulfoxide (49), NTB (48), and hydrogen peroxide.

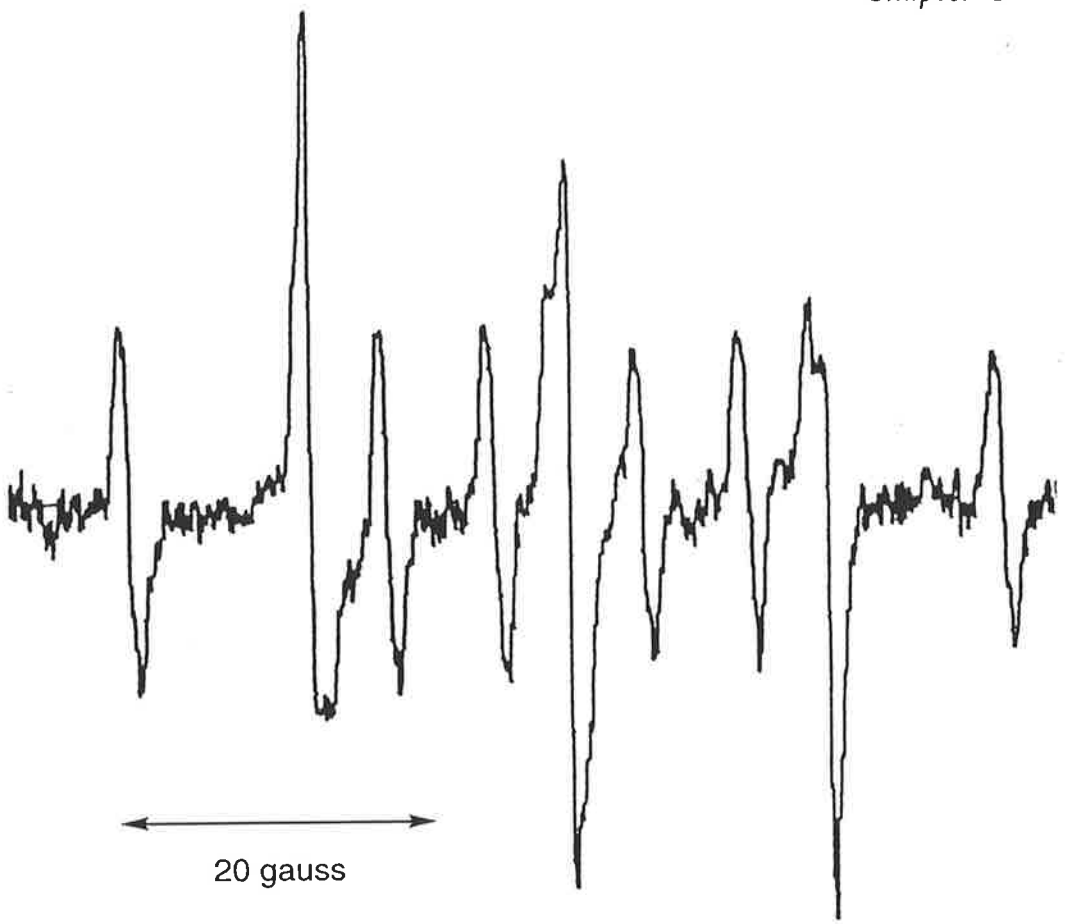
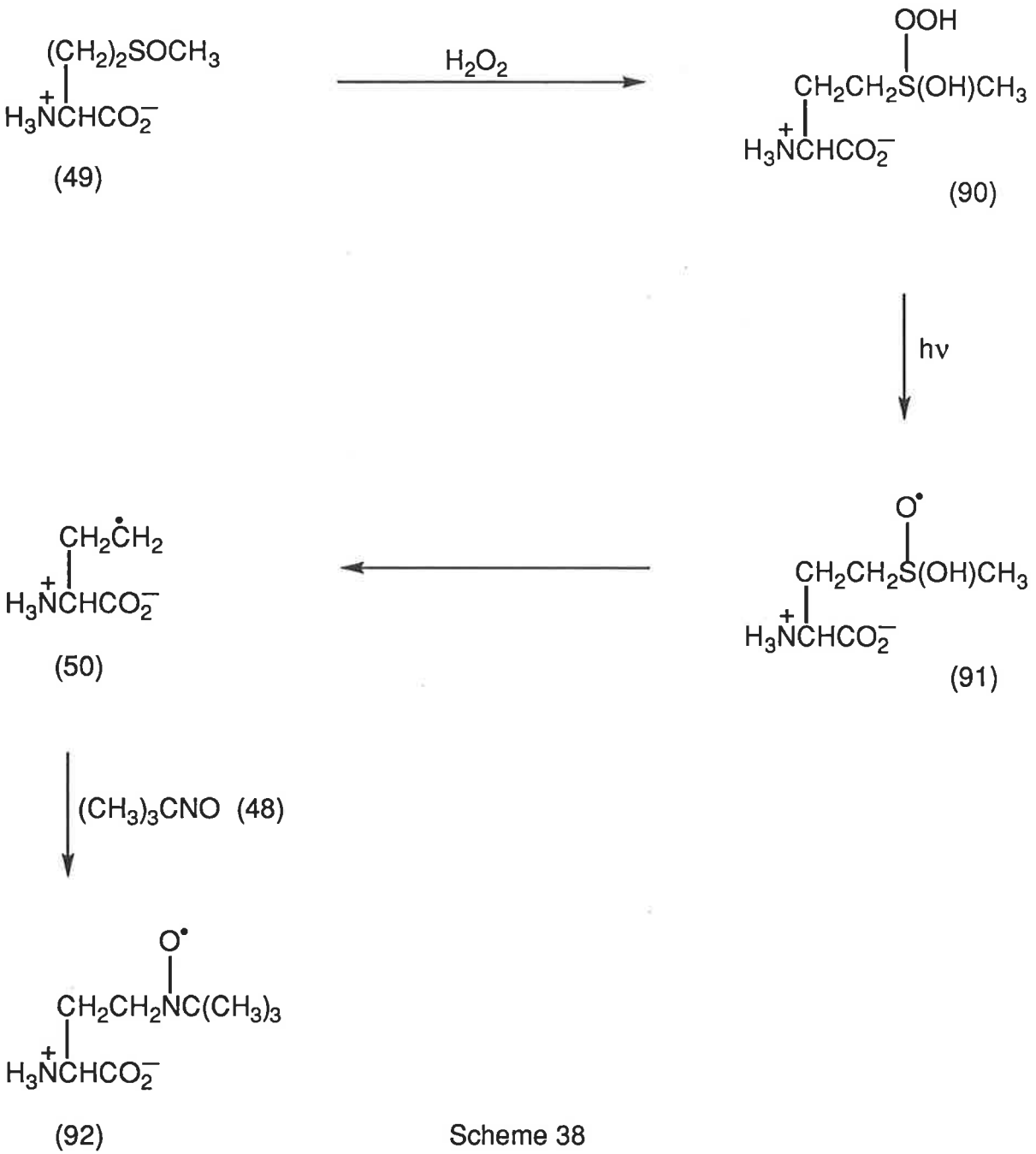


Figure 4: E.s.r. spectrum obtained from photolysis of methionine sulfoxide (49), NTB (48), and hydrogen peroxide followed by extraction with light petroleum.

di-*tert*-butyl nitroxide (87) and the adduct (92), formed by trapping of the γ-centred radical (50) by NTB (48) (Scheme 38), respectively. The nitroxide (87) was removed by carefully washing the reaction mixture with light petroleum, as reported,[87] and the resultant spectrum showed the triplet of triplets for the adduct (92) (Figure 4), comparable with the signal reported for the deprotonated form of the adduct (92).[88] The seventeen and twelve gauss triplets for the adduct (92) arise through the respective interactions of the unpaired spin density with the nitrogen and equivalent γ-hydrogens. Formation of the γ-centred radical (50) can be attributed to photolytic cleavage of the peroxide bond of the hydrogen peroxide-methionine sulfoxide adduct (90) and β-scission of the resultant oxygen-centred radical (91) (Scheme 38).

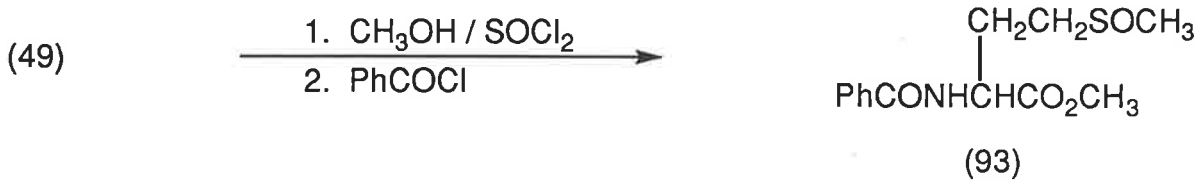


Scheme 38

As discussed in the Introduction, the proposed mechanisms for displacement of radicals from sulfoxides involved either reaction of a hydrogen peroxide-sulfoxide adduct, as outlined in Scheme 16, or substitution of hydroxyl radical on sulfur. The observation that decarboxylation of methionine sulfoxide (49) occurred in the presence of a relatively high concentration of hydroxyl radical (Fenton's reagent) whereas reaction at the sulfoxide moiety occurred with low

hydroxyl radical concentration (photolysis) indicated that the mechanism in Scheme 16 is correct.

The success in generating the γ-centred radical (50) from methionine sulfoxide (49) prompted attempts to produce the corresponding radical from a methionine sulfoxide derivative. A mixture of diastereomers of *N*-benzoyl-methionine sulfoxide methyl ester (93) was synthesized in 64% yield by addition of methionine sulfoxide (49) to methanol that had been pretreated with thionyl chloride, treatment of the resultant methyl ester with benzoyl chloride (Scheme 39), and crystallization of the resultant oil from ethyl acetate/light petroleum.

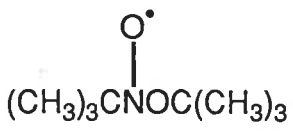


Scheme 39

Satisfactory microanalytical data was obtained for the sulfoxide (93) and the f.a.b. mass spectrum showed a protonated molecular ion at *m/z* 284. The ^{1}H n.m.r. spectrum of the diastereomeric mixture showed multiplets at δ 2.28-2.56 and δ 2.72-3.01 for the β-and γ-protons, respectively, singlets at δ 2.60 and 2.61 for the methylsulfoxy protons, singlets at δ 3.80 and 3.81 for the methyl ester protons, and a multiplet at δ 4.87-4.94 for the α-protons. Photolysis of a mixture of a solution of the sulfoxide (93) and NTB (48) in benzene and aqueous hydrogen peroxide gave rise to a fifteen gauss triplet in the e.s.r. spectrum, presumably for di-*tert*-butyl nitroxide (87). As it was likely that the heterogeneity of the solvent system again hindered reaction of the sulfoxide (93) with hydrogen peroxide, reactions of the sulfoxide (93) with an organic peroxide were investigated.

Alkyl radicals formed *via* carbon-sulfur bond homolysis have been detected by e.s.r. spectroscopy in photolytic reactions of di-*tert*-butyl peroxide with

sulfoxides.[57] Photolysis of a solution of the sulfoxide (93), NTB (48), and di-*tert*-butyl peroxide in benzene gave rise to two triplets (1:1:1, 16G and 27G) in the e.s.r spectrum. These were attributed to di-*tert*-butyl nitroxide (87) and *tert*-butoxy-*tert*-butyl nitroxide (94), the latter formed by trapping of *tert*-butoxy radical by NTB (48). The nitroxide (94) has a reported coupling constant of twenty-seven gauss in benzene.[81,89] The e.s.r. spectrum of the mixture obtained from photolysis of a suspension of the sulfoxide (93) and NTB (48) in di-*tert*-butyl peroxide showed the same two triplets. These triplets were generated during photolysis of a mixture of di-*tert*-butyl peroxide and NTB (48) in the absence of the sulfoxide (93), confirming that they were not associated with the sulfoxide (93). As formation of the nitroxides (87) and (94) dominated the above reactions, generation of radicals from the methionine sulfoxide derivative (93) was not pursued.

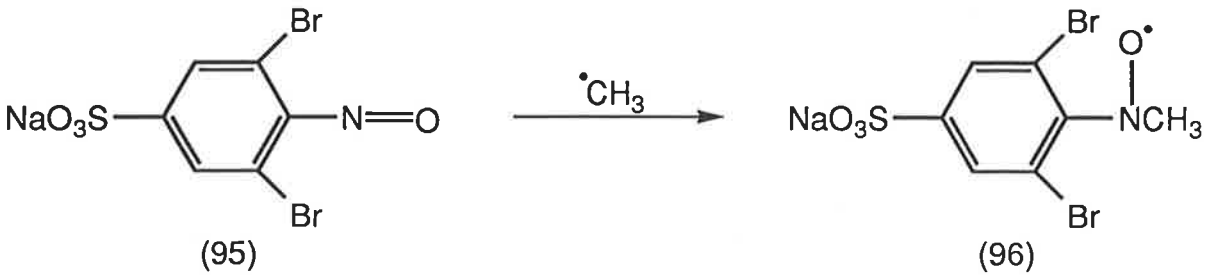


(94)

As e.s.r. spectroscopic evidence for production of radicals from sulfoxides was only obtained when a homogeneous aqueous solvent system was used, generation of radicals from water soluble sulfoxides was investigated further. It had been reported that aqueous solutions of NTB (48) were easily prepared by stirring overnight[87,88] but solutions prepared in this way were found to be not useful in obtaining e.s.r. spectra. Solutions were therefore prepared by lengthy sonication of suspensions of NTB (48) in water but were found to be useful for a short time only or not at all. As consistently useful solutions of NTB (48) in water could not be produced, a water soluble radical trap was required.

Sodium 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS) (95) is an efficient radical trap with a solubility of approximately one hundred grams per litre in

water at 20°C.[90] Unlike NTB (48), DBNBS (95) does not form a nitroxide when exposed to light,[91] presumably because of the relative instability of the aromatic radical formed by cleavage of the carbon-nitrogen bond of the sulfonate (95), compared with *tert*-butyl radical, formed on cleavage of NTB (48). In addition, no adduct of DBNBS (95) and hydroxyl radical has been observed by e.s.r. spectroscopy.[90,92] The ^{1}H n.m.r. spectrum of the trap (95), synthesized by oxidation of the sodium salt of 3,5-dibromosulfanilic acid, as reported,[90] showed a singlet at δ 8.33, consistent with the chemical shift reported for the two aromatic protons.[91] The radical trapping ability of the product (95) was tested by combining it with a mixture known to produce methyl radical,[54,55] namely aqueous hydrogen peroxide, dimethyl sulfoxide and ferrous sulfate. The e.s.r. spectrum of the resultant mixture showed mainly a triplet (1:1:1, 15G) of quartets (1:3:3:1, 14G) (Figure 5)#, comparable with the reported signal for the adduct (96), produced by trapping of methyl radical by DBNBS (95) (Scheme 40).[93] The triplet and quartet splittings arise *via* the respective interactions of the unpaired spin density with the nitrogen and methyl hydrogens. No e.s.r signals appeared in this experiment until the addition of ferrous ions, the final step, indicating that these ions induced peroxide bond homolysis of a sulfoxide-hydrogen peroxide adduct in the essential step of production of radicals *via* this method.



Scheme 40

Hyperfine splitting due to the aromatic protons was observed in some e.s.r. spectra of DBNBS (95) adducts. The effect was either additional splitting of signals, as in this case where triplet splitting (1:2:1, 1G) can be seen, or line broadening, and is not discussed further.

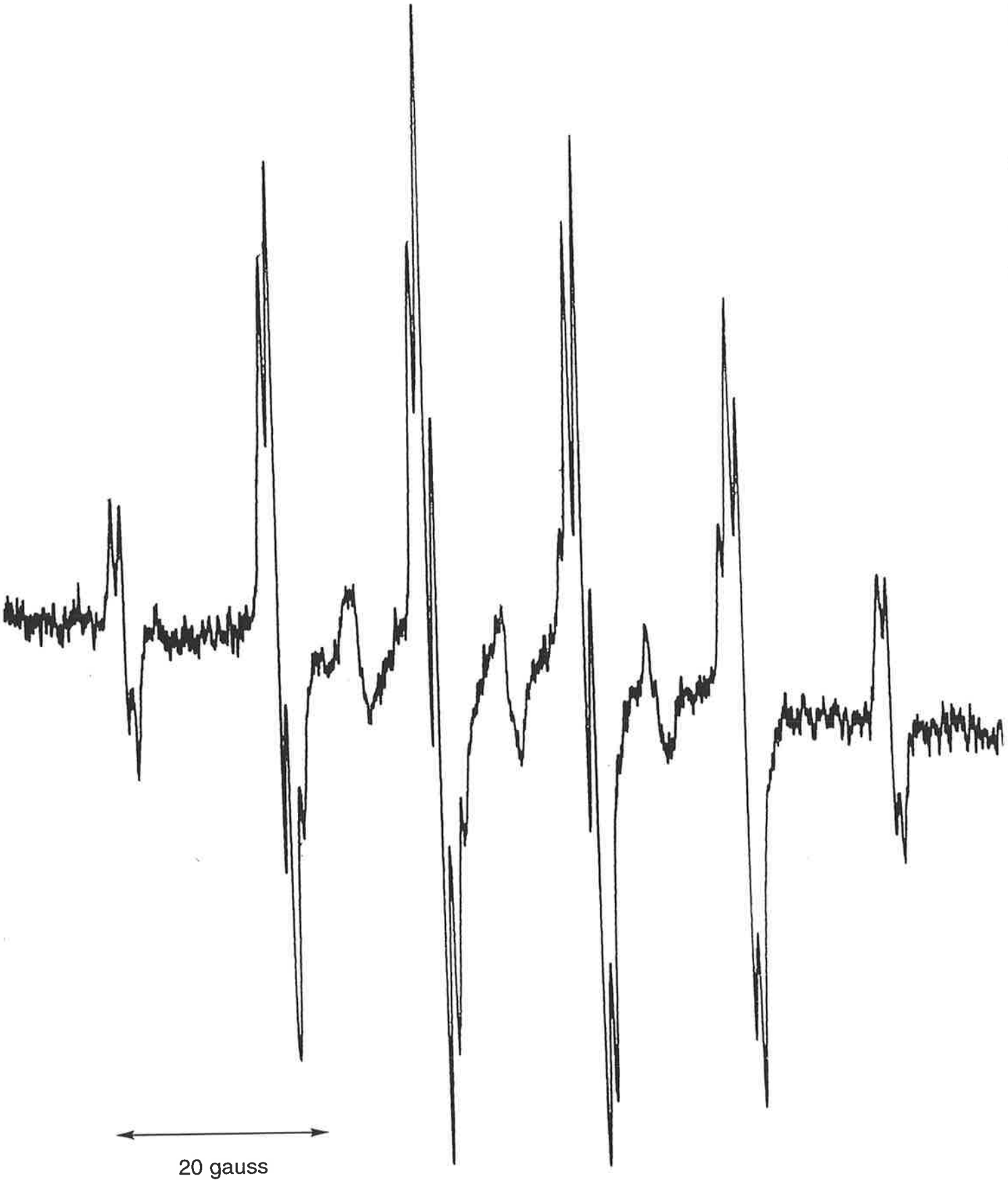


Figure 5: E.s.r. spectrum obtained from reaction of dimethyl sulfoxide, DBNBS (95), hydrogen peroxide and ferrous sulfate.

The utility of DBNBS (95) in the trapping of alkyl radicals generated from a variety of sulfides and sulfoxides was investigated. A summary of the results of reactions of sulfides and sulfoxides with hydrogen peroxide and ferrous sulfate in the presence of DBNBS (95) is given in Table 2. Sulfides were used as starting materials in most experiments, as they were usually readily available and sulfoxides were easily formed *in situ* on addition of hydrogen peroxide. In reference to Scheme 9, in which the proposed method for generation of radicals from cysteine residues in peptides is outlined, *in situ* oxidation of sulfide moieties would simplify the procedure by eliminating the need for a separate oxidation step.

Table 2: E.s.r. Parameters for Spin Adducts of DBNBS (95) and Radicals Produced by Reaction of Sulfides and Sulfoxides with H_2O_2 and $FeSO_4$.

Sulfide/Sulfoxide	Radical*	Adduct	$a_N^{t'}$ (gauss)	a_H^t (gauss)
CH_3SOCH_3	$^{\bullet}CH_3$	(96)	15	14[9]
$CH_3CH_2SCH_2CH_3$	$^{\bullet}CH_2CH_3$	(97)	15	13
$CH_3CH_2SCH_3$	$^{\bullet}CH_2CH_3$	(97)	15	13
$(CH_3)_3CSCH_3$	$^{\bullet}C(CH_3)_3$	(98)	15	-
$CH_3S(CH_2)_3CO_2H$ (104)	(105)	(107)	15	13
$CH_3SCH_2CH_2CO_2H$ (103)	(106)	(108)	15	13
$CH_3S(CH_2)_3NH_2.HCl$	(116)	(118)	15	13
$CH_3SCH_2CH_2NH_2.HCl$	(117)	(119)	14	14

Table 2 continued:

Sulfide/Sulfoxide	Radical*	Adduct	$a_N^{t'}$ (gauss)	a_H^{t} (gauss)
methionine (26) or methionine sulfoxide (49)	(50)	(99)	15	13
methionine hydrochloride	-	-	15	12
S-methylcysteine hydrochloride	(120)	(121)	15	13
glycylmethionylglycine (58)	(123)	(124)	15	12
methionylleucylphenyl-alanine (59)	-	-	15	12
S-methylglutathione hydrochloride	-	-	14	10

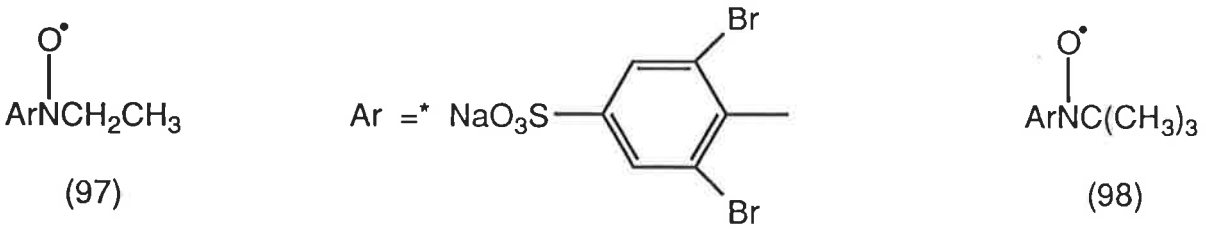
$^{t'}$ 1:1:1 triplet hyperfine splitting

^{t} 1:2:1 triplet hyperfine splitting except for the adduct (96)

^{q} quartet hyperfine splitting

* In cases where two radicals are generated, parameters for the adduct of the major radical are given.

Treatment of a mixture of diethyl sulfide and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate gave rise to a triplet (1:1:1, 15G) of triplets (1:2:1, 13G) in the e.s.r. spectrum (Figure 6). This signal can be attributed to the adduct (97), produced by trapping of ethyl radical by DBNBS (95). The fifteen and thirteen gauss triplet splittings arise *via* the respective interactions of the unpaired spin density with the nitrogen and methylene hydrogens. A similar signal would be expected for any DBNBS (95) adduct of a side-chain methylene radical generated from an *S*-methylcysteine (28) or methionine (26) residue.



*For the remainder of this Chapter, "Ar" is as defined above, representing the aromatic moiety of spin adducts of DBNBS (95).

The e.s.r. spectrum of the mixture obtained from treatment of a solution of ethylmethyl sulfide and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate showed a triplet (1:1:1, 15G) of triplets (1:2:1, 13G), for the ethyl radical adduct (97), and a triplet (1:1:1, 15G) of quartets (1:3:3:1, 14G), for the methyl radical adduct (96). The spectrum appeared as a combination of the spectra shown in Figures 5 and 6 and indicated, by comparisons of peak areas of the signals, that ethyl and methyl radicals had been produced in an approximate ratio of 6:1, reflecting the greater stability of the primary ethyl radical over methyl radical. No e.s.r. signals were observed when this experiment was repeated in the absence of hydrogen peroxide, verifying that the peroxide is an essential reagent in forming radicals from sulfides by this method.

Treatment of a mixture of *tert*-butylmethyl sulfide and a solution of DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate gave rise to a triplet (1:1:1, 15G) (Figure 7) in the e.s.r. spectrum. The triplet indicated that a tertiary radical had been trapped and can be attributed to the adduct (98), produced by trapping of *tert*-butyl radical by DBNBS (95). Fifteen gauss is consistent with the reported coupling constant for the adduct of *tert*-butyl radical and the deuterated form of DBNBS (95).[90] The absence of the signal for the methyl radical adduct (96) reflects the far greater stability of *tert*-butyl radical over methyl radical.

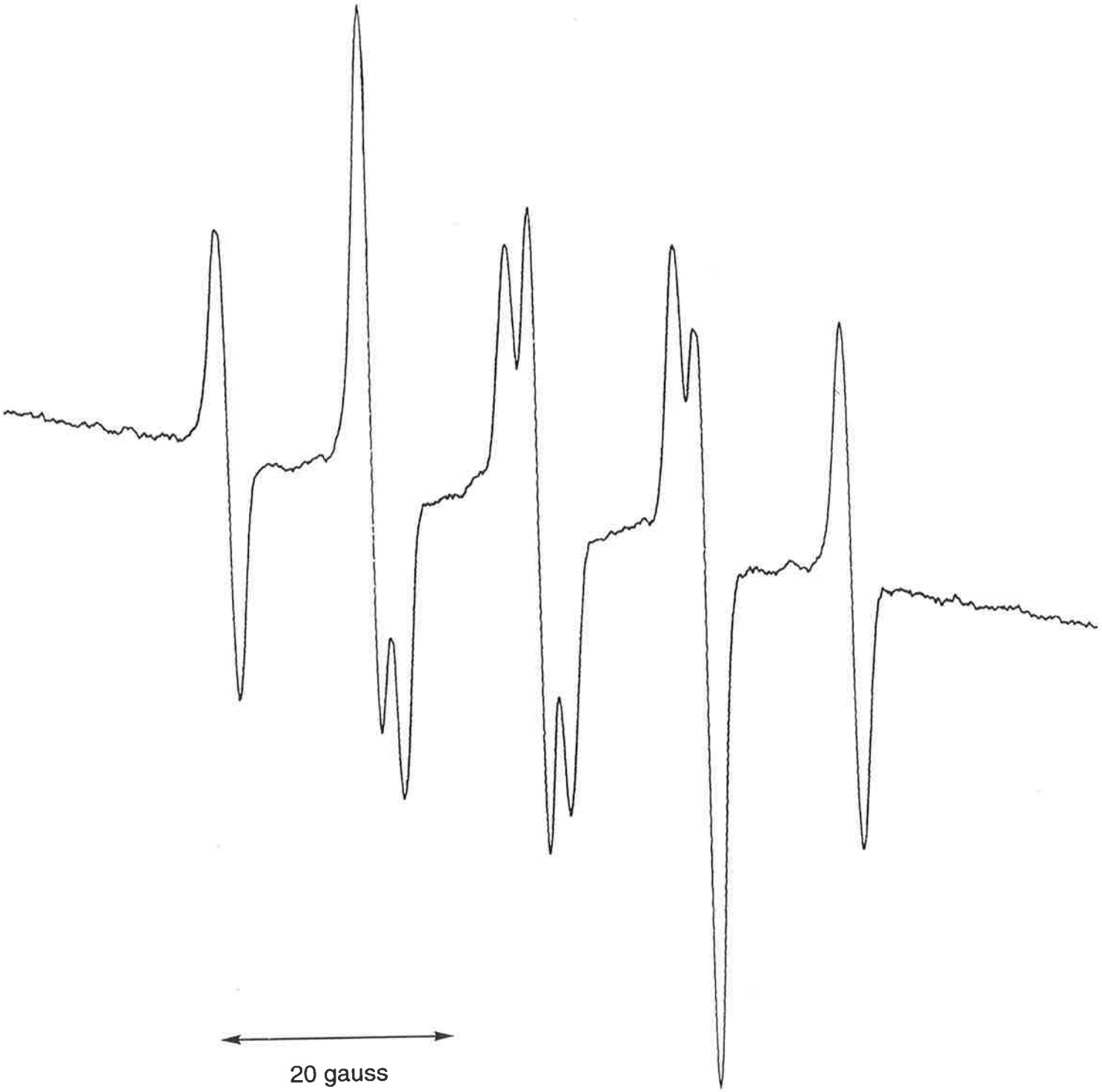


Figure 6: E.s.r. spectrum obtained from reaction of diethyl sulfide, DBNBS (95), hydrogen peroxide and ferrous sulfate.

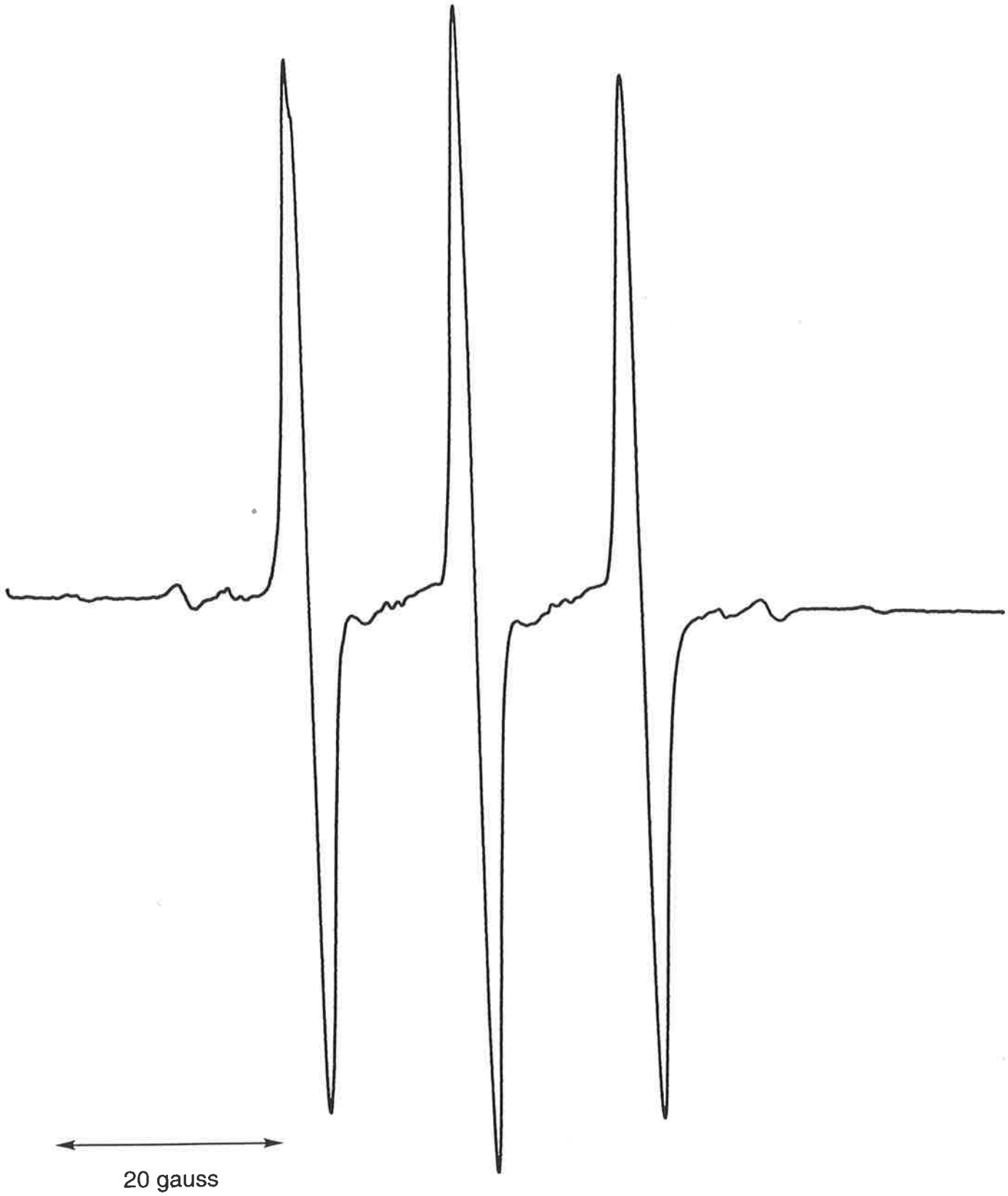
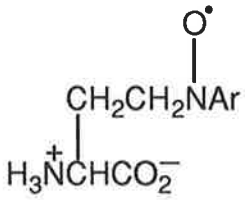


Figure 7: E.s.r. spectrum obtained from reaction of *tert*-butylmethyl sulfide, DBNBS (95), hydrogen peroxide and ferrous sulfate.

Once the generality of the procedure used above had been established, it was applied to amino acid sulfides. The e.s.r. spectrum of the mixture obtained from treatment of a solution of methionine (26) and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate was similar to that obtained from the experiment with ethylmethyl sulfide. It showed a triplet (1:1:1, 15G) of triplets (1:2:1, 13G), attributable to the adduct (99), produced by trapping of the γ-centred radical (50) by DBNBS (95), and a triplet (1:1:1, 15G) of quartets (1:3:3:1, 14G) for the methyl radical adduct (96) (Figure 8). Formation of the radical (50) can be attributed to the mechanism outlined in Scheme 38, except starting with methionine (26) and forming methionine sulfoxide (49) *in situ*. Homolysis of either of the carbon-sulfur bonds during β-scission of the oxygen-centred radical (91) gives rise to both the γ-centred radical (50) and methyl radical. The approximate 4:1 ratio of the signals for the adducts (99) and (96) reflects the greater stability of the primary radical (50). Formation of the radical (50) indicated that the methodology was potentially applicable to generation of carbon-centred side-chain radicals from methionine residues in peptides. No e.s.r. signals were observed when this experiment was repeated in the absence of hydrogen peroxide, verifying that the peroxide is an essential reagent for radical generation from sulfides by this method.



(99)

The e.s.r. spectrum of the mixture obtained from treatment of a solution of methionine sulfoxide (49) and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate was the same as that obtained from the same experiment with methionine (26), confirming that radicals could be generated from a sulfide or a sulfoxide by this method. In addition, a similar spectrum (signal ratio 3:1, Figure 9) was obtained after photolysis of a solution of methionine sulfoxide (49) and

DBNBS (95) in aqueous hydrogen peroxide, indicating that either photolysis or ferrous ion addition could be used to initiate the radical process.

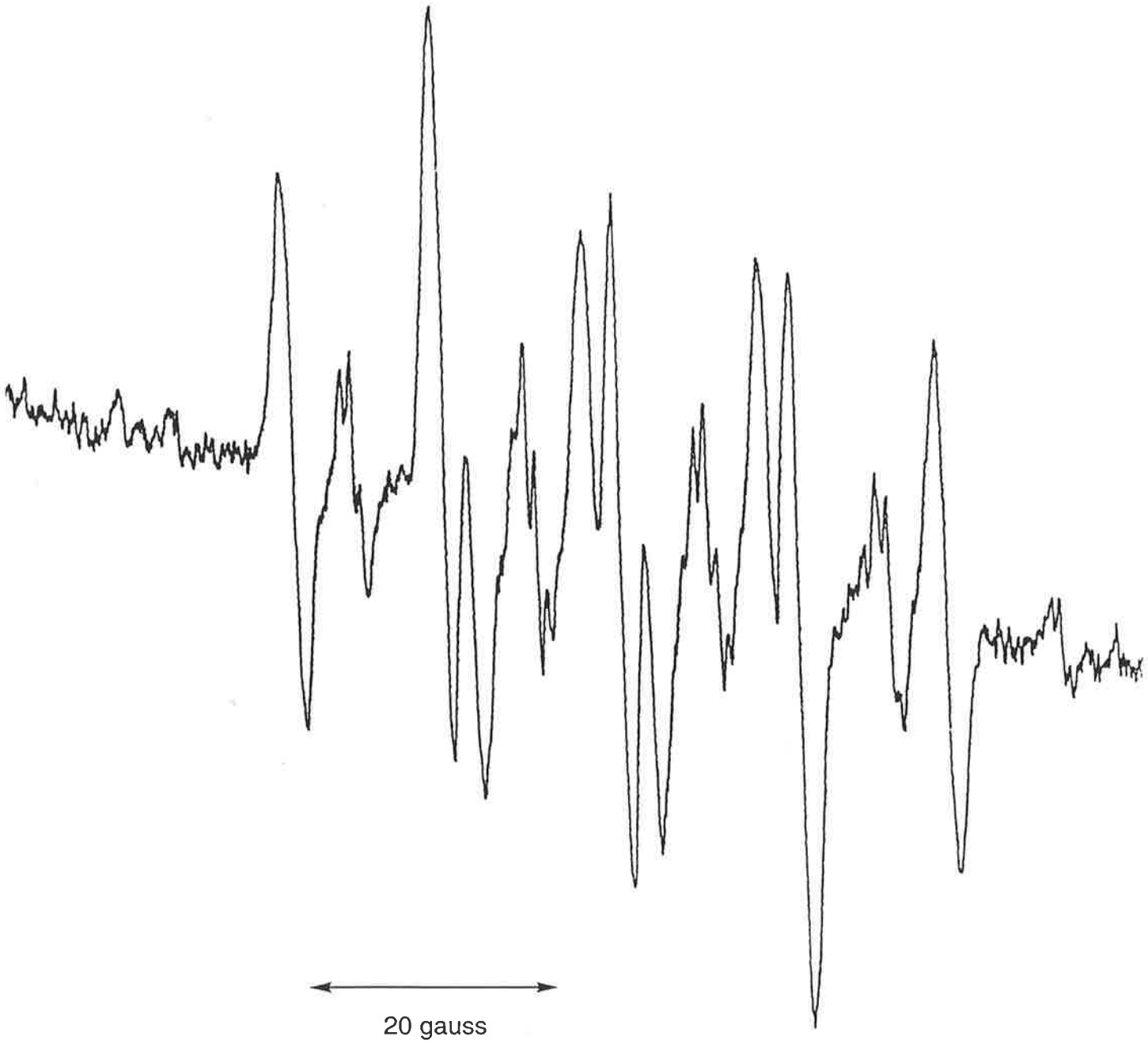


Figure 8: E.s.r. spectrum obtained from reaction of methionine (26), DBNBS (95), hydrogen peroxide and ferrous sulfate.

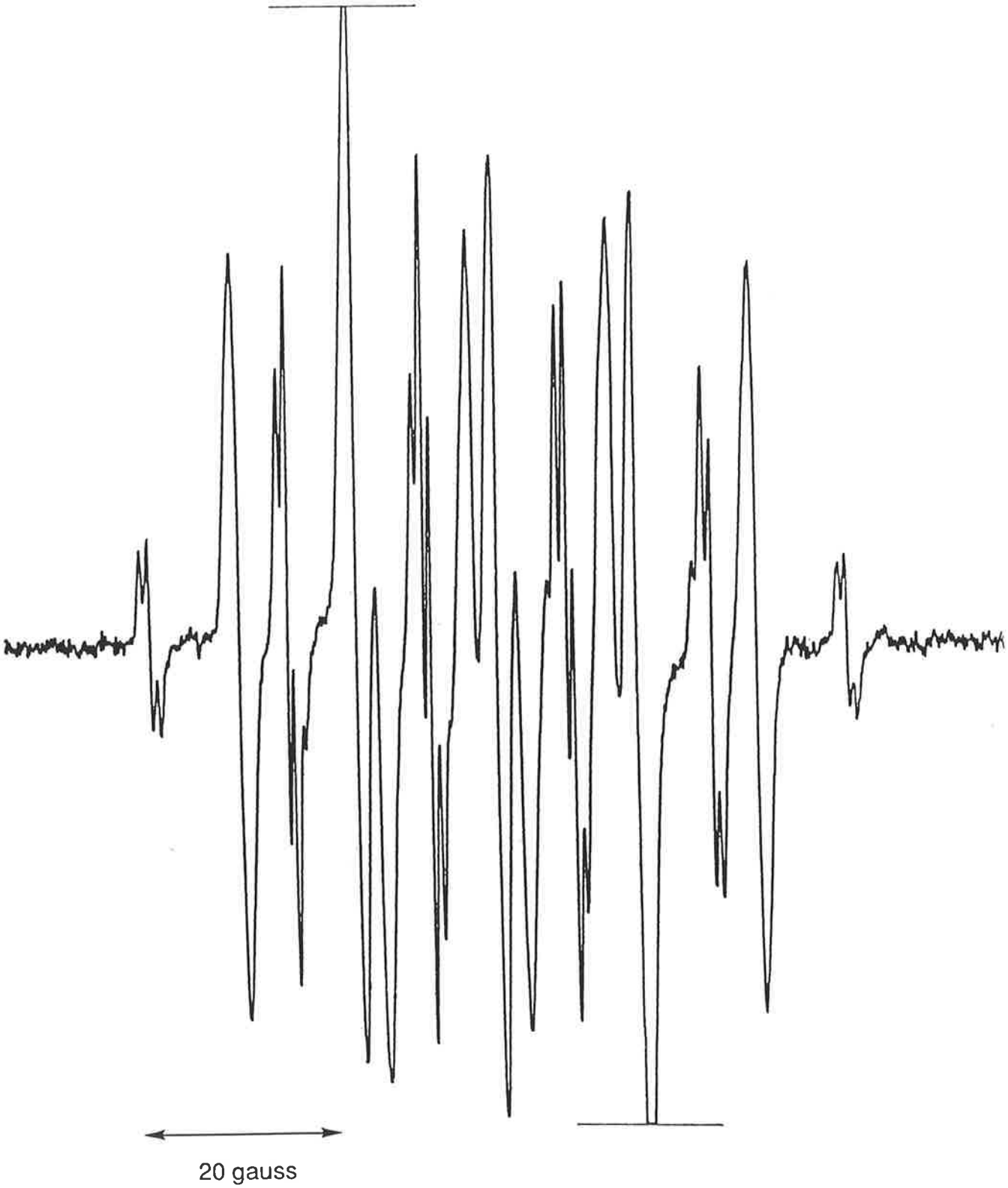
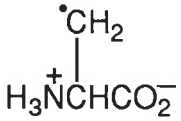
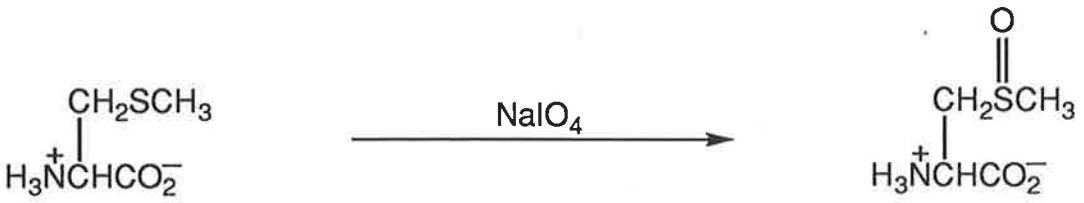


Figure 9: E.s.r. spectrum obtained from photolysis of methionine sulfoxide (49), DBNBS (95), and hydrogen peroxide.

As cysteine (25) is more abundant than methionine (26) in naturally-occurring peptides, application of the methodology used to generate the γ-centred radical (50) from methionine (26) or its sulfoxide (49) to generation of the β-centred amino acid radical (100) from *S*-methylcysteine (28) or its sulfoxide (57) was investigated. The e.s.r. spectrum of the mixture obtained from treatment of a solution of *S*-methylcysteine (28) and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate showed a complex pattern of several peaks, none of which could be assigned.



(100)



(28) (57)

Scheme 41

As the peculiar behaviour of *S*-methylcysteine (28) under the established reaction conditions may have been due to inefficient conversion of *S*-methylcysteine (28) to its sulfoxide (57), *S*-methylcysteine sulfoxide (57) was synthesized by treatment of the sulfide (28) with sodium periodate (Scheme 41). The ^{1}H n.m.r. spectrum of the mixture of two diastereomers of the sulfoxide (57) showed singlets at δ 2.80 and δ 2.81 for the methylsulfoxy protons. The f.a.b. mass spectrum showed a protonated molecular ion at *m/z* 152. No e.s.r. signals were observed, however, on photolysis of a solution of *S*-methylcysteine sulfoxide (57) and DBNBS (95) in aqueous hydrogen peroxide.

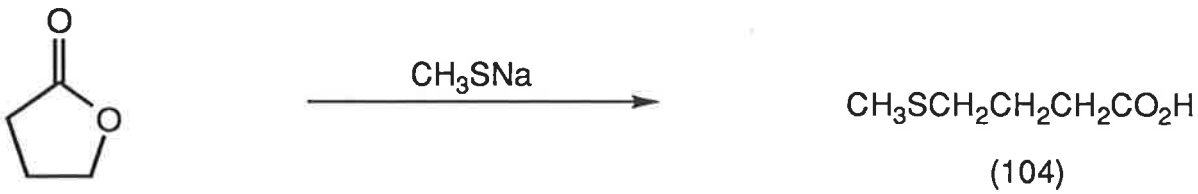
Inexplicably, generation of the β-centred radical (100) from *S*-methylcysteine (28) was inefficient compared with generation of the γ-centred radical (50) from

methionine (26). As the structural difference between the sulfides (28) and (26) is the proximity of the α-substituents to the methylthio moiety, the relationships between these groups and their effect on production of the radicals (100) and (50) was investigated. Generation of radicals from the amines (101) and (102), and the carboxylic acids (103) and (104), the structures of which are derived by decarboxylation or deamination of *S*-methylcysteine (28) or methionine (26), was studied by e.s.r. spectroscopy.

$CH_3SCH_2CH_2NH_2$ (101)

$CH_3SCH_2CH_2CH_2NH_2$ (102)

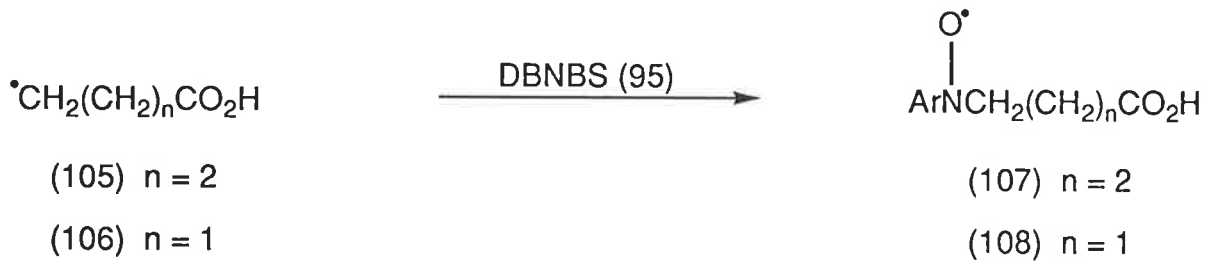
$CH_3SCH_2CH_2CO_2H$ (103)



Scheme 42

4-Methylthiobutanoic acid (104) was synthesized by treatment of butyrolactone with sodium thiomethoxide (Scheme 42), as reported.[94] A solution of the acid (104) and DBNBS (95) in aqueous hydrogen peroxide was treated with ferrous sulfate. The e.s.r. spectrum of the mixture showed a triplet (1:1:1, 15G) of quartets (1:3:3:1, 14G), corresponding to the methyl radical adduct (96), and a triplet (1:1:1, 15G) of triplets (1:2:1, 13G) in a ratio of 1:8. The presence of the signal for the methyl radical adduct (96) indicated that reaction had occurred at the methylthio moiety of the sulfide (104), as expected. The triplet of triplets can thus be attributed to the adduct (107), produced by trapping of the methylene radical (105) by DBNBS (95) (Scheme 43, n = 2). It is possible that the acid (104) decarboxylated, as has been reported for reaction of carboxylic acids with

hydroxyl radical,[84] to give an alternative methylene radical, the spin adduct of which would also give rise to a triplet of triplets in the e.s.r. spectrum. The extent to which this possible side-reaction occurred cannot be determined.



(105) n = 2
(106) n = 1

(107) n = 2
(108) n = 1

Scheme 43

The e.s.r. spectrum of the mixture obtained from treatment of a solution of 3-methylthiopropanoic acid (103) and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate was similar to that described above. It showed a 6:1 ratio of signals attributable to the adduct (108), produced by trapping of the radical (106) by DBNBS (95) (Scheme 43, n = 1), and the methyl radical adduct (96) (Figure 10). A similar e.s.r. spectrum was obtained on repeating the above experiment with the sodium salt of the acid (103), indicating that the deprotonated form of the radical (106) and methyl radical had been generated. These results indicated that the proximity of the methylthio and carboxyl or carboxylate groups of *S*-methyl-cysteine (28) was not hindering generation of the β-centred radical (100) from this amino acid (28).

The e.s.r. spectrum of the mixture obtained from treatment of a solution of 3-methylthiopropylamine (102) and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate showed a triplet (1:1:1, 14G) of doublets (4G), along with unassigned minor signals. The doublet hyperfine splitting indicated that a secondary radical had been trapped. For comparison, this experiment was

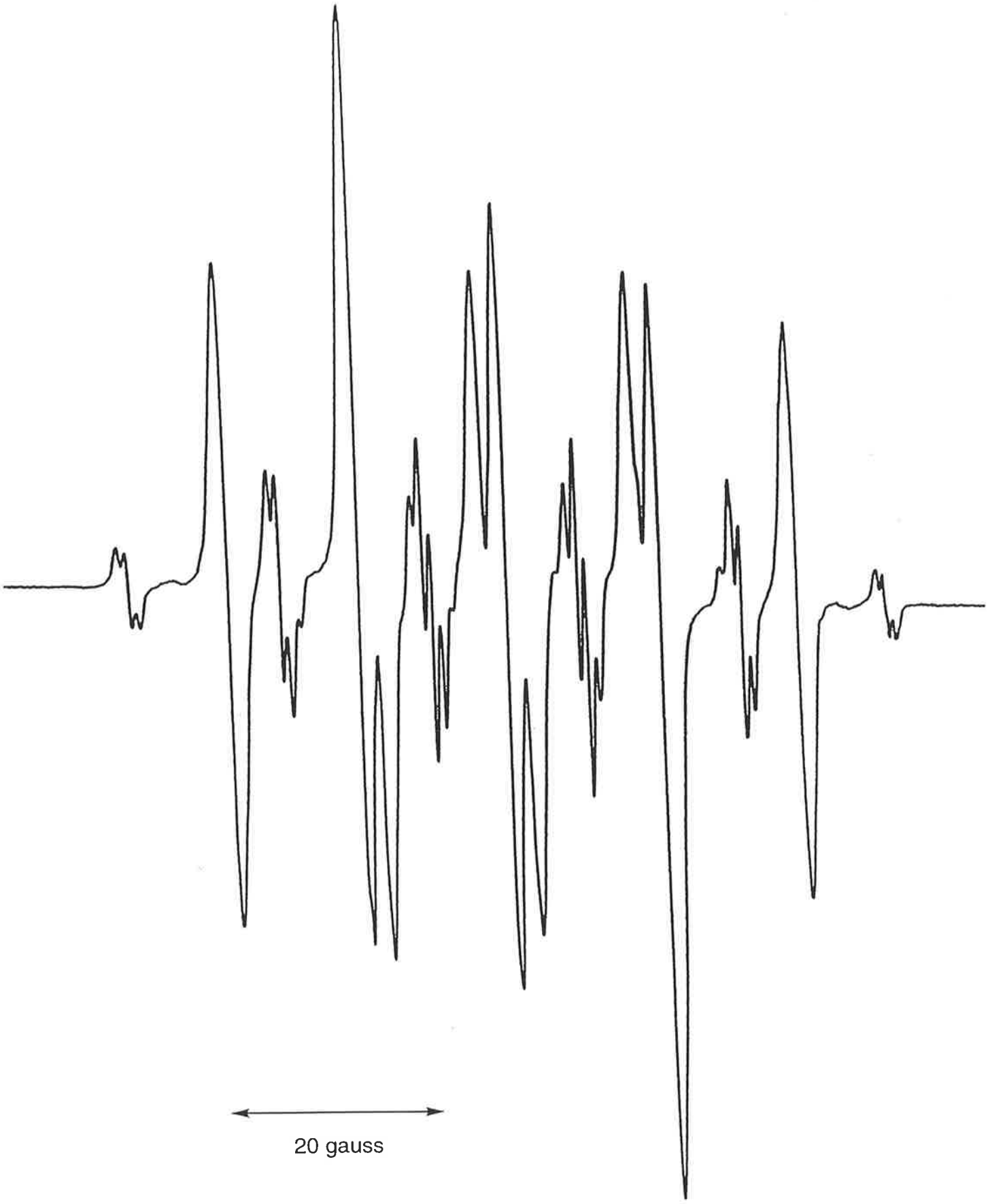
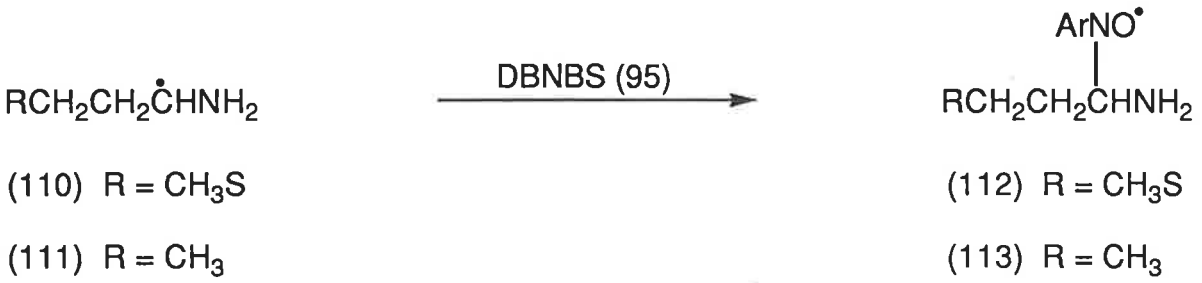


Figure 10: E.s.r. spectrum obtained from reaction of 3-methylthiopropanoic acid (103), DBNBS (95), hydrogen peroxide and ferrous sulfate.

$CH_3CH_2CH_2NH_2$

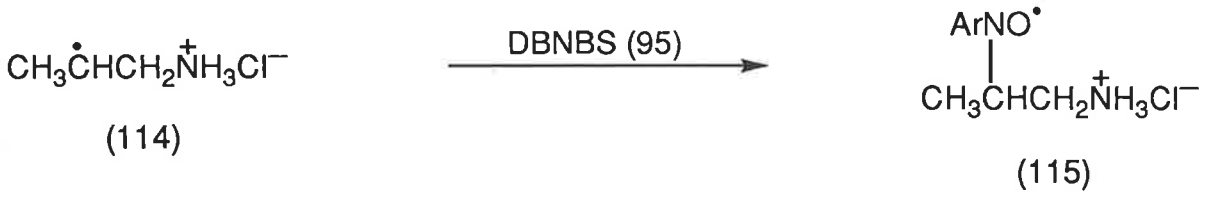
(109)

repeated with propylamine (109) and the resultant e.s.r. spectrum showed a triplet (1:1:1, 14G) of doublets (7G) as the major signal. The signals can be attributed to the adducts (112) and (113), produced by trapping of the radicals (110) and (111), respectively, by DBNBS (95) (Scheme 44). Formation of the radicals (110) and (111), stabilized by resonance through their nitrogens, can be attributed to hydrogen-atom abstraction from the amines (102) and (109), respectively, by hydroxyl radical. For the sulfide (102), hydrogen-atom abstraction must have occurred at a faster rate than radical generation *via* reaction at the methylthio group. The sulfide moieties of the radical (110) and the adduct (112) may have been in the form of their hydrogen peroxide complexes but they are represented as sulfides for simplicity.



Scheme 44

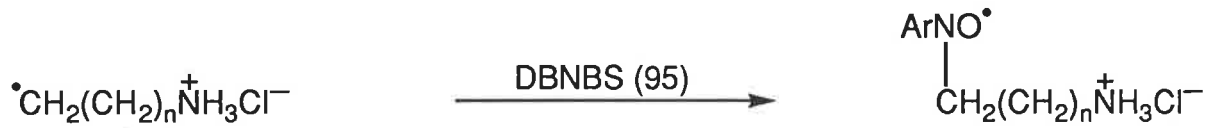
To avoid formation of these stabilized radicals (110) and (111), the nitrogens of the amines (102) and (109) were protonated by treatment with hydrochloric acid, producing the respective hydrochloride salts. The e.s.r. spectrum of the mixture obtained from treatment of a solution of propylamine hydrochloride and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate showed two signals. The major signal was a triplet (1:1:1, 15G) of doublets (11G), attributable to the adduct (115), produced by trapping of the radical (114) by DBNBS (95)



(114) (115)

Scheme 45

(Scheme 45). The minor signal was not well resolved but could be assigned as a triplet (1:1:1, 15G) of triplets (1:2:1, 13G), presumably arising from the adduct (118), produced by trapping of the radical (116) by DBNBS (95) (Scheme 46, n = 2). The approximate 2:1 ratio of the signals for the adducts (115) and (118) reflects the greater stability of the secondary radical (114) over the primary radical (116). Evidently, hydrogen-atom abstraction occurred at sites distant from the destabilizing positively charged nitrogen of propylamine hydrochloride, as expected.



(116) n = 2
(117) n = 1
(118) n = 2
(119) n = 1

Scheme 46

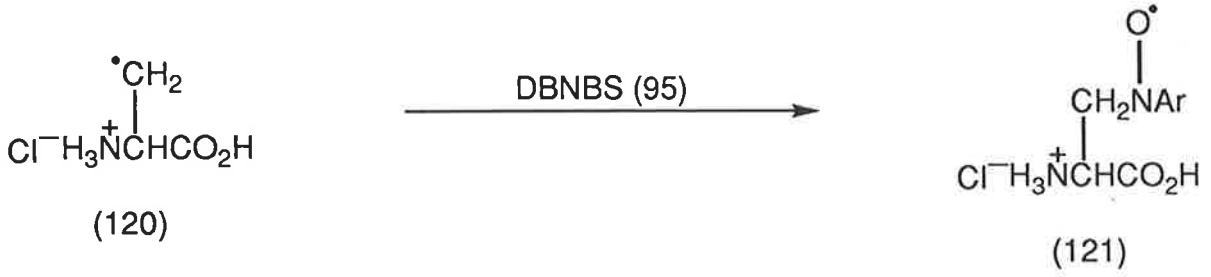
Protonation of the nitrogen of 3-methylthiopropylamine (102) was thus expected to promote reaction at the methylthio moiety. Accordingly, the e.s.r. spectrum of the mixture obtained from treatment of a solution of 3-methylthiopropylamine hydrochloride and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate showed a triplet (1:1:1, 15G) of triplets (1:2:1, 13G), attributable to the adduct (118), and the triplet of quartets for the methyl radical adduct (96). The approximate 5:1 ratio of the signals for the adducts (118) and (96) reflects the greater stability of the primary radical (116) over methyl radical. Evidently, amino group protonation is required to achieve reaction at the

methylthio moiety of the amine (102). As the amine (102) has a structure analogous to methionine (26) without its carboxyl group, this result indicated that the amino group of methionine (26) was protonated under the reaction conditions used to generate a γ-centred radical from this amino acid (26).

The e.s.r. spectrum of the mixture obtained from treatment of a solution of 2-methylthioethylamine (101) and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate showed a complex pattern of several peaks, none of which could be assigned. Some of the peaks decayed with time, indicating that the corresponding radicals were unstable. To determine the effect of protonation on reaction of this amine (101), 2-methylthioethylamine (101) was treated with hydrochloric acid to form its hydrochloride salt. The e.s.r. spectrum of the mixture obtained from treatment of a solution of this salt and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate showed two signals. The major signal was a triplet (1:1:1, 14G) of triplets (1:2:1, 14G) with several overlapping peaks due to the common coupling constant, attributable to the adduct (119), produced by trapping of the radical (117) by DBNBS (95) (Scheme 46, n = 1). The minor signal corresponded to the methyl radical adduct (96). The approximate 4:1 ratio of the signals for the adducts (119) and (96) reflects the greater stability of the primary radical (117) over methyl radical. As with 3-methylthiopropylamine (102), reaction at the methylthio moiety occurred on protonation of the amine.

The results obtained from reactions of the amines (101) and (102) and their hydrochloride salts indicated that reaction at the methylthio group of *S*-methylcysteine (28) did not occur on treatment with Fenton's reagent because the amino group of this amino acid (28) was deprotonated under the reaction conditions. It has been reported[95] that amino acids decarboxylate to give α-centred radicals *via* a reaction involving coordination of hydroxyl radical to deprotonated amino groups. It was therefore probable that *S*-methylcysteine (28) reacted *via* coordination of hydroxyl radical to its amino group.

To protonate the nitrogen, *S*-methylcysteine (28) was converted to its hydrochloride salt by treatment with hydrochloric acid. A solution of this salt and DBNBS (95) in aqueous hydrogen peroxide was treated with ferrous sulfate. The e.s.r. spectrum of the resultant mixture, although less well resolved than signals obtained previously, showed a triplet (1:1:1, 15G) of triplets (1:2:1, 13G) which can be attributed to the adduct (121) produced by trapping of the β-centred radical (120) by DBNBS (95) (Scheme 47). A minor signal corresponded to the methyl radical adduct (96) and the approximate ratio of the major to minor signals was 4:1. Presumably, protonation of the amino group of *S*-methylcysteine (28) abated reactions involving the deprotonated amino group and the β-centred radical (120) and methyl radical were generated *via* the general mechanism outlined in Scheme 16.

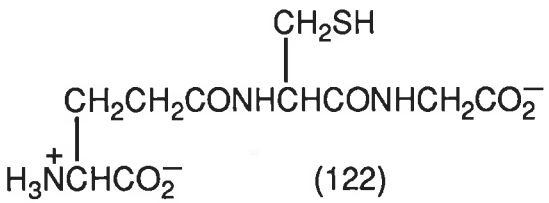


Scheme 47

For comparative purposes, a solution of methionine hydrochloride and DBNBS (95) in aqueous hydrogen peroxide was treated with ferrous sulfate. The e.s.r. spectrum showed a well resolved triplet (1:1:1, 15G) of triplets (1:2:1, 12G), indicating that the protonated form of the γ-centred radical (50) had been trapped, and a minor signal corresponding to the methyl radical adduct (96).

Evidently, carbon-centred side-chain amino acid radicals can be generated by treatment of sulfur containing amino acids with hydrogen peroxide and ferrous sulfate, provided the amino group is protonated. Extension of the methodology to peptides requires the ability to methylate thiol moieties of cysteine residues.

As mentioned in the Introduction, trimethylphosphate (TMP) is a methylating agent potentially selective for cysteine residues in peptides.[43] Reactions of cysteine (25) and glutathione (122), a tripeptide containing cysteine (25), with TMP were investigated with a view to developing conditions for selective methylation of thiols in peptides. Cysteine (25) was treated with TMP as in the reported method.[43] The ^{1}H n.m.r. spectrum of the reaction mixture indicated that quantitative conversion of cysteine (25) to *S*-methylcysteine (28) had occurred, by comparison with spectra of authentic samples of cysteine (25) and the product (28).



(122)

Similar treatment of glutathione (122), however, gave an approximately 1:1 ratio of *S*-methylglutathione (60) and a compound assigned as glutathione disulfide. Formation of the sulfide (60) was confirmed by comparison of t.l.c properties and ^{1}H n.m.r. spectral data of the reaction mixture with those of an authentic sample. The second product was assigned as glutathione disulfide because a doublet of doublets at δ 4.74 (J = 4.5, 9.5 Hz) and two doublets of doublets at δ 2.94 (J = 9.5, 14.5 Hz) and 3.28 (J = 4.5, 14.5 Hz) corresponded to the α- and β-protons, respectively, of a cystine residue. In addition, integrals in the n.m.r. spectrum of the reaction mixture indicated that only a third of the resonances for glycine and glutamic acid residues arose from *S*-methylglutathione (60). The complex f.a.b mass spectrum of the reaction mixture showed a peak at *m/z* 344 corresponding to the molecular ion plus sodium for *S*-methylglutathione (60). Small peaks at *m/z* 612 and 635 corresponded to the molecular ion of glutathione disulfide and this molecular ion plus sodium, respectively. Oxidation was not avoided when the reaction was repeated under nitrogen.

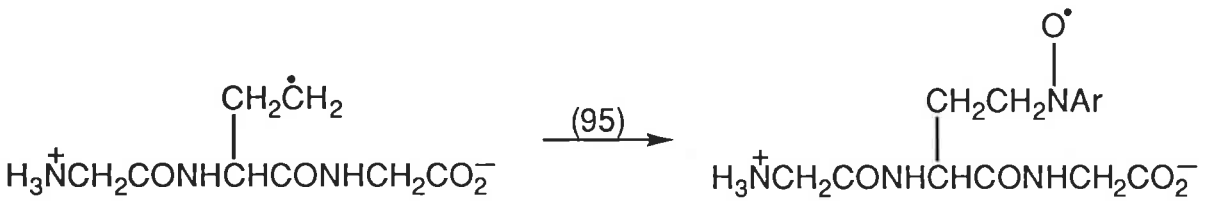
In the reported method for methylation of cysteine (25) with TMP,[43] the reaction solution was maintained at approximately neutral pH by occasional addition of 1.5N sodium hydroxide to quench the acidic byproduct dimethylphosphate. ^{1}H N.m.r. spectral analysis of mixtures obtained from solutions of glutathione (122) in water, acid, and base indicated that the peptide (122) was stable in neutral solution only. Oxidation of glutathione (122) occurred in acidic solution. Treatment of glutathione (122) with TMP in aqueous 0.1M pH 7.0 phosphate buffer, in an attempt to accurately control the pH and thus avoid oxidation, again gave both *S*-methylglutathione (60) and glutathione disulfide.

Treatment of commercial *S*-methylglutathione (60) with sodium periodate gave *S*-methylglutathione sulfoxide. The ^{1}H n.m.r. spectrum of the reaction mixture indicated that the conversion was almost quantitative. ^{1}H N.m.r resonances indicative of the formation of a 1:1 mixture of diastereomers of the sulfoxide were two singlets at δ 2.75 and 2.76 for the methylsulfoxy protons, and multiplets from δ 3.15-3.48 for the β-protons of the *S*-methylcysteine sulfoxide residue. The f.a.b mass spectrum showed peaks at *m/z* 338 and 360 for the protonated molecular ion and the molecular ion plus sodium, respectively, of the product sulfoxide.

In reference to the proposed method for generating radicals from cysteine residues in peptides (Scheme 9), the results of the above reactions showed that methylation of cysteine residues in peptides using TMP is achievable but oxidation is a competing reaction. In addition, sulfides can be converted to sulfoxides in peptides, if necessary, by use of sodium periodate.

Extension of the methodology used to generate β- and γ-centred amino acid radicals, to peptides, was investigated through reactions of small peptides containing *S*-methylcysteine or methionine residues. A solution of glycyl-methionylglycine (58) and DBNBS (95) in aqueous hydrogen peroxide was treated with ferrous sulfate. The e.s.r. spectrum of the mixture showed a triplet (1:1:1,

15G) of triplets (1:2:1, 12G) attributable to the adduct (124), formed by trapping of the γ-centred radical (123) by DBNBS (95) (Scheme 48), and a signal corresponding to the methyl radical adduct (96) (Figure 11), in a 6:1 ratio. The slope in the spectrum shown in Figure 11 was due to poor tuning of the e.s.r. spectrometer which in this case could not be improved. As discussed in the Introduction, selective hydrogen-atom abstraction from glycine residues often occurs in radical reactions of peptides. Work described in Chapter One indicated that *C*-terminal glycyl radicals are most readily formed. The experiment with glycylmethionylglycine (58) thus indicates that reaction of methionine residues in peptides to give γ-carbon-centred radicals can be achieved in preference to formation of α-centred radicals.



(123) (124)

Scheme 48

The e.s.r. spectrum of the mixture obtained from treatment of a solution of the more complex peptide, methionylleucylphenylalanine (59), and DBNBS (95) in aqueous hydrogen peroxide with ferrous sulfate showed two signals. Although the spectrum was not well resolved, a triplet (1:1:1, 15G) of triplets (1:2:1, 12G) could be seen along with a minor signal for the methyl radical adduct (96). These signals indicated that selective reaction of the methionine residue of the peptide (59) had occurred, giving rise to either a γ-centred radical or methyl radical.

A solution of *S*-methylglutathione (60), a tripeptide containing *S*-methylcysteine (28) and a *C*-terminal glycine residue, and DBNBS (95) in aqueous

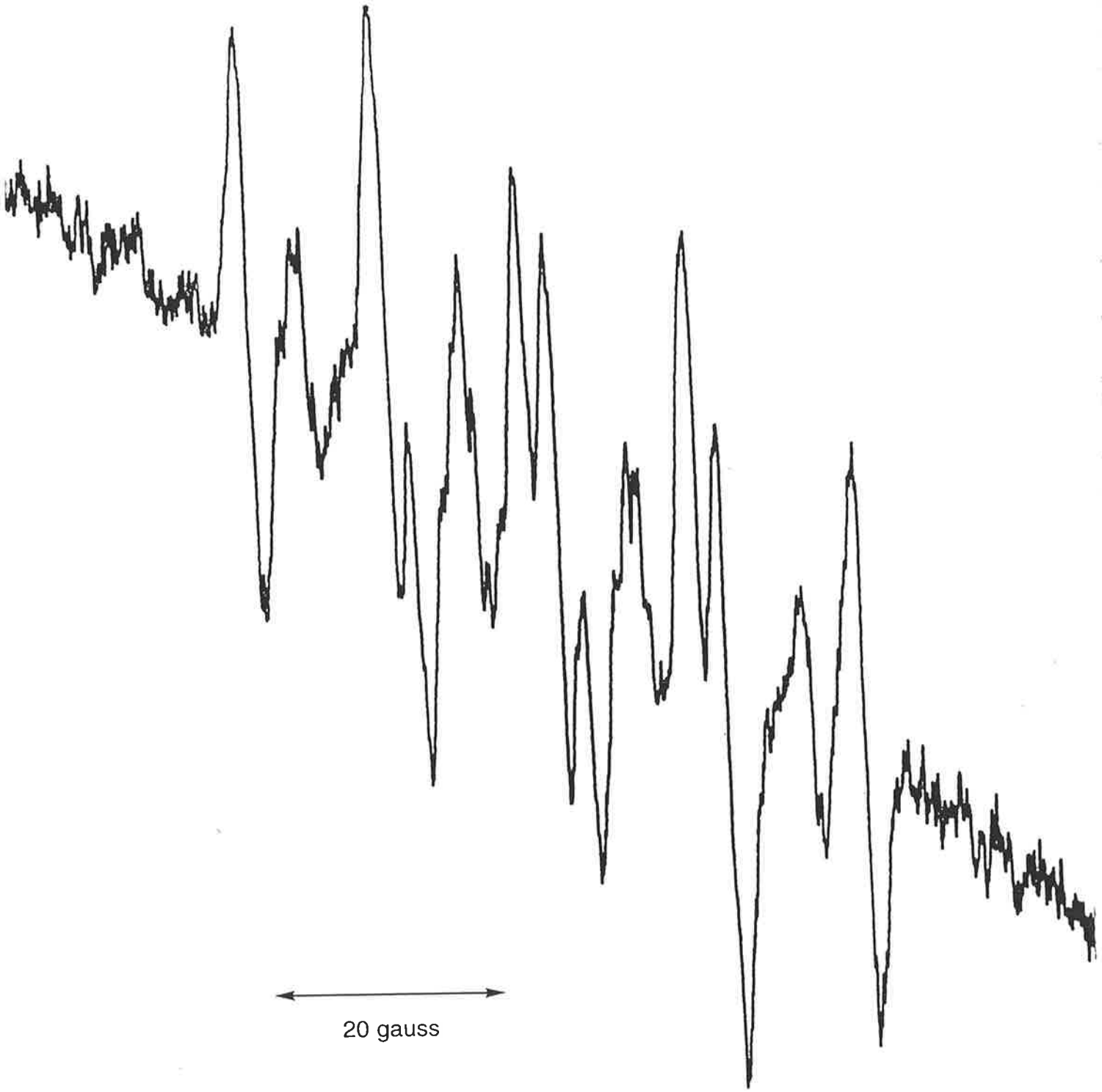


Figure 11: E.s.r. spectrum obtained from reaction of glycylmethionylglycine (58), DBNBS (95), hydrogen peroxide and ferrous sulfate.

hydrogen peroxide was treated with ferrous sulfate. The e.s.r. spectrum of the mixture showed three signals in a ratio of approximately 30:8:1. The major signal was a triplet (1:1:1, 13G) of doublets (17G), attributable to an adduct formed by trapping of a methine radical by DBNBS (95). A triplet (1:1:1, 14G) of triplets (1:2:1, 10G) was attributed to the adduct of a methylene radical and DBNBS (95) and a smaller signal corresponded to the methyl radical adduct (96). Evidently, reaction at the methylthio group of the *S*-methylcysteine residue occured to give a β-centred methylene radical and methyl radical but this was not the major process. The major radical formed in this reaction may have been the α-centred radical formed by decarboxylation of the glutamic acid residue of the peptide (60). Decarboxylation can occur *via* coordination of hydroxyl radical to the deprotonated amino group of this residue, as has been reported for deprotonated amino acids.[95]

To ensure that the amino group of *S*-methylglutathione (60) was protonated in this reaction, a solution of *S*-methylglutathione hydrochloride and DBNBS (95) in aqueous hydrogen peroxide was treated with ferrous sulfate. The e.s.r. spectrum showed a triplet (1:1:1, 14G) of triplets (1:2:1, 10G) attributable to an adduct of a methylene radical and DBNBS (95). The minor signal corresponded to the methyl radical adduct (96). Presumably, a β-centred radical and methyl radical were produced by reaction at the methylthio moiety of the *S*-methylcysteine residue. This result indicated that side-chain carbon-centred radicals can be generated in peptides through reactions of *S*-methylcysteine residues.

As discussed previously, selective hydrogen-atom abstraction from glycine residues often occurs in radical reactions of peptides and *C*-terminal glycine residues are most reactive. The result of the experiment with *S*-methylglutathione hydrochloride thus indicates that β-carbon-centred radicals can be generated in peptides in preference to α-centred radicals. In addition,

competing radical reactions involving deprotonated amino groups were shown to be easily avoided by protonation.

The work discussed in this Chapter established that carbon-sulfur bond homolysis to give alkyl radicals can be achieved by reaction of sulfides or sulfoxides with hydrogen peroxide and either ferrous ion addition or photolysis. Application of this methodology to sulfur-containing amino acids and tripeptides containing them indicated that side-chain carbon-centred radicals in peptides can be selectively generated *via* the mechanism outlined in the Introduction (Scheme 9). A proviso of the method is that amino groups must be protonated otherwise alternative reactions may occur.

Conclusion

The photolytic reaction of triglycine (24) with di-*tert*-butyl peroxide gave rise to crosslinked peptides and tripeptides containing glycine and alanine residues. Product studies on this reaction demonstrated the decreasing relative ease of formation of *C*-terminal, non-terminal, and *N*-terminal α-centred radicals in peptides. The reaction indicated that selective hydrogen-atom abstraction from glycine residues followed by carbon-carbon bond forming reactions of the resultant radicals (1) could be useful for modification of small or uncomplicated peptides. For larger, more complicated peptides it was evident that radical reactions of other functionality, such as reactive disulfide moieties, could interfere with generation of α-centred radicals.

These reactive disulfides, common in peptides, may be useful in producing side-chain carbon-centred radicals. A series of reactions of derivatized cystine, cysteine, *S*-methylcysteine, and *S*-methylcysteine sulfoxide indicated that manipulation of cystine or cysteine residues in peptides to generate β-centred radicals is achievable. Product studies on photolytic reactions of an *S*-methylcysteine sulfoxide derivative with di-*tert*-butyl peroxide provided evidence for generation of the corresponding β-centred radical. This reaction of the *S*-methylcysteine sulfoxide derivative is potentially useful in the synthesis of β-substituted amino acid derivatives. Substitution of alkoxy radicals on sulfur is potentially a general method for generation of carbon-centred radicals from sulfoxides.

A general procedure for alkyl radical generation from sulfides or sulfoxides has been established. Carbon-sulfur bond homolysis can be achieved by reaction of sulfides or sulfoxides with hydrogen peroxide, initiated by either ferrous ions or photolysis. A proviso of the method is that any amino groups must be protonated otherwise reactions other than carbon-sulfur bond homolysis may occur. The methodology was successfully applied to generation of β- and

γ-centred radicals from sulfur-containing amino acids and tripeptides containing them. The procedure is thus potentially useful for selective generation of side-chain carbon-centred radicals in larger peptides and proteins. Extensions of this work would involve applying the methodology to larger peptides and developing techniques for utilizing the radicals generated to selectively modify peptides.

In establishing the above method, sodium 3,5-dibromo-4-nitrosobenzene sulfonate (95) was shown to be an effective trap for radicals in aqueous solvent.

Experimental

General

Microanalyses were performed by Chemical and Micro Analytical Services Pty. Ltd., Victoria, Australia. Melting points were measured using a Kofler hot-stage melting point apparatus under a Reichert microscope and are uncorrected. Infrared spectra were recorded on a Hitachi 270-30 infrared spectrophotometer and data processor, as nujol mulls between NaCl plates. Electron impact (e.i.) mass spectra were recorded, and accurate mass measurement of ions was determined, on an AEI MS-3010 mass spectrometer. Fast atom bombardment (f.a.b.) mass spectra were recorded in positive ion mode on a Vacuum Generators ZAB 2HF spectrometer.

^{1}H N.m.r. spectra were recorded on a Bruker ACP-300 spectrometer, unless otherwise stated when a Varian T-60 spectrometer was used. Unless otherwise stated, n.m.r. spectra were recorded as dilute solutions in deuterochloroform, using tetramethylsilane as an internal reference. In cases where deuterium oxide, deuterium oxide/d_4-acetic acid, or d_4-acetic acid were used as n.m.r. solvents, sodium 3-(trimethylsilyl)propane-sulfonate (TSP) was used as an external reference. ^{13}C N.m.r. spectra were recorded on a Bruker ACP-300 spectrometer as dilute solutions in deuterochloroform, using *tert*-butanol as an external reference.

Unless otherwise stated, chromatography was performed on either a Chromatotron 7924T (Harrison Research, Palo Alto/TC Research, Norwich) or a squat column,[96] using Merck silica gel 60 PF_{254} containing gypsum. The eluting solvent system was a gradient of light petroleum/ethyl acetate, unless otherwise stated. Analytical t.l.c. was performed using Merck silica gel 60 F_{254} on aluminium backing plates. Magnesium sulfate was used for drying of organic solutions unless otherwise stated.

Solvents were purified and dried by standard procedures. Light petroleum refers to the fraction with boiling point 66-68°C.

Aqueous hydrogen peroxide refers to a 34% w/w solution of hydrogen peroxide in water purchased from Ajax Chemicals.

Experimental for Chapter One

Triglycine (24), glycylglycylalanine (68), alanylglycylglycine (69), cystine (27), and *S*-methylcysteine (28) were purchased from Sigma Chemical Co.. Glycylalanylglycine (70) was purchased from Bachem Feinchemikalien AG. Heptafluorobutyric acid (67) was purchased from Aldrich Chemical Co.. Tetrabutylammonium phosphate (66) was purchased as a standard solution in water from Millipore.

1,1,3,3-Tetramethylisoindolin-2-yloxyl (TMIO) (19)[97] was obtained as a gift.[98]

N-*Benzoylglycine Methyl Ester (13a)*[99] was prepared from glycine using standard methods.

Photolytic reactions were conducted with reaction solutions in quartz tubes in a Rayonet photochemical reactor containing 12 RPR 3500 or 3000 lamps.

N-*Benzoyl-α-(1,1,3,3-tetramethylisoindolin-2-yloxy)-glycine Methyl Ester (61)*

A solution of *N*-benzoylglycine methyl ester (13a) (50 mg, 0.26 mmol), TMIO (19) (50 mg, 0.26 mmol) and di-*tert*-butyl peroxide (0.25 ml, 1.36 mmol) in benzene (15 ml) under nitrogen was irradiated with ultraviolet light (λ = 350 nm) in a Rayonet photochemical reactor for 12 hours. The solvent was removed under reduced pressure and the residue was chromatographed on silica. The starting material (13a) was recovered as a colourless solid (33 mg, 66%). The adduct (61) was obtained as a colourless solid (14 mg, 14%; 42% based on recovered starting material (13a)). ^{1}H n.m.r. δ 1.34, s, CH_3; 1.38, s, CH_3; 1.52, br s, 2xCH_3; 3.84, s, OCH_3; 6.22, d, J 9.5 Hz, CH; 7.05-7.08, m, 2H, ArH; 7.19-7.23, m, 3H, ArH+NH; 7.42-7.53, m, 3H, Ph; 7.83-7.86, m, 2H, Ph. Mass spectrum (f.a.b.) *m/z* 383 ($(M+H)^+$, 50), 192 (100), 190 (36), 176 (89), 174 (44), 105 (94). This compound (61) was insufficiently stable for complete characterization.

*Photolysis of the Glycine Derivative (13a) and 1,1,3,3-Tetramethylisoindolin-2-yloxyl (19) with Di-*tert*-butyl Peroxide in* tert*-Butanol*

A solution of the glycine derivative (13a) (100 mg, 0.52 mmol), TMIO (19) (110 mg, 0.58 mmol), and di-*tert*-butyl peroxide (0.5 ml, 2.64 mmol) in *tert*-butanol (10 ml) under nitrogen was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 18 hours. The solvent was removed under reduced pressure and the residue was chromatographed on silica. The starting glycine derivative (13a) was recovered (44 mg, 44%).

2-Methoxy-1,1,3,3-tetramethylisoindoline (62) was isolated as a colourless oil (20 mg, 17%). ^{1}H n.m.r. δ 1.44, br s, 4xCH_3; 3.78, s, OCH_3; 7.08-7.11, m, 2H, ArH; 7.21-7.24, m, 2H, ArH, consistent with that reported.[38]

2-(2-Hydroxy-2-methylpropoxy)-1,1,3,3-tetramethylisoindoline (64) was isolated as a colourless oil (23 mg, 15%), (Found: C, 72.9; H, 9.6; N, 5.3. $C_{16}H_{25}NO_2$ requires C, 73.0; H, 9.6; N, 5.3%). υ_{max} 3384, 1410, 1376, 1360, 1318, 1164, 1060, 1002, 750 cm^{-1}. ^{1}H n.m.r. δ 1.31, s, 2xCH_3; 1.46, br s, 4xCH_3; 2.50, br, OH; 3.83, s, CH_2; 7.09-7.12, m, 2H, ArH; 7.23-7.26, m, 2H, ArH. ^{13}C n.m.r. δ 25.22, 26.48, 30.12, 67.50, 70.67, 84.89, 121.39, 127.25, 144.88. Mass spectrum (e.i.) *m/z* 263 (M^+, 4), 248 (54), 190 (13), 177 (13), 176 (100), 160 (23), 158 (18), 145 (15), 144 (15), 59 (10).

2-(2-Hydroxy-2-methylpropoxy)-1,1,3,3-tetramethylisoindoline (64)

A solution of TMIO (19) (100 mg, 0.53 mmol) and di-*tert*-butyl peroxide (0.5 ml, 2.64 mmol) in *tert*-butanol (15 ml) under nitrogen was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 18 hours. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate, washed with water, dried and concentrated under reduced pressure. The residue was chromatographed on silica to give the colourless oils 2-(2-hydroxy-2-methylpropoxy)-1,1,3,3-tetramethylisoindoline (64) (55 mg, 40%), and 2-methoxy-

1,1,3,3-tetramethylisoindoline (62) (24 mg, 22%), each with ^{1}H n.m.r. spectral data as described above.

Photolysis of the Glycine Derivative (13a) and 1,1,3,3-Tetramethylisoindolin-2-yloxyl (19) in Acetone

A solution of the glycine derivative (13a) (100 mg, 0.53 mmol) and TMIO (19) (107 mg, 0.56 mmol) in acetone (10 ml) under nitrogen was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 14 days. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate, washed with water, dried, and concentrated under reduced pressure. The residue was chromatographed on silica and the starting materials (19) (70 mg, 65%) and (13a) (69 mg, 69%) were recovered. Another fraction, which was not further purified, contained 2-(2-oxopropoxy)-1,1,3,3-tetramethylisoindoline (65) (Found: *m/z* 247.1579. $C_{15}H_{21}NO_2$ requires 247.1572). ^{1}H n.m.r. δ 1.46, br s, 4xCH_3; 2.27, s, $COCH_3$; 4.57, s, CH_2; 7.10-7.14, m, 2H, ArH; 7.23-7.27, m, 2H, ArH. Mass spectrum (e.i.) *m/z* 247 (M^+, 5), 232 (100), 204 (8), 190 (25), 176 (35), 160 (27), 158 (58), 145 (31), 115 (18), 91 (19), 43 (74). This ^{1}H n.m.r. spectral data is consistent with that reported for the compound (65).[37] The ^{1}H n.m.r. spectrum of the crude reaction mixture indicated that the ketone (65) constituted approximately 15% of the product mixture.

Thermolysis of the Adduct (61)

N-Benzoyl-α-(1,1,3,3-tetramethylisoindolin-2-yloxy)-glycine methyl ester (61) (2 mg) was dissolved in toluene (0.5 ml) and heated in an oil bath (130°C) for 18 hours. T.l.c. analysis of the reaction mixture indicated that the adduct (61) decomposed to form several compounds. The ^{1}H n.m.r. spectrum showed five

peaks from δ 3.5-4.0 corresponding to methyl ester singlets but structures of the decomposition products could not be elucidated.

Photolysis of the Adduct (61)

N-Benzoyl-α-(1,1,3,3-tetramethylisoindolin-2-yloxy)-glycine methyl ester (61) (1 mg) was dissolved in toluene (1 ml) and irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 5 days. T.l.c. and ^{1}H n.m.r. analysis of the reaction mixture indicated that the starting material (61) made up the majority of the reaction mixture.

Reactions of Triglycine (24)

In the remaining experimental work for Chapter One, all mass spectra were obtained using positive ion fast atom bombardment with a glycerol matrix and trifluoroacetic acid. Deuterium oxide was the n.m.r. solvent. TSP was used as an external reference unless otherwise stated, in which case the residual HOD peak was used as an internal reference and was set at δ 4.80 before being removed from spectra by presaturation.

Triglycine (24) was recrystallized from water/methanol and its purity was established using t.l.c. (eluant: 2-butanone/ methanol/ water, 7:3:3) and ^{1}H n.m.r. spectroscopy.

General Procedure for Acetylation of Peptides and Reaction Mixtures

A solution of the peptide or mixture of peptides (2-5 mg) in acetic anhydride/acetic acid (2:1) was stirred at room temperature for 1.5 hours. The solution was diluted with water and concentrated under reduced pressure.

N-Acetyltriglycine was recrystallized from water/ethanol as a colourless solid, single peak by h.p.l.c.. ^{1}H n.m.r. δ 2.06, s, CH_3; 3.96, s, CH_2; 3.99, s, 4H, CH_2. Mass spectrum *m/z* 232 $(M+H)^+$.

N-Acetylglycylglycylalanine, synthesized from commercial glycylglycylalanine (68), was a single peak by h.p.l.c.. ^{1}H n.m.r. δ (HOD reference) 1.36, d, J 7.5 Hz, CH_3; 2.00, s, $COCH_3$; 3.88, s, CH_2; 3.90, s, CH_2; 4.31, q, J 7.5 Hz, CH. Mass spectrum (MIKES on 246 $(M+H)^+$) *m/z* 246, 228, 191, 157, 147, 100, 90.

H.p.l.c. Assay for Triglycine (24)

H.p.l.c. was carried out using either a Waters 501 h.p.l.c. pump connected to a Lambda-Max Model 481 LC spectrophotometer or an ICI LC1110 h.p.l.c. pump connected to a LC 1200 UV/VIS detector. Detectors were connected to an ICI

DP700 data station. A Waters RCM 8 x 10 was used to house a Waters Nova-Pak® C_{18} Radial-Pak cartridge (8 x 100 mm) h.p.l.c. column. The eluting solvent was an aqueous solution of either TFA, HFBA (67), or TBAP (66), with a flow rate of 1.0-2.0 ml/minute. The detection wavelength ranged from λ = 200-220 nm.

Retention Times of Triglycine (24) and N-Acetyltriglycine

Retention was dependent on solvent flow rate. Values are given for a flow rate of 1.5 ml/min.

0.05% TFA: R_t (triglycine (24)) ~ 1.5-2 min. R_t (*N*-acetyltriglycine) ~ 3.5 min.

0.1% HFBA: R_t (triglycine (24)) ~ 5 min. R_t (*N*-acetyltriglycine) ~3.5 min.

5mM TBAP: R_t (*N*-acetyltriglycine) ~ 4.5 min.

*Photolytic Reactions of Triglycine (24) with Di-*tert*-butyl Peroxide (λ = 300 nm)*

1) A solution of triglycine (24) (300 mg, 1.58 mmol) and di-*tert*-butyl peroxide (2.9 ml, 15.8 mmol) in water (25 ml) and *tert*-butanol (30 ml) was sonicated for 15 minutes and, under nitrogen, irradiated with ultraviolet light (300 nm) in a Rayonet photochemical reactor for 1 hour and 45 minutes. A portion of the reaction solution (~5 ml) was photolysed for a further 75 minutes. Reaction solutions were concentrated under reduced pressure to give viscous yellow residues. Both portions were analysed by h.p.l.c. (aqueous 0.1% HFBA (67)) which showed a major peak, which coeluted with an authentic sample of triglycine (24) (R_t ~ 5 min.), and 5 minor peaks of approximately equal size. From this point on, work was conducted using the 1 hour and 45 minute reaction mixture, the h.p.l.c. trace of which is shown in Figure 1.

Glycylglycylalanine (68), glycylalanylglycine (70), and alanylglycylglycine (69) were each coinjected with the reaction mixture onto the h.p.l.c. column. Of these, glycylglycylalanine (68) (R_t ~ 10.5 min.) and glycylalanylglycine (70) (R_t ~ 8.5 min.) coeluted with peaks in the reaction mixture. A small amount of the reaction mixture was acetylated using the procedure described on page 101. In either aqueous 0.1% HFBA or 5mM aqueous TBAP (66), *N*-acetylglycylglycylalanine and *N*-acetylglycylalanylglycine coeluted with peaks in the acetylated reaction mixture. (*N*-Acetylglycylalanylglycine, single peak by h.p.l.c., was synthesized from commercial glycylalanylglycine (70) using the procedure described above.)

The ^{1}H n.m.r. spectrum of the reaction mixture showed three singlets for triglycine (24) as its major peaks. Minor resonances were at δ 1.0-2.5 and δ 3.5-5.0. Glycylglycylalanine (68) was added to a sample of the reaction mixture and the doublet at δ 1.37, J 7.5 Hz, increased in size relative to the other resonances. Addition of glycylalanylglycine (70) to a sample of the reaction mixture caused a relative increase in the size of the doublet at δ 1.45, J 7.5 Hz. In the n.m.r. spectrum of the reaction mixture, the relative peak heights of the doublets at δ 1.37 and 1.45 indicated that the approximate ratio of the peptides (68) and (70) was 5:1. Addition of alanylglycylglycine (69) to a sample of the reaction mixture gave rise to a doublet at δ 1.63, J 7.5 Hz, which was not present in the spectrum of the reaction mixture.

A small amount of the 1 hour, 45 minute reaction mixture was chromatographed by numerous injections of small quantities onto the analytical size h.p.l.c. column (0.1% aqueous HFBA (67)). The fractions collected were concentrated under reduced pressure and the products were repurified several times to afford the compounds described below. For the dimers (71A,B) and (72A,B) the existence and nature of peaks for α-protons which were near the residual HOD peak in ^{1}H n.m.r. spectra was confirmed by obtaining spectra in d_4-acetic acid/deuterium oxide (1:1) or d_4-acetic acid using TSP as an external reference. In these cases, resonances for the α-proton in the alternative solvent are given.

H.p.l.c. retention times (R_t) of products are given for a solvent flow rate of 1.5 ml/min..

The major component of the reaction mixture was triglycine (24), R_t ~ 5 min.. ^1H n.m.r. δ 3.97, s, CH_2; 4.09, s, CH_2; 4.13, s, CH_2, comparable with that of an authentic sample. Mass spectrum *m/z* 190 ($(M+H)^+$, 96), 146 (23), 133 (58), 115 (100), 87 (43), 76 (39), 57 (56). MIKES on 190: *m/z* 172, 146, 133, 115, 76.

The relative yields of the following products, shown in Table 1, were determined from the h.p.l.c. trace shown in Figure 1 with the yield of glycylglygylalanine (68) set as unity.

R_t ~ 8.5 min.: A mixture of the C-Terminal Symmetric Dimer (71A) of Triglycine (24) and Glycylalanylglycine (70).
For the product (71A): Relative yield: 0.4. ^1H n.m.r. δ 3.95, s, 2xCH_2; 4.06, d, J 17.0 Hz, 2xCH and 4.10, d, J 17.0 Hz, 2xCH (AB quartet); 5.16, s, 2xCH. δ (CD_3CO_2D/D_2O) 5.19, s, 2xCH. Mass spectrum (MIKES on 377 $(M+H)^+$) *m/z* 377, 359, 320, 313, 285, 263, 245.
For the product (70): Relative yield: 0.2. ^1H n.m.r. δ 1.45, d, J 7.5 Hz, CH_3; 3.89, s, CH_2; 4.04, s, CH_2; 4.45, q, J 7.5 Hz, CH, consistent with that of an authentic sample of glycylalanylglycine (70). The peak at R_t ~ 8.5 min. in the reaction mixture coeluted from the h.p.l.c. column with an authentic sample of glycylalanylglycine (70).

C-Terminal Symmetric Dimer (71B) of Triglycine (24): R_t ~ 9 min.. Relative yield: 0.5. ^1H n.m.r. δ 3.95, s, 2xCH_2; 4.08, s, 2xCH_2; 4.70, s, 2xCH. δ (CD_3CO_2D/D_2O) 5.09, s, 2xCH. Mass spectrum (MIKES on 377 $(M+H)^+$) *m/z* 377, 359, 313, 285, 263.

Glycylglycylalanine (68): R_t ~ 10.5 min.. Relative yield: 1.0. ^1H n.m.r. δ 1.44, d, J 7.5 Hz, CH_3; 3.93, s, CH_2; 4.06, s, CH_2; 4.39, q, J 7.5 Hz, CH, consistent with that of an authentic sample of glycylglycylalanine (68). Mass spectrum (MIKES on 204

$(M+H)^+$ *m/z* 204, 186, 160, 147, 115, 90. The peak at R_t ~ 10.5 min. in the reaction mixture coeluted from the h.p.l.c. column with an authentic sample of glycylglycylalanine (68).

Non-symmetric Dimer (72A) of Triglycine (24): R_t ~ 12 min.. Relative yield: 0.5. ^1H n.m.r. δ 3.75, d, J 17.0 Hz, 1H, CH_2 and 3.85, d, J 17.0 Hz, 1H, CH_2 (AB quartet); 3.95, s, CH_2; 3.97, s, CH_2; 4.08, s, CH_2; 4.83, d, J 4.5 Hz, CH; 5.08, d, J 4.5 Hz, CH. δ (CD_3CO_2D/D_2O) 5.03, d, J 4.5 Hz, CH; 5.18, d, J 4.5 Hz, CH. Mass spectrum (MIKES on 377 $(M+H)^+$) *m/z* 377, 359, 313, 303, 285, 263, 245.

Non-symmetric Dimer (72B) of Triglycine (24): R_t ~ 13.5 min.. Relative yield: 0.7. ^1H n.m.r. δ 3.84, d, J 17.5 Hz, 1H, CH_2 and 3.94, d, J 17.5 Hz, 1H, CH_2 (AB quartet); 3.88, d, J 16.0 Hz, 1H, CH_2 and 3.95, d, J 16.0 Hz, 1H, CH_2 (AB quartet); 3.97, s, CH_2; 4.07, d, J 17.5 Hz, 1H, CH_2 and 4.11, d, J 17.5 Hz, 1H, CH_2 (AB quartet); 4.82, d, J 4.5 Hz, CH; 5.19, d, J 4.5 Hz, CH. δ (CD_3CO_2D) 5.18, br, CH; 5.38, br, CH (poor resolution). Mass spectrum (MIKES on 377 $(M+H)^+$) *m/z* 377, 359, 313, 285, 263, 245.

2) A solution of triglycine (24) (30 mg, 0.16 mmol) and di-*tert*-butyl peroxide (0.29 ml, 1.6 mmol) in water (2 ml) and *tert*-butanol (3 ml) was sonicated (15 mins) and irradiated with ultraviolet light (300 nm) in a Rayonet photochemical reactor under nitrogen for 2 days with samples removed at 6, 9, 12, 23, and 30 hours, and di-*tert*-butyl peroxide (0.15 ml, 0.8 mmol) added after 23 hours. The samples were concentrated under reduced pressure. H.p.l.c. analysis (aqueous 0.1% HFBA (67)) of the 6, 9, and 12 hour samples showed that the triglycine (24) had reacted faster than in reactions described below (using wavelength 350 nm), because the samples contained far less triglycine (24) than samples described below. The sizes of the product peaks in the h.p.l.c. traces and the complexity of the reaction mixture increased with time. There was no peak for triglycine (24) in the 23 hour sample and the 30 hour and 2 day samples were very complex.

*Photolytic Reactions of Triglycine (24) with Di-*tert*-butyl Peroxide (λ = 350 nm)*

General: A solution of triglycine (24) (100 mg, 0.53 mmol) and di-*tert*-butyl peroxide (0.5 ml, 2.72 mmol) in water (8 ml) and *tert*-butanol (12 ml) was sonicated for 15 minutes and, under nitrogen, irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor. Additional di-*tert*-butyl peroxide (0.5 ml, 2.72 mmol) was added to the reaction mixture during irradiation. The reaction mixture was concentrated under reduced pressure and 1,2-dichloroethane (10 ml) was added. The resultant suspension was stirred at room temperature for 1 hour and filtered to give an orange solid which was dissolved in water. The solution was filtered and the filtrate was concentrated under reduced pressure. The reaction mixtures and products were analysed using h.p.l.c., ^{1}H n.m.r. spectroscopy, and mass spectrometry.

1) Samples were removed periodically during a 24 hour reaction, during which additional di-*tert*-butyl peroxide was added after 8 hours. The ^{1}H n.m.r. spectra of all samples showed three singlets corresponding to triglycine (24) as the major peaks. Minor peaks in the regions δ 1.2-1.5 and δ 3.6-4.2 increased in size with reaction time. The mass spectrum of an 8 hour sample was complex but showed a peak at *m/z* 377 which corresponds to the protonated molecular ion for a dimer of triglycine (24). Samples acetylated by the procedure described above were analysed by h.p.l.c. (5mM aqueous TBAP (66)). When separate coinjections of acetylated reaction samples with *N*-acetyltriglycine and *N*-acetylglycylglycyl-alanine were made, *N*-acetyltriglycine coeluted with the major peak in all samples and *N*-acetylglycylglycylalanine coleuted with a minor peak in the 24 hour sample.

2) The above general procedure was carried out with photolysis for 48 hours, during which additional di-*tert*-butyl peroxide was added after 24 hours. The reaction mixture was coinjected onto the h.p.l.c. column (0.1% aqueous HFBA (67)) with glycylglycylalanine (68) and alanylglycylglycine (69), separately.

Glycylglycylalanine (68) coeluted with one product peak. Alanylglycylglycine (69) did not coelute with any product peaks. Attempted separation of the components of the reaction mixture using a preparative h.p.l.c. column was unsuccessful.

3) The general procedure described on page 106 was carried out with photolysis for 46 hours, during which additional di-*tert*-butyl peroxide was added after 24 hours. Separation of products was achieved by preparative h.p.l.c. (0.1% aqueous HFBA (67)) involving numerous injections of small quantities of the reaction mixture onto an analytical size h.p.l.c. column. The major component of the reaction mixture was shown to be triglycine (24) by comparison of its ^{1}H n.m.r. spectrum with that of an authentic sample and its mass spectrum which showed a protonated molecular ion at *m/z* 190. Minor components that were identified are described below.

Glycylglycylalanine (68) was identified by comparison of its ^{1}H n.m.r. spectrum with that of an authentic sample and its coelution from the h.p.l.c. column with an authentic sample.

Product of coupling an α-centred radical of triglycine (24) with acetonyl radical. ^{1}H n.m.r. δ (HOD reference) 2.21, s, CH_3; 3.16, d, J 5.5 Hz, CH_2CO; 3.88, s, CH_2; 4.00, s, CH_2; δ 4.73, m, CH.

The ^{1}H n.m.r. spectrum of another fraction showed peaks corresponding to the diastereomers of the triglycine dimer (71), described in detail previously.

*Photolytic Reaction of Triglycine (24) and Cystine (27) with Di-*tert*-butyl Peroxide*

A mixture of triglycine (24) (50 mg, 0.26 mmol) and cystine (27) (64 mg, 0.26 mmol) was suspended in water (40 ml) and *tert*-butanol (30 ml). Concentrated hydrochloric acid (1 ml) was added to form a homogeneous solution. Di-*tert*-butyl peroxide (0.24 ml, 1.3 mmol) was added and the solution was sonicated for 15 minutes. The resultant solution under nitrogen was irradiated with ultraviolet light (300 nm) in a Rayonet photochemical reactor for 21 hours. T.l.c. analysis of the reaction mixture (eluant: acetic acid/water/chloroform, 8:1:3) indicated that no cystine (27) remained but triglycine (24) and one product were present. The solution was concentrated under reduced pressure and a small amount of the residual oil was chromatographed using h.p.l.c. (0.1% aqueous HFBA (67)). The unknown product was rechromatographed to give pure *S*-methylcysteine (28) with ^{1}H n.m.r. spectral data and t.l.c. properties consistent with those of an authentic sample. ^{1}H n.m.r. δ 2.14, s, CH_3; 2.98, dd, J 7.5, 15.0 Hz, 1H, CH_2; 3.08, dd, J 4.5, 15.0 Hz, 1H, CH_2; 3.93, dd, J 4.5, 7.5 Hz, CH. T.l.c. (eluant: 2-butanone/methanol/water, 14:3:5) showed that the product had the same R_f and ninhydrin development colour as an authentic sample of *S*-methylcysteine (28).

Experimental for Chapter Two

The cystine derivatives (76) and (78),[100] the alanine derivative (13b),[101] the phenylalanine derivative (83),[102] and the β-chloroalanine derivative (86)[103] were obtained as gifts. The dehydroalanine derivative (80) was synthesized by treatment of the corresponding β-nitroalanine derivative, obtained as a gift,[104] with base, as reported.[78] *S*-Methylcysteine (28) was purchased from Sigma Chemical Co.. Cysteine hydrochloride was purchased from Fluka Chemie AG.

Photolytic reactions were conducted with reaction solutions under nitrogen in quartz tubes in a Rayonet photochemical reactor containing 12 RPR 3500 or 3000 lamps, unless otherwise stated.

N-*Benzoyl*-S-*methylcysteine Methyl Ester (55)*

Method 1: *S*-Methylcysteine (28) (3.0 g, 22 mmol) was added to methanol (50 ml) which had been pretreated with thionyl chloride (2.4 ml, 33 mmol) and the resulting solution was stirred overnight under anhydrous conditions. As t.l.c. analysis of the mixture indicated that esterification was incomplete, additional thionyl chloride was added after 17 hours and stirring was continued for another 4 hours. The solution was concentrated under reduced pressure and the residue was diluted with methanol (20 ml). The resultant solution was concentrated to dryness under reduced pressure to give crude *S*-methylcysteine methyl ester hydrochloride as a yellow solid. Potassium carbonate (7.5 g, 54 mmol) was added to a stirred mixture of ice (30 g) and the crude *S*-methylcysteine methyl ester hydrochloride. The resulting solution was cooled in an ice bath while benzoyl chloride (3.2 ml, 27 mmol) was added and the suspension that formed was stirred at room temperature overnight. The reaction mixture was extracted with dichloromethane (x3) and the combined organic extracts were washed with saturated sodium bicarbonate, dried and concentrated under reduced pressure. The colourless solid residue was recrystallized from ethyl acetate/light petroleum

to yield the sulfide (55) as colourless crystals (4.91 g, 87%), m.p. 80-81.5°C (Found: C, 57.1; H, 6.1; N, 5.4. $C_{12}H_{15}NO_3S$ requires C, 56.9; H, 6.0; N, 5.5%). υ_{max} 3336, 1736, 1636, 1522, 1270, 1236, 1018, 732 cm^{-1}. ^1H n.m.r. δ 2.10, s, SCH_3; 3.01, dd, J 6.0, 14.0 Hz, 1H, CH_2; 3.10, dd, J 5.0, 14.0 Hz, 1H, CH_2; 3.74, s, OCH_3; 4.97-5.03, m, CH; 7.36-7.86, m, 6H, Ph+NH. Mass spectrum (e.i.) *m/z* 253 (M^+, 55), 206 (55), 194 (6), 132 (51), 122 (27), 117 (14), 105 (100), 100 (10), 77 (43).

Method 2 : Reduction of the disulfide (78) was based on the reported method.[76] *N,N'*-Dibenzoylcystine (78) (3.80 g, 8.5 mmol) was suspended in acetic acid (8 ml) and aqueous ethanol (26%, 30 ml) and stirred at 40°C while zinc powder (0.80 g, 12 mmol) was added portionwise over 40 minutes. Acetic acid (5 ml) and aqueous ethanol (26%, 20 ml) were added to the mixture to allow ease of stirring. After 2 hours, 3N hydrochloric acid (10 ml) was added and the resultant solution was stirred at 40°C for 2.5 hours. As t.l.c. analysis of the mixture indicated that reaction was not complete, zinc powder (0.20 g, 3 mmol) was added and the mixture was stirred at room temperature overnight. Most of the solvent was removed under reduced pressure and the remaining suspension was filtered and extracted with ethyl acetate (x3). The organic fractions were dried, filtered and concentrated under reduced pressure. The residue was dissolved in methanol (20 ml) and added to methanol (25 ml) which had been pretreated with thionyl chloride (1.3 ml, 18 mmol). The resultant solution was stirred overnight under anhydrous conditions and concentrated under reduced pressure. The residue was diluted with methanol (15 ml) and the solution was concentrated to dryness to give crude *N*-benzoylcysteine methyl ester (79) (3.4 g). A portion of this crude ester (79) (250 mg, ~80% pure, ~0.8 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (0.17 ml, 1.1 mmol) were dissolved in benzene (15 ml) and the mixture was stirred under nitrogen while methyl iodide (0.07 ml, 1.1 mmol) was added. After 3 hours stirring, the solvent was removed under reduced pressure and the residue was partitoned between ethyl acetate and water. The aqueous fraction was extracted with ethyl acetate and the combined organic fractions were washed

with 10% hydrochloric acid and water, dried, filtered and concentrated under reduced pressure. The residue was chromatographed on silica to give three products as colourless oils.

*The percentage yields given were calculated by scaling up the quantities of products obtained to the quantities that would have been obtained if all of the crude cysteine derivative (79) had been used in the methylation reaction.

N-Benzoyl-*S*-methylcysteine methyl ester (55) (48 mg, 30%*). ^{1}H n.m.r. δ 2.13, s, SCH_3; 3.05, dd, J 6.0, 14.0 Hz, 1H, CH_2; 3.13, dd, J 5.0, 14.0 Hz, 1H, CH_2; 3.79, s, OCH_3; 4.99-5.06, m, CH; 7.17, br d, J 7.0 Hz, NH; 7.41-7.55, m, 3H, Ph; 7.82-7.85, m, 2H, Ph, consistent with the ^{1}H n.m.r. spectrum of an authentic sample of the sulfide (55) (see Method 1).

N-Benzoyldehydroalanine methyl ester (80) (49 mg, 38%*). ^{1}H n.m.r. δ 3.88, s, OCH_3; 5.99, s, CH; 6.80, s, CH; 7.44-7.54, m, 3H, Ph; 7.82-7.85, m, 2H, Ph; 8.55, br s, NH. This data is comparable with that reported for the dehydroalanine derivative (80).[78]

N,N'-Dibenzoylcystine dimethyl diester (76) (53 mg, 18%*), with t.l.c. properties and ^{1}H n.m.r. spectral data consistent with those of an authentic sample. ^{1}H n.m.r. (60 MHz) δ 3.1-3.2, m, CH_2; 3.8, s, OCH_3; 4.8-5.2, m, CH; 7.1-8.0, m, 6H, Ph+NH.

Method 3 : *S*-Methylcysteine (28) was synthesized by treatment of a solution of the hydrochloride salt of cysteine (25) in methanol with sodium and methyl iodide, as reported.[79] The crude *S*-methylcysteine (28) was converted to its methyl ester and benzoyl amide using standard procedures described in Method 1. Yield (29%), ^{1}H n.m.r. spectral data as described above.

N-*Benzoyl*-S-*methylcysteine Sulfoxide Methyl Ester (56)*

Method 1: A solution of sodium metaperiodate (2.6 g, 12 mmol) in water (35 ml) was cooled in an ice bath. A solution of *N*-benzoyl-*S*-methylcysteine methyl ester (55) (3.0 g, 12 mmol) in methanol (40 ml) was added dropwise over 20 minutes. The mixture was stirred at ice-bath temperature for 1 hour and the precipitate was removed by filtration and washed with methanol. The filtrate was concentrated under reduced pressure until the methanol was removed and the remaining aqueous solution was extracted with dichloromethane (x3). The combined organic extracts were dried, filtered and concentrated under reduced pressure to leave a colourless solid which recrystallized from ethyl acetate/light petroleum as a 1:1 mixture of diastereomers of the sulfoxide (56) (2.63 g, 82%), m.p. 121-127°C (Found: C, 53.5; H, 5.4; N, 5.2. $C_{12}H_{15}NO_4S$ requires C, 53.5; H, 5.6; N, 5.2%). υ_{max} 3292, 1742, 1644, 1530, 1218, 1026, 698 cm^{-1}. ^1H n.m.r. δ 2.68, s, 0.5x3H, $SOCH_3$; 2.69, s, 0.5x3H, $SOCH_3$; 3.27, dd, J 4.0, 13.5 Hz, 0.5x1H, CH_2; 3.38, dd, J 6.0, 13.5 Hz, 0.5x1H, CH_2; 3.47, dd, J 7.5, 13.5 Hz, 0.5x1H, CH_2; 3.52, dd, J 5.5, 13.5 Hz, 0.5x1H, CH_2; 3.80, s, 0.5x3H, OCH_3; 3.82, s, 0.5x3H, OCH_3; 5.07, apparent q, J 5.5 Hz, 0.5x1H, CH; 5.10-5.16, m, 0.5x1H, CH; 7.40-7.56, m, 3H, Ph; 7.75, br d, J 7.0 Hz, 0.5x1H, NH; 7.84-7.87, m, 2H, Ph; 8.21, br d, J 7.5 Hz, 0.5x1H, NH. ^{13}C n.m.r. δ 38.9, 39.1, 49.3, 49.7, 53.0, 53.1, 54.3, 55.3, 127.2, 127.3, 128.5, 128.6, 132.0, 132.1, 132.91, 132.94, 167.3, 167.4, 170.3, 170.4. Mass spectrum (f.a.b.) *m/z* 270 ($(M+H)^+$, 21), 243 (7), 206 (29), 105 (100), 77 (21).

Method 2: A solution of *N*-benzoyl-*S*-methylcysteine methyl ester (55) (100 mg, 0.40 mmol) in acetone (2 ml) was stirred in an ice bath while aqueous hydrogen peroxide (0.05 ml, ~0.6 mmol) was added dropwise. After 15 minutes the ice bath was removed and the mixture was heated at reflux for 4 hours. The solvent was removed under reduced pressure and the residue was partitioned between ethyl acetate and water. The layers were separated and the aqueous fraction was washed with ethyl acetate. The combined organic fractions were dried, filtered and concentrated under reduced pressure to give a yellow oil. The ^1H n.m.r.

spectrum of this residue showed two sets of resonances in a ratio of ~3:1. The major set was for a 1:1 mixture of the diastereomers of the sulfoxide (56): δ 2.68, s, 0.5x3H, $SOCH_3$; 2.69, s, 0.5x3H, $SOCH_3$; 2.30-3.57, m, CH_2; 3.78, s, 0.5x3H, OCH_3; 3.80, s, 0.5x3H, OCH_3; 5.05-5.15, m, CH; 7.38-7.54, m, 3H, Ph; 7.80-7.87, m, 2H, Ph; 8.28, br d, J 7.0 Hz, NH. The minor set corresponded to *N*-benzoyl-*S*-methyl-cysteine sulfone methyl ester (81): δ 2.97, s, SO_2CH_3; 2.30-3.57, m, CH_2; 3.84, s, OCH_3; 5.05-5.15, m, CH; 7.38-7.54, m, 3H, Ph; 7.80-7.87, m, 2H, Ph; 8.08, br d, J 7.0 Hz, NH. The f.a.b. mass spectrum of the mixture showed protonated molecular ions at *m/z* 270 and 286 for the products (56) and (81), respectively.

*Photolysis of the Sulfide (55) and Di-*tert*-butyl Peroxide*

A solution of the sulfide (55) (100 mg, 0.4 mmol) and di-*tert*-butyl peroxide (0.73 ml, 4.0 mmol) in benzene (5 ml) was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 24 hours. More di-*tert*-butyl peroxide (0.73 ml) was added after seven hours. The solvent was removed under reduced pressure. The ^1H n.m.r. spectrum of the residue showed that little of the sulfide (55) had reacted. A small doublet at δ 1.52 (J 7.0 Hz) may have corresponded to the β-protons of the alanine derivative (13b). The above reaction was repeated on half the scale using ultraviolet light of wavelength 300 nm and similar results were obtained.

*Photolysis of the Sulfoxide (56) and Di-*tert*-butyl Peroxide in Benzene*

1) A solution of the sulfoxide (56) (50 mg, 0.19 mmol) and di-*tert*-butyl peroxide (0.2 ml, 1.1 mmol) in benzene (10 ml) was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for three days. The solution was washed with water (x2), dried, filtered and concentrated under reduced pressure. The ^1H n.m.r. spectrum of the residue showed that a complex mixture had formed, with

a doublet at δ 1.51 (J 7.0 Hz) corresponding to the β-protons of the alanine derivative (13b). An authentic sample of the alanine derivative (13b) was added to a sample of the reaction mixture and the ^{1}H n.m.r. spectrum showed relative increases in the size of the doublet at δ 1.51 (J 7.0 Hz), the singlet at δ 3.78, and the multiplet centred at δ 4.81, which correspond to the β-, methyl ester, and α-protons, respectively, of the product (13b). Comparisons of integrals in the ^{1}H n.m.r. spectrum of the reaction mixture indicated that the ratio of the product (13b) to the starting material (56) was ~2:1 and that the product (13b) was ~20% of the amino acid derived material.

2) A solution of the sulfoxide (56) (250 mg, 0.93 mmol) and di-*tert*-butyl peroxide (0.9 ml, 4.90 mmol) in benzene (25 ml) was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor. Di-*tert*-butyl peroxide (0.9 ml) was added after 5.5 hours. Approximately one third of the reaction mixture was removed after 21 hours. Di-*tert*-butyl peroxide (0.5 ml, 2.72 mmol) was added to the remaining reaction mixture which was photolysed for a further 27 hours, during which more di-*tert*-butyl peroxide (0.5 ml) was added after seven hours. The two portions were washed with water, dried, filtered and concentrated under reduced pressure. T.l.c. analysis of the residues showed that complex reaction mixtures had formed and some starting material (56) remained. The residue from the 21 hour portion was chromatographed on silica eluting with ethyl acetate/light petroleum followed by ethyl acetate/methanol. Three compounds were identified by comparison of ^{1}H n.m.r. spectral data and t.l.c. properties of the partially purified fractions obtained with those of authentic samples. Isolation of pure products was difficult due to the complexity of the reaction mixture and similar chromatographic properties of compounds within.

One compound was the starting material (56). The second compound was *N*-benzoylphenylalanine methyl ester (83): ^{1}H n.m.r. δ 3.24, dd, J 5.5, 14.0 Hz, 1H, CH_2; 3.31, dd, J 6.0, 14.0 Hz, 1H, CH_2; 3.78, s, CH_3; 5.10, dt, J 5.5, 7.5, Hz, CH; 6.57, br d, J 7.5 Hz, NH; 7.12-7.15, m, 2H, Ph; 7.27-7.33, m, 3H, Ph; 7.43-7.52, m, 3H, Ph; 7.71-

7.74, m, 2H, Ph. The third compound was the alanine derivative (13b): ^{1}H n.m.r. δ 1.53, d, J 7.0 Hz, CH_3; 3.80, s, OCH_3; 4.82, m , CH; 6.81, br, NH; 7.40-7.55, m, 3H, Ph; 7.80-7.83, m, 2H, Ph.

Integrals of peaks in the ^{1}H n.m.r. spectrum of the crude reaction mixture indicated that the alanine derivative (13b) was approximately 30% of the amino acid derived material and the ratio of the products (13b) and (83) and the starting material (56) was ~2:1:1. The ^{1}H n.m.r. spectrum of the 48 hour reaction mixture indicated that although little of the starting material (56) remained, the ratio and yields of the major products (13b) and (83) had not changed.

3) A solution of the sulfoxide (56) (100 mg, 0.37 mmol) and di-*tert*-butyl peroxide (0.35 ml, 1.90 mmol) in benzene (10 ml) was irradiated with ultraviolet light of wavelength 300 nanometres in a Rayonet photochemical reactor for 24 hours. Additional di-*tert*-butyl peroxide (0.35 ml) was added after 7 hours. The solution was washed with water, dried , filtered, and concentated under reduced pressure. The ^{1}H n.m.r. spectrum of the complex reaction mixture indicated that little of the starting material (56) remained and that the alanine derivative (13b) was produced in approximately 30% yield. The ratio of the major products (13b) and (83) was ~2:1.

4) A solution of the sulfoxide (56) (20 mg, 0.07 mmol) and di-*tert*-butyl peroxide (0.13 ml, 0.70 mmol) in benzene (3 ml) was irradiated with ultraviolet light (300 nm) in a Rayonet photochemical reactor for 23 hours. Di-*tert*-butyl peroxide (0.13 ml) was added after seven hours. The solution was concentated under reduced pressure. The ^{1}H n.m.r. spectrum of the complex reaction mixture indicated that the ratio of the compounds (13b), (83), and (56) was 2:1:1, as estimated by comparison of the respective methyl ester peak heights. The alanine derivative (13b) made up approximately 30% of the amino acid derived material.

Photolysis of N-*Benzoyl*-S-*methylcysteine Sulfoxide Methyl Ester (56)*

1) *Iso*-propanol (0.1 ml, 1.3 mmol) was added to a solution of the sulfoxide (56) (50 mg, 0.19 mmol) in benzene (5 ml) which was then irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 52 hours. Catalytic amounts of the radical initiators benzoyl peroxide, AIBN, and acetone were added after 9 hours, 24 hours, and 32 hours, respectively, because t.l.c. analysis indicated that no reaction was occurring. The solvent was removed under reduced pressure. The ^{1}H n.m.r. spectrum of the reaction mixture showed that at least 90% of the starting material (56) remained. A small singlet at δ 2.98, corresponding to the methylsulfonyl protons of the sulfone (81), indicated that a small amount of the sulfoxide (56) had been oxidized.

2) A solution of the sulfoxide (56) (20 mg, 0.07 mmol) in *iso*-propanol (3 ml) was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for four days. The solvent was removed under reduced pressure and both t.l.c. and ^{1}H n.m.r. spectroscopic analysis of the residue showed that the sulfoxide (56) was the only material present.

*Photolysis of the Sulfoxide (56) and Di-*tert*-butyl Peroxide in Carbon Tetrachloride/Benzene*

A solution of the sulfoxide (56) (100 mg, 0.37 mmol) and di-*tert*-butyl peroxide (0.7 ml, 3.7 mmol) in benzene/carbon tetrachloride (12 ml, 11:1) was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 30 hours. More di-*tert*-butyl peroxide (0.7 ml) was added after 7.5 hours and 23 hours. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate. The solution was washed with water, dried, filtered and concentrated under reduced pressure. The complex reaction mixture was chromatographed on silica and the partially purified fractions obtained were

shown to contain *N*-benzoyl-β-chloroalanine methyl ester (86), the alanine derivative (13b), the phenylalanine derivative (83), and the dehydroalanine derivative (80), by comparison of ^{1}H n.m.r. spectral data and t.l.c. properties with those of authentic samples. ^{1}H n.m.r. for the product (86): δ 3.86, s, OCH_3; 4.04, dd, J 3.5, 11.5 Hz, 1H, CH_2; 4.09, dd, J 3.0, 11.5 Hz, 1H, CH_2; 5.20, apparent dt, J 7.0, 3.0 Hz, CH; 7.06, br d, J 6.0 Hz, NH; 7.43-7.58, m, 3H, Ph; 7.84-7.86, m, 2H, Ph.

The ^{1}H n.m.r. spectrum of the crude reaction mixture showed that the major products, the β-chloroalanine derivative (86) and the alanine derivative (13b), formed in about 30% and 15% yield, respectively. The phenylalanine derivative (83) was a minor product. Signals for the alkene (80) were not present in the ^{1}H n.m.r. spectrum of the crude reaction mixture and formation of the alkene (80) can be attributed to elimination of hydrogen chloride from the chloride (86) during chromatography.

*Photolysis of the Sulfoxide (56), Chlorotriphenylmethane, and Di-*tert*-butyl Peroxide*

A solution of the sulfoxide (56) (50 mg, 0.19 mmol), chlorotriphenylmethane (110 mg, 0.39 mmol) and di-*tert*-butyl peroxide (0.35 ml, 1.90 mmol) in benzene (10 ml) was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 50 hours. More di-*tert*-butyl peroxide (0.35 ml) was added after 17.5 hours, 26 hours, and 41 hours because t.l.c. analysis of the mixture indicated that significant amounts of the starting materials remained. The solvent was removed under reduced pressure and the complex reaction mixture was chromatographed on silica. The partially purified fractions were shown to contain the β-chloroalanine derivative (86), the alanine derivative (13b), and the phenylalanine derivative (83), by comparison of ^{1}H n.m.r. spectral data and t.l.c. properties with those of authentic samples. Chlorotriphenylmethane (66 mg, 60%) was recovered from the reaction mixture.

*Photolysis of the Sulfoxide (56) and Di-*tert*-butyl Peroxide in α,α,α-Trichlorotoluene*

1) A solution of the sulfoxide (56) (100 mg, 0.37 mmol), and di-*tert*-butyl peroxide (0.7 ml, 3.7 mmol) in α,α,α-trichlorotoluene (7 ml) was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 30 hours. The solvent was removed under reduced pressure. The ^{1}H n.m.r. spectrum of the residue indicated, by comparison with n.m.r. spectra of authentic samples, that no starting material (56) remained, the major product was the β-chloroalanine derivative (86) and the alanine derivative (13b) was a minor product. Comparisons of integrals in the n.m.r. spectrum indicated that the approximate yield of the chloride (86) was 50% and the ratio of the products (86) and (13b) was ~6:1.

*Photolysis of the Sulfoxide (56) and Di-*tert*-butyl Peroxide in α,α,α-Trichlorotoluene with External Standard*

A solution of the sulfoxide (56) (30 mg, 0.11 mmol), and di-*tert*-butyl peroxide (0.2 ml, 1.1 mmol) in α,α,α-trichlorotoluene (2.5 ml) was irradiated with ultraviolet light (350 nm) in a Rayonet photochemical reactor for 34 hours. *Tert*-butyl benzamide (4 mg, 0.022 mmol) was dissolved in the reaction solution which was then concentrated under reduced pressure. The ^{1}H n.m.r. spectrum of the residue showed peaks characteristic of the sulfoxide (56), the β-chloroalanine derivative (86), and the alanine derivative (13b). Comparisons of integrals of these peaks with that of the *tert*-butyl singlet of *tert*-butyl benzamide indicated that the yields of the products (86) and (13b) were approximately 45% and 4%, respectively. Approximately 35% of the starting material (56) remained and the yield of the chloride (86) based on remaining starting material (56) was 70%.

Reaction of the Sulfoxide (56) with Benzoyl Peroxide

A solution of the sulfoxide (56) (50 mg, 0.19 mmol) and benzoyl peroxide 90 mg, 0.37 mmol) in benzene (3 ml) was heated at reflux for 5 hours and then concentrated under reduced pressure. The ^{1}H n.m.r. spectrum of the complex reaction mixture showed resonances of the starting material (56), and two singlets for the olefinic protons of the dehydroalanine derivative (80)[78] at δ 6.00 and 6.81. Among many other peaks, a small doublet at δ 1.50, J 7.0 Hz, indicated that the alanine derivative (13b) had possibly formed, as judged by comparison with the n.m.r. spectrum of an authentic sample. Chromatographic attempts to isolate the compound with the doublet at δ 1.50 were unsuccessful as separation of components of the complex mixture proved to be difficult.

Photolysis of the Sulfoxide (56), TMIO (19), and Hydrogen Peroxide

The sulfoxide (56) (20 mg, 0.07 mmol) and TMIO (19) (14 mg, 0.07 mmol) were dissolved in benzene (2 ml) and aqueous hydrogen peroxide solution (0.1 mmol) was added. The vigorously stirred mixture was irradiated through pyrex with a 250W mercury lamp for three hours under nitrogen. The layers were separated and the organic layer was dried and concentrated under reduced pressure. Chromatography of the complex reaction mixture on silica, eluting with ethyl acetate/light petroleum followed by ethyl acetate/methanol, gave partially purified fractions. Compounds identified were TMIO (19), the sulfoxide (56), and *N*-benzoylalanine methyl ester (13b), by comparison of t.l.c. properties and peaks in the ^{1}H n.m.r. spectra of the fractions with those of authentic samples. ^{1}H n.m.r. resonances for the product (13b) were δ 1.53, d, J 7.0 Hz, CH_3; 3.80, s, OCH_3; 4.75-4.85, m, CH; 6.80, br, NH; 7.42-7.55, m, 3H, Ph; 7.81-7.86, m, 2H, Ph. An isolated yield of this product was not obtained due to the complex nature of the reaction mixture, which caused difficulty in obtaining pure fractions by chromatography.

Reaction of the Sulfoxide (56), TMIO (19), Hydrogen Peroxide and Ferrous Sulfate

Procedures are based on that used for synthesis of 2-methoxy-1,1,3,3-tetramethyl-isoindoline (62).[38]

1) In Benzene/Water: To a stirred mixture of the sulfoxide (56) (100 mg, 0.37 mmol) and TMIO (19) (71 mg, 0.37 mmol) in benzene (15 ml) was added a solution of ferrous sulfate heptahydrate (155 mg, 0.56 mmol) in water (5 ml). Aqueous hydrogen peroxide (0.04 ml, ~0.5 mmol) was diluted with water (5 ml) and added dropwise over thirty minutes. The mixture was stirred under nitrogen for 2 hours at which time half of the reaction mixture was removed. Aqueous hydrogen peroxide (0.04 ml)/water (5 ml) was added to the other half which was stirred for a further hour. For both portions, the benzene and aqueous layers were separated and the aqueous fraction was washed with ethyl acetate and light petroleum. The ethyl acetate fractions were combined with the benzene layers and dried, filtered and concentrated under reduced pressure. The two mixtures obtained were complex but similar by t.l.c. and their f.a.b. mass spectra showed peaks at *m/z* 397 which can be attributed to the protonated molecular ion of the adduct (82). The two mixtures were combined and chromatographed on silica eluting with ethyl acetate/light petroleum followed by ethyl acetate/methanol. T.l.c. and ^{1}H n.m.r. spectroscopic analysis of the resultant partially purified fractions showed that some of the TMIO (19) and the sulfoxide (56) remained and that *N*-benzoyldehydroalanine methyl ester (80) had formed. No compound with a molecular weight of 396 was identified.

2) In Methanol/Water: A stirred mixture of the sulfoxide (56) (100 mg, 0.37 mmol), TMIO (19) (71 mg, 0.37 mmol), and ferrous sulfate heptahydrate (155 mg, 0.56 mmol) in methanol/water (15 ml, 2:1) was treated with aqueous hydrogen peroxide (0.1 ml, ~1 mmol) added dropwise over ten minutes. The suspension was stirred for eight hours during which time more aqueous hydrogen peroxide (0.1 ml) and ferrous sulfate heptahydrate (155 mg) were added because t.l.c.

analysis of the mixture indicated that no products had formed. The mixture was heated at reflux overnight followed by removal of most of the solvent under reduced pressure. The remaining aqueous suspension was extracted with dichloromethane (x2) and light petroleum (x2) and the combined organic extracts were dried, filtered and concentrated under reduced pressure. The ^{1}H n.m.r. spectrum of the residue showed that the major compound was the sulfoxide (56) and a minor product was the dehydroalanine derivative (80), as shown by the characteristic olefinic signals at δ 5.99 and 6.78.[78] T.l.c. analysis of the mixture indicated that no TMIO (19) remained but the structures of any products derived from TMIO (19) could not be elucidated from the n.m.r. spectrum.

3) In tert-*Butanol/Water*: To a stirred mixture of the sulfoxide (56) (50 mg, 0.19 mmol) and TMIO (19) (36 mg, 0.19 mmol) in *tert*-butanol (10 ml) was added a solution of ferrous sulfate heptahydrate (80 mg, 0.29 mmol) in water (2 ml). Aqueous hydrogen peroxide (0.1 ml, ~1 mmol) was diluted with water (1 ml) and added dropwise over thirty minutes. The mixture was stirred under nitrogen for three hours. The *tert*-butanol was removed under reduced pressure and the aqueous residue was extracted with ethyl acetate. This organic extract was dried, filtered, and concentrated under reduced pressure. T.l.c. analysis of the residue indicated that no TMIO (19) remained and showed a spot with the same R_f as an authentic sample of the alcohol (64). The ^{1}H n.m.r. spectrum of the residue showed resonances of the alcohol (64): δ 1.31, s, 2xCH_3; 1.46, br s, 4xCH_3; 2.45, br, OH; 3.83, s, CH_2; 7.09-7.12, m, 2H, ArH; 7.23-7.26, m, 2H, ArH. This data is consistent with that of the fully characterized sample of the alcohol (64) described on page 98. Singlets at δ 2.69 and 2.97, in a 2:1 ratio, were attributable to methyl protons of the starting material (56) and the sulfone (81), respectively, indicating that the only reaction of the sulfoxide (56) was oxidation.

Reactions of the Sulfoxide (56) and the Sulfide (55) with Tributyltin Hydride

1) In the reactions of the sulfoxide (56) with tributyltin hydride and aryl and alkyl halides, the sulfoxide (56) (50 mg, 0.19 mmol) and the halide (0.19 mmol) were dissolved in benzene (5-10 ml) and AIBN (2 mg, catalytic) was added. For the reaction with bromobenzene, the mixture was heated at reflux. For the reactions with iodobenzene and methyl iodide, the mixtures were irradiated through pyrex using a 250W mercury lamp. A solution of tributyltin hydride (0.05 ml, 0.19 mmol) in benzene (2 ml) was added dropwise over 1 hour. Heating or irradiation of the resultant mixture was continued for 2-5 hours followed by concentration of the reaction mixture under reduced pressure.

By comparison with t.l.c. properties of an authentic sample of *N*-benzoyldehydroalanine methyl ester (80), t.l.c. analysis of the heated reaction mixture indicated that the only reaction was partial conversion of the sulfoxide (56) to the alkene (80). This reaction mixture was stirred with aqueous potassium fluoride in order to remove stannyl compounds and the ^1H n.m.r. spectrum of the resultant mixture indicated that exposure to base resulted in conversion of all of the starting material (56) to the dehydroalanine derivative (80), the characteristic[78] olefinic signals of which were seen at δ 6.00 and 6.81.

T.l.c. or ^1H n.m.r. spectroscopic analysis of the irradiated reaction mixtures indicated that the only amino acid derived material present was the starting material (56).

2) In reactions of the sulfide (55) or the sulfoxide (56) with tributyltin hydride only, a mixture of the substrate (50 mg), tributyltin hydride (1.2 equivalents) and AIBN (2 mg, catalytic) in benzene (10 ml) was heated at reflux for 8 hours. Reaction mixtures were stirred overnight with an equal volume of 20% aqueous potassium fluoride to remove stannyl compounds. The organic layers were washed with water, dried, filtered, and concentrated under reduced pressure. The ^1H n.m.r. spectrum of the mixture obtained from reaction of the sulfide (55) showed resonances for the starting material (55) along with a similar set of peaks

with no corresponding methyl ester singlet. These peaks indicated that hydrolysis of the ester moiety of the sulfide (55) may have occurred. T.l.c. analysis of the sulfoxide (56) reaction mixture before stirring with potassium fluoride indicated that most of the starting material (56) remained and that one of the minor compounds present was the dehydroalanine derivative (80). Exposure of this reaction mixture to potassium fluoride resulted in conversion of all of the sulfoxide (56) to the dehydroalanine derivative (80), as shown by ^{1}H n.m.r. spectroscopy.

3) A solution of the sulfoxide (56) (50 mg, 0.19 mmol), tributyltin hydride (0.05 ml, 0.19 mmol), and di-*tert*-butyl peroxide (0.2 ml, 1.1 mmol) in benzene (10 ml) was irradiated (300 nm) in a Rayonet photochemical reactor (300 nm) for 7 hours. T.l.c. analysis of the reaction mixture indicated that no reaction had occurred. The mixture was stirred with an equal volume of 10% aqueous potassum fluoride solution for 2 days. The organic layer was washed with water, dried, filtered, and concentrated under reduced pressure. The ^{1}H n.m.r. spectrum of the resultant mixture indicated that all of the sulfoxide (56) was converted to the dehydroalanine derivative (80) on stirring with potassium fluoride.

Experimental for Chapter Three

Nitroso-*tert*-butane (48), methionine (26), methionine sulfoxide (49), *S*-methylcysteine (28), glutathione (122), *S*-methylglutathione (60), and methionylleucylphenylalanine (59) were purchased from Sigma Chemical Co.. 3-Methylthiopropionic acid (103) and 3-methylthiopropylamine (102) were purchased from TCI Tokyo Kasei Kogyo Co.. 2-Methylthioethylamine (101) was purchased from Fluka Chemie AG. Ethylmethyl sulfide and *tert*-butylmethyl sulfide were purchased from Aldrich Chemical Co.. Glycylmethionylglycine (58) was purchased from Bachem Feinchemikalien AG. Dimethyl sulfoxide and diethyl sulfide were purchased from BDH Chemical Co..

E.s.r. spectra were recorded on a Varian E9 e.s.r. spectrometer. All hyperfine splitting constants are given in gauss (G).

General Procedures for E.s.r. Experiments using Nitroso-tert-*butane (NTB) (48)*

The procedure and quantities were as described below unless otherwise stated. All photolyses were carried out through quartz.

In water: Saturated aqueous solutions of NTB (48) in water were prepared by sonicating a suspension of NTB (48) (10 mg) in water (1 ml) for 1-1.5 hours and were used immediately. The sulfoxide (2-5 mg) was dissolved in an aqueous solution of NTB (48) (0.25 ml) and aqueous hydrogen peroxide (0.25 ml) was added. The resultant mixture was either treated with ferrous sulfate heptahydrate (catalytic) or irradiated through quartz with a 250W mercury lamp for 2-3 minutes, before being transferred to a flat e.s.r. cell and analysed by e.s.r. spectroscopy.

In benzene or benzene/water: Di-*tert*-butyl peroxide or aqueous hydrogen peroxide (0.25 ml) was added to a solution of the sulfoxide (2-5 mg) and NTB (48)

(2-5 mg) in benzene (0.25 ml) and the resultant combination was thoroughly mixed. The mixture was either treated with ferrous sulfate heptahydrate (catalytic) or irradiated with ultraviolet light for 2-3 minutes using either a 250W mercury lamp or an Oriel 1000W high pressure mercury lamp. The 1000W lamp was sharply focussed on the sample in the cavity of the e.s.r. spectrometer. The 250W lamp was a source of diffuse light directed at the sample before it was placed in the spectrometer. Mixtures were analysed by e.s.r. spectroscopy in either a flat e.s.r. cell (benzene/water) or a quartz e.s.r. tube (benzene).

Photolysis of NTB (48)

A solution of NTB (48) (2 mg) in benzene (0.5 ml) was irradiated (1000W). The e.s.r. spectrum of the resultant mixture showed a 1:1:1 triplet, a_N = 15G, consistent with the reported spectral data for di-*tert*-butyl nitroxide (87) in benzene.[81]

E.s.r. Experiments with N-*Benzoyl*-S-*methylcysteine Sulfoxide Methyl Ester (56)*

1) A solution of the sulfoxide (56) and NTB (48) in benzene was mixed with aqueous hydrogen peroxide and irradiated (250W). The e.s.r. spectrum of the resultant mixture showed a 1:1:1 triplet, a_N = 15 G, consistent with the reported spectral data for di-*tert*-butyl nitroxide (87).[81]

2) Ferrous sulfate heptahydrate was added to a mixture of a solution of the sulfoxide (56) and NTB (48) in benzene and aqueous hydrogen peroxide. The e.s.r. spectrum of the resultant mixture showed two 1:1:1 triplets of a_N = 15G and a_N = 27G, consistent with the reported spectral data for di-*tert*-butyl nitroxide (87)[81] and deprotonated *tert*-butylhydroxy nitroxide (88),[82] respectively.

Reaction of Dimethyl Sulfoxide with Fenton's Reagent and NTB (48)

Ferrous sulfate heptahydrate was added to a solution of NTB (48) in dimethyl sulfoxide (0.25 ml)/aqueous hydrogen peroxide (0.25 ml). The e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, a_N = 17G) and a triplet (1:1:1, a_N = 17G) of quartets (1:3:3:1, a_H = 14G), consistent with the reported signals for di-*tert*-butyl nitroxide (87)[81] and *tert*-butylmethyl nitroxide (89),[82] respectively.

E.s.r. Experiments with Methionine Sulfoxide (49) and NTB (48)

1) Aqueous hydrogen peroxide and ferrous sulfate heptahydrate were added to an aqueous solution of the sulfoxide (49) and NTB (48). The e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, a_N = 16G) of doublets (a_H = 2G) (Figure 2), attributable to the adduct of NTB (48) and the α-centred radical formed by decarboxylation of the amino acid (49).

2) The above procedure was repeated with photolysis instead of ferrous sulfate heptahydrate addition. The e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, a_N = 17G) of triplets (1:2:1, a_H = 12G) and a triplet (1:1:1, a_N = 17G) (Figure 3), attributable to the adduct (92) and di-*tert*-butyl nitroxide (87), respectively. To extract the nitroxide (87), the mixture was washed with light petroleum (x2). The e.s.r. spectrum of the aqueous fraction showed a triplet (1:1:1, a_N = 17G) of triplets (1:2:1, a_H = 12G) (Figure 4).

E.s.r. Experiments with N*-Benzoylmethionine Sulfoxide Methyl Ester (93)*

1) A solution of the sulfoxide (93) and NTB (48) in benzene was mixed with aqueous hydrogen peroxide and irradiated (1000W). The e.s.r. spectrum of the resultant mixture showed a 1:1:1 triplet, a_N = 15G, consistent with the reported signal for di-*tert*-butyl nitroxide (87).[81]

2) A solution of the sulfoxide (93), NTB (48), and di-*tert*-butyl peroxide in benzene was irradiated (1000W). The e.s.r. spectrum of the resultant mixture showed two 1:1:1 triplets, a_N = 16G and a_N = 27G, consistent with the reported signals for di-*tert*-butyl nitroxide (87)[81] and *tert*-butoxy-*tert*-butylnitroxide (94)[81,89] in benzene, respectively.

3) A suspension of the sulfoxide (93) and NTB (48) in di-*tert*-butyl peroxide was irradiated (1000W). The e.s.r. spectrum of the resultant mixture showed the two triplets described immediately above in experiment 2).

*Photolysis of NTB (48) with Di-*tert*-butyl Peroxide*

A mixture of NTB (48) (2 mg) and di-*tert*-butyl peroxide (0.5 ml) was irradiated with a 1000W lamp. The e.s.r. spectrum of the resultant mixture showed the two triplets described in experiment 2) on this page, attributable to the nitroxides (87) and (94).

Sodium 3,5-Dibromo-4-nitrosobenzene Sulfonate (DBNBS) (95) was synthesized by oxidation of the sodium salt of 3,5-dibromosulfanilic acid, as reported.[90] ^{1}H n.m.r. (D_2O) δ 8.33, s, ArH, consistent with that reported.[91]

General Procedure for E.s.r. Experiments using DBNBS (95)

The procedure and quantities were as described below unless otherwise stated. All photolyses were carried out through quartz.

Sulfides: A mixture of the sulfide (2-5 mg (solid) or 0.02 ml (liquid)) and aqueous hydrogen peroxide (0.5 ml) was stirred for at least 10 minutes. Addition of

DBNBS (95) (5 mg) was followed by addition of ferrous sulfate heptahydrate (catalytic) or irradiation with a 250W mercury lamp for 2-3 minutes.

Sulfoxides: The above procedure was carried out without stirring for 10 minutes.

Ratios of e.s.r. signals were estimated by comparing peak areas.

Hyperfine splitting due to the aromatic protons was observed in some e.s.r. spectra of DBNBS (95) spin adducts. The effect, which was either additional splitting of signals (~1G only) or line broadening, is not discussed in regard to individual spectra.

Reaction of Dimethyl Sulfoxide, DBNBS (95), and Fenton's Reagent

1) Ferrous sulfate heptahydrate was added to a solution of DBNBS (95) in aqueous hydrogen peroxide (0.25 ml)/dimethyl sulfoxide (0.25 ml). The e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G) (Figure 5), attributable to the adduct (96), as the major signal.

2) A solution of DBNBS (95) in water (0.5 ml), a solution of DBNBS (95) in dimethyl sulfoxide/water (1:1, 0.5 ml), and a solution of DBNBS (95) in dimethyl sulfoxide/aqueous hydrogen peroxide (1:1, 0.5 ml) were analysed by e.s.r. spectroscopy and no signals were observed. The triplet of quartets described immediately above was observed in the e.s.r. spectrum of the mixture obtained when ferrous sulfate heptahydrate was added to the latter solution.

Reaction of Diethyl Sulfide, DBNBS (95), and Fenton's Reagent

A mixture of diethyl sulfide and aqueous hydrogen peroxide was stirred for 30 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The

e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 13 G) (Figure 6), attributable to the adduct (97).

Reaction of Ethylmethyl Sulfide, DBNBS (95), and Fenton's Reagent

A mixture of ethylmethylsulfide and aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 6:1 ratio of a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 13 G and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G), attributable to the adducts (97) and (96), respectively.

Reaction of tert-*Butylmethyl Sulfide, DBNBS (95), and Fenton's Reagent*

A mixture of *tert*-butylmethyl sulfide and aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, a_N = 15 G) (Figure 7), attributable to the adduct (98).

E.s.r. Experiments with Methionine (26), Methionine Sulfoxide (49), and Methionine Hydrochloride using DBNBS (95)

A solution of methionine (26) in aqueous hydrogen peroxide was stirred for 15 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 4:1 ratio of a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 13G) and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G) (Figure 8), attributable to the adducts (99) and (96), respectively.

The e.s.r. spectrum obtained from repetition of the above experiment starting with methionine sulfoxide (49) and without stirring for 15 minutes was the same

as that obtained from methionine (26), as described immediately above. This experiment with methionine sulfoxide (49) was repeated with photolysis with a 250W mercury lamp for 2 minutes instead of addition of ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 3:1 ratio of the two signals described immediately above (Figure 9).

A solution of methionine hydrochloride in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 6:1 ratio of a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 12G) and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G), attributable to the protonated form of the adduct (99) and the adduct (96), respectively.

E.s.r. Experiments with S-Methylcysteine (28), S-Methylcysteine Sulfoxide (57), and S-Methylcysteine Hydrochloride using DBNBS (95)

A solution of *S*-methylcysteine (28) in aqueous hydrogen peroxide was stirred for 15 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The complex e.s.r. spectrum of the resultant mixture showed several peaks, none of which could be assigned.

A solution of *S*-methylcysteine sulfoxide (57) and DBNBS (95) in aqueous hydrogen peroxide was irradiated with ultraviolet light for 2 minutes. The resultant mixture did not give rise to any e.s.r. signals.

A solution of *S*-methylcysteine hydrochloride in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 4:1 ratio of a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 13G) and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G), attributable to the adducts (121) and (96), respectively.

Reaction of 4-Methylthiobutanoic Acid (104), DBNBS (95), and Fenton's Reagent

A solution of 4-methylthiobutanoic acid (104) in aqueous hydrogen peroxide was stirred for 15 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed an 8:1 ratio of a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 13G) and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G), attributable to the adducts (107) and (96), respectively.

Reaction of 3-Methylthiopropanoic Acid (103), DBNBS (95), and Fenton's Reagent

A solution of 3-methylthiopropanoic acid (103) in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 6:1 ratio of a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 13G) and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G) (Figure 10), attributable to the adducts (108) and (96), respectively. A similar e.s.r. spectrum was obtained on repeating this experiment with the sodium salt of the acid (103).

Reaction of 3-Methylthiopropylamine (102), DBNBS (95), and Fenton's Reagent

A solution of 3-methylthiopropylamine (102) in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, a_N = 14G) of doublets (a_H = 4G), attributable to the adduct (112), along with unassigned minor signals.

Reaction of Propylamine (109), DBNBS (95), and Fenton's Reagent

A solution of propylamine (109) in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a triplet (1:1:1, a_N = 14G) of doublets (a_H = 7G), attributable to the adduct (113), along with unassigned minor signals.

Reaction of Propylamine Hydrochloride, DBNBS (95), and Fenton's Reagent

A solution of propylamine hydrochloride in aqueous hydrogen peroxide was stirred for 15 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 2:1 ratio of a triplet (1:1:1, a_N = 15G) of doublets (a_H = 11G) and a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 13G), attributable to the adducts (115) and (118), respectively.

Reaction of 3-Methylthiopropylamine Hydrochloride, DBNBS (95), and Fenton's Reagent

A solution of 3-methylthiopropylamine hydrochloride in aqueous hydrogen peroxide was stirred for 15 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 5:1 ratio of a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 13G) and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G), attributable to the adducts (118) and (96), respectively.

Reaction of 2-Methylthioethylamine (101), DBNBS (95), and Fenton's Reagent

A solution of 2-methylthioethylamine (101) in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The complex e.s.r. spectrum of the resultant mixture showed several peaks, none of which could be assigned.

Reaction of 2-Methylthioethylamine Hydrochloride, DBNBS (95), and Fenton's Reagent

A solution of 2-methylthioethylamine hydrochloride in aqueous hydrogen peroxide was stirred for 15 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 4:1 ratio of a triplet (1:1:1, a_N = 14G) of triplets (1:2:1, a_H = 14G) and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G), attributable to the adducts (119) and (96), respectively.

Reaction of Glycylmethionylglycine (58), DBNBS (95), and Fenton's Reagent

A solution of glycylmethionylglycine (58) in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 6:1 ratio of a triplet (1:1:1, a_N = 15G) of triplets (1:2:1, a_H = 12G) and a triplet (1:1:1, a_N = 15G) of quartets (1:3:3:1, a_H = 14G) (Figure 11), attributable to the adducts (124) and (96), respectively.

Reaction of Methionylleucylphenylalanine (59), DBNBS (95), and Fenton's Reagent

A solution of methionylleucylphenylalanine (59) in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture was not well resolved but a triplet (1:1:1, 15G) of triplets (1:2:1, 12G) could be seen along with a minor signal corresponding to the methyl radical adduct (96). The major signal can be attributed to the adduct of DBNBS (95) and a γ-centred methylene radical, generated from the methionine residue of the peptide (59).

Reaction of S*-Methylglutathione (60), DBNBS (95), and Fenton's Reagent*

A solution of *S*-methylglutathione (60) in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 30:8:1 ratio of a triplet (1:1:1, a_N = 13G) of doublets (a_H = 17G), a triplet (1:1:1, a_N = 14G) of triplets (1:2:1, a_H = 10G), and a signal corresponding to the methyl radical adduct (96). The major signals corresponded to DBNBS (95) adducts of a methine radical and a methylene radical, respectively. The methylene radical can be assigned as the β-centred radical generated from the *S*-methylcysteine residue of the peptide (60).

Reaction of S*-Methylglutathione Hydrochloride, DBNBS (95), and Fenton's Reagent*

A solution of *S*-methylglutathione hydrochloride in aqueous hydrogen peroxide was stirred for 10 minutes. DBNBS (95) was added followed by ferrous sulfate heptahydrate. The e.s.r. spectrum of the resultant mixture showed a 6:1 ratio of a triplet (1:1:1, a_N = 14G,) of triplets (1:2:1, a_H = 10 G) and a signal attributed to the

methyl radical adduct (96) by comparison with other spectra. The major signal corresponded to a DBNBS (95) adduct of a methylene radical which can be assigned as the β-centred radical generated from the *S*-methylcysteine residue.

N-Benzoylmethionine Sulfoxide Methyl Ester (93)

Methionine sulfoxide (49) (0.5 g, 3.0 mmol) was added to methanol (20 ml) which had been pretreated with thionyl chloride (0.35 ml, 4.6 mmol) and the resulting solution was stirred for 3 hours under anhydrous conditions. Thionyl chloride (0.35 ml) was added and the solution was stirred overnight. The solution was concentrated under reduced pressure and the residue was diluted with methanol (15 ml). The resultant solution was concentrated to dryness under reduced pressure to give crude methionine sulfoxide methyl ester hydrochloride. Potassium carbonate (0.85 g, 6.2 mmol) was added to a stirred mixture of ice (10 g) and the crude ester. The resulting solution was cooled in an ice bath while benzoyl chloride (0.45 ml, 3.9 mmol) was added and the suspension that formed was stirred at room temperature for 4 hours. The reaction mixture was extracted with dichloromethane (x4) and the combined organic extracts were washed with saturated sodium bicarbonate, dried and concentrated under reduced pressure. The residue was chromatographed on silica eluting with a gradient of methanol/ethyl acetate to yield a 1:1 mixture of diastereomers of the sulfoxide (93) as a colourless oil (0.63 g, 73%) which crystallized from ethyl acetate/light petroleum (64%), m.p. 109-113°C (Found: C, 54.9; H, 6.2; N, 4.9. $C_{13}H_{17}NO_4S$ requires C, 55.1; H, 6.1; N, 4.9%). υ_{max} 1732, 1634, 1542, 1532, 1378, 1036, 1016 cm^{-1}. ^1H n.m.r. δ 2.28-2.56, m, CH_2; 2.60, s, 0.5x3H, $SOCH_3$; 2.61, s, 0.5x3H, $SOCH_3$; 2.76, ddd, J 6.0, 7.5, 13.5 Hz, 0.5x1H, CH_2SO; 2.89, t, J 7.0 Hz, 0.5x2H, CH_2SO; 2.96, dt, J 13.5, 7.5 Hz, 0.5x1H, CH_2SO; 3.80, s, 0.5x3H, OCH_3; 3.81, s, 0.5x3H, OCH_3; 4.87-4.94, m, CH; 7.43-7.53, m, 3H, Ph; 7.69, br d, J 7.5 Hz, NH; 7.86-7.91, m, 2H, Ph. Mass spectrum (f.a.b.) *m/z* 284 ($(M+H)^+$, 68), 220 (53), 160 (10), 105 (100), 77 (10).

S-*Methylcysteine Sulfoxide (57)*

A solution of *S*-methylcysteine (28) (1.0 g, 7.4 mmol) in water (15 ml) was added to a solution of sodium metaperiodate (1.6 g, 7.4 mmol) in water (15 ml) in an ice bath. The combination was stirred until the bath warmed to room temperature. The mixture was concentrated under reduced pressure and methanol (30 ml) was added to the residue. After repeated shaking of the resultant suspension, the sodium iodate was removed by filtration. The filtrate was concentrated under reduced pressure to give an off-white solid residue which began to colour and oil on standing in air. Methanol (10 ml) was added to dissolve the coloured material and the undissolved solid was filtered off in a sealed system under nitrogen as it was not stable until completely dry. The sulfoxide (57) was recrystallized from ethanol/water as a mixture of diastereomers (0.75 g, 67%), m.p. 170-171°C (dec.). ^{1}H n.m.r. (D_2O) for one diastereomer: δ 2.80, s, CH_3SO; 3.21, dd, J 7.5, 14.0 Hz, 1H, CH_2; 3.46, dd, J 6.0, 14.0 Hz, 1H, CH_2; 4.18, dd, J 6.0, 7.5 Hz, CH; for the other diastereomer: δ 2.81, s, CH_3SO; 3.27, m, CH_2; 4.24, dd, J 4.5, 7.0 Hz, CH. Mass spectrum (f.a.b.) *m/z* 152 ($(M+H)^+$, 50), 88 (63), 75 (50), 57(63), 44(100). MIKES on 152: *m/z* 88.

4-Methylthiobutanoic Acid (104)[94]

A solution of butyrolactone (0.50 g, 5.8 mmol) and sodium thiomethoxide (0.50 g, 7.1 mmol) in dry dimethyl sulfoxide under nitrogen was stirred for four days. Hydrochloric acid (1N, 20 ml) was added and the resultant solution was extracted with dichloromethane (x3) and diethyl ether (x1). The combined organic extracts were dried, filtered, and concentrated under reduced pressure. As it was evident that the residue contained dimethyl sulfoxide, it was dissolved in diethyl ether, and the resultant solution was washed with water (x5). The organic solution was dried, filtered and concentrated under reduced pressure to give the acid (104) as a colourless liquid (0.31 g, 40%). ^{1}H n.m.r. δ 1.94, p, J 7.0 Hz, CH_2; 2.10, s, CH_3S; 2.51,

t, J 7.0 Hz, CH_2; 2.56, t, J 7.0 Hz, CH_2; 11.20, br s, CO_2H, comparable with the reported spectrum for the acid (104).[94] Mass spectrum (e.i.) *m/z* 134 (M^+, 100), 87 (35), 75 (51), 74 (84), 61 (93).

Sodium Salt of 3-Methylthiopropanoic Acid (103)

A solution of 3-methylthiopropanoic acid (103) (0.5 g, 4.2 mmol) in water was stirred while 1.0M NaOH (4.2 ml, 4.2 mmol) was added portionwise. The resultant solution was stirred at room temperature for one hour after which the solvent was removed under reduced pressure to give a colourless sodium salt.

Amine Hydrochlorides

Propylamine (109), 3-methylthiopropylamine (102), 2-methylthioethylamine (101), *S*-methylcysteine (28), methionine (26), and *S*-methylglutathione (60) were converted to their hydrochloride salts by stirring in dilute hydrochloric acid for ten minutes. The resultant solutions were concentrated under reduced pressure to give colourless salts.

Methylation of Cysteine (25) and Glutathione (122) with Trimethylphosphate (TMP)

Cysteine (25): Cysteine (25) was treated with TMP (2 equivalents), as reported.[43] The ^1H n.m.r. spectrum of the crude reaction mixture indicated, by comparison with n.m.r. spectra of authentic samples of the amino acids (25) and (28), that all of the cysteine (25) had been converted to *S*-methylcysteine (28). Resonances for the product (28) were δ (D_2O) 2.14, s, CH_3; 2.98, dd, J 7.5, 15.0 Hz, 1H, CH_2; 3.08, dd, J 4.5, 15.0 Hz, 1H, CH_2; 3.93, dd, J 4.5, 7.5 Hz, CH.

Glutathione (122): A solution of glutathione (122) (0.5 g, 1.6 mmol) and TMP (0.38 ml, 3.2 mmol) in water (15 ml) was stirred at 40°C for 22 hours. As reported for methylation of cysteine (25) using TMP,[43] aqueous sodium hydroxide (1.5N) was periodically added to the solution to maintain approximately neutral pH. The mixture was concentrated under reduced pressure to give an ~1:1 mixture of *S*-methylglutathione (60) and a compound assigned as glutathione disulfide. *S*-Methylglutathione (60) was identified by comparison of t.l.c. properties and ^{1}H n.m.r. spectral data of the reaction mixture with those of an authentic sample of the peptide (60). Integrals in the ^{1}H n.m.r. spectrum of the reaction mixture showed that not all of the resonances for glycine and glutamic acid residues arose from *S*-methylglutathione (60). There were also resonances corresponding to the β- and α- protons of a cystine residue: δ (D_2O) 2.94, dd, J 9.5, 14.5 Hz, 2H, β-CH; 3.28, dd J 4.5, 14.5 Hz, 2H, β-CH; 4.74, dd J = 4.5, 9.5 Hz, 2H, α-CH. The complex f.a.b. mass spectrum of the reaction mixture contained a peak at *m/z* 344 for the molecular ion plus sodium of *S*-methylglutathione (60) and smaller peaks at *m/z* 612 and 635 which can be assigned to the molecular ion of glutathione disulfide and this molecular ion plus sodium, respectively.

When the above reaction was repeated in aqueous 0.1M pH 7.0 phosphate buffer, the same result as above was obtained.

S-*Methylglutathione Sulfoxide*

Commercial *S*-methylglutathione (60) (20 mg, 0.06 mmol) was added to a solution of sodium periodate (14 mg, 0.07 mmol) in water (0.2 ml) in an ice bath. The resultant suspension was allowed to warm to room temperature and stirred for 20 hours. The mixture was concentrated under reduced pressure to give colourless solid material which was assigned as a mixture of *S*-methylglutathione sulfoxide and sodium iodate. The ^{1}H n.m.r. spectrum of the solid indicated that no starting material (60) remained and showed resonances for glycine and

glutamic acid residues. ^{1}H N.m.r (D_2O) resonances indicative of the formation of a 1:1 mixture of diastereomers of *S*-methylglutathione sulfoxide were two singlets at δ 2.75 and 2.76 for the methylsulfoxy protons, and four doublets of doublets at δ 3.19 (8.5, 13.5 Hz), 3.24 (11.0, 13.5 Hz), 3.35 (4.0, 13.5 Hz), and 3.45 (5.5, 13.5 Hz) for the β-protons of an *S*-methylcysteine sulfoxide residue. The f.a.b mass spectrum of the reaction mixture showed peaks at *m*/z 338 and 360 for the protonated molecular ion and the molecular ion plus sodium, respectively, of the product sulfoxide.

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