Enzymatic Synthesis of 4-Amino-3,5-diethylphenyl Sulfate, a Rodent Metabolite of Alachlor

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Rat liver tissue homogenates were utilized for in vitro enzymatic conversion of 2,6-diethylaniline (DEA) to the important alachlor metabolite 4-amino-3,5-diethylphenyl sulfate (ADEPS), which was also generated as a radiolabeled standard for use in metabolism studies. ADEPS formation in rodents is associated with the production of other reactive metabolites implicated in alachlor rodent carcinogenesis, making dependable access to an ADEPS standard highly desirable. ^{14}C-DEA was oxidized by rat liver microsomes to ^{14}C-4-amino-3,5-diethylphenol, which was further converted to ADEPS via addition of the phosphosulfate transferase cofactor adenosine-3′-phosphate-5′-phosphosulfate. Microprobe NMR was used in conjunction with high-resolution mass spectrometry to fully characterize the resulting ^{14}C-ADEPS and confirm its structure. Because microgram quantities sufficed for full characterization, the enzymatic transformation provides a viable alternative to radiosynthesis of ^{14}C-ADEPS.

Keywords: *Alachlor; enyzmes; metabolite; synthesis; microprobe NMR; FAB-MS*

INTRODUCTION

Alachlor [**I**, 2-chloro-*N*-(methoxymethyl)-*N*-(2,6-diethylphenyl)acetamide] is a preemergence herbicide widely used for the control of weeds in many crops, including corn and soybeans. A number of studies have elucidated the mammalian metabolism of alachlor, which in rats is extensively degraded via a highly complex network of metabolic pathways (Sharp, 1988). Additional in vitro studies have further characterized these pathways as including conjugation via glutathione *S*-transferases (Feng and Patanella, 1988) and oxidation via cytochrome P-450 mixed function oxygenases (Feng and Patanella, 1989). A third pathway of alachlor metabolism in rats (see Figure 1) has been found to involve N-dealkylation of alachlor to form 2-chloro-(2,6-diethylphenyl)acetamide, **II**, followed by hydrolysis of the amide bond to form 2,6-diethylaniline (DEA), **III** (Kimmel et al., 1986; Feng et al., 1989). Further oxidation of DEA leads to formation of 4-amino-3,5-diethylphenol (ADEP), **IV** (Feng and Wratten, 1987). Additional in vitro studies have demonstrated that rat nasal tissues catalyze the conversion of **III** to the reactive 3,5-diethylbenzoquinone-4-imine, which is the proposed intermediate leading to alachlor carcinogenesis (Feng et al., 1990; Li et al., 1992). This DEA-mediated pathway also likely accounts for the formation of 4-amino-3,5-diethylphenyl sulfate (ADEPS), **V**, which is a minor urinary metabolite identified in rats and mice (Sharp, 1988). Should ADEPS arise from the same precursor **IV** that is postulated to lead to the reactive quinone imine intermediate, then the presence of ADEPS in metabolite profiles may serve as a useful indicator of the presence of a pathway implicated in tumor formation, making dependable access to an ADEPS standard highly desirable. The study described here was therefore conducted to demonstrate that (1) ADEPS can be formed from DEA via enzyme preparations similar to those catalyzing formation of the quinone imine intermediate and (2) such enzyme preparations can be

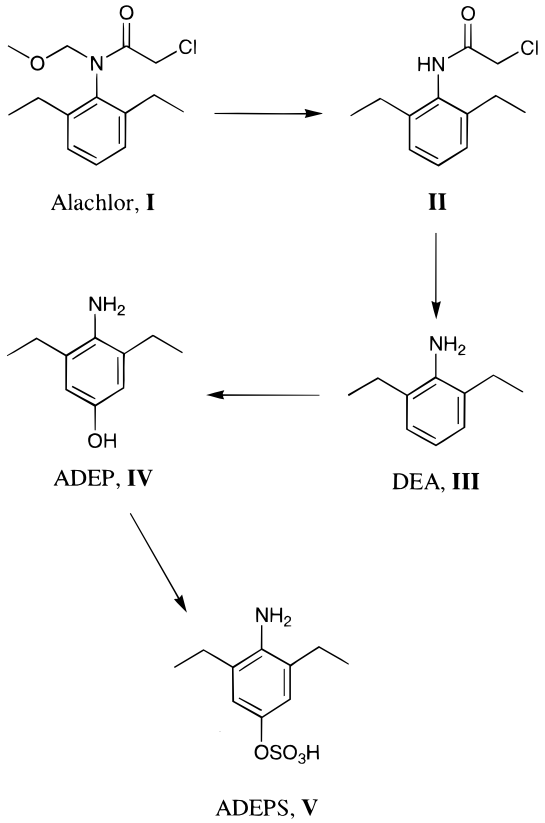

Figure 1. Proposed metabolic pathway for the conversion of alachlor to ADEPS.

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employed to develop an effective enzymatic synthesis of radiolabeled ADEPS for use as a standard to detect minor amounts of this important indicator metabolite, in lieu of costly multistep radiosynthesis.

EXPERIMENTAL PROCEDURES

Chemicals. Uniformly labeled (^{14}C-phenyl)diethylaniline·HCl (19.6 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, GSH, nicotinamide adenine dinucleotide phosphate (β-NADP), adenosine-5′-triphosphate (ATP), adenosine-3′-phosphate 5′phosphosulfate (PAPS), uridine-5′-diphosphoglucuronic acid (UDPGA), nicotinamide, magnesium chloride, and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (St. Louis, MO). *N*,*N*-Dimethylformamide dimethyl acetal (DMF·DMA) was obtained from Aldrich Chemical Co. (St. Louis, MO). Acetonitrile (Optima HPLC grade) and methanol (Optima HPLC grade) were obtained from Fisher Scientific Co. (St. Louis, MO) and were vacuum degassed and filtered (0.45 μm) prior to use. Atomlight and Atomflow scintillation cocktails were obtained from New England Nuclear. Deuterium oxide (99.996% D) was obtained from Cambridge Isotope Laboratories (Andover, MA).

Chemical Synthesis of ADEPS. ADEPS was synthesized from 4-amino-3,5-diethylphenol (ADEP). The synthesis of ADEP was reported previously (Feng and Wratten, 1987). ADEP (9 mg, 0.054 mmol) was dissolved in pyridine/methylene chloride 1:10 (1.5 mL) and mixed with a solution of pyridine/SO_3 (8 mg, 0.05 mmol) in methylene chloride (1 mL). The mixture was stirred at 25 °C and monitored for unreacted ADEP periodically using TLC (silica, ether solution). Additional portions of pyridine/SO_3 in methylene chloride (8 mg/mL each) were added after 3 h, so that a total of 3.7 equiv was used. Even though small amounts of unreacted phenol were still detectable, the reaction was stopped after 48 h by evaporation. The residue was partitioned between ether (2 × 5 mL) and water (2 × 10 mL). The combined water layers were lyophilized to yield a solid (32 mg), which was triturated with 95% ethanol (2 × 10 mL). The ethanol soluble material was dissolved in methanol/water (1:1, 1 mL) and washed through a column (2 mL) of AG50 W-X2 cation-exchange resin (100−200 mesh, ammonium form) in the same solvent. Fractions (1 mL) were collected and monitored by HPLC [isocratic 1 mL/min of 10% acetonitrile in 0.1 M ammonium acetate buffer (pH 3.25) on μ-Bondapak C-18 (3.9 × 300 mm)]. The third fraction contained the desired ADEPS (1 mg, 8% theoretical yield) as a clear oil that slowly solidified. NMR spectra were recorded on a Varian XL-300 spectrometer in $CDCl_3$. The following chemical shifts were assigned relative to $CHCl_3$ at δ 7.26: δ 6.97 (s, 2H), 2.61 (q, 4H), 1.30 (t, 6H). Negative ion FAB/MS showed *m*/*z* 244 (M − H). High-resolution negative ion FAB/MS showed 244.0642; $C_{10}H_{14}NO_4S$ requires 244.0644.

Liver Homogenate Preparation. The enzymatic conversions were conducted using rat liver 9000*g* (S-9), 100000*g* (cytosolic), and microsomal fractions. The enzyme fractions were prepared from the pooled organs of male Sprague-Dawley rats or Long Evans rats (Charles River Laboratories; average body weight = 250 g) using a previously published procedure (Feng and Wratten, 1987). The animals were sacrificed, and the livers were removed immediately. The pooled organs were rinsed with cold buffer (0.01 M phosphate, pH 7.4, 1.15% KCl) and homogenized using a Teflon pestle in cold buffer (3 mL/g of tissue). The homogenate was centrifuged at 9000*g* for 30 min, and the supernatant was filtered through cheesecloth to remove lipids. The resultant preparation was the S-9 fraction. Further centrifugation of the S-9 fraction at 100000*g* for 1 h produced a pellet that was suspended to produce the microsomal fraction. All procedures were performed at 0 °C, and the enzyme fractions were stored at −80 °C. The concentration of protein in the microsomal fraction was ∼24 mg/mL.

In Vitro Incubations. ^{14}C-DEA (0.07 μmol, 2.0 mCi/mmol) was incubated with rat liver microsomal fraction (1 mg of protein) in the presence of NADPH (1.5 μmol), $MgCl_2$ (3 μmol), phosphate buffer pH 7.4 (0.04 mmol), and water made up to a final volume of 0.3 mL. The conversion of ADEP to ADEPS was conducted as follows. ^{14}C-ADEP (0.07 μmol, 2.0 mCi/mmol) was incubated with rat liver cytosolic fraction (4 mg of protein), PAPS (2 μmol), phosphate buffer pH 6.5 (0.04 mmol), and water made up to a final volume of 0.3 mL. Incubations were also conducted with the liver S-9 fraction at pH 7.4 and in the presence of both NADPH and PAPS. The concentrations of the cofactors were the same as described above.

Enzymatic Preparation of ^{14}C-ADEPS. β-NADP was transferred in 1.0 mL of phosphate buffer (0.4 M; pH 7.4) to a 20 mL scintillation vial and mixed with 0.100 mL of $MgCl_2$ (0.25 M), 0.100 mL of nicotimamide (0.25 M), 0.100 mL of glucose-6-phosphate (0.50 M), 0.050 mL of glucose-6-phosphate dehydrogenase (285 units/mL), and 3 mL of rat liver microsomes (∼72 mg of protein). An additional 0.45 mL of buffer was used to bring the final volume to 4.8 mL. A micro stir bar was added to the vial, and the solution was incubated in a water bath at 37 °C with slow stirring for 6 min. To the solution was slowly added 0.100 mL of (^{14}C-phenyl)diethylaniline·Cl (0.0496 mCi; 19.6 mCi/mmol) in methanol. The oxidation of ^{14}C-DEA was monitored by sampling 0.025 mL aliquots and precipitating the protein with 0.025 mL of cold methanol. Following centrifugation, the supernatant was analyzed by HPLC/RAD. After 12 h of incubation, 0.5 mL of adenosine-3′-phosphate 5′-phosphosulfate in buffer (2 mg/mL) was added to the solution. After an additional 9.5 h at 37 °C, a new polar product representing 14% of the total radioactivity was observed by HPLC/RAD. The entire solution was transferred in a total volume of 8 mL to a centrifuge tube and mixed with 8 mL of methanol. Following centrifugation, 8 mL of the supernatant was reduced to a volume of 1.0 mL, which was a crude solution containing the phenyl sulfate.

A total of 1.0 mL (10^7 dpm/mL) of the crude phenyl sulfate solution was processed using HPLC/LSC. Two 0.5 mL aliquots were injected, and eluent was collected in 1.0 min fractions into a single set of 20 mL scintillation vials. The amount of radioactivity in each vial was determined by mixing 0.005 mL from each fraction with 15 mL of Atomlight and analyzing the solutions using liquid scintillation counting (LSC). A total of 1.38×10^6 dpm (7.8 μg) of the phenyl sulfate was isolated.

Instrumentation. HPLC analysis of the in vitro liver enzyme incubation mixtures was performed using a Waters μBondapak C-18, 10 μm, column (3.9 × 300 mm). A linear gradient was used, programmed from 20% acetonitrile in 5 mM acetate buffer (pH 4.6) to 100% acetonitrile in 15 min at 1 mL/min. Under these conditions, baseline peak resolution was achieved for all metabolites, with the following observed retention times: alachlor, 17.0 min; 2-chloro-(2,6-diethylphenyl)acetamide, **II**, 8.2 min; DEA, **III**, 12.0 min; ADEP, **IV**, 7.8 min; and ADEPS, **V**, 4.4 min. HPLC analysis for the metabolite standard preparation was conducted with equipment manufactured by Waters Associates, Inc. (Milford, MA), including a model U6K injector, a model 484 variable wavelength detector, two model 510 pumps, and a model 680 controller. HPLC analysis with radioactive flow detection (HPLC/RAD) was performed with a Radiomatic Flo-One Beta model BD radioactivity flow detector equipped with a 2.5 mL flow cell (Packard Instrument Co., Downers Grove, IL), interfaced in series with the UV detector. Atomflow liquid scintillation cocktail was introduced via a mixing tee and was pumped at 3.0 mL/min using a model E-120-S metering pump manufactured by Eldex Laboratories, Inc. (San Carlos, CA). Separation was achieved using a Beckman Altex 5 μm ODS column (4.6 mm × 25 cm) and a gradient program with a flow rate of 1.0 mL/min using 0.1% TFA and acetonitrile. The program started with 10% acetonitrile, which was held constant for the first 5 min. A linear gradient from 10 to 100% acetonitrile in 0.1% TFA was run in 20 min. Data from both detectors were collected and processed using the Monsanto Automated Chromatography System (MACS), an automated chromatography data system developed by Monsanto.

To isolate the ^{14}C-ADEPS metabolite standard, HPLC with liquid scintillation counting (HPLC/LSC) was performed using a Beckman Altex 5 μm ODS column (10 mm × 25 cm) and a

gradient program with a flow rate of 3.0 mL/min. The program started with 10% acetonitrile in aqueous acetic acid (1.0%), which was held constant for the first 5 min. A linear gradient from 10 to 100% acetonitrile was run in 20 min. LSC was performed on collected fractions collected every minute using Tracor Analytic Mark III model 6881 counters interfaced with the TRACE (The RadioActivity Counting SystEm), an automated data handling system developed by Monsanto. The standard counting time was 1 min per pass, and three valid passes were collected to generate a set of data. Valid passes were determined by applying an outlier test to the data generated from multiple passes. Under these conditions, baseline peak resolution was achieved for all compounds, with the following observed retention times: DEA, **III**, 18.2 min; ADEP, **IV**, 8.9 min; and ADEPS, **V**, 4.7 min.

Mass Spectrometry. Gas chromatography/mass spectrometry (GC/MS) data were obtained with a Finnigan 4515 quadrupole mass spectrometer and were processed with a Data General Nova 4 computer using SUPER INCOS software. The samples were introduced via a Finnigan 9610 gas chromatograph, which was equipped with a J&W Scientific DB-5 capillary column (15−30 m × 0.32 mm). Chemical ionization of the gas chromatograph eluent (GC/CI/MS) was performed with isobutane (0.5 Torr). The mass spectrometer was scanned generally from 200 to 650 amu in 1 s. Positive or negative ions were analyzed.

Dynamic FAB mass spectra (low resolution) were recorded on a VG ZAB-HF double-focusing mass spectrometer (Manchester, U.K.) and processed with a Digital VAXstation 4000.60 workstation and VG OPUS software (v 2.1A). The microbore HPLC system consisted of an Applied Biosystems Inc. model 140B syringe pump, an Applied Biosystems Inc. model 785A programmable absorbance detector (monitoring 254 nm), a Rheodyne model 8125 injector, and a VG dynamic FAB probe (M 416334D). The eluent of a Brownlee Spheri-5 ODS column (1 mm × 250 mm) was split 6:1 (v/v). The major portion flowed through a UV detector, where metabolites could be recovered if necessary. The remainder was directed into the mass spectrometer through a fused-silica capillary (50 μm i.d. × 150 μm o.d.). Water/methanol/glycerol, 7:2:1 (v/v), was added at a flow rate of 1 μL/min via another fused-silica capillary (200 μm × 375 μm o.d.) coaxial with the first so that the mixing did not occur until each component reached the ionization target. Chromatographic separation of the metabolites was carried out with the Spheri-5 column using gradient programming. The organic solvent was acetonitrile; the aqueous phase was 1% formic acid in water. The column was eluted at 40 μL/min with 5% acetonitrile and 95% aqueous held for 8 min followed by a linear gradient from 5 to 100% acetonitrile in 15 min. The FAB source temperature was maintained at ~40 °C, and the source potential was applied at −6000 eV for negative ion analysis. Ionization was achieved with an Ion Tech Saddle Field fast atom gun producing 7 kV of xenon atoms at a 1 mA emission current. The mass spectrometer was normally adjusted to a resolution of 1000 and scanned from 190 to 800 amu at a rate of 10 s/decade with a 1 s interscan time. The mass spectrum of the mobile phase just prior to elution of the sample was subtracted from the sample spectrum to obtain the mass spectra. Analysis of the phenyl sulfate solution resulted in a molecular ion cluster observed at *m*/*z* 244 (M − H).

For static (probe) high-resolution FAB/MS analysis, samples were deposited with a syringe on a thin layer of glycerol coating a FAB target and then introduced into the mass spectrometer. Ionization was achieved with an Ion Tech Saddle Field fast atom gun producing 7 kV of xenon atoms at a 1 mA emission current. The mass spectrometer was adjusted to 5000 resolution, and the peak matching technique using the decade unit on the spectrometer was applied for high-resolution analysis. The acceleration potential was 6000 V. The authentic ADEPS standard was used as reference for the *m*/*z* 244 ion. Negative ions were analyzed. The observed mass for the sample was 244.06597. The calculated mass for the phenyl sulfate of DEA is 244.06435, a difference of 0.0016 amu. The phenyl sulfate solution was then derivatized with DMF·DMA and analyzed. The derivatized phenyl sulfate produced an ion cluster at *m*/*z* 299, consistent with derivatization of the aniline to the imine.

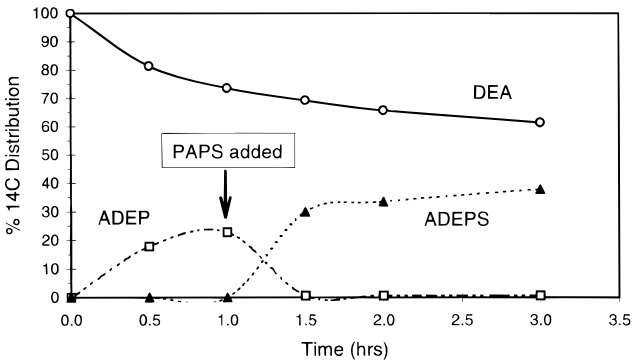
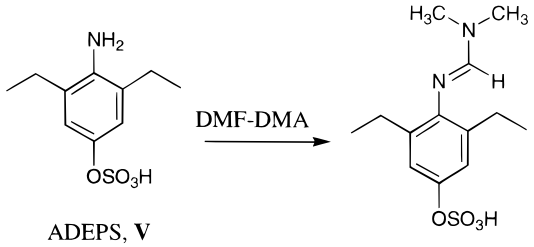


Figure 2. Incubation of DEA with rat liver S-9 preparations in the presence of NADPH and PAPS.



ADEPS, **V**

Figure 3. Reaction of ADEPS with DMF·DMA.

Microprobe NMR Analysis of ^{14}C-ADEPS. Less than 2 μg of the phenyl sulfate was exchanged with 99% D_2O and dissolved in 0.140 mL of 99.996% D_2O. The sample was transferred with an additional 0.010 mL of D_2O into a 3 mm NMR tube (327-PP) from Wilmad Glass Co., Inc. (Buena, NJ). NMR spectra were collected at 30 °C on a Varian Unity 500 MHz spectrometer equipped with a MIDT 500-3 micro indirect detection probe from Nalorac, Inc. (Martinez, CA). The sample volume was ~150 μL. The HOD resonance was suppressed with simple c.w. presaturation. The spectra required up to 5.5 h of signal averaging using a π/2 pulse every 3 s. The HOD resonance was assigned a chemical shift of 4.77 ppm.

RESULTS AND DISCUSSION

The conversion of DEA to ADEPS was initially investigated using a rat liver crude S-9 fraction to study the hydroxylation reaction in conjunction with sulfate conjugation (Figure 2). DEA was incubated with the S-9 enzymes in the presence of only NADPH. In 1 h, 23% of the initial DEA was converted to ADEP, as determined by HPLC/RAD. The PAPS cofactor was then added, and rapid conversion of ADEP to ADEPS was observed within 30 min. Two hours after the addition of PAPS, 62% of the initial DEA remained and 38% was converted to ADEPS. The phenol was barely detected. These results suggest that the aryl hydroxylation is the rate-limiting step in the formation of ADEPS from DEA. The DEA did not react directly to produce the *N*-sulfamate in these incubations.

The conversion of DEA to ADEP was then investigated using rat liver microsomal enzymes. Initially, DEA was incubated with microsomes in the presence of reduced NADPH. One major metabolite was produced, which coeluted by HPLC with a sample of authentic ADEP generated via chemical synthesis. The radiolabeled metabolite was purified by HPLC and derivatized with acetic anhydride/pyridine. GC/CI/MS analysis of the acetylated derivative produced a base

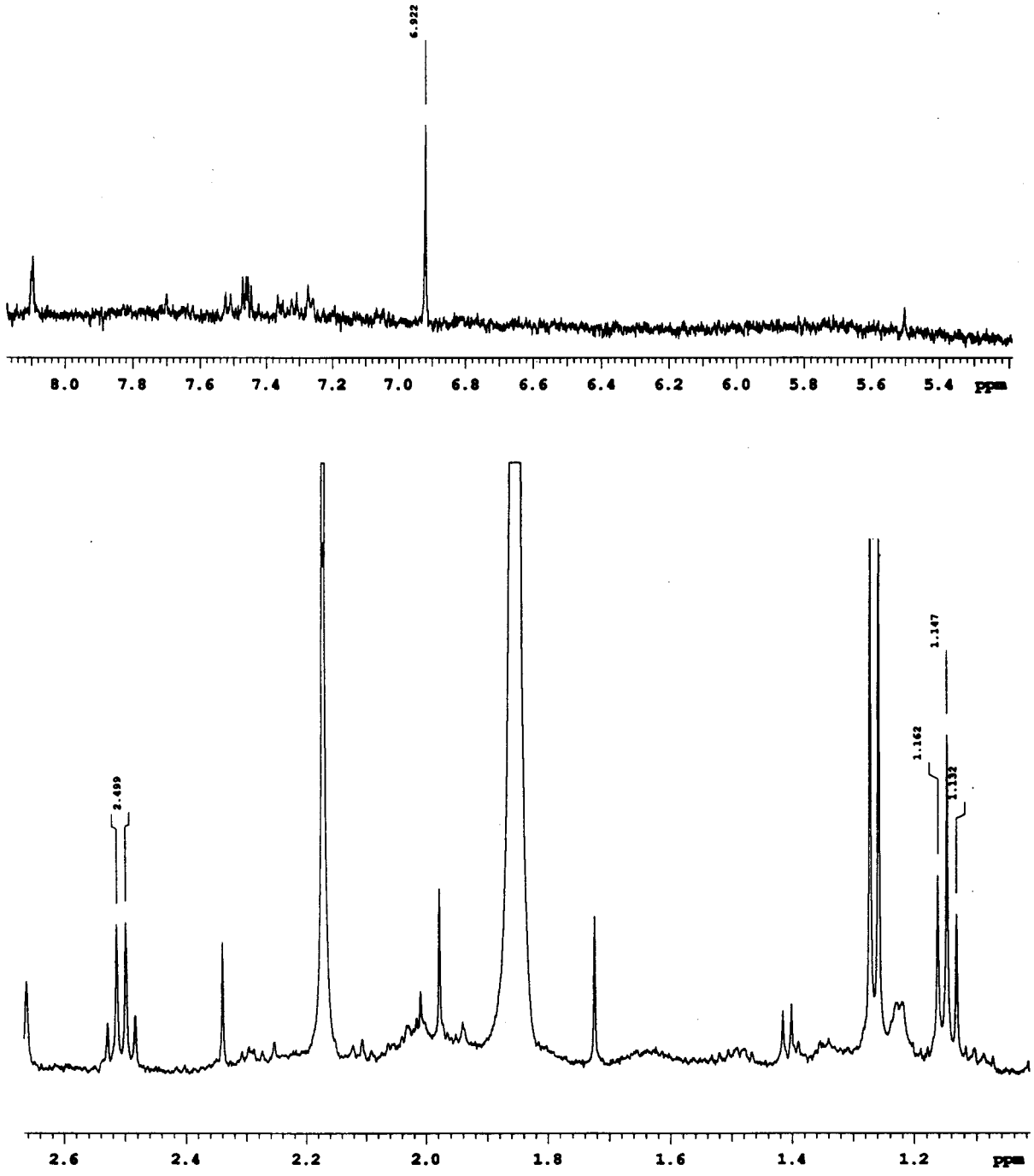


Figure 4. 500 MHz proton NMR spectrum of ^{14}C-ADEPS using a 3 mm microprobe.

peak at *m*/*z* 250 (M + H), consistent with the assignment as *N*-(4-acetoxy-2,6-diethylphenyl)acetamide. It should be noted that the HPLC/RAD and mass spectral properties of the derivatized metabolite also matched those of the acetylated authentic standard of ADEP. Two other minor metabolites were observed in the crude incubation mixture but not characterized. These were presumably the products of hydroxylation either on the nitrogen or on the alkyl side chains. The conversion of DEA to ADEP did not occur when heat-denatured enzymes were used or in the absence of NADPH, suggesting that aryl hydroxylation was catalyzed by microsomal cytochrome P-450 enzymes. Good recovery of the applied radioactivity indicates that very little covalent binding to the precipitated protein occurred during this conversion.

Liver cytosolic sulfotransferases were next used to convert ADEP to ADEPS. Crude sulfotransferase preparations are known to function effectively in vitro for the introduction of sulfate groups onto xenobiotic phenol groups in the presence of the phosphosulfate transferase cofactor PAPS (Dauterman, 1984). We found that incubation of ADEP with rat liver cytosolic enzymes in the presence of PAPS led to the formation of a single, more polar metabolite. The metabolite was isolated and purified by HPLC/RAD and analyzed by negative ion FAB mass spectrometry. The spectrum showed a base peak at *m*/*z* 244, consistent with the addition of SO_3.

The results and conditions from the smaller scale in vitro incubations were used to prepare a ^{14}C-labeled metabolite standard of ADEPS. ^{14}C-DEA was converted to ^{14}C-ADEPS using rat liver microsomes in a 17.3%

yield. The product (7.8 μg) was successfully isolated and purified using HPLC chromatography, with >98% radiochemical purity as demonstrated by HPLC reinjection analysis. FAB-MS analysis of the isolated product resulted in a molecular ion cluster at *m*/*z* 244, and high-resolution FAB was consistent with the formula for ADEPS. However, because the radiolabeled metabolite was needed as a standard in EPA guideline studies, additional characterization was considered desirable.

The sulfate conjugation of ADEP could theoretically occur at the amino group to form a sulfamate or at the phenol to produce a sulfate ester (Jakoby et al., 1980). On the basis of the molecular weight alone, it is not possible to distinguish between the two isomers. However, dimethylformamide dimethylacetal (DMF·DMA) is a reagent that reacts specifically with primary amines to produce dimethylamidino derivatives (Knapp, 1979), to the exclusion of hydroxyl functionalization. Derivatization of the phenyl sulfate with DMF·DMA produced a base peak at *m*/*z* 299 as determined by negative ion FAB. The increase of 54 amu after derivatization indicates that a dimethylamidino derivative of the sulfate metabolite had been formed (Figure 3). This reaction therefore confirmed that sulfate conjugation had occurred at the phenol to produce ADEPS.

NMR spectroscopy in combination with MS data is a definitive means to establish the structure of metabolite standards. Unfortunately, NMR spectroscopy is intrinsically insensitive and typically plays a limited role in metabolism studies. Until recently, the amount of material required for routine ^{1}H NMR analysis was typically 20−50 μg. With the development of higher field magnets and recent advances in probe technology, routine NMR analysis is now possible with much smaller sample sizes (Crouch and Martin, 1992a,b). Microprobe technology uses either 2.5 or 3 mm sample tubes with a solvent volume of 100−140 μL. Because the sample is more concentrated by at least a factor of 5, the gain in signal averaging efficiency is striking. The sample occupies a much smaller volume in the magnetic field, which simplifies optimization of the field homogeneity. The commercial availability of microprobe technology has made possible the collection of ^{1}H NMR spectra for sub-microgram samples.

Approximately 1−2 μg of the isolated product was analyzed by NMR using a 3 mm microprobe at 500 MHz (Figure 4). The proton spectrum showed three sets of resonances, indicating a high degree of symmetry in the molecule. The aliphatic region exhibited the characteristic triplet (δ 1.15) and quartet (δ 2.50) for the ethyl hydrogens. A downfield singlet was observed at δ 6.92 and was consistent with a symmetrical trisubstituted benzene ring. The chemical shifts matched those of the authentic sample of ADEPS and thus confirmed the identity of the radiolabeled standard as ^{14}C-ADEPS.

SUMMARY

We have demonstrated here that ADEPS, an in vivo rodent metabolite of alachlor, can be obtained from DEA via the same in vitro pathway that produces ADEP (Figure 1). This enzymatic transformation involves a sequential N-dealkylation and amide hydrolysis of alachlor to yield DEA. The aniline is further oxidized to ADEP, which is then converted to ADEPS. Because this oxidative pathway is associated with formation of a reactive metabolic intermediate that might contribute to the mechanism of alachlor carcinogenesis (Feng et al., 1990), the presence and/or abundance of ADEPS in metabolite profiles may serve as a useful indicator of the contribution of this pathway in a given metabolism study. We have also shown that a radiolabeled standard of ADEPS can be readily prepared via cell-free rodent liver extracts as a convenient alternative to a costly multistep radiosynthesis. Although the approach illustrated here utilizes precursor materials that are specific for alachlor metabolic transformations, it is apparent that such methods can be used to prepare metabolite standards and explore in vitro oxidation and sulfation reactions of other chloroacetanilide herbicides such as metolachlor.

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