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^{13}C-NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AS A PROBE OF ENZYME ENVIRONMENT—II[1]

EFFECT OF SOLVENT AND pH ON ^{13}C-CHEMICAL SHIFTS IN DERIVATIZED AMINO-ACID MODELS

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Abstract—The synthesis and characterization of side-chain derivatives of amino- and carboxy-protected lysine, serine and cysteine, and of two tripeptides is reported. Broad-band proton-decoupled ^{13}C-nuclear magnetic resonance spectra have been determined and in almost all cases, each carbon resonance has been unambiguously assigned by a combination of off-resonance and specific decoupling techniques. The effect of solvent and pH on chemical shifts is discussed. The objective of these studies is to provide models relevant to the use of ^{13}C-labelled electrophilic inhibitors as probes of enzyme active-site environment.

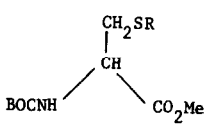
In recent years, much attention has been paid to the uses of ^{13}C-nuclear magnetic resonance spectroscopy (CMR) in structural studies of proteins.[2] These investigations have been assisted by ^{13}C-chemical shift data obtained by studying model amino-acids,[3] and oligopeptides[4] at various pH-values.

A major method used to study the mechanism of the catalytic action of enzyme systems is the covalent labelling of active-site functional groups by reaction with appropriate electrophilic irreversible inhibitors.[5] Degradative and separative procedures must then be developed to locate and identify the specific amino-acids which have reacted. For unambiguous identification, synthetic model derivatives are then required for comparison,[6] and for structurally-complex active-site directed inhibitors such amino-acid derivatives represent a considerable synthetic effort.

We report here the results of a systematic CMR investigation of derivatized amino-acids soluble in either organic or aqueous phases. The study has been carried out in the course of our investigations of enzyme active-site environment using ^{13}C-labelled electrophilic inhibitors, (e.g. Br^{13}CH$_2$CO$_2$H).[7] In order to enable unambiguous interpretation of the spectra of the resulting covalently-labelled enzyme, a knowledge of changes in chemical shift values in varying solution conditions is required. Information can then be provided on reactive catalytic functional groups at the active site without the need for full degradative and separative procedures. Indeed, we have shown with bovine pancreatic ribonuclease A that the specificity and sequence of functional-group alkylation can be followed over a time period by experiments performed in an NMR tube.[8] Furthermore, the effects of denaturation and pH on the ^{13}C NMR spectrum provide additional information on the native enzyme active-site environment.

We have chosen to synthesise a range of derivatised amino-acids to test the sensitivity of key CMR chemical shifts to a wide range of conditions, including the effects of pH change on zwitterionic species, and solvent variation. Observations were extended to include measurements on tripeptides. The results with these models show that it is generally possible to distinguish successfully between reaction at thiol-, hydroxy-, amino-, and imidazolyl functions in the enzymatic context. Furthermore, observation of the effect of pH on the key carbon resonance frequently enables determination of the state of ionization of a zwitterionic species of a polybasic acid.

Model Derivatives Soluble in Organic Solvents. The four amino-acids which formed the basis of this study were L-cysteine, L-histidine, L-lysine and L-serine, chosen to provide examples of the thio-, imidazo-, amino- and hydroxy-functionalities which are so frequently involved in enzymatic catalysis. The amino-terminus of each was protected as the t-butyloxycarbonyl (BOC) derivative and the carboxyl-terminus esterfied as the methyl ester (i.e. **1a**, **2a**, **3a**, **4a**) by combinations and modifications of conventional methods fully described in the Experimental Section. Such derivatisation provides compounds which are soluble in most organic solvents, thus allowing spectroscopic determinations over a range of solution conditions. Furthermore, the derivatising groups provide the added advantage of appearing as



1

a: R = H
b: R = CH_2CH_2CN
c: R = $CH_2CH_2CONH_2$
d: R = $CH_2CH_2CO_2Me$
e: R = CH_2CONH_2
f: R = CH_2CO_2Me
g: R = Me

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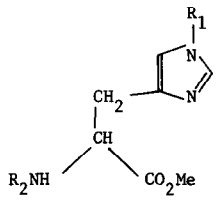
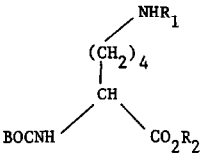


2

a: R_1 = H; R_2 = BOC
b: R_1 = CH_2CH_2CN; R_2 = BOC
c: R_1 = CH_2CH_2CN; R_2 = H

[Nb. differentiation of Nτ and Nw substitution is discussed in the text]



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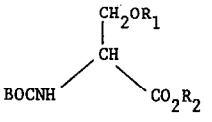
a: R_1 = H; R_2 = Me
b: R_1 = CH_2CH_2CN; R_2 = Me

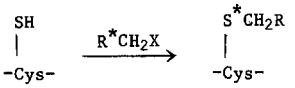


4

a: R_1 = H; R_2 = Me
b: R_1 = CH_2CH_2CN; R_2 = Me

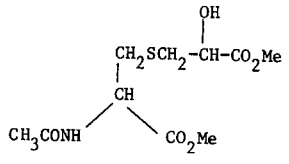
single lines in the PMR spectrum, and are uncomplicated in the CMR spectrum. This considerably facilitates full spectroscopic characterisation of the derivative and additionally provides good internal references for proton integration values, thus allowing a simple assay of the purity of transformation products.

The key carbon resonance of interest in the context of probing the mechanism of enzymatic catalysis in inhibitor studies is that adjacent to a reacting amino-acid side chain functional group e.g. with cysteine:



where R^*CH_2X = $BrCH_2CONH_2$
$BrCH_2COOH$
CH_2 = CH-CN
CH_2 = CH-$CONH_2$

In order to probe the sensitivity of this key resonance (i.e. C*) to chemical environment in the absence of complications due to ionization in zwitterionic structures, the protected derivatives (**1a**, **2a**, **3a**, **4a**) were reacted with a number of potentially useful electrophilic aklylating agents. These included acrylonitrile, methyl acrylate, acrylamide, β-bromopropionic acid, iodoacetic acid and iodoacetamide. The widest range of derivatives was prepared in the cysteine series by reactions of **1a**, which provided derivatives (**1b–1g**, and also **5** in a related series) showing widely different side-chain moieties. The effect of such changes in sub-structure on the key-resonance can only be fully resolved by examining a series of compounds in which the remainder of the molecule remains constant. Additional compounds in the histidine, lysine and serine series were prepared by a similar reaction of **2a**, **3a** and **4a** with acrylonitrile to afford respectively the cyanoethylated derivatives **2b**, **3b** and **4b**. The effects of changing the heteroatom on the key resonance (–S–*CH_2–, –NH–*CH_2–, –Im–*CH_2– and –O–*CH_2) were investigated in this series. In order to compare the key resonance in the cyanoethylated derivative **1b** to a model which bore a close structural relationship to an enzyme, the tripeptide t-butoxycarbonylglycyl (s → S-(2-cyanoethyl))-L-cysteinylglycine ethyl ester **6** was prepared by the method shown in Fig. 1.



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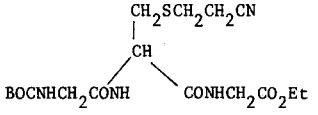


6

CMR spectra of model derivatives in organic solvents. Table 1 and Fig. 1 summarise the CMR data recorded in organic solvents for all model derivatives of this type, and appropriate intermediates used in synthetic schemes. In each case, both proton noise decoupled spectra and off-resonance decoupled spectra were determined. This information together with comparison of chemical shift-values within a species allowed unambiguous assignment of all resonances to specific carbon atoms. In certain cases, further NMR experiments (e.g. specific proton decoupling) were necessary in order to fully interpret the spectra. For example, the two glycine methylene carbon atoms in the tripeptide **6** (Fig. 1) were attributed to resonances at 44.69 δ and 41.54 δ. They were individually assigned by specific frequency proton decoupling by low power irradiation of the appropriate methylene protons in the proton spectra (irradiation at 380 Hz (Boc-Gly-methylene) downfield from TMS resulting in decoupling the triplet centred at 44.69 δ in the CMR spectrum).

Another useful technique in this context is the observation of the magnitude of the apparent coupling constant in the off-resonance decoupled spectrum (J_{app}). For

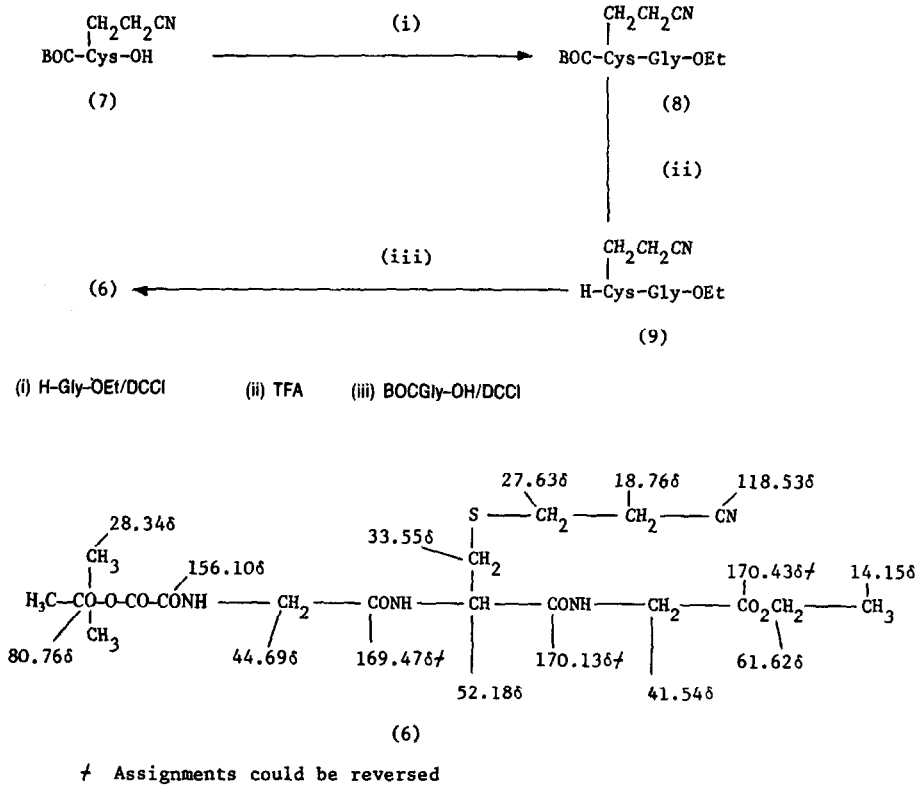


Fig. 1. Preparative route to **6** *and CMR assignments.*

example in the S-carbamoylmethyl derivative **1e** the two methylene carbon atoms adjacent to the sulphur atom were observed 36.04 and 35.54 δ. The magnitude of J_{app} in off-resonance decoupled spectra is proportional to the difference (Δf) between the proton resonance frequency and the decoupler frequency, and inversely proportional to the power of the decoupling irradiation.[9] In the PMR spectrum of **1e**, the SCH_2CO proton resonance was at lower field (i.e. larger Δf) than the $-CH-CH_2-S$ resonance. Therefore, in the off-resonance decoupled CMR spectrum the triplet at 35.54 δ (J_{app} = 70 Hz) can be assigned to the $-CH-CH_2-S$ carbon whilst that at 36.04 δ (J_{app} = 80 Hz) to the $S-CH_2-CO$ carbon atom.

A number of general trends observed for the chemical shift variation are apparent from examination of Table 1. Firstly, the resonances common to all amino-acid derivatives are largely invariant to within 1 ppm throughout a series irrespective of side chain derivatisation (e.g. **1b–1g**). Not surprisingly, the C-3 carbon displays the greatest chemical shift sensitivity. However, the point of crucial interest to this study is the sensitivity of the side-chain resonances throughout a series, and in particular the key resonance adjacent to a heteroatom. The three derivatives **1b**, **1c** and **1d** having a $-S-*CH_2-CH_2-R$ moiety show this resonance at a consistent value viz 28.27, 28.34 and 27.67 δ respectively indicating that the effect of R is not overwhelming. However, in cases where the R- functionality is β- to sulphur (i.e. $-S-*CH_2-R$), e.g. **1e**, **1f** and to a lesser extent **5**, there is much greater variation in value. From the viewpoint of identification of a reacting functionality in an enzyme system, these results suggest that the introduction of a $-*CH_2CH_2-R$ moiety (e.g. by Michael addition of an acrylate derivative) would provide the most definitive information.

Table 2 provides a comparison of key resonance (C*) chemical shift values obtained for the four cyanoethyl adducts prepared. The large difference in chemical shift of the C*-resonance in this series of compounds should allow the reacting side-chain functionality in an enzyme to be easily determined. It is also encouraging to note that there is a significant difference between the C* chemical shift values for the two nitrogen derivatives **2b** and **3b**.

However, in order to further limit any ambiguity, experiments were performed to examine the effect of solvent and pH on these key resonances. This required the study of unprotected, side-chain modified amino-acid derivatives.

Water-soluble derivatives. Side-chain derivatized zwitterionic derivatives of cysteine, histidine, lysine and methionine were prepared, in general, by direct reaction of the free amino-acid with the appropriate electrophilic reagent. In the case of cysteine, direct reaction with acrylonitrile, iodoacetamide and iodoacetic acid under suitable conditions produced good yields of **10**, **10b** and **10c**, respectively. In the histidine series, the cyanoethyl derivative **11a** was prepared from the protected derivative **2b** by N-terminus deprotection using anhydrous TFA to afford **2c**, followed by acid-catalysed ester saponification performed in an NMR tube with dilute aqueous hydrochloric acid. However, the carbonyl methyl derivative **11b** and the bis-carboxymethyl derivative **12** were prepared by reaction of N_α-acetyl-L-his-

Table 1. Carbon chemical shift values for organic-soluble amino-acid derivatives

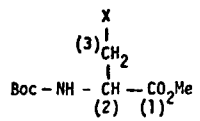
Compound	Chemical shift δ[a]													
	Resonances Common to all Amino-acid Derivatives							Side-Chain Resonances "X"						
L-Cysteine	C-1	C-2	C-3	$(CH_3)_3C$	$(CH_3)_3C$	NHC=O	OCH_3	SCH_2	SCH_2CH_2	CN	C=O	OCH_3	SCH_3	CH
Boc-Cys-Ome (1a)	170.79[b] s	54.98 d	27.30 t	28.27 q	80.33 s	155.07 s	52.64 q							
CH_2CH_2CN Boc-Cys-Ome (1b)	171.05 s	53.49 d	34.57 t	28.27 q	80.45 s	155.00 s	52.77 q	28.27 t	18.27 t	117.89 s				
$CH_2CH_2CONH_2$ Boc-Cys-Ome (1c)	171.63 s	53.68 d	34.83 t	28.34 q	80.40 s	155.26 s	52.64 q	28.34 t	36.00 t		173.72 s			
$CH_2CH_2CO_2Me$ Boc-Cys-Ome (1d)	171.38 s	53.49 d	34.70† t	28.34 q	80.26 s	155.07 s	52.51 q	27.67 t	34.57† t		172.03 s	51.80 q		
CH_2CONH_2 Boc-Cys-Ome (1e)	171.45 s	53.44 d	35.54 t	28.32 q	80.58 s	155.42 s	52.80 q	36.04 t			171.45 s			
CH_2CO_2Me Boc-Cys-Ome (1f)	171.38 s	53.29 d	35.03 t	28.34 q	80.35 s	155.06 s	52.58† q	33.86 t			170.47 s	52.45† q		
CH_3 Boc-Cys-Ome (1g)	171.70 s	53.30 d	36.90 t	28.34 q	80.20 s	155.13 s	52.51 q						16.25 q	
$CH_2CH(OH)CO_2Me$ Boc-Cys-Ome (5)	171.35 s	52.76 d	35.32 t	23.03[c] q		173.33 s	52.35 q	36.90 t			170.42 s	52.35 q		70.35 71.05
L-Histidine								C'-2	C'-4	C'-5	NCH_2	CH_2CN	CN	
Boc-His-Ome (2a)	172.74 s	53.88 d	29.83 t	28.34 q	80.07 s	155.65 s	52.32 q	135.37 d	134.07 s	116.14 d				
CH_2CH_2CN Boc-His-Ome (2b)	172.55 s	56.68 d	30.35 d	28.40 q	79.74 s	155.59 s	52.19 q	136.87 d	138.75 s	116.27 d	42.57 t	20.54 t	116.72 s	

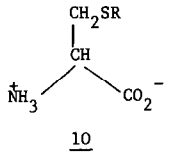
L-Lysine								C-4	C-5	C-6	NCH_2	$\underline{CH_2CN}$	CN
Boc-Lys-Ome (3a)	173.39 s	53.29 d	28.92 m	28.40 q	79.87 s	155.91 s	52.91 q	22.68 t	32.30 t	42.11 t			
CH_2CH_2CN Boc-Lys-Ome (3b)	173.26 s	53.49 d	29.44 t	28.34 q	79.94 s	155.39 s	52.19 q	22.94 t	32.63 t	48.81 t	45.10 t	18.72 t	118.61 s
L-Serine								OCH_2	$\underline{CH_2CN}$	CN			
Boc-Ser-ome (4a)	171.50 s	56.02 d	63.30 t	28.34 q	80.39 s	155.85 s	52.58 q						
CH_2CH_2 Boc-Ser-OmeCN (4b)	171.10 *	53.70 *	71.36 t	28.33 q	80.26 s	155.37 *	52.40 *	65.96 t	18.85 t	119.00 s			

(a) chemical shift downfield from TMS in $CDCl_3$ solution.

(b) multiplicity in off-resonance proton decoupled spectra
s singlet d doublet
m multiplet, etc.

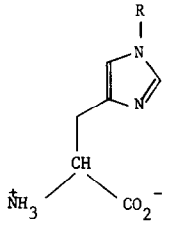
(c) acetyl amino group.
* not observed
† assignment could be reversed.





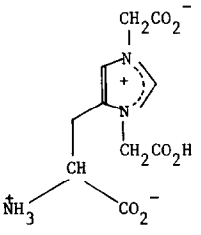
10

a: R = CH_2CH_2CN
b: R = CH_2CONH_2
c: R = CH_2CO_2H



11

a: R = CH_2CH_2CN
b: R = CH_2CO_2H



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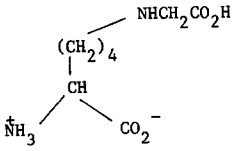

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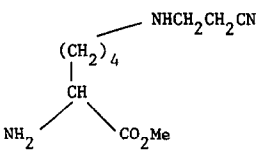

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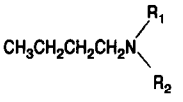


15

a: R_1 = CH_2CH_2CN; R_2 = H
b: R_1 = R_2 = CH_2CH_2CN

Table 2. *C-Chemical shift values for organic-soluble cyanoethyl derivatives

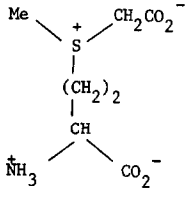
Functionality	*C Chemical Shift δ
S-*CH_2CH_2CN (1b)	28.27
N_{imid}-*CH_2CH_2CN (2b) [a]	42.57
N_{amino}-*CH_2CH_2CN (3b)	45.10
O-*CH_2CH_2CN (4b)	65.96

[a] It is not possible to say whether this is the Nτ or Nπ - monocyanoethyl derivative from spectroscopic evidence. However, literature precedent suggests this to be the Nτ - adduct.

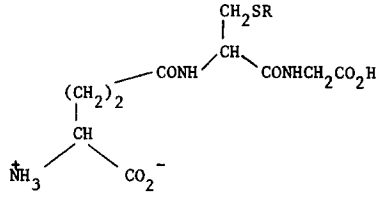
tidine with iodoacetic acid, essentially by the method of Crestfield.[10] N_ϵ-carboxymethyl lysine **13a** was prepared by direct alkylation of the appropriate cupric salt with iodoacetic acid followed by destruction of the resulting complex in aqueous suspension with H_2S. However, difficulty was experienced in the preparation of a cyanoethylated derivative, both by deprotection of **3b** and by direct alkylation of the cupric salt. The former method was complicated by the inherent instability of the deprotected amino-ester derivative **14**. As an alternative series of models, n-butyl-amine was cyanoethylated by reaction with neat acrylonitrile at 85°, affording both the monocyanoethylated n-butylamine **15a**,[11] and the dicyanoethyl product **15b**, purified by distillation. In addition to the comparative data provided by these derivatives in acidic aqueous solution, both were additionally soluble in organic solvents, thereby allowing direct comparison of NMR data in aqueous and non-aqueous media. L-Methionine at pH 4.5 was reacted with a 5 molar excess of iodoacetic acid for 24 hr at 25° to afford the S-carboxymethylsulphonium salt **16**.

Finally, in order to examine these effects in models which were more representative of enzyme structure, the tripeptides S(2-cyanoethyl)-glutathione **17a** and S-carboxymethyl-glutathione **17b** were prepared by reaction of glutathione with acrylonitrile and bromoacetic acid respectively in aqueous solution.

CMR spectra of water-soluble derivatives. Table 3 summarises the CMR data recorded in aqueous solution for the water-soluble derivatives. Again, all carbon resonances have been unambiguously assigned with the aid of partially-decoupled spectra. A good example of



16



17

a: R = CH_2CH_2CN
b: R = CH_2CO_2H

the use of off-resonance and fully noise decoupled spectra in which a tightly coupled system is observed is afforded by those obtained for S-(2-cyanoethyl)-L-cysteine **10a** at pH 7.0 (Fig. 2). Resonances at 174.24, 122.06 (not shown), 55.24 and 19.83 δ were assigned directly from chemical shift considerations to carbonyl, cyanide, methine and $-\underline{CH_2}CN$ carbons respectively. Two resonances at 33.67 and 28.54 δ were assigned to the $-CH_2SCH_2-$carbons. The off-resonance decoupled spectrum enabled individual assignation. Figure 2(a) shows the high field section of this spectrum. The off-resonance decoupled spectrum (Fig. 2(b)) contains a doublet assignable to the methine carbon, a triplet attributable to a methylene carbon, and two other resonances which appear as triplets with additional multiplicity of the outer lines. In the PMR spectrum of **1b**, the $-SCH_2CN_2CN$ protons form a tightly coupled system (the resonance is observed as a broad singlet as a result of the nearly identical chemical shift values for the two sets of protons). Therefore, the off-resonance decoupled CMR spectrum would be expected to show second-order coupling effects for the carbon nuclei which have directly attached tightly coupled protons.[12] The resonances at 28.54 and 19.83 δ can therefore be assigned to $-S\underline{CH_2}CH_2CN$ and $-\underline{CH_2}-CN$ carbons respectively, whilst the resonance at 33.67 δ is attributable to the $-CH\underline{CH_2}S-$ carbon.

S-Carboxymethyl-L-cysteine **10c** is only sparingly soluble in water at pH 7.0. Therefore, CMR spectra were recorded both in sodium hydroxide at pH 14.0 and in hydrochloric acid at pH 1.0. At pH 14.0 the spectrum showed resonances at 55.83, 38.64 and 38.02 δ assignable to methine, methylene and methylene carbon respectively. The two methylene resonances ($-CH_2SCH_2-$) were unambiguously assigned by the magnitude of J_{app} in

Table 3. Carbon chemical shift values for water-soluble amino-acid derivatives

Compound**	Ph	Chemical Shift δ[(a)] Common Resonances						Side-Chain Resonances "X"					
L-Cysteine		C-1	C-2	C-3	SCH_2	$\underline{CH_2}CN$	CN	C=O					
$\underline{CH_2}CH_2CN$ Cys (10a)	7.0	174.24[(b)] s	55.24 d	33.67 t	28.54 t	19.83 t	122.06 s						
$\underline{CH_2}CH_2CN$ Cys (10a)	14.0	182.37	56.68	38.47	28.41	19.80	122.20						
$\underline{CH_2}CONH_2$ Cys (10a)	1.0	171.90	54.01	32.83	28.86	19.96	121.99						
$\underline{CH_2}CONH$ Cys (10b)	1.0	170.86 s	52.97 d	32.92 t	35.74 t								
$\underline{CH_2}CO_2H$ Cys (10c)	14.0	*	55.83 d	38.64 t	38.02 t			*					
$\underline{CH_2}CO_2H$ Cys (10c)	1.0	171.03†	52.92	32.80	34.73			174.95†					
L-Histidine					C'-2	C'-4	C'-5	N_τ-CH_2	N_π-CH_2	$\underline{CH_2}CN$	CN	C=O	OCH_3
$\underline{CH_2}CH_2CN$ His-OMe (2c)	7.0	174.52 s	55.67 d	29.47 t	*	*	119.77 d	43.14 t		20.49 t	119.77 s		54.12 q
$\underline{CH_2}CH_2CN$ His (11a)	1.0	170.33 s	52.72 d	25.94 t	136.50 *	128.57 *	122.03 *	45.77 t		20.02 t	118.74 *		
$\underline{CH_2}CO_2H$ His (11b)	4.5	173.40† s	54.35 d	26.72 t	136.91 d	128.81 s	122.54 d	52.66 t	50.52 *			172.09† s	
$\underline{CH_2}CO_2H$ His (11b)	14.0	183.41 s	57.03 d	34.00 *	139.14 d	138.34 s	199.48 d	50.92 t				176.52 s	
$\underline{CH_2}CO_2H$ His (11b)	1.0	170.82† s	52.53 d	25.85 t	137.57 d	127.92 s	123.12 d	50.69 t				170.66† s	
$(\underline{CH_2}CO_2H)_2$ His (12)	4.0	172.47† s	53.13 d	25.13 t	139.27 d	130.41 s	123.26 d	52.18 t	50.10 t			171.98† s	
$(\underline{CH_2}CO_2H)_2$ His (12)	1.0	*	51.83 d	24.53 t	140.10 *	130.16 *	123.73 *	51.02 t	48.75 t			*	
L-Lysine					C-4	C-5	C-6	NCH_2	C=O				
$\underline{CH_2}CO_2H$ Lys (13a)	7.2	175.50†	55.34	25.98	22.36	30.71	47.86	50.03	172.24†				
$\underline{CH_2}CO_2H$ Lys (13b)	1.0	172.57†	53.33	25.74	22.26	29.98	47.97	47.00	172.57†				
L-Methionine					C-4	SCH_2	SCH_3	C=O					
$\underline{CH_2}CO_2H$ Met (16)	7.0	173.34 s	53.70 d	26.14	38.38 t	48.34 t	24.57 q	169.03 s					
$\underline{CH_2}CO_2H$ Met (16)	1.0	172.30 s	52.91 d	25.83 t	38.28 t	47.78 t	24.70 q	168.80 s					

(a) chemical shift references to dioxan = 67.4 δ

(b) multiplicity in off-resonance proton decoupled spectra s singlet, d doublet etc.

(c)* not observed † assignments could be reversed.

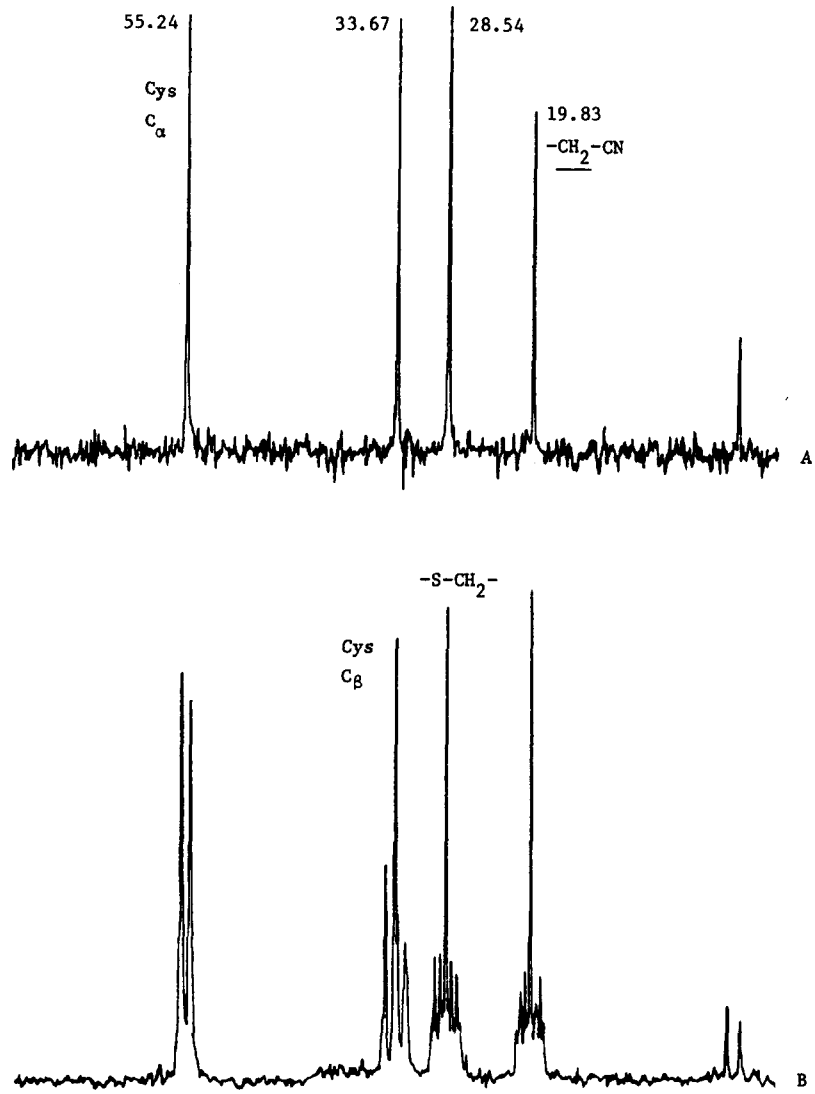
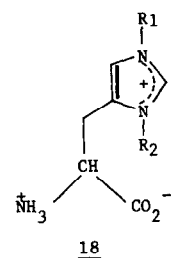


Fig. 2. CMR spectra of S-(C-cyanoethyl)-L-cysteine **10a** at pH 7.0. (High Field Region Only) A: Proton-noise decoupled spectrum; 1024 pulses, 4 sec recycle time. B: Off-resonance decoupled spectrum; 58,300 pulses, 1 sec recycle time.

the off-resonance decoupled spectrum.[9] The $-CHCH_2S-$ protons in the PMR spectrum are at higher field (smaller Δf) than the $-SCH_2CO-$ protons. Consequently, the triplet centred at 38.64 δ ($J_{app} = 70$ Hz) in the off-resonance decoupled CMR spectrum can be assigned to the $-\underline{C}HCH_2S-$ carbon, whereas, the triplet centred at 38.02 δ ($J_{app} = 80$ Hz) can be attributed to the $-S\underline{C}H_2CO-$ carbon nucleus.

Functionalization of histidine moieties is complicated by the possibility of reaction at either or both of the heteroaromatic N-atoms ($N\tau$ and $N\pi$). The major reaction product has spectroscopic properties which are to be attributed to the zwitterionic N^{τ}-isomer (18τ).[13] Whereas the CMR spectrum of (18τ) in water at pH 4.5 showed a resonance at 52.66 δ assignable to the C*-resonance (NH_2CO), a very weak resonance at 50.52 δ could be attributed to the C* resonance of the $N\pi$-isomer (18π) obtained as a minor product. Nigen *et al*[14] have reported these two chemical shift values at 51.5 and 49.7 δ respectively at pH 6.2 for the same compounds. Clearly, the shift on these resonances will be sensitive to the state of ionization of the whole molecule, and these effects will be discussed below in terms of pH variance.



18

π: $R_1 = H$; $R_2 = CH_2CO_2^-$

τ $R_1 = CH_2CO_2^-$; $R_2 = H$

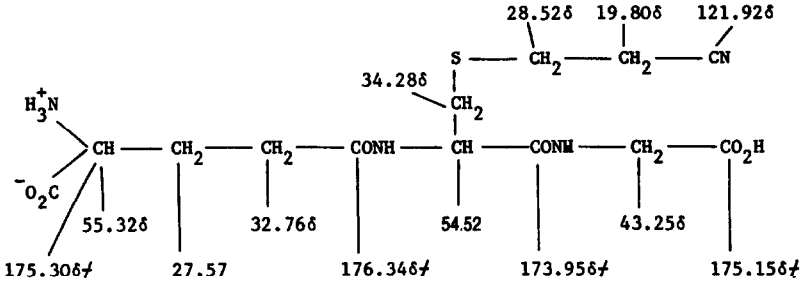
Due to problems experienced in preparation and separation of mono- and dicyanoethylated L-lysine derivatives, coupled with their inherent instability, n-butylamine was employed as a simple model in order to obtain further information of the C* chemical shift values for mono- and dicyanoethylated amines in aqueous media. Table 4 summarises the chemical shift values obtained. Figure 3 shows the CMR chemical shift values, and the assignments of S-(2-cyanoethyl) glutathione **17a** dissolved in D_2O at pH 4.1. The spectrum was assigned by comparison with a published and fully interpreted spectrum of reduced glutathione.[15] Resonance positions numbered 1 and 2 on Fig. 4(a) were tentatively assigned as $CysC_\alpha$ and $GluC_\alpha$ respectively, by direct comparison with the spectrum of gluthione. However, specific frequency decoupling at 380 Hz downfield from TSP in the 100 MHz PMR spectrum at the $GluC_\alpha$ proton, produced two singlets (1 and 3 in Fig. 4b) in the CMR spectrum, with the remainder of the resonances being observed as partially coupled multiplets. Unexpectedly, resonance 1 can therefore be unequivocally assigned to $GluC_\alpha$, and resonance 2 to $CysC_\alpha$, reversing the initial assignments. This clearly underlines the precautions that must be taken for unambiguous assignment of CMR spectra. Resonance 3 (Fig. 4b) was also a singlet in the specific frequency decoupled spectrum, because the two proton resonances $GlyC_\alpha$ and $GluC_\alpha$ are within 10 Hz of each other in the PMR spectrum, and the power required to fully decouple resonance 1 also decoupled resonance 3. The initial $CysC_\alpha$ and $GluC_\alpha$ assignments made by Jung *et al.*[15] were checked on reduced glutathione by the same specific decoupling technique, and were confirmed.

This technique was also used to unambiguously assign the $GluC_\beta$ and $-SCH_2CH_2CN$ resonances. Low power irradiation at 248 Hz downfield from TSP in the 100 MHz PMR spectrum at the resonance position of the $-SCH_2CH_2CN$ protons, produced two singlets at 28.52 and 19.80 δ in the CMR spectrum able to be unambiguously assigned to $-SCH_2CH_2CN$ and $-CH_2CN$ carbon nuclei respectively. Therefore, the resonance at 27.57 δ was attributable to $GluC_\beta$. Thus the C* chemical shift ($-SCH_2CH_2CN$) was 28.52 δ. This is in close agreement with the value of 28.54 δ obtained for the equivalent cysteine derivative (**10a**).

Table 4. Carbon chemical shift values for butylamine and the cyanoethyl derivatives

Compound	Solvent†	Chemical Shift δ							
		CH_3	CH_2	CH_2	CH_2	N	CH_2	CH_2	CN
$BuNH_2$	A	14.18	20.38	36.52	42.23				
$BuNH_2$	B	14.13	20.48	35.52	41.50				
$BuNH_2$	C	13.91	19.96	29.62	40.37				
$BuNHCH_2CH_2CN$ (15a)	Neat	14.28	20.73	32.58	49.23		45.63	18.91	119.55
$BuNHCH_2CH_2CN$ (15a)	A	14.38	20.82	32.64	49.26		45.67	19.00	119.44
$BuNHCH_2CH_2CN$ (15a)	C	14.79	20.03	28.23	48.61		43.54	15.79	118.44
$BuN(CH_2CH_2CN)_2$ (15b)	A	14.34	20.64	29.93	53.47		49.80	17.04	119.59
$BuN(CH_2CH_2CN)_2$ (15b)	C	13.87	19.97	25.69	54.17		49.12	14.26	118.32

† A = $CDCl_3$; B = D_2O pH 7.0; C = D_2O/HCl pH 1.0



/ Assignments could be reversed.

$CysC_\alpha$ refers to the methine carbon of cysteine, $CysC_\beta$ refers to the methylene adjacent to the thiol in cysteine etc.

Fig. 3. CMR assignments of S-(2-cyanoethyl)-glutathione **17a**.

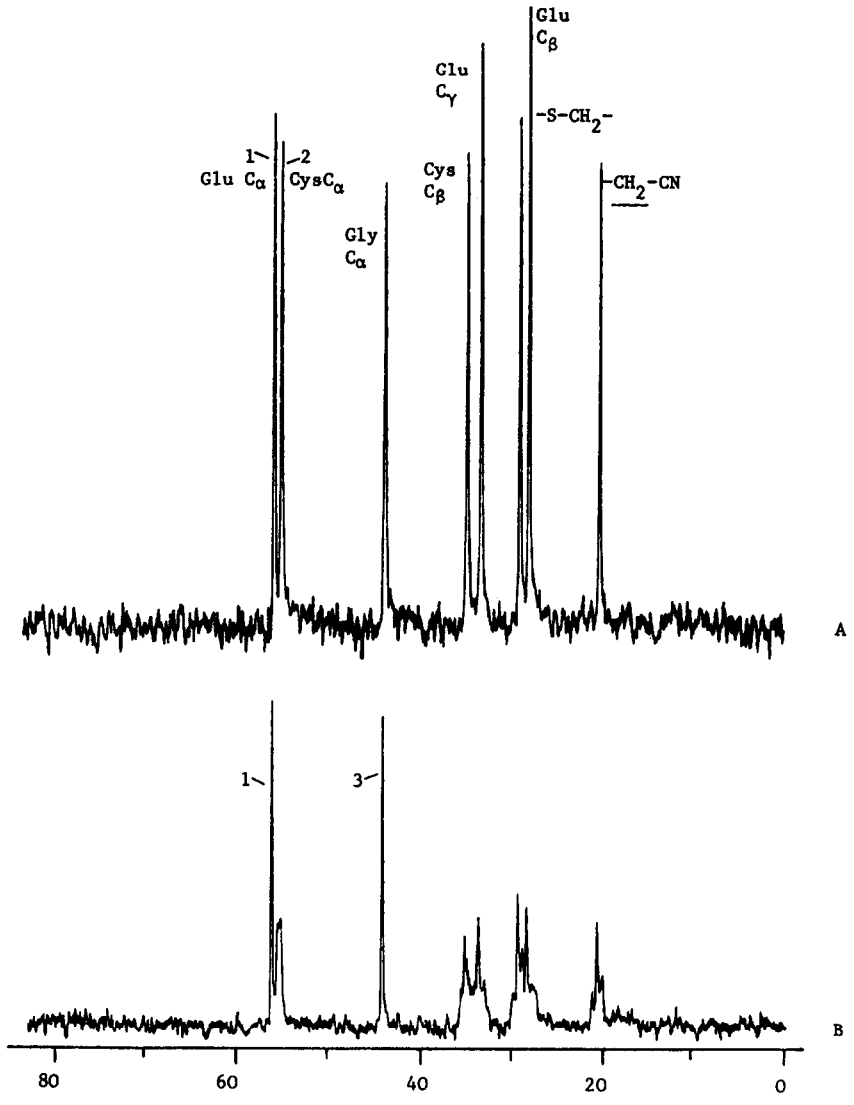


Fig. 4. CMR spectra of S-(2-cyanoethyl)-glutathione **17a** at pH 4.1. A: Proton-noise decoupled spectrum; 1574 pulses, 0.8 sec recycle time. B: Specifically decoupled spectrum at 380 Hz downfield from TSP in the 100 MHz PMR spectrum; 926 pulses, 0.8 sec recycle time.

Comparison of C chemical shifts.* The key C* resonance has been unambiguously assigned in all cases and a comparison of the C* values obtained for the equivalent organic- and water-soluble derivatives within a series is presented below.

Effect of solvent on the C chemical shift.* The C* chemical shift values for the organic-soluble S-cyanoethyl derivative **1b** and its water-soluble equivalent **10a** at pH 7.0 are 28.27 and 28.54 δ respectively. This same resonance in S-(2-cyanoethyl) glutathione **17a** at pH 7.3 is observed at 28.52 δ. These values are all in close agreement. The C* value for the organic-soluble tripeptide **6** is 27.63 δ, indicating a minor but significant difference which may be due to an orientation, or steric effect causing a slight upfield shift.

The C* chemical shift values of the cyanoethylated histidine derivatives are observed at 42.57 δ for the organic-soluble derivative (**2b**), and at 43.14 δ for the water-soluble compound **11a** at pH 7.0. These values are again in good agreement.

The water-soluble S-carboxymethyl-L-cysteine derivative (**10a**) was not sufficiently soluble in water at pH 7.0 to allow determination of the CMR spectrum. In base, the C* chemical shift of (**10c**) was observed at 38.02 δ and in acid at 34.73 δ, reflecting the state of ionization of the carboxyl group. The equivalent carbon resonance in the S-carboxymethyl glutathione derivative **17b** at pH 7.0 was at 37.92 δ consistent with an ionized carboxyl group in a zwitterionic structure. As expected, there is agreement between the C* resonance value obtained for **10c** in acid (34.73 δ) and the organic-soluble derivative **14** (33.86 δ), both values reflecting the shift of a non-ionized carboxyl group.

Cyanoethyl derivatives. The values obtained for the C* chemical shift (Table 5) in this series of compounds at pH 7.0 allows a clear differentiation between electrophilic reaction at sulphur and nitrogen. A tentative assignment can also be made between different nitrogen functionalities *viz* amino and imidazole moieties. The C* resonance adjacent to an imidazole functionality shifts

Table 5. *C-Chemical shift values for water-soluble cyanoethyl derivatives at different pH values

Functionality		*C Chemical Shift δ		
		pH 7.0	pH 1.0	pH 14.0
S-*CH_2CH_2CN	(10a)	28.54	28.86	28.41
N_{imid}-*CH_2CH_2CN	(11a)	43.14	45.77	
N_{amino}-*CH_2CH_2CN	(15a)	45.67[a]	43.54	

a: in $CDCl_3$ solution

Table 6. *C-Chemical shift values for water-soluble carboxymethyl derivatives at different pH values

Functionality		*C Chemical Shift δ		
		pH 1.0	pH 7.0	pH 14.0
S-*CH_2CO_2H	(10c)	34.73		38.02
-$\overset{+}{S}(CH_3)$*CH_2CO_2H	(16)	47.78	48.34	
τ-N_{imid}-*CH_2CO_2H	(18a)	50.92	52.66[a]	50.69
π-N_{imid}-*CH_2CO_2H	(18b)		50.52[a]	
N_{imid}-(*CH_2CO_2H)$_2$	(12)	51.02 48.75	τ 52.18[b] π 50.10[b]	
N_{amino}-*CH_2CO_2H	(13a)	47.00	50.03	

a: at pH 4.5; b: at pH 4.0

downfield to 45.77 δ in acidic solution, whereas an upfield shift to 43.54 δ occurs when adjacent to an amino group, thus enabling differentiation. The chemical shift of the key resonance adjacent to a thio-ether moiety will be unaffected by changes in solution pH, thus providing a simple asignment of this functionality.

Carboxymethyl derivatives. Table 6 summarises the C* chemical shift values for the carboxymethyl derivatives prepared. The values obtained in this series of compounds again allows differentiation between reaction at sulphur and nitrogen functionalities. Additionally, the difference in values obtained for the two sulphur derivatives **10c** and **16** allows them to be unambiguously identified. In this series, absolute assignment of the individual nitrogen functionalities is difficult due to the similarity of the C* chemical shifts. Protonation of the adjacent nitrogen atom does not simplify these assignments in this series, since the chemical shift of the key resonance moves upfield when adjacent to both amino and imidazole functionalities. This overlap and upfield shift on protonation, is due to the dominating effect of the carboxyl group immediately adjacent to the key resonance.

CONCLUSIONS

Results obtained with these model compounds indicate that a Michael acceptor such as acrylonitrile would be the most useful inhibitor as a diagnostic ^{13}C-covalent label. Its major advantages are:

(a) Most importantly, the C* chemical shift is largely dependent on the reacting functionality; pH and solution effects are less important.

(b) In addition to recognition of (reaction at) cysteine, a positive distinction can be made between reaction at lysine and histidine.

(c) Due to the reversible nature of the Michael addition, alkylated products from reaction with methionine (or with histidine to form the diadduct) are not formed.

Bromoacetic acid would also be a useful inhibitor for this approach but provides less information than acrylonitrile because the chemical shift of the key resonance is dominated by the effect of the adjacent carboxyl group. Furthermore, reaction occurs with methionine and histidine to form a sulphonium salt and diadduct respectively. However, these additional reactions could provide useful information concerning these reactive functionalities within an enzyme. A considerable advantage with bromoacetic acid is that the literature contains many reports of enzyme inhibition reactions performed with bromo- or iodo-acetic acid whereas acrylonitrile has not been well studied in this respect.

Nigen *et al.*[14] have reported some initial experiments using {2-^{13}C}-bromoacetic acid as a covalent label. They reacted seal and sperm whale myoglobins with ^{13}C-enriched bromoacetic acid at pH 7. This procedure resulted in non-specific incorporation of the ^{13}C-label by reaction at lysine, histidine and the amino terminus of the peptide chain. However, the experiment showed that this approach was viable in terms of sensitivity, but the absence of chemical shift data on model compounds at various pH values, and the partial overlap of resonances prevented complete assignment of the ^{13}C-enriched resonance positions.

EXPERIMENTAL

General. IR spectra were recorded on a Perkin–Elmer 157G Infrared Spectrometer. PMR spectra were recorded at 60 MHz on a Perkin–Elmer R-12A Spectrometer, or at 100 MHz on a Varian Associates XL-100-12 instrument (deuterium lock) in deuteriochloroform solution with TMS as internal reference, or in deuterium oxide with 3-trimethylsilyl-1-propanesulphonic acid sodium salt (TSP) as internal reference. CMR spectra were recorded at 22.6 MHz on a Brucker HFX-90E or at 25.2 MHz on a Varian Associates XL-100-12 Spectrometer, both operating in the Fourier transform mode. Amino-acid solutions were prepared for CMR spectroscopy in deuteriochloroform solution with TMS as internal reference, or in deuterium oxide with dioxan as internal reference as noted.

Low resolution mass spectra were recorded on an AEI MS12 Spectrometer equipped with a VG Digispec Data Aquisition System. High resolution mass spectra were recorded on an AEI MS50 Spectrometer at the Physico-Chemical Measurement Unit, Aldermaston. Melting points (m.p.) were determined with a Kofler hot stage apparatus and are uncorrected. Organic solutions were dried over anhydrous magnesium sulphate and evaporated at $< 40°$ on a rotary evaporator at *ca.* 18 mm Hg.

In cases where known compounds were prepared by modifications of reported procedures, additional information is provided. Hitherto unreported data on known compounds (e.g. CMR data) is included where appropriate.

L-*Cysteine derivatives*

N - t - Butoxycarbonyl - methyl ester **1a**. 1a was prepared by way of N-t-butoxycarbonyl L-cysteine, which was in turn prepared by debenzylation of N-t-butoxycarbonyl-S-benzyl-L-cysteine.[16] Diazomethane in ether[17] was added to an ethereal solution of BOC-L-cysteine (1.0 g) and allowed to react for 5 min at 25°. The solution was extracted with 1M sodium bicarbonate solution (3 × 10 ml), the organic phase dried, and evaporated to

yield a red oil. The aqueous phase was taken to pH 3 with citric acid, extracted with ether (3 × 10 ml), the organic phase being further reacted with diazomethane as above. The methyl ester **1a** was purified by column chromatography on silica gel affording a colourless oil eluted by chloroform (90%). (Found M-56, 179.0251; $C_5H_9NO_4S$ requires 179.0253). ν_{max} 3360 (NH), 2570 (SH), 1750 (C=O ester), 1700 (C=O urethane), 1395 and 1365 (t-BU), 1160 (CO) cm^{-1}; PMR τ, ($CDCl_3$) 4.4 (1H, bd, NH), 5.4(1H, m, H-2), 6.23 (3H, s, OCH_3), 7.04 (2H, m, H-2), 8.53 (10H, s, SH and 3 × CH_3); CMR δ ($CDCl_3$) 170.79 (C-1), 155.07 (C=O urethane), 80.33 (C(CH$_3$)$_3$), 54.98 (C-2), 52.64 (OCH_3), 28.27 (3 × CH_3), 27.30 (C-3); *m/z* 179 (M-56, 10%), 135 (179-44, 30%), 118 (135-17, 25%), 76 (14%), 59 (CO_2Me, 17%), 57 (t-Bu, 100%), 41 (57-16, 35%).

N-t-Butoxycarbonyl-S-(2-cyanoethyl)-L-cysteine methyl ester **1b**. N-t-Butoxycarbonyl-L-cysteine methyl ester (1a) (0.10 g, 0.43 mmol), triethylamine (0.2 ml) methanol/water (2:1, 2 ml), and acrylonitrile (0.03 g, 0.57 mmol) were maintained at 25° for 5 hr. The methanol was then evaporated, water (5 ml) was added and the solution extracted with ether (5 × 4 ml). The organic phase was dried, evaporated, and the residue purified by column chromatography on silica gel. The S-2-cyanoethyl) adduct (1b), a pale yellow oil, was eluted by chloroform (72%). (Found: M, *m/z* 288.1148; $C_{12}H_{20}N_2O_4S$ requires 288.1144) ν_{max} 3360 (NH), 2260 (CN), 1745 (C=O ester), 1710 (C=O urethane), 1395 and 1370 (t-Bu), 735 (CH_2SCH_2) cm^{-1}; PMR τ, ($CDCl_3$), 4.40 (1H, bd $J_{NH\text{-}CH}$ 8 Hz, NH), 5.35 (1H, bm, H-2), 6.15 (3H, s, OCH_3), 6.88 (2H, bd, H-3), 7.12–7.55 (4H, A_2B_2 multiplet, SCH_2CH_2), 8.50 (9H, s, 3 × CH_3); CMR δ, ($CDCl_3$), 171.05 (C-1), 155.00 (C=O urethane), 117.89 (CN), 80.45 (C(CH$_3$)$_3$), 53.49 (C-2), 52.77 (OCH_3), 34.57 (C-3), 28.27 (3 × CH_3), 28.27 (SCH_2CH_2), 18.22 (CH_2CN); *m/z* 288 (M, < 1%), 232 (M-56, 13%), 188 (232-44, 9%), 139 (12%), 129 (NH_2=$CHCH_2SCH_2CH_2CN$, 20%), 88 (NH_2 = $CHCO_2Me$, 33%), 57 (t-Bu, 100%), 44 (22%).

N-t-Butoxycarbonyl-S-(2-carbamoylethyl)-L-cysteine methyl ester **1c**. N-t-Butoxycarbonyl-L-cysteine methyl ester **1a** (0.096 g, 0.41 mmol), methanol (0.5 ml), triethylamine (0.2 ml) and acrylamide (0.044 g, 0.62 mmol) were maintained at 25° for 18 hr, the methanol was then evaporated. The residue was dissolved in water (6 ml) and extracted with ether (4 × 8 ml). The organic phase was dried and evaporated to give (**1c**) the S-(2-carbamoylethyl) derivative as a yellow oil (79%). (Found: M, *m/z* 306.1247; $C_{12}H_{22}N_2O_5S$ requires 306.1250). ν_{max} 3410 and 3180 (primary amide NH), 3340 (NH), 1740 (C=O ester), 1700 (C=O urethane), 1660 (C=O amide), 1395 and 1365 (t-Bu), 755 (CH_2SCH_2) cm^{-1}; PMR τ, ($CDCl_3$), 3.75 (2H, bs, NH_2), 4.40 (1H, bd $J_{NH\text{-}CH}$ 8 Hz, NH), 5.50 (1H, m, H-2), 6.22 (3H, s, OCH_3), 7.00–7.60 (6H, m, H-3 and SCH_2CH_2), 8.53 (9H, s, 3 × CH_3); CMR δ, ($CDCl_3$), 173.72 (C=O amide), 171.63 (C-1), 155.26 (C=O urethane), 80.40(C)$CH_3)_3$), 53.68 (C-2), 52.64 (OCH_3), 36.00 (CH_2CO), 34.83 (C-2), 28.34 (3 × CH_3), 28.34 (SCH_2CH_2); *m/z* 306 (M < 1%), 250 (M-56, 2%), 233 (250-17, 4%), 189 (250-45-16, 30%), 118 (CH_2=$SCH_2CH_2CONH_2$, 22%), 104 (HS=$CHCH_2CONH_2$, 13%), 72 (17%), 57 (t-Bu, 100%), 44 (30%), 41 (57-16, 43%).

N-t-Butoxycarbonyl-S-(2-methoxycarbonylethyl)-L - cysteine methyl ester **1d**. N-t-Butoxycarbonyl-L-cysteine methyl ester (**1a**) (0.045 g, 0.19 mmol), methanol (1 ml), triethylamine (0.2 ml) and 3-bromopropionic acid (0.035 g, 0.23 mmol) were maintained at 25° for 18 hr. After this time the methanol was evaporated, the residue dissolved in ether, filtered, and the filtrate treated with diazomethane in ether as described in the preparation of **1a**. The S-(2-methoxycarbonylethyl) derivative **1d** was purified by preparative tlc eluting with carbon tetrachloride: chloroform:ethanol (10:40:1) and was a colourless oil (72%), R_f 0.25 ν_{max} 3360 (NH), 1740 (C=O ester), 1680 (C=O urethane), 1390 and 1365 (t-Bu), 11.0 (CO), 760 (CH_2SCH_2) cm^{-1}; PMR τ, ($CDCl_3$), 4.65 (1H, bm, NH), 5.45 (1H, m, H-2), 6.23 (3H, s, OCH_3), 6.31 (3H, s, OCH_3), 7.00 (2H, bd, H-3), 7.30 (4H, A_2B_2 multiplet, SCH_2CH_2), 8.55 (9H, s, 3 × CH_3); CMR δ, ($CDCl_3$), 172.03 (C=O ester), 171.38 (C=O ester), 155.07 (C=O urethane), 80.26 (C(CH$_3$)$_3$), 53.49 (C-2), 52.51 (OCH_3), 51.80 (OCH_3), 34.70 and 34.57 (C-3 and CH_2CO), 27.34 (3 × CH_3) 27.67 (SCH_2CH_2); *m/z* (M, <1%), 265 (M-56, 3%), 204 (265-44-17, 46%, 172 (204-32, 32%), 162 (204-58, 11%), 133 (CH_2=$SCH_2CH_2CO_2Me$, 44%), 88 (NH_2=$CHCO_2Me$, 16%), 59 (CO_2Me, 20%), 57 (t-Bu, 100%), 45 (51%), 44 (21%), 41 (57-16, 53%).

N-t-Butoxycarbonyl-S-carbamoylmethyl-L-cysteine methyl ester **1e**. N-t-Butoxycarbonyl-L-cysteine methyl ester **1a** (0.062 g, 0.26 mmol), methanol (3 ml), triethylamine (0.2 ml) and iodacetamide (0.064 g, 0.36 mmol) were maintained at 25° for 12 hr, then evaporated. The residue was dissolved in chloroform and purified by preparative tlc eluting with ethyl acetate. The S-carbamoylmethyl derivative (**1e**) was obtained as a colourless oil (81%). R_f 0.52. ν_{max} 3380 and 3180 (prim. amide NH), 3340 (NH), 1740 (C=O ester), 1680 (C=O urethane), 1645 (C=O amide), 1395 (t-Bu) cm^{-1}; PMR τ, ($CDCl_3$), 3.28 (1H, b, amide NH), 3.91 (1H, b, amide NH), 4.48 (1H, bd, $J_{NH\text{-}CH}$ 8 Hz, urethane NH), 5.48 (1H, bm, H-2), 6.23 (3H, s, OCH_3), 6.76 (2H, s, SCH_2CO), 6.80-7.27 (2H, AB region of ABX multiplet J_{AB} 14.5 Hz, H-3), 8.55 (9H, s, 3 × CH_3); CMR δ, ($CDCl_3$), 171.45 (C-1), 155.42 (C=O urethane), 80.58 (C(CH$_3$)$_3$), 53.44 (C-2), 52.80 (OCH_3), 36.04 (SCH_2CO), 35.54 (C-3), 28.34 (3 × CH_3); *m/z* 292 (M, < 1%), 236 (M-56, 7%), 219 (236-17, 5%), 175 (236-44-17, 27%), 158 (175-17, 31%), 133 (14%), 104 (CH_2=SCH_2CONH_2, 15%), 88 (NH_2=$CHCO_2Me$, 20%), 59 (CO_2Me, 84%), 57 (t-Bu, 100%), 41 (57-16, 24%).

N - t - Butoxycarbonyl-S-methyoxycarbonylmethyl-L- cysteine methyl ester **1f**. N-t-Butoxycarbonyl-L-cysteine methyl ester **1a** (0.110 g, 0.47 mmol), methanol (0.5 ml) triethylamine (0.2 ml) and iodacetic acid (0.105 g, 0.56 mmol) were maintained at 25° for 4 hr, then evaporated. The residue was dissolved in ether, filtered and treated with diazomethane in ether as described in the preparation of **1a**. The resulting oil was dissolved in chloroform and purified by preparative tlc eluting with 2% ethanol in chloroform. The S-methoxycarbonylmethyl derivative **1f** was obtained as a colourless oil (80%). R_f 0.11 (Found M, *m/z* 307.1088; $C_{12}H_{12}NO_6S$ requires 307.1090) ν_{max} 3365 (NH), 1740 (C=O ester), 1680 (C=O urethane), 1395 and 1365 (t-Bu), 1160 (CO), 760 (CH_2SCH_2) cm^{-1}; PMR τ, ($CDCl_3$), 4.58 (1H, bm, NH), 5.45 (1H, bm, H-2), 6.24 and 6.28 (3H and 3H, s and s, 2 × OCH_3), 6.72 (2H, s, SCH_2CO), 6.90 (2H, AB region of ABX multiplet, H-3), 8.52 (9H, s, 3 × CH_3); CMR δ, ($CDCl_3$), 171.38 (C=O ester), 170.47 (C=O ester), 155.06 (C=O urethane), 80.35 (C(CH$_3$)$_3$), 53.29 (C-2), 52.58 (OCH_3), 52.45 (OCH_3), 35.03 (C-3), 33.86 (SCH_2CO), 28.34 (3 × CH_3); *m/z* 307 (M, <1%), 251 (M-56, 10%), 206 (251-45, 4%), 190 (251-44-17, 15%), 158 (190-32, 30%), 119 (CH_2=SCH_2CO_2Me, 10%), 88 (NH_2=CH-CO_2Me, 17%), 74 (NH_2=CH-CO_2H, 11%), 59 (CO_2Me, 6%), 57 (t-Bu, 100%), 41 (57-16, 27%).

N-t-Butoxycarbonyl-S-methyl-L-cysteine methyl ester **1g**. N-t-Butoxycarbonyl-L-cysteine methyl ester **1g** (0.124 g, 0.53 mmol), methanol (0.5 ml), triethylamine (0.2 ml) and methyl iodide (40 μl, 0.61 mmol) were maintained at 25° for 15 hr, then evaporated. The residue was dissolved in water (5 ml), and extracted with ether (4 × 10 ml). The organic phase was dried and evaporated. The residue was dissolved in water (5 ml), and (82%). (Found M, *m/z* 249.1036; $C_{10}H_{19}NO_4S$ requires 249.1035). ν_{max} 3360 (NH), 1740 (C=O ester), 1685 (C=O urethane), 1390 and 1365 (t-Bu), 1160 (CO) cm^{-1}; PMR τ, ($CDCl_3$), 4.50 (1H, bd $J_{NH\text{-}CH}$ 8Hz, NH), 5.45 (1H, m, H-2), 6.24 (3H, s, OCH_3), 7.08 (2H, bd $J_{CH_2\text{-}CH}$ 6 Hz, H-3), 7.87 (3H, s, SCH_3), 8.54 (9H, s, 3 × CH_3); CMR δ, ($CDCl_3$), 171.70 (C-1), 155.13 (C=O urethane), 80.20 (C(CH$_3$)$_3$), 53.29 (C-2), 52.51 (OCH_3), 36.85 (C-3), 28.34 (3 × CH_3), 15.99 (SCH_3); *m/z* 249 (M, 2%), 193 (M-56, 3%), 148 (193-45, 8%), 132 (193-44-17, 54%), 90 (24%), 88 (NH_2=$CHCO_2Me$, 26%), 62 (CH_3SCH_3, 10%), 61 (CH_2=SCH_3 and NH_2CO_2H, 43%), 59 (CO_2Me, 14%), 57 (t-Bu, 100%), 56 (CH_2=$C(CH_3)_2$, 17%), 47 (HS=CH_2, 10%), 44 (20%), 43 (23%), 42 (21%), 41 (57-16, 60%).

N - Acetyl-S-(2-methoxycarbonyl-2-hydroxyethyl)-L-cysteine methyl ester **5**. N-Acetyl-L-cysteine (0.30 g, 1.8 mmol) was dissolved in 0.1M sodium hydroxide solution (2.0 ml) and taken to pH 6 with 5M sodium hydroxide solution. Bromopyruvic acid (0.30 g, 1.8 mmol) was dissolved in 0.1M sodiumhydroxide (1.5 ml) and taken to pH 6 with 5M sodium hydroxide solution. The bromopyruvic acid solution was added to the N-acetyl-L-cysteine solution over a period of 10 min, maintaining pH 6 by addition of 0.1M sodium hydroxide solution. After 20 min at 25°, no further decrease in pH was observed. The solution was maintained at 25° for 1 hr, sodium borohydride (0.070 g, 0.018 mol) was then added. After 30 min, sodium ions were removed from solution by passage through a Dowex 50 W-X8

with water. The eluent was evaporated, methanol (8 ml) was added and evaporated. This addition and evaporation of methanol was repeated 5 times to remove boric acid and last traces of water from the solute. The mobile yellow oil was dissolved in chloroform and purified by column chromatography on silica gel. The S-(2-methoxycarbonyl-2-hydroxyethyl) derivative **5** was eluted by 4% ethanol in chloroform as a colourless oil (38%), R_f 0.52. ν_{max} 3450 (OH), 3340 (NH), 3280 (OH), 1740 (C=O ester), 1650 (C=O amide), 1100 (OH) cm^{-1}; PMR τ, ($CDCl_3$), 3.10 (1H, bd, NH), 5.10, (1H, bm, H-2), 5.55 (1H, t J_{CH-CH_2} 5 Hz., $\underline{CH_2}CHOH$), 6.20, 6.22 and 6.20 (7H, s, s and bm, $2\times OCH_3$ and OH), 6.74–7.14 (4H, m, CH_2SCH_2), 7.94 (3H, s, CH_3); CMR δ, ($CDCl_3$), 173.33 (C=O amide), 171.35 (C-1), 170.42 (C=O ester), 71.05 and 70.85 ($CH_2\underline{CH}(OH)$, 2 diastereoisomers present), 52.76 (C-2), 53.35 ($2\times OCH_3$), 36.90 (C-3), 35.32 ($S\underline{CH_2}CHOH$), 23.03 (CH_3); *m/z* 260 (M-18, 7%), 220 (M-59, 10%), 202 (260-59, 42%), 143 (202-59, 11%), 131 (19%), 101 (12%), 89 (CH_2=SCH_2CHO, 23%), 88 (NH_2=$CHCO_2Me$, 25%), 74 (NH_2=$CHCO_2H$, 17%), 60 (18%), 59 (CO_2Me, 11%), 45 (20%), 43 (CH_2=CO, 100%), 42 (21%), 41 (11%).

N - t-Butoxycarbonylglycyl-S-(2-cyanoethyl)-L-cysteinylglycine ethyl ester **6**. This tripeptide was prepared from appropriately protected amino-acids via two dicyclohexylcarbodi-imide coupling. reactions. S-(2-cyanoethyl)-L-cysteine **10a** was prepared by the method of Friedmann *et al.*[18] by reaction of acrylonitrile with L-cysteine at pH 8.0 to yield white crystals (80%). m.p. 179–181° dec (lit[18] 180–181°). S-(2-cyanoethyl)-L-cysteine (10a) (12.40 g, 0.071 mol), t-butanol (50 ml), triethylamine (19.7 ml, 0.075 mmol) and t-butoxycarbonyl azide (14.2 ml, 0.099 mol) were maintained at 50-55° for 18 hr. The solvent was evaporated, water (50 ml) added and taken to pH at 0° with citric acid. The solution was extracted with ethyl acetate (1×80, 2×20 ml), the organic phase dried and evaporated to yield N-t-butoxycarbonyl-S-(2-cyanoethyl)-L-cysteine **7** as a yellow oil (89%). **7** (9.0 g, 0.033 mol), glycine ethyl ester hydrochloride (4.6 g, 0.033 mol) and triethylamine (4.1 ml, 0.033 mol), were dissolved in dry dimethylformamide (DMF) (50 ml) and cooled to 0°. To the cooled solution was added dicyclohexylcarbodi-imide (6.7 g, 0.033 mol) in DMF (15 ml) over 15 min. The solution was stirred at 0° for 2 hr then at 25° overnight. Dilute acetic acid (20 ml) was added, after 30 min the solvent was evaporated, ether (30 ml) added to the residue, filtered, and the filtrate evaporated. A further volume of ether (30 ml) was added, filtered and the filtrate washed successively with citric acid (1M, 2×30 ml), water (2×20 ml), sodium bicarbonate (0.5 M, 2×30 ml) and finally almost saturated sodium carbonate solution (2×10 ml). The organic phase was dried and evaporated. The residue, a yellow oil, was crystallised from ether/petroleum ether to give white crystals of the dipeptide **8** (62%). PMR τ, ($CDCl_3$), 2.65 (1H, bt J_{NH-CH_2} 6 Hz, NH amide), 4.181H, d, J_{NH-CH} 8Hz, NH urethane), 5.55 (1H, m, $NHC\underline{H}CO$), 5.76 and 5.97 (4H, q, 4H, $J_{CH_2-CH_3}$ 7 Hz and d J_{CH_2-NH} 6 Hz, $\underline{OCH_2}$ and $NHC\underline{H_2}CO$), 7.00 and 7.20 (6H, m and m, $SC\underline{H_2}CH$ and SCH_2CH_2), 8.52 and 8.71 (12H, s and t $J_{CH_3-CH_2}$ 7Hz, $\overline{3\times CH_3}$ and $OCH_2\underline{CH_3}$).

The dipeptide **8** was used without further purification. N-t-Butoxycarbonyl-S-(2-cyanoethyl)-L-cysteineylglycine ethyl ester (8) (0.63 g, 180 mmol) was dissolved in anhydrous trifluoroacetic acid (10 ml) and stirred for 1 hr at 25°, then evaporated. A 1 : 1 mixture of potassium carbonate (1M) and ethyl acetate at 0° was added (40 ml) to the residue, the organic phase removed and the aqueous phase further extracted with ethyl acetate (2×15 ml). The combined organic phases were dried and evaporated to give the amino-free dipeptide **9** as a yellow oil (94%). PMR τ, ($CDCl_3$), 1.84(1H, bm, $\underline{NH}CH_2$), 5.80 and 5.95 (4H, q $J_{CH_2-CH_3}$ 7 Hz and d J_{CH_2-NH} 5 Hz, $\underline{O}CH_2$ and $NHCH_2CO$), 6.38 (1H, bt, NH_2CH), 6.90-7.40 (6H, m, $CH_2SCH_2CH_2$), 7.60 (2H, bm, NH_2), 8.71 (3H, t $J_{CH_3-CH_2}$ 7 Hz, $OCH_2\underline{CH_3}$).

The dipeptide **9** was used without further purification, S-(2-cyanoethyl) - L-cysteineylglycine ethyl ester **9** (0.22 g, 0.83 mmol), and t-butoxycarbonylycine (0.15 g, 0.083 mmol) were dissolved in dry DMF (10 ml) and cooled to 0°. Dicyclohexylcarbodi-imide (0.017 g, 0.84 mmol) was added over 15 min in DMF (3 ml). The solution was stirred at 0° for 2 hr and allowed to warm to 25° overnight. The workup procedure was the same as that used for the preparation of the dipeptide **8**, and gave a yellow oil. This oil was purified by preparative tlc (3, 20×20 cm plates) eluting with ethyl acetate to yield the tripeptide (6) as a yellow oil (60%), R_f 0.55 (Found M, *m/z* 416.1733; $C_{17}H_{28}N_4O_6S$ requires 416.1730.) ν_{max} 3340 (NH urethane), 3300 (NH amide), 2250 (CN), 1740 (C=O ester), 1680 (C=O urethane), 1650 (C=O amide, 1395 and 1365 (t-Bu), 1160 (CO), 760 (CH_2SCH_2) cm^{-1}; PMR τ, ($CDCl_3$), 2.60 (2H, m, $2\times$NH amide), 4.46 (1H, t J_{NH-CH_2} 6 Hz. NH urethane), 5.30 (1H, m, $NH\underline{CH}CO$), 5.87 (2H, q J_{CH-CH_3} 7 Jz, OCH_2), 6.03 (2H, d J_{CH-NH} 5 Hz, $NHCH_2CO_2Et$), 6.22 (2H, d J_{CH_2-NH} 6Hz, $BocNH\underline{CH_2}$), 7.03 (2H, m, $\overline{CHCH_2S}$), 7.25 (4H, A_2B_2 multiplet $SCH_2\underline{CH_2}$), 8.59 and 8.76 (12H, s and t $J_{CH_3-CH_2}$ 7Hz, $3\times CH_3$ and $OCH_2\underline{CH_3}$); CMR δ ($CDCl_3$), 170.43 (C=O), 170.13 (C=O), 169.47 (C=O), 156.10 (C=O urethane), 118.53 (CN), 80.76 ($\underline{C}(CH_3)_3$), 61.62 (OCH_2), 52.18 ($NH\underline{CH}CO$), 44.69 ($BocNHCH_2$), 41.54 ($NH\underline{CH_2}CO_2$), 33.55 ($\overline{CHCH_2S}$), 28.34 ($3\times CH_3$), 27.63 ($S\underline{CH_2}CH_2$), 18.76 ($\underline{CH_2}CN$), 14.15 ($OCH_2\underline{CH_3}$); *m/z* 416 (M, <1%), 360 (M-56, 3%), 243 (11%), 242 (15%), 189 (11%), 188 (16%), 129 (NH_2=$CHCH_2SCH_2CH_2CN$, 44%), 104 (46%), 76 (21%), 57 (tBu, 68%), 56 ($(CH_3)_2C$=CH_2, 50%), 44 (64%), 41 (57–16, 100%).

S-carbamoylmethyl-L-cysteine (10b) and S-carboxymethyl-L-cysteine **10c** were prepared by the method of Goodman *et al.*[19]

S-(2-cyanoethyl)glutathione **17a**. To reduced glutathione (γ-glutamyl-L-cysteinylglycine) (0.24 g, 0.78 mmol) in water (10 ml) at pH 8 (adjusted by addition of solid sodium bicarbonate) was added acrylonitrile (0.082 g, 1.56 mmol) and the solution maintained at 25° for 18 hr, then taken to pH 4 with Dowex 50-X8(H^+) ion-exchange resin, filtered and the filtrate evaporated. The residue was recrystallised from ethanol/water at 0°, filtered and dried *in vacuo* at 60° (80%). ν_{max} 3340 and 3365 (NH), 2250 (CN), 1670 and 1635 (C=O amide and carboxylic acid) cm^{-1}; PMR τ, (D_2O), 5.40 (shoulder on HOD, m, $CysC_\alpha H$), 6.19 and 6.21 (3H, s and t J_{CH-CH_2} 6Hs, $GlyCH_2$ and $GluC_\alpha H$), 6.90 (2H m, $CHCH_2S$), 7.16 (4H, bs, SCH_2CH_2), 7.44 (2H, m, $GluCH_2CONH$), 7.80 (2H, m, $GluCH\underline{CH_2}$); CMR δ, (D_2O, pH 4.1), 176.34, 175.30, 175.15, 173.95 ($4\times$C=O), 121.92 (CN), 55.32 ($GluC_\gamma$), 54.52 ($CysC_\gamma$), 43.25 ($GlyC_\alpha$), 34.28 ($CysC_\beta$), 32.76 ($GluC_\gamma$), 28.52 ($S\underline{CH_2}CH_2$), 27.57 ($GluC_\beta$), 19.80 ($\underline{CH_2}CN$).

S-Carboxymethylglutathione **17b**. Prepared by the same procedure as that to prepare S-(2-cyanoethyl)-glutathione **17a**, substituting iodoacetic acid for the acrylonitrile, in 60% yield. ν_{max} 3345 and 3365 (NH), 1670 and 1635 (C=O) cm^{-1}; PMR τ, (D_2O), 5.40 (shoulder of HOD, m, $CysC_\alpha H$), 6.22 and 6.18 (3H, s and m, $GlyCH_2$ and $GluC_\alpha H$), 6.72 (2H, s, SCH_2CO), 6.81-7.20 (2H, AB region of ABX multiplet, $CH\underline{CH_2}S$), 7.48 (2H, m, $GluCH_2CONH$), 7.81 (2H, m, $GluCH\underline{CH_2}$); CMR δ, (D_2O/NaOH, pH 7.3), 178.53, 175.84, 175.18, 175.03 ($4\times$C=O), 54.99 and 53.84 ($CysC_\alpha$ and $GluC_\alpha$), 44.29 ($GlyC_\alpha$), 37.92 ($S\underline{CH_2}CO$), 34.51 and 32.18 ($CysC_\beta$ and $GluC_\gamma$), 27.10 ($GluC_\beta$).

L-Histidine derivatives

L-Histidine methyl ester dihydrochloride and N_α-*t*-butoxycarbonyl-L-histidine methyl ester **2a** were both prepared by the method of Handford.[20] The latter compound had m.p. 123–125° (lit[20] 123–125°). (Found M, *m/z* 269.1368 $C_{12}H_{19}N_3O_4$ requires 269.1375) and showed the following CMR data: δ, ($CDCl_3$), 172.74 (C-1), 155.65 (C=O urethane), 135.37 (C'-2), 134.07 (C'-4), 116.14 (C'-5), 80.07 ($\underline{C}(CH_3)_3$), 53.88 (C-2), 52.32 (OCH_3), 29.83 (C-3), 28.34 ($3\times CH_3$).

N_α-t-Butoxycarbonyl-N_{im}-(2-cyanoethyl)-L-histidine methyl ester **2b**. N_α-t-Butoxycarbonyl-L-histidine methyl ester **2a** (0.106 g, 0.40 mmol), methanol (1.0 ml) and acrylonitrile (0.013 g, 0.48 mmol) were maintained at 40–45° for 24 hr then evaporated to give a yellow oil. The oil was purified by alumina chromatography, the N_{im}-(2-cyanoethyl) adduct **2b**, a colourless oil, was eluted by 10% chloroform in methylene chloride (35%). ν_{max} 3350 (NH), 2265 (CN), 1740 (C=O ester), 1690 (C=O urethane), 1390 and 1365 (t-Bu), 1155 (CO) cm^{-1}; PMR τ, ($CDCl_3$), 2.60 (1H, bs, C'-2H), 3.23 (1H, bs, C'-5H), 4.10 (1H, bd J_{NH-CH} 8 Hz, NH urethane), 5.55 (1H, m, H-2), 5.87 (2H, $J_{CH_2-CH_2}$ 6Hz, NCH_2), 6.38 (3H, s, OCH_3), 7.02 (2H, m, H-3), 7.25 (2H, t $J_{CH_2-CH_2}$ 5 Hz, CH_2CN), 8.62 (9H, s, $3\times CH_3$); CMR δ, ($CDCl_3$), 172.55 (C-1), 155.65 (C=O urethane), 138.75 (C'-4), 136.87 (C'-2), 116.72 (CN), 116.27 (C'-5), 79.74 ($\underline{C}(CH_3)_3$), 53.68 (C-2), 52.19 (OCH_3), 42.57 (NCH_2), 30.35 (C-3), 28.40 ($3\times CH_3$), 20.54 ($\underline{CH_2}CN$).

N_α-t-butoxycarbonyl-N^{im}-(2-cyanoethyl)-L-histidine was eluted (see above) with 100% chloroform. PMR τ, ($CDCl_3$), 2.56 (1H, bs, C'-2H), 3.20 (1H, bs, C'-5H), 4.21 (1H, bm, NH), 5.60–6.03 (3H, m, H-2 and NCH_2), 6.90–7.40 (4H, m and bt $J_{CH_2-CH_2}$ 6Hz, H-2 and CH_2CN), 8.58 (9, s, $3\times CH_3$).

N_{im}-(2-Cyanoethyl)-L-histidine methyl ester **2c**. N_α-t-Butoxycarbonyl-N_{im}-(2-cyanoethyl)-L-histidine methyl ester **2b** (0.3 g, 0.93 mmol) was dissolved in anhydrous trifluoroacetic acid (7 ml) and after 30 min at 25° the solvent was evaporated. The residue was dissolved in water (10 ml) and stirred vigorously with Amberlite IRA 400 (OH^-) until the solution attained pH 8.5. The solution was then filtered and the filtrate evaporated to yield free amine **2c** (90%). ν_{max} 3400 (NH), 2265 (CN), 1740 (C=O) cm^{-1}; PMR τ, (D_2O), 5.71 (3H, bt $J_{CH_2-CH_2}$ 6 Hz, and m, NCH_2 and H-2), 6.30 (3H, s, OCH_3), 7.20 (4H, bt $J_{CH_2-CH_2}$ 6 Hz and m, CH_2CN and H-3); CMR δ, (D_2O/NaOH, pH 7.0), 174.52 (C-1), 119.77 (CN), 119.77 (C'-5), 55.67 (C-2), 54.12 (OCH_3), 43.14 (NCH_2), 29.47 (C-3), 20.49 (CH_2CN).

N_{im}-(2-Cyanoethyl)-L-histidine **11a**. N_{im}-(2-cyanoethyl)-L-histidine methyl ester **2c** was hydrolysed in acidic aqueous solution in the probe of the NMR spectrometer at 60° to the acid **11a**. The compound was not fully characterised, but CMR data was recorded: CMR δ, (D_2O/HCl, pH 1.0), 170.33 (C-1), 136.50 (C'-2), 128.57 (C'-4), 122.03 (C'-5), 118.74 (CN), 52.72 (C-2), 45.77 (NCH_2), 25.94 (C-3), 20.02 (CH_2CN).

N_τ-Carboxymethyl-L-histidine **11b**. N_τ-Carboxymethyl-L-histidine was prepared from N_α-acetyl-L-histidine by the method of Crestfield *et al.*[10] with modification of their separation procedure. Nα-Acetyl-L-histidine and iodoacetic acid were reacted, and the acetyl group removed by acid hydrolysis. The reaction mixture was chromatographed on Amberlite IR 120 (20×3.5 cm diam) ion-exchange resin eluting with 0.2N sodium citrate buffer pH 2.78. The diadduct **12** (*vide infra*) eluted immediately, and was detected by the ninhydrin colour test for amino-acids. Elution with 0.2N sodium citrate buffer (pH 5.0) gave the N-carboxylmethyl derivative **11b** which was desalted and crystallised by the published procedure (30%). m.p. 256–258° dec. ν_{max} 3450 (NH), 3360 (NH), 1940 (NH_3), 1670 (C=O), 1630 (C=N), 915, 865 and 785 (substituted imidazole ring) cm^{-1}; PMR τ, (D_2O), 1.26 (1H, bs, C'-2H), 2.56 (1H, bs, C'-5H), 5.18 (shoulder of HOD, s, NCH_2CO), 5.97 (1H, bm, H-2), 6.61–6.73 (2H, AB region of ABX multiplet, H-3); CMR τ, (D_2O/NaOH, pH 14.0), 183.41 (C-1), 176.52 (C=O), 139.14 (C'-2), 138.34 (C'-4), 119.48 (C'-5), 57.03 (C-2), 50.92 (NCH_2CO), 34.00 (C-3); τ (D_2O, pH 4.5), 173.40 and 172.09 ($2\times$C=O), 136.91 (C'-2), 128.81 (C'-4), 122.54 (C'-5), 54.35 (C-2), 52-66 (NCH_2CO), 26.72 (C-3); τ, (D_2O/HCl, pH 1.0), 170.82 and 170.66 ($2\times$C=O), 137.57 (C'-2) 127.92 (C'-4), 123.12 (C'-5), 52.53 (C-2), 50.69 (NCH_2CO), 25.85 (C-3).

$N_\pi N_\tau$-Dicarboxymethyl-L-histidine **12**. This product was formed in the reaction. The fractions containing **12** from the IR-120 ion-exchange column were combined, desalted and crystallised according to the published procedure[10] (21%). m.p. 260-70° dec. ν_{max} 3450 (NH), 3360 (NH), 1680–1620 (broad C=O) cm^{-1}; PRM τ, (D_2O), 1.12 (1H, d $J_{CH\text{-}N\text{-}CH}$ 2 Hz, C'-2H), 2.46 (1H, bs, C'-5H), 5.03 (4H, s, $2\times NCH_2CO$), 5.68–5.91 (1H, X region of ABX multiplet, H-2), 66.2–6.70 (2H, AB region of ABX multiplet, H-3); CMR δ, (D_2O, pH 4.0), 172.47 and 171.98 (C=O), 139.27 (C'-2), 130.41 (C'-4), 123.26 (C'-5), 53.13 (C-2), 52.18 ($N\tau CH_2$), 50.10 ($N\pi CH_2$), 25.13 (C-3); δ, (D_2O/HCl, pH 1.0), 140.10 (C'-2), 130.16 (C'-4), 123.73 (C'-5), 51.83 (C-2), 51.02 ($N\tau CH_2$), 48.75 ($N_\pi CH_2$), 24.53 (C-3).

L-Lysine derivatives

N_αt-Butoxycarbonyl-N_ϵ-benzyloxycarbonyl-L-lysine was prepared from N_ϵ-benzyloxycarbonyl-L-lysine[21] essentially by the method of Schnabel[22] (see foregoing), with a modified workup procedure. After the reagents had been maintained at 50–55° for 20 hr, the solvent was evaporated, water (50 ml) was added, taken to pH 8 with solid sodium bicarbonate and extracted with ether (3×10 ml). The aqueous phase was then taken to PH 3 with solid citric acid, extracted with ether (3×20, 2×10 ml), the combined organic phases dried and evaporated to give a yellow oil (70%). (PMR τ, ($CDCl_3$), 0.04 (1H, s, COOH), 2.65 (5H, s, Ph), 5.70 (1H, bm, H-2), 6.80 (2H, bm, H-6), 8.10–8.50 (15H, bm and s, H-3 H-4 H-5 and $3\times CH_3$)). Material of this purity was found to be suitable for the next stage.

N_α-t-Butoxycarbonyl-N_ϵ-benzyloxycarbonyl-L-lysine methyl ester was prepared by the method used to prepare t-butoxycarbonyl-L-cysteine methyl ester **1a** (see above). The methyl ester was obtained as a pale yellow oil (93%). (Found M, *m/z* 394.2111; $C_{20}H_{30}N_2O_6$ requires 394.2104). ν_{max} 3360 (NH), 1740 (C=O ester), 1685 (C=O urethane), 1605 and 1590 (arom. C=C). 1395 and 1365 (t-Bu), 1160 (CO) cm^{-1}; PMR τ, ($CDCl_3$), 2.58 (5H, s, Ph), 4.91 and 4.90 (4H, s and bm, CH_2Ph and $2\times$NH), 5.75 (1H, m, H-2), 6.30 (3H, s, OCH_3), 6.83 (2H, bm, H-6), 8.10-8.80 and 8.57 (15H, bm and s, H-3 H-4 H-5 and $3\times CH_3$); *m/z* (M, < 1%), 338 (M-56, 2%), 294 (338–44, 8%), 218 (15%), 174 (14%), 142 (25%), 108 (22%), 81 (C_7H_7, 100%), 84 (NH_2=$CHCH_2CH_2CH$=CH_2, 21%), 59 (CO_2Me, 15%), 57 (t-Bu. 74%). 41 (57-16, 31%).

N_α-t-Butoxycarbonyl L-lysine methyl ester **3a**. N_α-t-Butoxycarbonyl-N_ϵ-benzyloxycarbonyl-L-lysine methyl ester (4.30 g, 11.0 mmol) in methanol (40 ml) and 5% palladium on charcoal (400 mg) were stirred at 25°,with hydrogen bubbling through the solution for 2 days. The solution was filtered and evaporated to give the α-amino derivative **3a** as a green very viscous oil. This was dissolved in 1M citric acid solution (20 ml) and extracted with ether (3×2 ml). The aqueous phase was then taken to pH 9 with solid sodium bicarbonate, extracted with ethyl acetate (3×20, 2×10 ml), the combined organic phases dried and evaporated to give (3a) as an unstable pale green oil (90%). ν_{max} 3360 (NH), 3280 (NH), 1735 (C=O ester), 1685 (C=O urethane), 1395 and 1365 (t-Bu) cm^{-1}; PMR τ, ($CDCl_3$), 6.26 (3H, bm, NH and NH_2), 5.80 (1H, bm, H-2), 7.00 (2H, bm, H-6), 8.00–8.60 and 8.55 (15H, bm and s, H-3 H-4 H-5 and $3\times CH_3$); CMR δ, ($CDCl_3$), 173.39 (C-1), 155.91 (C=O), 79.87 ($\underline{C}(CH_3)_3$), 53.29 (C-2), 52.91 (OCH_3), 42.11 (C-6), 32.30 (C-5), 28.92 (C-3), 28.40 ($3\times CH_3$), 22.68 (C-4); *m/z* 260 (M, 2%), 204 (M-56, 11%), 187 (204-17, 14%), 160 (204–44, 7%), 143 (19%), 142 (21%), 84 (NH_2=$CHCH_2CH_2CH$=CH_2, 71%), 57 (t-Bu, 100%), 56 ((CH_3)$_2$C=CH_2, 20%), 41 (57-16, 33%).

N_α-t-Butoxycarbonyl-N_ϵ-(2-cyanoethyl)-L-lysine methyl ester **3b**. N_α-t-Butoxycarbonyl-L-lysine methyl ester (3a) (0.163 g, 0.63 mmol), methanol (1 ml) and acrylonitrile (0.40 g, 0.75 mmol) were maintained at 25° for 5 h then evaporated. The resulting oil was purified by preparative tlc eluting with 50% ether in chloroform containing a trace of concentrated ammonia solution, to give the N_ϵ-(2-cyanoethyl) adduct **3b**, a pale yellow oil (31%), R_f 0.47, which decomposed on standing in solution. ν_{max} 3360 (NH), 2260 (CN), 1735 (C=O ester), 1685 (C=O urethane), 1395 and 1365 (t-Bu), 1160 (CO) cm^{-1}; PMR τ, ($CDCl_3$), 4.76 (1H, bd, NH), 5.70 (1H, bm, H-2), 6.26 (3H, s, OCH_3), 6.92–7.63 (6H, bm, H-6 and CH_2CH_2CN), 8.05–8.70 and 8.45 (15H, bm and s, H-3 H-4 H-5 and $3\times CH_3$); CMR δ, ($CDCl_3$), 173.26 (C-1), 155.39 (C=O), 118.61 (CN), 79.94 ($\underline{C}(CH_3)_3$), 53.49 (C-2), 52.19 (OCH_3), 48.81 (C-6), 45.10 (NCH_2), 32.63 (C-5), 29.44 (C-3), 28.34 ($3\times CH_3$), 22.94 (C-4), 18.72 ($\underline{CH_2}CN$); *m/z* 313 (M, < 1%), 257 (M-56, 12%), 213 (257–44, 5%), 142 (10%), 84 (NH_2=$CHCH_2CH_2CH$=CH_2, 58%), 69 (NH_2=$CHCH_2CN$, 18%), 57 (t-Bu, 100%), 56 ((CH_3)$_2$C=CH_2, 23%), 41 (57-16, 46%).

N_ϵ-Carboxymethyl-L-lysine **13**. L-lysine (0.5 g, 3.56 mmol) was dissolved in boiling water and cupric carbonate (0.7 g) was added. After 2 min the solution was cooled and filtered. The filtrate was taken to pH 10 with 5 M sodium hydroxide solution, iodoacetic acid (0.79 g, 4.3 mmol) was added and the solution heated to 90° for 19 hr, maintaining pH 10 by periodic addition of sodium hydroxide. The solution was then acidified to pH 2 with hydrochloric acid, hydrogen sulphide bubbled through the solution for 5 min, filtered and the filtrate evaporated. The residue was dissolved in 0.2 M sodium citrate buffer, pH 3.2 and chromatographed on a column of Amberlite IR 120 (20×3.5 cm dia.) eluting with the same citrate buffer. After 100 ml of eluant has passed through the column, the buffer was changed to 0.2 M sodium citrate, pH 4.6. A ninhydrin positive component was eluted almost immediately. These pooled fractions were desalted on Dowex 2-X8 according to the method published by Crestfield *et al.*[10] N_ϵ-carboxymethyl-L-lysine **13** was crystallised from ethanol/water at 0° (35%). m.p. 180° dec. ν_{max} 3350 (NH), 1580 (carboxylate anion), 8.45 (NH_2) cm^{-1}: PMR τ (D_2O), 6.26 (1H, bt

$J_{CH\text{-}CH_2}$ 6Hz, H-2), 6.39 (2H, s, $NHCH_2CO$), 6.84-7.00 (2H, m, H-6), 7.95–8.60 (6H, m, H-3 H-4 H-5); CMR δ, (D_2O, pH 7.2), 175.50 and 172.24 (C=O), 55.34 (C-2), 50.03 (NCH_2), 47.86 (C-6), 30.71 (C-5), 25.98 (C-3), 22.36 (C-4). δ. (D_2O/HCl, pH 1.0), 172.57 (C=O), 53.33 (C-2), 47.97 (C-6), 47.00 (NCH_2), 29.98 (C-5), 25.74 (C-3), 22.26 (C-4).

L-Serine derivatives

N-t-Butoxycarbonyl-L-serine methyl ester **4a** was prepared from N-t-butoxycarbonyl-L-serine[20] by the same esterification method as used to prepare **1a** (see above). **4a** was purified by column chromatography on silica gel eluting with 50% ethyl acetate in chloroform and was obtained as a colourless oil (90%). ν_{max} 3500–3300 (OH), 3340 (NH), 1735 (C=O), 1395 and 1365 (T-Bu) cm^{-1}; PMR τ, ($CDCl_3$), 4.35 (1H, bd $J_{NH\text{-}CH}$ 8 Hz, NH), 5.65 (1H, m, H-2), 6.05 and 6.23 (5H, bm and S, H-3 and OCH_3), 6.91 (1H, bm OH), 8.54 (9H, s, $3\times CH_3$); CMR δ, ($CDCl_3$), 171.50 (C-1), 155.85 (C=O), 80.39 ($\underline{C}(CH_3)_3$), 63.30 (C-3), 56.02 (C-2), 52.58 (OCH_3), 28.34 ($3\times CH_3$); *m/z* 219 (M, < 1%), 188 (M-31, 4%), 163 (M-56, 2%), 159 (M-60, 16%), 145 ($BocNHCH_2CH_3$, 16%), 132 (25%), 104 (163-59, 10%), 101 (11%), 88 (NH_2=$CHCO_2Me$, 17%), 59 (CO_2Me, 23%), 57 (t-Bu, 100%), 44 (16%), 41 (57-16, 36%).

N-t-Butoxycarbonyl-O-(2-cyanoethyl)-L-serine methyl ester **4b**. N-t-butoxycarbonyl-L-serine methyl ester **4a** (0.10 g, 0.45 mmol) was dissolved in freshly prepared potassium t-butoxide (approx. 2 mmol) in t-butanol, cooled to 10° and acrylonitrile (0.12 g, 2.3 mmol) added over 20 min maintaining the temperature below 15°. After 18 hr at 25°, sufficient concentrated citric acid was added to take the solution to pH 7. The aqueous solution was extracted with ethyl acetate (4×10 ml), the organic phase dried and treated with diazomethane in ether until there was just a persistent yellow colour (i.e. slight excess of diazomethane). After 5 min the solution was evaporated. The resultant oil was purified by column chromatography on alumina, the O-(2-cyanoethyl) adduct **4b**, a pale yellow oil, was eluted by methylene chloride (33%). ν_{max} 3360 (NH), 2260 (CN, 1740 (C=O ester), 1680 (C=O urethane), 1390 and 1365 (t-Bu), 1155 (CO) cm^{-1}; PMR τ, ($CDCl_3$), 4.60 (1H, bm, NH), 5.55 (1H, bm, H-2), 6.22 (7H, bm, OCH_3 H-3 OCH_2), 7.40 (2H, m, CH_2CN), 8.53 (9H, s, $3\times CH_3$); CMR δ, ($CDCl_3$), 171.10 (C-1), 155.37 (C=O), 119.00 (CN), 80.26 ($\underline{C}(CH_3)_3$), 71.36 (C-3), 65.96 (OCH_2), 53.70 (C-2), 52.40 (OCH_3), 28.33 ($3\times CH_3$), 18.85 (CH_2CN); *m/z* 215 (M-56, 3%), 171 (215-44, 13%), 113 (43%), 88 (NH_2=$CHCO_2Me$, 33%),59 (CO_2Me, 29%), 57 (t-Bu, 100%), 54 (34%), 41 (57-16, 69%).

Other derivatives

S-Carboxymethyl-L-methionine sulphonium salt **16**. L-Methionine (0.5 g, 3.3 mmol) was dissolved in dil HCl (8 ml) and taken to pH 4.5 with c. HCl. Iodoacetic acid (3.1 g, 16.5 mmol) in sodium hydroxide solution (8 ml) at pH 4.5 was added to the solution of L-methionine and maintained at 25° for 24 hr. The solution was taken to pH 2 and extracted with ether (3×20 ml), the aqueous phase was then lyophilised. Analysis by CMR spectroscopy was performed without further purification. CMR δ, (D_2O/NaOH, pH 7.0), 173.33 (C=O), 169.03 (C=O), 53.72 (C-2), 48.34 ($\underline{S}CH_2CO$), 38.38 (C-4), 26.14 (C-3), 24.57 (SCH_3); δ, (D_2O/HCl, pH 1.0), 172.30 (C=O), 168.80 (C=O), 52.91 (C-2), 47.78 ($\underline{S}CH_2CO$), 38.28 (C-4), 25.83 (C-3), 24.70 (SCH_3).

N-(2-cyanoethyl)-n-butylamine **15a** *and NN-Bis(2-cyanoethyl)-n-butylamine* **15b**. n-Butylamine (2.92 g, 0.04 mmol) and acrylonitrile (4.77 g, 0.09 mol) were heated at 85° for 15 hr and then distilled. The monoadduct **15a** distilled at 83–86°/1.6 mmHg[11] and the diadduct **15b** at 164–166° 1.5 mmHg (28%). CMR see Table 4.

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