Labelling of Amino-acid Side-chains with ^{13}C-Labelled Electrophiles; Potential Application to the Probing of Active Sites of Enzymes

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Summary Side-chain groups in functional amino-acids appropriately protected at both terminii have been treated with electrophilic inhibitors (*e.g.* acrylonitrile), ^{13}C n.m.r. spectroscopy allowing unambiguous elucidation of the reacting side-chain functionality; an extension of this technique using ^{13}C-enriched inhibitors to the study of enzyme active-site environment is discussed.

A MAJOR method used to study the mechanism of catalytic action of enzyme systems is the covalent labelling of active-site functional groups by reaction with appropriate electrophilic inhibitors.[1] Lengthy degradative and separative procedures are then required to locate and identify specific amino-acid residues which have reacted. For unambiguous identification, synthetic model derivatives are required for comparison, and for structurally complex active-site directed inhibitors, the synthesis of model derivatives requires much effort.

This investigation is directed towards elucidation of active-site functional groups using ^{13}C-labelled Michael acceptors as irreversible inhibitors (*e.g.* $^{13}CH_2$=CH–CN). The chemical shift of the enriched ^{13}C resonance should allow determination of catalytic functional groups without degradative procedures.

We have synthesised various amino-acids with functional side chains with the amino-terminus protected as the t-butoxycarbonyl derivative and with the carboxyl-terminus esterified [*e.g.* (**1**)]. Reaction of side chain groups with electrophilic reagents [*e.g.* (**1**) → (**2**)] was next accomplished to produce covalently labelled model compounds (**2**)—(**6**).‡

For compounds (**2**)—(**5**), addition occured readily with 1·2—1·4 molar excess of electrophile at ambient tempera-

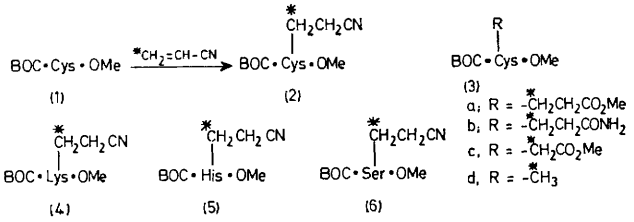
TABLE

Electrophile	Derivative	^{13}C Resonance[a] (C*) in protected amino-acid (A) Cys	Lys	His	Ser
CH_2=CHCN	A–$\overset{*}{C}H_2CH_2CN$	28·27	45·10	42·57	65·96
$BrCH_2CH_2CO_2H$	A-$\overset{*}{C}H_2CH_2CO_2Me$	27·67			
CH_2=CHCONH$_2$	A–$\overset{*}{C}H_2CH_2CONH_2$	28·34			
ICH_2CO_2H	A–$\overset{*}{C}H_2$–CO_2Me	35·03			
MeI	A–$\overset{*}{C}H_3$	16·25			

[a] Chemical shifts in p.p.m. downfield from Me_4Si in $CDCl_3$ solution.

‡ All new compounds have been fully characterized. It is not possible to distinguish between the two possible imidazole N^1- and N^3-isomers using the data available.

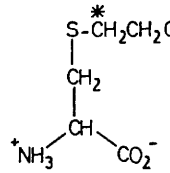
ture in methanol or aqueous methanol during 24 h. Addition of 1 mol. equiv. of triethylamine facilitated reaction in the case of the cysteine derivatives. As expected, BOC·Ser·OMe did not undergo addition under these conditions, but reaction in the presence of $KOBu^t$ in Bu^tOH with slow addition of acrylonitrile afforded a low yield of (**6**). Although the conditions for preparation of derivatives (**2**)—(**5**) are notably mild, these reactions should not be taken as fully valid models for enzyme active-site alkylation. However, there are numerous reports of enzyme alkylation with Michael acceptors and related derivatives under physiological conditions.[1]



Broad-band proton-decoupled ^{13}C n.m.r. spectra at natural abundance have been determined for these and related derivatives and, in conjunction with proton off-resonance decoupled spectra, resonances have been assigned to each individual carbon atom. In the Table, the chemical shifts of the key resonances [*i.e.* C* in (**2**)—(**6**)] are listed.

The large difference in chemical shift of the C*-resonance in the series (**2**)—(**6**) allows the reacting side-chain functionality in these model compounds to be determined easily and it is noteworthy that a significant distinction between the lysine and histidine derivatives is observed. Furthermore, the C*-resonance in the cysteine derivative (**7**) is relatively insensitive to pH and solvent effects.

The large differences in chemical shifts of the key ^{13}C-resonances confirm the potential of this technique and encourage the extension of these model experiments to native enzyme systems. The importance of the method is emphasised in experiments designed to use active-site directed inhibitors since synthesis of the model derivatives required for degradative comparison can be avoided. In addition to information on active-site functionality, the effect of denaturation on the enriched ^{13}C-resonance can be expected to provide insight into active-site environment.



(**7**)

pH 1 (DCl-D_2O)	δ 29·96[a]
pH 7 (D_2O)	δ 29·64
pH 14 ($NaOD$-D_2O)	δ 29·51

[a] Chemical shifts in p.p.m. downfield from 3-trimethylsilyl-1-propanesulphonic acid sodium salt.

Nigen *et al.*[2] have recently published results obtained for covalent labelling of seal myoglobin using [2-^{13}C]bromoacetic acid. Although their experiments showed that this type of approach is viable with regard to sensitivity, there was insufficient distinction between chemical shifts to allow unambiguous assignments. Overlap of resonances is almost certainly due to the dominating effects of the CO_2H group immediately adjacent to the ^{13}C-label. In our experiments using Michael acceptors, this effect has been eliminated.

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