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^{13}C-Enriched Methyl Alkanesulfonates: New Lipophilic Methylating Agents for the Identification of Nucleophilic Amino Acids of Proteins by NMR.

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Abstract: ^{13}C labelled methyl alkylsulfonates are low cost and easily accessible methylating agents. They were used as NMR probes, in association with DEPT ^{13}C {^{1}H} NMR and 2D reversed {^{1}H,^{13}C} correlation NMR for the identification of nucleophilic residues of proteins. This labelling technique allows to visualize the nature of the reactive amino acids and to distinguish between lysine and histidine nucleophilic residues.

There has been considerable interest in the recent years in the development of aminoacid specific modifying reagents as probes for the identification of reactive residues in proteins[1]. Numerous such reagents, mainly substituted aryl halides or sulfonates[2], have thus been developed for the nucleophilic sulfhydryl and amino groups. This approach was successfully applied in the study of protein structures, but most of the reported data were based on indirect evidence, e.g. kinetic studies, amino acid composition, etc.

Nuclear magnetic resonance spectroscopy (NMR) can provide direct and rapid qualitative and semi-quantitative informations on reactive and accessible amino-acids. Such attempts have been achieved using fluorine NMR and 2,6-dinitro-4-trifluoromethyl phenyl group as an NMR probe[3] however the CF_3 group being far from the protein active site, no precise information was obtained. Despite the low sensitivity of the ^{13}C-NMR and the extra-signals due to the protein structure, this technique should be much more powerful, the carbon directly attached to the aminoacid heteroatom being potentially observable. Thus ^{13}C formaldehyde bonds with Bovine Serum Albumin (BSA) were directly observed using ^{13}C-NMR spectroscopy[4] and ^{13}C-labelled cyanoethyl groups have shown some potential on aminoacid models[5].

The use of a methyl probe should be most useful for the NMR analysis of nucleophilic residues of proteins. The large range of chemical shifts of methyl groups would lead to a precise and easy identification of the side chain functional group to which they are linked. Furthermore, a labelled methyl group does not lead to ^{13}C-^{13}C extra coupling and the presence of 3 equivalent protons should allow the use of enhancement techniques by polarization transfer and give good {^{1}H,^{13}C} correlations.

Methyl alkanesulfonates were recently reported to cause severe Allergic Contact Dermatitis (ACD) in guinea pigs[6] and mice[7]. These compounds were suspected to act as lipophilic methylating agents, transferring a methyl group to nucleophilic sites of aminoacids in proteins.

We now report the use of ^{13}C-labelled methyl alkanesulfonates in association with DEPT 135 and 2D reversed $\{^1H,^{13}C\}$ correlation NMR as very promising techniques for the characterization of nucleophilic amino acid residues of proteins.

Compounds **1** and **2**, labelled at C'1 (99 atom % ^{13}C) were synthesized[6] as shown below. The physicochemical properties of such methylating agents can be easily modulated, by modifying the length or the nature of the alkyl chain, in order to adapt them to more or less lipophilic proteins. Thus, we used compound **1** to label Human Serum Albumin (HSA) (as illustrated below) but found the use of **2** more efficient in the case of Hen Egg white Lysozyme (HEL) or Ribonuclease A.

$$RSO_3Na \xrightarrow{a} RSO_3Ag \xrightarrow{b} RSO_3\,{}^{13}CH_3$$

$R = C_{12}H_{25}$ **1**

$R = C_8H_{17}$ **2**

Reagents: (a) HNO_3 2M / EtOH (1:1), $AgNO_3$, 25°C, 30 min; (b) $^{13}CH_3I$ / CH_3CN, 70°C, 30 min, 82% yield.

Incubation of HSA with an excess of **1** in a phosphate buffer (0.1 M) at pH 7.4 (48 hours; 50 °C) led to the appearance of five signals beside those of the starting material (Fig 1, part A). The three signals between 33.2 and 36.2 ppm were interpreted as arising from amine-methyl adducts (lysine or histidine) while the signal at 43.1 ppm arose from a dimethylated lysine adduct. The signal at 25.2 ppm was assigned to the methyl sulfonium salt derivative of methionine[10].

This assignment was supported by the chemical shifts of methyl groups on model nucleophilic amino acids (Table). This result is in complete agreement with the number of the most reactive sites of HSA estimated to be 2 to 3 [2,8]. Reduction of the disulfide bridges under denaturing conditions[1] (8 M urea, dithiothreitol; Fig 1, part B) led to an increase of the number of accessible amino groups by "opening" of the tertiary structure and to the appearance of a new large peak at 15.2 ppm (Me on cystein).

Table: Chemical shift[(a)] of S-Me and N-Me groups in model methylated nucleophilic aminoacids[(b)].

Aminoacids	Methylated products	$\delta\ ^{13}C$	$\delta\ ^1H$
Nα-Ac-Cys-OMe	Nα-Ac-S-methyl-Cys-OMe	16.2	2.13
Met	Methionine methylsulfonium chloride[(c)]	25.1	2.80
Nα-Ac-His-OMe	Nα-Ac-N_{im}-methyl-His-OMe	33.1	3.63
Nα-Boc-Lys-Ala-OMe	Nα-Boc-Nε-methyl-Lys-Ala-OMe	33.6	2.72
	Nα-Boc-Nε-dimethyl-Lys-Ala-OMe	44.2	2.90
Nα-Boc-Tyr-OMe	Nα-Boc-O-methyl-Tyr-OMe	55.0	3.70

(a) Spectra were run on a Bruker 200 MHz instrument. $CDCl_3$ was used as solvent except for the methionine derivative (D_2O).
(b) Model aminoacids were methylated either with methyl alkanesulfonates or with methyl iodide.
(c) Commercially available compound.

The use of a DEPT ^{13}C $\{^1H\}$ sequence[8] with a Θ angle of 135° allowed us to eliminate the likelihood of much of the extraneous interference[9], to increase the signal to noise ratio and use a narrow width sweep. As illustrated, methyl alkanesulfonates in association with a DEPT sequence allowed to get rapid qualitative and semi-quantitative informations on amino or thio nucleophilic groups but did not differenciate between possible

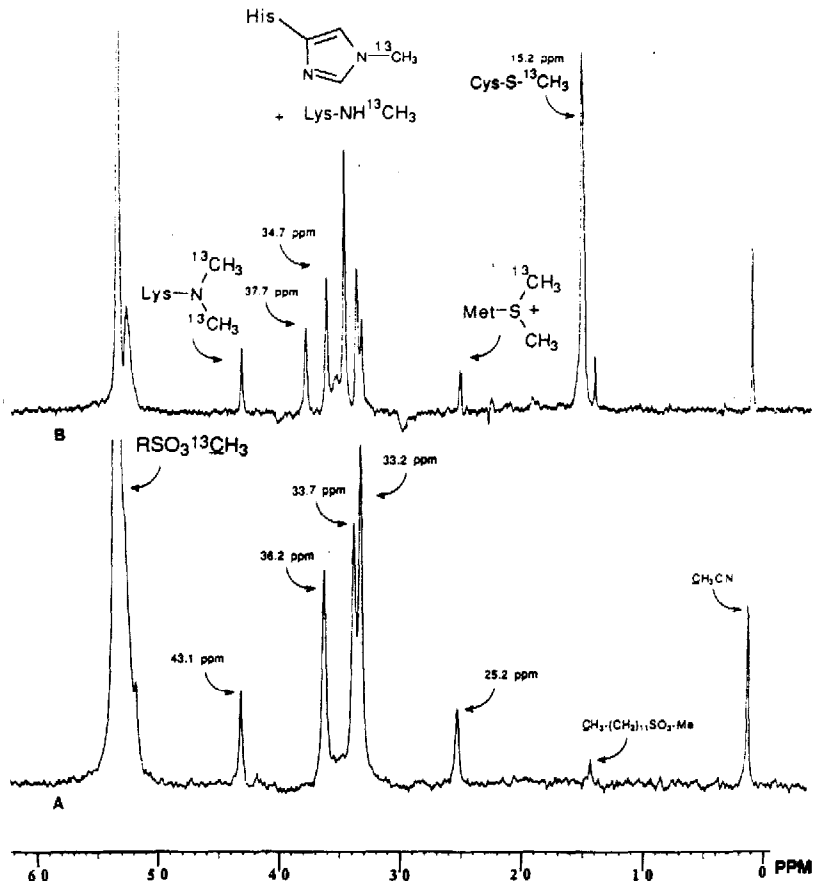

Figure 1. DEPT ^{13}C $\{^1H\}$ NMR spectra (Θ = 135°) of HSA incubated with labelled 1. A: 1 mM HSA incubated with 1 at pH 7.4; B: 1 mM HSA incubated with 1 after reduction of the disulfide bridges. DEPT ^{13}C $\{^1H\}$ NMR spectra were obtained by the method of Doddrell et al.[8] on a Bruker 200 MHz instrument using the following conditions: 400 scans, sweep width 5050 Hz, acquisition time 1.622 s, memory size 16K (digital resolution 0.6 Hz/pt). Exponential multiplication was used for Fourier Transform spectra using a line broadening factor of 5 Hz. Samples were dissolved in 2 mL deuterium oxide (99.8 atom % excess ^2H) and the methyl signal of a trace of acetonitrile was used as internal reference at 1.3 ppm.

lysine and histidine adducts as their ^{13}C chemical shift are very close.

As the ^1H chemical shifts of methylated lysines and histidines must be quite different, we tried to use 2D- reversed $\{^1H,^{13}C\}$ correlation NMR in order to distinguish between lysine and histidine methyl adducts. As shown on Fig. 2, these two methylated species appeared clearly in two zones distant by about 1 ppm on the proton chemical shift scale. Moreover, this correlation experiment confirmed the assignement of the other peaks.

In summary, ^{13}C labelled methyl alkylsulfonates are low cost and easily accessible methylating agents. They can be used as NMR probes, in association with DEPT ^{13}C $\{^1H\}$ NMR and 2D reversed $\{^1H,^{13}C\}$ correlation NMR, to get rapid qualitative and semi-quantitative informations about the accessible reactive aminoacids of proteins. This labelling technique allows one to visualize the nature of the reactive amino acids, to

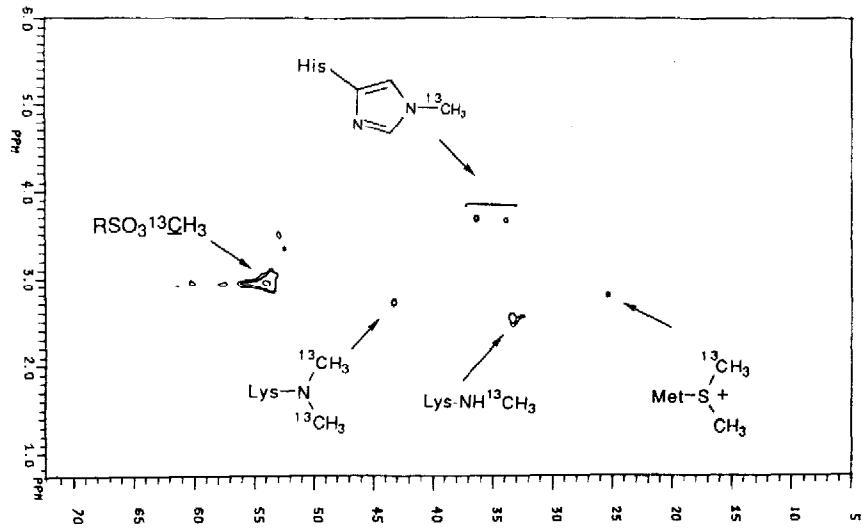

Figure 2. 2D reversed {^{1}H,^{13}C} correlation experiment based on a BIRD sequence of HSA incubated with 1 at pH 7.4. This correlation was run on a Bruker 400 MHz instrument.

have a good idea of the number of reactive sites and to compare the relative reactivity of sites under various reaction conditions. This should find numerous applications in enzymatic and protein studies and the same technique could find extensions in the field of nucleic acids.

Warning: Skin contact with the methylalkylsulfonates must be avoided[6,7]. As methylating agents, these substances must be handled with care.

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   In a typical procedure: to silver dodecanesulfonate (1.78 g; 5 mmol) in CH_3CN (70 mL) was added CH_3I (1 g; 7 mmol). The mixture was heated to 70°C for 30 min, filtered and the solvant removed under vacuum to give 1. ^{1}H-NMR ($CDCl_3$) δ 0.88 (t, 3H); 1.05-1.98 (m, 20H); 3.08 (t, 2H); 3.86 (d, 3H, J_{H-C} = 148 Hz); ^{13}C-NMR δ: 13.9; 22.5; 23.3; 28.1; 28.9; 29.2; 29.3; 24.4 (2C); 29.5; 31.8; 49.7; 55.0.
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