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Characterization of Covalent Protein Conjugates Using Solid-State ^{13}C NMR Spectroscopy

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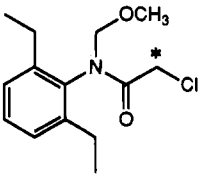
ABSTRACT: Cross-polarization magic-angle spinning (CPMAS) ^{13}C NMR spectroscopy has been used to characterize covalent conjugates of alachlor, an α-chloroacetamide hapten, with glutathione (GSH) and bovine serum albumin (BSA). The solid-state NMR method demonstrates definitively the covalent nature of these conjugates and can also be used to characterize the sites of hapten attachment to proteins. Three different sites of alachlor binding are observed in the BSA system. Accurate quantitation of the amount of hapten covalently bound to GSH and BSA is reported. The solid-state ^{13}C NMR technique can easily be generalized to study other small molecule/protein conjugates and can be used to assist the development and refinement of synthetic methods needed for the successful formation of such protein alkylation products.

Antibody-based immunoassay techniques provide rapid and sensitive methods for the quantitation of small target molecules and are particularly useful as screening methods prior to instrumental analysis of xenobiotic residues in the environment (Wratten & Feng, 1990; Van Emon & Mumma, 1990). In order to elicit antibody production, small molecules must be attached covalently as haptens to carrier proteins. These larger protein conjugates are then used to immunize animals that produce antibodies for immunoassay development. We have been interested for some time in the development of new spectroscopic techniques for the characterization of such small molecule–protein conjugates. We report here on the use of solid-state ^{13}C NMR spectroscopy as a versatile and effective tool that can be used to characterize hapten–protein conjugates and guide the development of new synthetic approaches for their formation.

Conventional analytical methods such as GC, HPLC, and mass spectrometry are not well suited for characterizing hapten–protein conjugates or for directly detecting covalent bonding between small organic molecules and proteins. While hapten radiolabeling and UV detection have been used to characterize protein alkylation products, no general method short of protein sequencing is available for deriving information about the nature of the attachment (Lundblad & Noyes, 1989). The latter technique is both time consuming and inapplicable to heterogeneous systems such as variably substituted hapten–protein conjugates. Since solid-state ^{13}C NMR spectroscopy has been used extensively to characterize the structure and dynamics of a variety of synthetic (Fedotov & Schneider, 1989; Komoroski, 1986) and naturally occurring (Garbow & Stark, 1990; Chirlian & Opella, 1990; Garbow et al., 1989; Smith et al., 1989) macromolecular systems, it occurred to us that this technique should be ideally suited to the characterization of hapten–protein conjugates. We considered that solid-state ^{13}C NMR, in conjunction with selective hapten ^{13}C isotope enrichment, could be used for direct observation of nucleophilic protein heteroatom displacement at the α-position of α-halocarbonyl substrates.



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Effective methods for covalently attaching α-haloacetamides to thiolated protein carriers have recently been developed in our laboratories and were crucial to the elaboration of an immunoassay for the detection of alachlor (**1**), one of the most widely used of the chloroacetanilide herbicides (Feng et al. 1990a,b). The development of these methods was guided by a powerful new application of solid-state ^{13}C NMR spectroscopy for the characterization of covalent small molecule–protein conjugates. This NMR technique can provide quantitative measurement of the stoichiometry of hapten–protein conjugation, as well as chemical-shift information useful in identifying the sites of small molecule attachment to proteins. The solid-state ^{13}C NMR technique thus provides a novel approach for the direct observation and characterization of covalent binding of reactive molecules to proteins and should find wide application for the study of protein modification. In particular, may of the reagents used in protein labeling contain an α-haloketone moiety that reacts with protein nucleophiles via halogen displacement (Lundblad & Noyes, 1989). Furtermore, various strategies for mechanism-based enzyme inactivation ("suicide inhibition") rely on enzyme-catalyzed unmasking of highly reactive α-halocarbonyl al-

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kylating species at the active site (Walsh, 1982).

Materials and Methods

Materials. The [^{13}C]alachlor used in this study was enriched to 90 atom % ^{13}C in the chloromethyl carbon. Covalent conjugates were formed between alachlor and (1) glutathione (GSH), (2) bovine serum albumin (BSA), (3) BSA activated by thiolation of lysine residues with *S*-acetylmercaptosuccinic anhydride (AMSA), and (4) poly(DL-lysine). GSH (as the free acid, 98–100% purity), BSA (RIA grade, 96–99% albumin), poly(DL-lysine) (HBr salt, MW = 30 000–70 000) and *S*-acetylmercaptosuccinic anhydride (AMSA), were obtained from Sigma Chemical Co. and were used without further purification. Although radioimmunoassay-grade BSA was used in the present study, the conjugation method described below is effective for serum albumins of varying degress of purity. For each conjugate, two samples were prepared under identical conditions, one using [^{13}C]alachlor and one using natural-abundance alachlor. All samples were prepared as lyophilized solids after dialysis.

Preparation of Alachlor/Glutathione Conjugates. For preparation of [^{13}C]alachlor/GSH conjugates, [^{13}C]alachlor was mixed with natural-abundance alachlor to yield a material that was enriched to 19 atom % ^{13}C in the chloromethyl carbon position. GSH (155 mg, 0.5 mmol) and alachlor (136 mg, 0.5 mmol) were suspended in 35 mL of methanol. After the addition of 4 mL of 0.5 M $NaOCH_3/CH_3OH$ at room temperature, the suspension became clear. The reaction was stirred for 2.5 h at room temperature, and solvent was removed by rotary evaporation. The crude product was redissolved in 20 mL of water, and the pH of the solution was adjusted to 2–3 with trichloroacetic acid. Unreacted alachlor (yellow liquid) was removed by pipet, and the product was lyophilized. The resulting white crystals were analyzed by high-resolution FAB/MS and by solution ^{1}H and ^{13}C NMR to confirm both the identity of the alachlor/GSH products as well as the ^{13}C-enrichment level of the [^{13}C]alachlor/GSH conjugate. High-resolution ^{1}H and ^{13}C NMR spectra of the alachlor/GSH conjugate in D_2O were collected on Varian VXR-500 and VXR-300 spectrometers, respectively.

The mass spectrum of the alachlor/GSH conjugate showed *m/e* 541.2322 (MH, 541.2332 calculated for $C_{24}H_{37}N_4O_8S$), arising from the natural-abundance alachlor conjugate, and *m/e* 542.2365 (MH, 541.2332 calculated for $C_{23}{}^{13}CH_{37}N_4O_8S$), arising from the [^{13}C]alachlor conjugate. The ^{1}H NMR spectrum of this conjugate shows a signal at 3.95 ppm arising from CH_3O, which was used to adjust the pH of the sample. Because of restricted rotation about its amide bond, alachlor exists in two different rotameric forms (Chupp & Olin, 1967). This is reflected in both the ^{1}H and ^{13}C NMR spectra of the alachlor/GSH conjugate, which show duplicated peaks corresponding to both rotameric forms of the conjugated alachlor moiety.

^{1}H NMR (500 MHz) for the major rotamer (67.7%): δ 1.11 (t, 6H, $CH_2C\underline{H}_3$), 2.03 (m, 2H, $CH_2C\underline{H}_2CH$), 2.39 (t, 2H, $C\underline{H}_2CH_2CH$), 2.44 (q, 4H, $C\underline{H}_2CH_3$), 2.73–2.94 (m, 2H, $SC\underline{H}_2CH$), 3.04 (d, 81% of 2H, J = 4 Hz, for protons attached to ^{12}C of $COC\underline{H}_2S$; 19% of 2H, J = 142 Hz for protons attached to ^{13}C of $COC\underline{H}_2S$), 3.33 (s, 3H, $CH_2OC\underline{H}_3$), 3.75 (t, 1H, $CH_2CH_2C\underline{H}(NH_2)COOH$, 3.79 (s, 2H, $CONHC\underline{H}_2COOH$), 4.35 (q, 1H, $SCH_2C\underline{H}(NH)CONH$), 4.86 (s, 2H, $NC\underline{H}_2OCH_3$), 7.15–7.40 (m, 3H, aromatic); ^{13}C NMR (75 MHz) δ 32.1 (^{13}C-enriched $CO\underline{C}H_2S$).

^{1}H NMR (500 MHz) for the minor rotamer (32.3%): δ 1.03 (t, 6H, $CH_2C\underline{H}_3$), 2.08 (m, 2H, $CH_2C\underline{H}_2CH$), 2.34–2.50 (t, q, 6H, $C\underline{H}_2CH_2CH$ and $C\underline{H}_2CH_3$), 3.00–3.21 (m, 2H, $SC\underline{H}_2CH$), 3.26 (s, 3H, $CH_2OC\underline{H}_3$), 3.71 (d, 81% of 2H, J = 4 Hz, for protons attached to ^{12}C of $COC\underline{H}_2S$; 19% of 2H, J = 142 Hz for protons attached to ^{13}C of $COC\underline{H}_2S$), 3.76 (t, 1H, $CH_2CH_2C\underline{H}(NH_2)COOH$, 3.90 (s, 2H, $CONHC\underline{H}_2COOH$), 4.59 (q, 1H, $SCH_2C\underline{H}(NH)CONH$), 4.92 (s, 2H, $NC\underline{H}_2OCH_3$), 7.15–7.40 (m, 3H, aromatic); ^{13}C NMR (75 MHz) δ 3.10 (^{13}C-enriched $CO\underline{C}H_2S$).

Preparation of Alachlor/Activated BSA Conjugates. The procedure for preparing alachlor conjugates with activated BSA has been described previously (Feng et al., 1990a). BSA (200 mg, 3 nmol) and AMSA (13 mg, 25 mol equiv) were dissolved in 12 mL of water at 0 °C. A solution of alachlor (20 mg, 25 mol equiv; natural abundance or 90% ^{13}C enriched, with or without an added trace of ^{14}C-labeled alachlor) in 2 mL of dioxane was slowly added. The pH of the solution was adjusted to 11 with 1 M sodium bicarbonate, and the reaction mixture was stirred for 15 min at 0 °C. After being stirred for 2 h at room temperature, the reaction mixture was neutralized with HCl. Initially, the alachlor/BSA conjugate was purified by dialysis overnight against water and lyophilized. When the solid-state ^{13}C NMR spectrum of this sample showed two relatively sharp signals, consistent with its containing low molecular weight material, the sample was redialyzed overnight against 1:1 acetonitrile/water and again lyophilized.

Preparation of Alachlor/BSA Conjugates. Alachlor (20 mg, 0.07 mmol; natural abundance or 90% ^{13}C enriched, with or without an added trace of ^{14}C-labeled alachlor) was dissolved in 2 mL of dioxane. This solution was then added slowly to a solution of BSA (200 mg, 3 nmol) dissolved in 12 mL of water at 0 °C. The pH of the solution was adjusted to 11 with 1 M sodium bicarbonate; the reaction mixture was stirred for 15 min at 0 °C and was maintained for 2 h at room temperature. The mixture was then neutralized with HCl, purified by dialysis overnight against 1:1 acetonitrile/water, and lyophilized.

Preparation of Alachlor/Poly(lysine) Conjugates. Poly(DL-lysine) (300 mg, 5.5 μmol) was dissolved in 18 mL of water at 0 °C. Alachlor (151.5 mg, 100 mol equiv) dissolved in 3 mL of dioxane was slowly added. The pH of the solution was adjusted to 11 with 1 M sodium carbonate, and the reaction mixture was stirred for 15 min at 0 °C. After being stirred for 2 h at 50 °C, the reaction mixture was neutralized with HCl and the poly(lysine)/alachlor conjugate was purified by dialysis against 1:1 acetonitrile/water. The acetonitrile was removed by further dialysis against water, and the conjugate was lyophilized.

Experimental NMR. Cross-polarization magic-angle spinning (CPMAS) ^{13}C NMR spectra (Schaefer & Stejskal, 1976; Mehring, 1983; Fyfe, 1983) were obtained at 31.94 MHz by using 2-ms 50-kHz ^{1}H–^{13}C spin-lock contacts with high-power (65 kHz) proton dipolar decoupling. Samples were spun at the magic angle (54.7°) with respect to the static magnetic field in a double-bearing rotor system (Schaefer et al., 1987) at a rate of 3 kHz. Spinning side bands were eliminated from the CPMAS ^{13}C NMR spectrum of alachlor (Figure 1a) by using the TOSS (Dixon, 1982) pulse sequence. Sample weights in this study ranged from 100 to 300 mg, and each ^{13}C NMR spectrum required one to two days of data collection. Under the described CPMAS conditions, line intensities within each spectrum accurately represent the *relative* amount of each carbon type present in the sample (Stejskal et al., 1979; Fukamizo et al., 1986). Tabulated carbon compositions determined from NMR spectra were obtained from

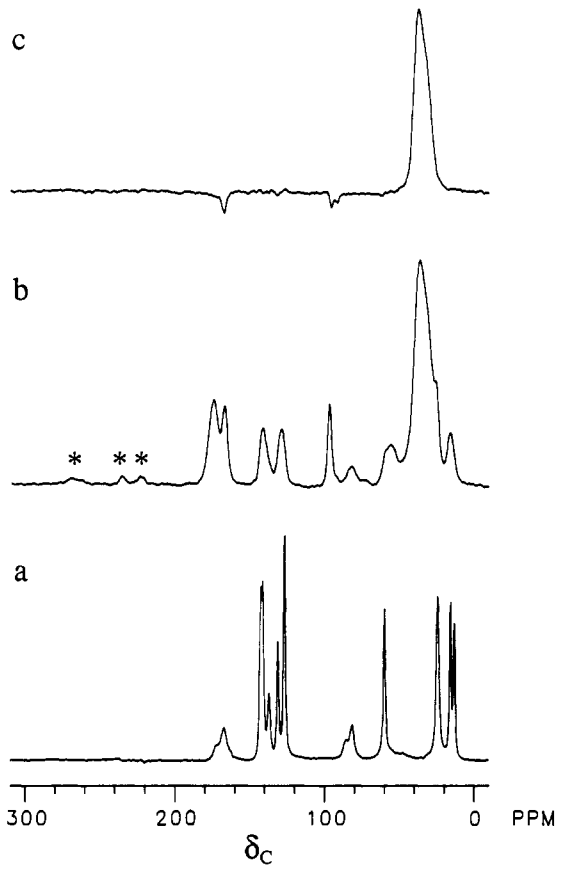


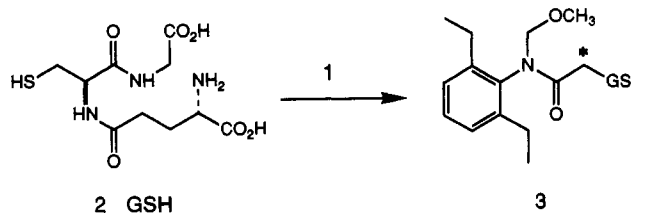
FIGURE 1: CPMAS ^{13}C NMR spectra of crystalline alachlor and lyophilized GSH/alachlor covalent conjugates. (a) Natural-abundance alachlor. Spinning side bands were eliminated in this spectrum only by using the TOSS pulse sequence. Line assignments are $-CH_3$ (δ_C 12, 15 ppm); $-\underline{C}H_2-CH_3$ (δ_C 24 ppm); $-O-CH_3$ (δ_C 59 ppm): $-N-CH_2-O$ (δ_C 81 ppm); aromatic (δ_C 126–142 ppm); carbonyl (δ_C 167 ppm). (b) [^{13}C]Alachlor/GSH conjugate. Asterisks denote spinning side bands that arise from the mechanical sample rotation. (c) Difference, showing signals arising from enriched alachlor carbon.

peak areas measured by deconvolution and integration. Deconvolution of peaks in experimental spectra was accomplished on a Nicolet-1280 computer using Nicolet's NMCCAP Program. Spinning side-band intensities were included in all integrations.

Results and Discussion

GSH/Alachlor Conjugates. Figure 1a illustrates the natural-abundance CPMAS ^{13}C NMR spectrum of crystalline alachlor. Spinning side bands were eliminated from this spectrum by using the total suppression of side bands (TOSS) (Dixon, 1982) pulse sequence. All carbon atoms, except the chloromethyl carbon, are visible and assignable in this spectrum. The chloromethyl carbon resonance is not observed because of broadening caused by dipolar coupling to the quadrupolar chlorine nucleus, an effect previously observed in halogenated polymers (Schaefer et al., 1984; Poliks & Schaefer, 1990). Signals arising from carbons bonded to quadrupolar ^{14}N nuclei are broadened into "asymmetric doublet" line shapes (Hexem et al., 1981; Olivieri et al., 1987). The "invisibility" of carbons that are bonded to chlorine is advantageous for studying alachlor–protein conjugates. Covalent conjugation of alachlor with a peptide or protein involves the nucleophilic displacement of Cl^- and formation of a heteroatom covalent bond between the α-methylene carbon and the peptide/protein. *Detection of signals arising from the ^{13}C-enriched methylene carbon in the spectra of alachlor conjugates therefore provides direct evidence that chlorine has been displaced and a covalent bond has formed.*

Scheme I



2 GSH 3

To test the utility of the solid-state ^{13}C NMR method in a simple system, we prepared covalent conjugates of alachlor with glutathione (GSH), **2**. Reaction of alachlor with GSH results in a covalently bonded conjugate, **3**, containing a newly formed C-S bond (Scheme I). Two conjugates were prepared by using either natural-abundance (^{12}C) or ^{13}C-enriched alachlor. The latter was enriched to 19 atom % ^{13}C in the chloromethyl carbon position. Figure 1b illustrates the solid-state ^{13}C NMR spectrum of the GSH/[^{13}C]alachlor covalent conjugate, which shows both natural-abundance signals and the signal arising from the enriched alachlor carbon. The line widths observed in this spectrum are broadened due to dispersions of chemical shifts that reflect the amorphous nature of this lyophilized sample. Figure 1c is the difference spectrum that results from subtracting the spectrum of a GSH/[^{12}C]-alachlor conjugate (not shown) from that of Figure 1b (taking into account differences in sample weight and total number of transients collected). The major peak in this difference spectrum is located at 35 ppm, consistent with the formation of a carbon–sulfur bond (Breitmaier & Voelter, 1989). Minor, negative-amplitude peaks in this difference spectrum are assigned to the carboxyl carbon of Cl_3CCOOH and to CH_3O^-, which were used to adjust the pH and which are present in sightly differing amounts in the two samples.

By comparing the integrated areas of the signals arising from the enriched alachlor carbon with the four natural-abundance carbonyl carbons of the conjugated GSH moiety, we can calculate the average number of alachlor molecules bound per molecule of GSH. From deconvolution of the spectra of Figure 1, the ratio of the ^{13}C-enriched alachlor carbon peak area (35 ppm signal; 19 atom % ^{13}C) to the natural-abundance carbonyl carbon peak area (175 ppm signal; 1.1 atom % ^{13}C), including spinning side-band intensities, is calculated to be 3.1:1. The 175 ppm signal receives contributions from the carbonyl carbons of both alachlor (1 per molecule) and GSH (4 per molecule). Using this information and accounting for the 19% ^{13}C enrichment of the alachlor, we calculate 0.9 mol of alachlor bound per mole of GSH. This NMR finding was also verified by mass spectrometry and agrees well with the expected 1:1 stoichiometry of this reaction.

BSA/Alachlor Conjugates. In preparing alachlor/protein conjugates for animal immunization, the protein is first activated with *S*-acetylmercaptosuccinic anhydride, AMSA, **4**, which reacts with side-chain NH_2 groups to yield terminal thiols of type **5** (Scheme II) (Feng et al., 1990a,b). Covalent binding of alachlor then occurs through the formation of a thiol ether bond to the chloromethyl carbon, as shown in structure **6**. Figure 2a shows the CPMAS ^{13}C NMR spectrum of the covalent conjugate formed from activated BSA and [^{12}C]-alachlor. Because of the relative molecular weights of the protein and alachlor, this spectrum is, in essence, the natural-abundance ^{13}C NMR spectrum of BSA itself. The broad lines in this spectrum reflect dispersions of chemical shifts due both to the heterogeneity of local sites within the protein chains and to the effects of interchain packing in the lyophilized solid. The spectrum of the activated BSA/[^{13}C]alachlor covalent

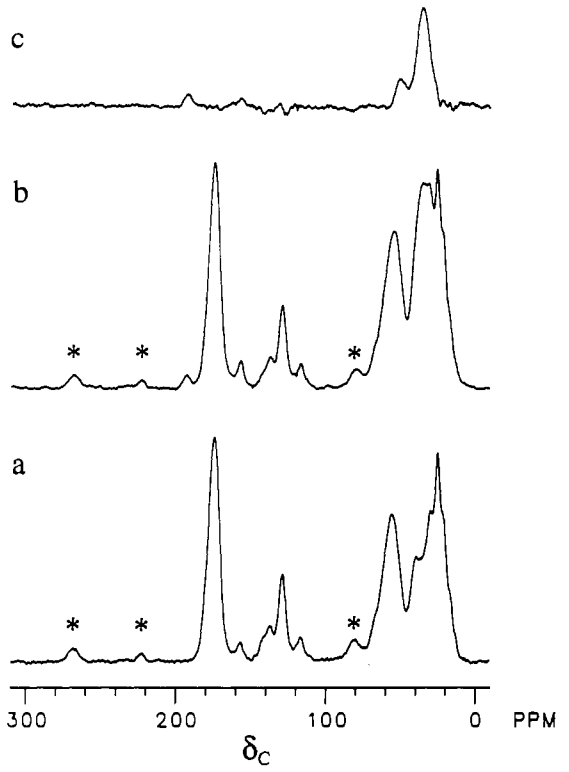
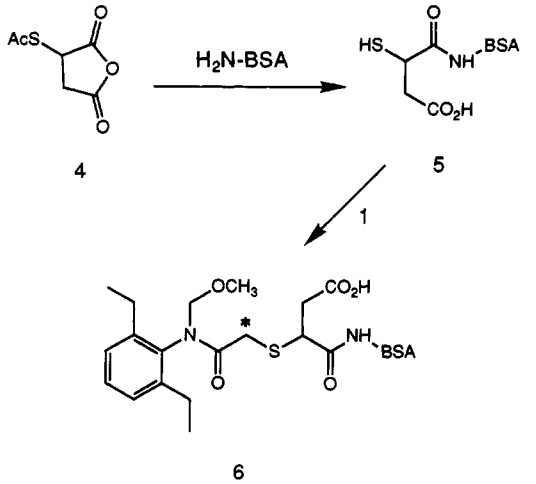


FIGURE 2: CPMAS ^{13}C NMR spectra of alachlor/activated BSA covalent conjugates following denaturation/dialysis. (a) Natural-abundance alachlor conjugate. Line assignments are aliphatic carbon (δ_C 10–40 ppm); C_α (δ_C 40–60 ppm); aromatic/olefinic carbon (δ_C 110–150 ppm); carbonyl carbon (δ_C 160–185 ppm). (b) [^{13}C]alachlor conjugate. (c) Difference, showing signals arising from enriched alachlor carbon. Asterisks denote spinning side bands.

Scheme II



conjugate is shown in Figure 2b, while Figure 2c illustrates the difference spectrum resulting from the subtraction of spectra of enriched and natural-abundance alachlor samples.

The difference spectrum of the BSA/[^{13}C]alachlor conjugate shows two partially resolved lines at 35 and 50 ppm, respectively. In contrast to the spectrum of crystalline alachlor (Figure 1a), the lines in this difference spectrum are broad, because the aforementioned heterogeneity of the protein is transferred to the bound alachlor. The 35 ppm chemical shift corresponds well with the signal observed in the difference spectrum of the GSH/alachlor covalent conjugates (Figure 1c); the 50 ppm chemical shift is characteristic of a nitrogen-bonded carbon (Breitmaier & Voelter, 1989). This result indicates that, besides a link through attached AMSA, there

Table I: Stoichiometry of Alachlor/BSA Conjugates from Deconvolution of Solid-State ^{13}C NMR Spectra (Figures 2 and 3)

	activated BSA		nonactivated BSA	
resonance	peak area[a]	bound alachlor[b]	peak area[a]	bound alachlor[b]
alachlor[^{13}C]–S (35 ppm)	45.5	4.0	6.6	0.6
alachlor[^{13}C]–N (50 ppm)	8.9	0.8	17.8	1.6
natural-abundance carbonyl (175 ppm)	100		100	

[a] Arbitrary units; spinning side bands included. [b] Mole equivalents per mole of BSA.

is a second site of attachment of alachlor to BSA, most likely via a direct reaction of the alachlor with a lysine side chain. In support of this idea, we measured a chemical shift of 52 ppm for the alachlor α-methylene carbon when covalently attached to lysine nitrogen in a [^{13}C]alachlor/poly(lysine) conjugate (spectrum not shown).

Stoichiometry of BSA/Alachlor Conjugates. The spectra of Figure 2 can be used to determine the average number of alachlor molecules covalently bound per molecule of BSA. This requires that the relative areas of the signals arising from the ^{13}C-enriched alachlor carbon (90 atom % ^{13}C) and natural-abundance carbonyl carbons (1.1 atom % ^{13}C) be determined. To convert these integrated areas into an average number of bound alachlors/BSA, the molecular composition of BSA must be considered. Since BSA is a 606-residue protein containing 12 asparagines, 17 glutamines, 36 aspartic acids, and 58 glutamic acids (Peters, 1983; Brown, 1975), each molecule of BSA has a total of 729 carbonyl carbons (peptide carbons + side-chain acids and amides). The ^{13}C enrichment of the chloromethyl carbon of the alachlor used for conjuation is 90/1.1 = 82 times that of natural abundance. Using the peak areas determined by deconvoluting and integrating the spectra shown in Figure 2 (Table I) and considering both the composition of BSA and the ^{13}C enrichment, we calculate an average of 4.8 mol equiv of alachlor bound per mole of BSA. Of these 4.8 mol; 4.0 are bound via the activator C–S bond and 0.8 are bound at an alternate site (C–N bond).

To compare the quantitative results determined by NMR with those obtained via radioisotopic methods, we prepared samples of the activated BSA/alachlor conjugate using a mixture of [^{13}C]alachlor and [^{14}C]alachlor, under the conditions reported in Figure 2 and Table I. NMR quantitation of bound alachlor hapten per equivalent of BSA was performed by deconvoluting a difference spectrum (not shown) of this radiolabeled conjugate, as described above. A total of 6.3 mol equiv of covalently bound alachlor per mole of activated BSA was found for the radiolabeled sample. The amount of bound alachlor in this sample was also determined independently by ^{14}C liquid-scintillation counting. The latter method provided a conjugation ratio of 8.3 mol equiv of alachlor per mole of activated BSA, in reasonable agreement with the NMR result. The difference in conjugation ratio between radiolabeled and nonradiolabeled conjugates (6.3 vs 4.8) reflects the variability in conjugate preparation commonly encountered in our laboratory and considerably exceeds the quantitation errors of the NMR experiment.

Sites of Alachlor Attachment in Nonactivated BSA/Alachlor Conjugates. The solid-state ^{13}C NMR results with AMSA-activated BSA samples indicate that alachlor is covalently bound to at least two different sites. One of these is identified by the ^{13}C chemical shift of the sulfur-bound

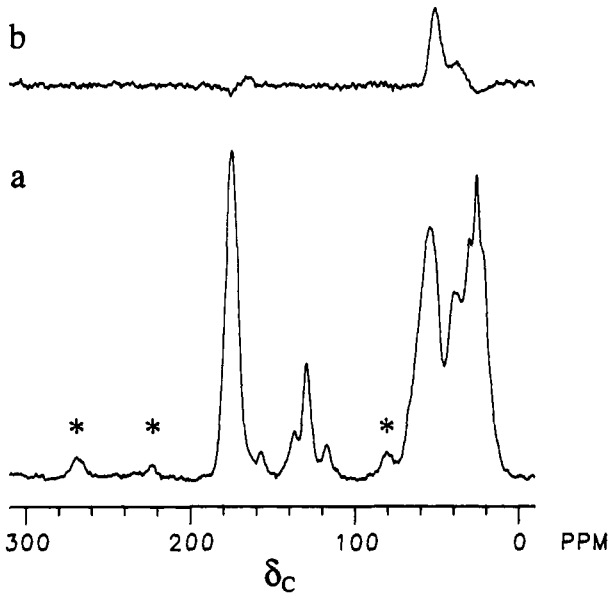
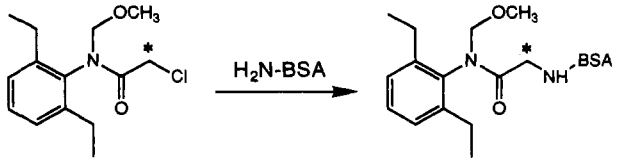


FIGURE 3: CPMAS ^{13}C NMR spectra of alachlor/BSA covalent conjugates. (bottom) [^{13}C]Alachlor/BSA conjugate. (top) Difference, showing signals arising from enriched alachlor carbon. Asterisks denote spinning side bands.

Scheme III



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carbon as being the thiol group of the protein activator. The other is characterized by a ^{13}C chemical shift indicative of a C–N covalent bond. Under basic conditions, alachlor can bind directly to lysine NH_2 groups, resulting in the formation of a carbon–nitrogen bond (Scheme III). To further investigate the reactivity of this site, we prepared conjugates of alachlor (^{13}C enriched and natural abundance) with BSA in the *absence* of AMSA. Figure 3 shows the CPMAS ^{13}C NMR spectra of these samples. The difference spectrum of Figure 3b demonstrates that alachlor binds to the nonactivated BSA at two different sites. The 50 ppm signal matches that of the downfield peak in Figure 2b and corresponds to alachlor attachment to a nitrogen atom. In addition, the chemical shift of the 35 ppm signal coincides with that of the C–S bond formed between alachlor and GSH (Figure 1c). This latter signal suggests a direct alkylation of BSA's cysteine residues by alachlor in the absence of activation. By deconvoluting and integrating the spectra in Figure 3 (Table I), we calculate an average of 2.2 mol of alachlor bound per mole of nonactivated BSA.

In conclusion, we have described a novel application of CPMAS ^{13}C NMR for characterizing the covalent attachment of α-chloroacetamide haptens to proteins. The solid-state ^{13}C NMR technique provides a unique detection method that directly visualizes the carbon atoms undergoing covalent binding to protein heteroatom residues. The solid-state ^{13}C NMR method permits accurate quantitation of alachlor covalently bound to glutathione and to bovine serum albumin and also distinguishes the sites of hapten attachment to protein residues. The solid-state ^{13}C NMR method can easily be used to study other small molecule conjugate systems in which a halogenated carbon atom undergoes protein heteroatom displacement and can be used to guide the development of effective methods for conjugate formation. Solid-state ^{13}C NMR should thus be generally applicable to the characterization of covalently modified proteins, yielding information about reaction stoichiometry as well as site attachment.

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