Registry No. NBD-Cl, 10199-89-0; methanethiol, 74-93-1; ethanethiol, 75-08-1; 2-propanethiol, 75-33-2; 1-propanethiol, 107-03-9; 2-methyl-2-propanethiol, 75-66-1; 2-butanethiol, 513-53-1; 2-methyl-1-propanethiol, 513-44-0; 1-butanethiol, 109-79-5.

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Determination of Aminodibenzothiophenes in a Coal Liquid

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Polycyclic aromatic compounds containing both nitrogen and sulfur heteroatoms in a coal liquid (SRC II HD) were determined by capillary column gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). Gas chromatography with a flame photometric detector and a 25% biphenyl polysiloxane stationary phase was applied to the nitrogen-containing polycyclic aromatic compound fraction. All aminodibenzothiophene isomers were positively identified by comparison with the retention times of newly synthesized standard compounds. The aminodibenzothiophenes were the major nitrogen/sulfur-containing heterocycles in the SRC II HD. The four aminodibenzothiophenes were assayed for genotoxicity by two methods: the Ames test and unscheduled DNA synthesis. The 2- and 3-aminodibenzothiophenes demonstrated significant genotoxicities in both assays.

Coal-derived liquids are extremely complex mixtures of organic chemicals, the majority of which are polycyclic aromatic compounds (PAC). While the polycyclic aromatic hydrocarbons (PAH) comprise the largest chemical class fraction of most coal liquids, there are substantial amounts of nitrogen-, sulfur-, and oxygen-containing PAC. All of these fractions have been extensively characterized using a variety of techniques (*1-3*) including gas chromatography (GC), high-performance liquid chromatography (HPLC), and mass spectrometry (MS).

The major objective for much of the previous work on detailed characterization of coal liquids, and the main motivation for this work, was to provide the necessary base line data for proper evaluation of the health effects of synthetic fuel production from coal. In this respect, chemical class separation of coal-derived products, followed by microbial mutagenicity and mouse skin tumorigenicity assays revealed that the PAH were mainly responsible for the observed tumorigenicity (*4, 5*), while the nitrogen-containing PAC contributed to the overall tumorigenic activity and were the principal microbial mutagens (*3*). While the tumorigenicity of the sulfur heterocycles (PASH) is presently unknown, the PASH were found generally to be more toxic and to bioconcentrate to a greater extent in various aquatic organisms than their analogous PAH (*6, 7*).

After fractionation of the nitrogen-containing PAC fraction, it was found that the amino polycyclic aromatic hydrocarbons (APAH), which are present in low concentrations in coal liquids (usually less than 1%), were responsible for the major part of the mutagenicity demonstrated by the nitrogen-containing PAC (*8-11*). The nitrogen heterocycles were generally much less mutagenic than the APAH. This result has prompted the investigation of other possible trace components in coal liquids, particularly the PAC that contain two heteroatoms, that may be responsible for some of the observed biological activity.

In this paper, the analysis of a solvent-refined coal liquid for PAC which contain both nitrogen and sulfur heteroatoms is described. Capillary column gas chromatography with sulfur-selective flame photometric detection and mass spectrometry was used to identify several new compounds which were previously unreported. Pure reference compounds were also synthesized and tested for genotoxicity.

EXPERIMENTAL SECTION

Materials. The solvent-refined coal heavy distillate (SRC II HD: 260-450 °C boiling point range) was collected during the processing of a West Virginia coal from the Pittsburgh Seam, and obtained from the Fort Lewis, WA, pilot plant which was operated by the Pittsburgh & Midway Coal Mining Co. This material is of pilot plant origin and should not necessarily be considered as representative of products that may eventually be produced on a commercial scale.

The four aminodibenzothiophene isomers used in this study were not commercially available and, therefore, had to be synthesized in our own laboratories. The synthetic procedures are reported elsewhere (*12*).

Fractionation and Derivatization Procedures. The SRC II HD material was fractionated into chemical classes by adsorption chromatography on neutral alumina and silicic acid

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according to the procedure of Later et al. (*13*). The third fraction (A-3) which was composed of the nitrogen-containing PAC and the second silicic acid fraction (S-2) which was composed of the APAH were analyzed in this study.

A portion of the A-3 fraction (≈30 mg) was dissolved in 4 mL of methylene chloride and extracted twice with 2 mL each of 10% H_2SO_4 and twice with 2 mL each of 20% H_2SO_4 using the method of Burchill et al. (*14*). The H_2SO_4 portions were combined, adjusted to a pH of 12 by the addition of 8 N NaOH dropwise, and back washed three times with 1.5 mL each of methylene chloride. All methylene chloride fractions were combined and concentrated to 0.2 mL, and a portion was derivatized with pentafluoropropionic anhydride (PFPA) as previously described (*15*).

Gas Chromatography and GC/MS. A Hewlett-Packard Model 5880 gas chromatograph equipped with a sulfur-selective flame photometric detector (FPD) and operated in the splitless injection mode was used to obtain chromatographic retention data. Hydrogen was used as the carrier gas at a linear velocity of 100 cm s^{-1}, and the sensitivity was set to give a full-scale response for 60 ng of benzo[*b*]naphtho[1,2-*d*]thiophene. Semiquantitation of the aminodibenzothiophenes was accomplished by comparing sample component peak areas with that of the standard 3-aminodibenzothiophene.

The capillary column used in this study was prepared by coating a 20 m × 0.32 mm i.d. length of fused silica tubing (Hewlett-Packard, Avondale, PA) with a 25% biphenyl polymethylsiloxane stationary phase (0.25 μm film thickness) (*16*). The stationary phase was cross-linked by purging with azo-*t*-butane vapors in nitrogen gas from a bubbler at room temperature for 1 h, followed by sealing the ends with a microtorch and heating in an oven at 220 °C for 1 h. After conditioning by programming from 40 °C to 280 °C at 1 °C min^{-1} and holding at the upper temperature for 10 h under a slow nitrogen gas purge, the column was ready for use.

A Hewlett-Packard Model 5982A GC/MS system was used to obtain mass spectral confirmation of identified compounds. The same chromatographic column was used as for gas chromatography, and the mass spectrometer was operated in the electron impact mode at 70-eV electron energy.

Genotoxicity Testing. The Ames mutagenicity assay was performed as described by Ames et al. (*17*) with minor modifications. These modifications consisted of using 20 mL of medium and 4 mL of top agar per plate instead of 30 mL and 2 mL per plate, respectively. *Salmonella typhimurium* strain TA98 was exposed to varying concentrations of the compounds dissolved in dimethyl sulfoxide. The compounds were tested for mutagenicity at 0 and 4% S9 rat liver homogenate concentrations (0.04 mL of S9/mL of phosphate buffer solution). Solvent controls as well as positive controls were included with each test. TA98 was provided by Bruce Ames, Berkeley, CA. The S9 rat liver homogenate used for microsomal activation of the fractions was purchased from Litton Bionetics, Kensington, MD.

The number of revertants per plate was determined by manual counting (direct or indirect). Indirect counting was performed by counting a 5 cm^2 plate area. The number obtained was divided by five to give the count/cm^2. The count/cm^2 was then multiplied by 56.7 (the total area per plate in cm^2) to give an estimate of the total number of revertants per plate. The direct counting method was used when the number of revertants per plate was less than 500.

Unscheduled DNA synthesis (UDS) was measured in primary hepatocytes of male Fisher 344 rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) which were 7 to 9 weeks old and weighed 170–235 g. The liver cultures were obtained as described by Williams (*18*). This included the two-step liver perfusion with 0.5 M ethylene glycol bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in Hank's balanced salt solution followed by perfusion with 100 units/mL collagenase (Type I, Sigma Chemical Co., St. Louis, MO) in Williams' medium E (Flow Laboratories, Inc., McLean, VA). The liver cells were isolated and checked for viability by trypan blue exclusion. Hepatocytes were seeded into each well of six-well culture plates at a concentration of 4 × 10^5 viable cells in 4 mL of Williams' medium E supplemented with 10% fetal bovine serum. After a 90-min attachment period, the cell cultures were washed and incubated for 18 h in 2 mL of Williams' medium E containing 10 μCi mL^{-1} [^{3}H]thymidine (New England Nuclear) and the test compound. After incubation, the cells were washed, fixed, and prepared for autoradiography. Each culture was dipped in Kodak NTB-2 emulsion diluted 1:1 with water, dried, and placed in a lighttight box with desiccant for 10 days at −15 °C. After exposure, the emulsion was developed in D-19 developer, fixed, and washed. Finally the cells were stained with methyl green pyronin Y for 25 s. Stock solutions of dibenzothiophene and the four aminodibenzothiophene isomers were prepared at a concentration of 5 mM in dimethyl sulfoxide (Me_2SO). The highest concentration of Me_2SO in the cell media was 1%. Negative solvent and media controls were included as well as a positive control, dimethylnitrosamine (DMN). Thirty cells per slide and three slides per concentration were scored for UDS by automatic grain counting as described by Mirsalis et al. (*19*). The number of net grains (NG) was determined by counting the silver grains over the nucleus minus the highest of two nuclear sized areas over the cytoplasm. A cell with more than 5 NG was considered to be in repair.

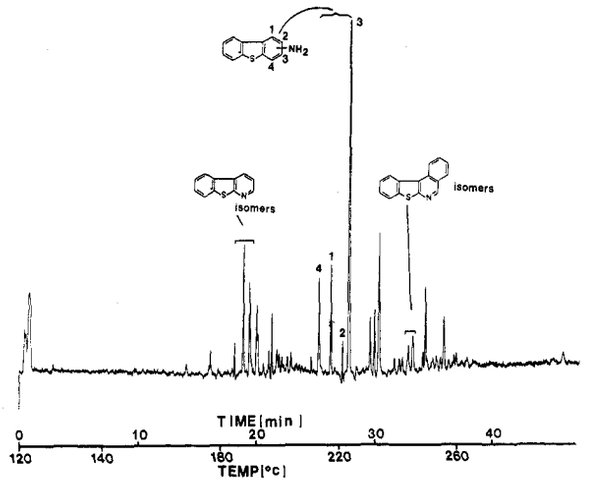

Figure 1. FPD chromatogram of the acid-extracted SRC II HD A-3 fraction on a 25% biphenylpolysiloxane stationary phase: temperature program from 120 °C to 265 °C at 4 °C min^{-1}; hydrogen carrier gas at 100 cm s^{-1}.

RESULTS AND DISCUSSION

Recent studies (*20*) have demonstrated that the 25% biphenylpolysiloxane stationary phase is superior to other stationary phases for the separation of polar polycyclic aromatic compound isomers such as alkylated carbazoles and dibenzothiophenes. The polarizable biphenyl group in the stationary phase is more sensitive to structural differences in these isomers than are other functional groups. Therefore, the 25% biphenyl polysiloxane was selected for the gas chromatography of the A-3 fraction which was enriched with the nitrogen-containing PAC. Numerous polycyclic aromatic thiophenes (PASH) have been identified in the A-2 fraction of the SRC II HD using a sulfur-selective detector (*21*). In the present work, the nitrogen-containing PASH in the A-3 fraction were studied using capillary column gas chromatography with sulfur-selective detection.

The chromatogram of the acid extracted A-3 fraction on the 25% biphenyl methylpolysiloxane stationary phase is shown in Figure 1. Retention times were compared with the newly synthesized standard samples, and the 1- to 4-aminodibenzothiophenes were identified. Since the secondary nitrogen heterocycles (2°-PANH) are not extracted by acid, the chromatogram in Figure 1 shows the sulfur-containing 3°-PANH (3°-PANSH) and amino-PASH (APASH). Several small peaks (most likely sulfur-containing 2°-PANH) were eliminated after acid extraction of the fraction. A modification of the method described by Later et al. (*22*) was used to distinguish the 3°-PANSH and APASH. In Later's work, yields exceeding 95% for the conversion of amino-PAH to

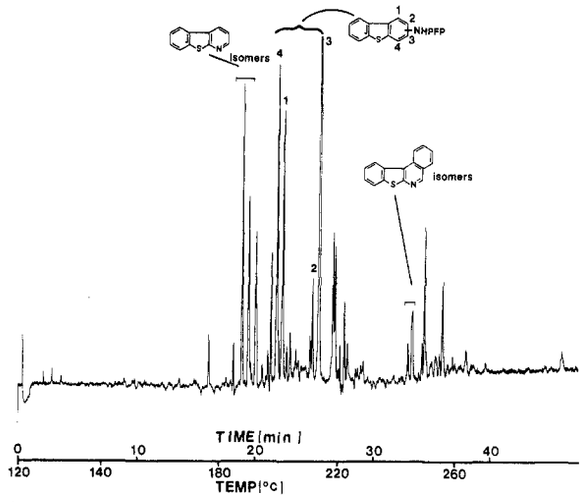

Figure 2. FPD chromatogram of the PFP-derivatized SRC II HD A-3 fraction. Conditions are given in Figure 1.

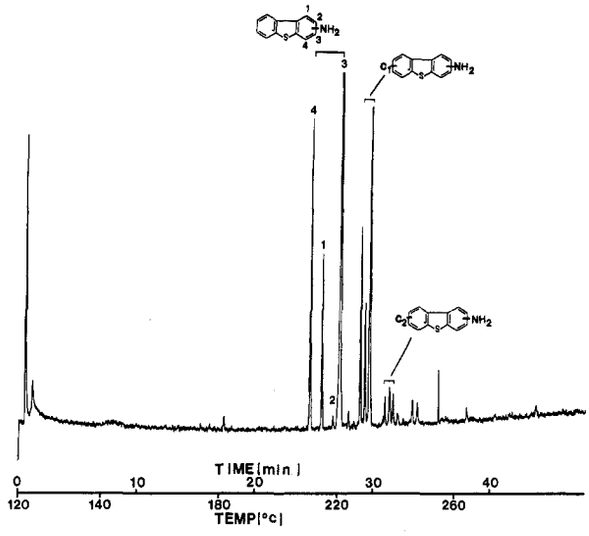

Figure 3. FPD chromatogram of the SRC II HD S-2 fraction. Conditions are given in Figure 1.

PFP-amide derivatives and no detectable PFP-amide derivatives for 3°-PANH were reported. Figure 2 shows the chromatogram of the PFP derivatives of the acid extract of the A-3 fraction. Comparison of Figure 2 with Figure 1 shows that some of the peaks shifted in retention time after derivatization. Retention times for the PFP amide derivatives decreased because the polar amino groups were blocked by the PFP groups, causing less dipole/induced dipole interactions with the polarizable biphenyl stationary phase. Identification of the four aminodibenzothiophene isomers was confirmed by comparing the retention times of the PFP-derivatized standards with the retention times of the shifted peaks in the chromatogram of the PFP-derivatized A-3 fraction. A chromatogram of the S-2 APAH fraction of the SRC II HD is shown in Figure 3. Only aminodibenzothiophenes and alkylated aminodibenzothiophenes were detected in this chromatogram. Further confirmation of peak identities was obtained by GC/MS analysis. The distinctive mass spectral fragmentation pattern of the PFP-amide derivatives greatly aided the identification of the derivatives, as was reported previously (*22*).

Table I lists the relative retention times of the standard aminodibenzothiophenes and their PFP-amide derivatives. Semiquantitation of these compounds in the samples analyzed is also given in Table I. Since the FPD used in this study did not have a linearizer, and quenching of the sulfur emission for some of the compounds which overlap the APAH and

Table I. Relative Retention Times and Semiquantitation of the Aminodibenzothiophenes and Their PFP-amide Derivatives in an SRC II Heavy Distillate Coal Liquid

compound	rel retention times[a] underivatized	rel retention times[a] PFP-amides	concn,[b] μg/g
1-aminodibenzothiophene	1.526	1.289	0.32
2-aminodibenzothiophene	1.581	1.436	0.11
3-aminodibenzothiophene	1.613	1.466	2.5
4-aminodibenzothiophene	1.464	1.256	0.33

[a] Retention relative to phenanthrene. These values are the average of two measurements. Both measurements agreed to within ±0.003. [b] Concentration in μg/g in the crude SRC II heavy distillate.

Table II. Ames Mutagenicity of the Aminodibenzothiophenes

compound	rev/μg[a]	R^2
1-aminodibenzothiophene		
0% S9[b]	c	
4% S9[d]	2.36	0.96
2-aminodibenzothiophene		
0% S9[b]	9.07	0.98
4% S9[e]	1.94×10^4	0.97
3-aminodibenzothiophene		
0% S9[b]	4.74	0.90
4% S9[f]	3.43×10^3	0.97
4-aminodibenzothiophene		
0% S9[b]	c	
4% S9[d]	c	

[a] Linear response region used to calculate dose response by linear regression curve fitting. [b] Solvent control value (0% S9), 21 ± 4. [c] Response <2× solvent control values. [d] Solvent control value (4% S9), 38 ± 5. [e] Solvent control value (4% S9), 29 ± 4. [f] Solvent control value (4% S9), 31 ± 4.

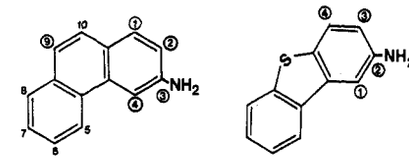

Figure 4. Structures of the aminophenanthrenes and aminodibenzothiophenes. The isomeric positions are labeled; circled numbers give the only specific isomer possibilities.

PANH in the sample could be expected, only semiquantitative values were obtained.

The mutagenicities of the 1- to 4-aminodibenzothiophenes are listed in Table II. The 2- and 3-aminodibenzothiophenes are strongly mutagenic, and their average mutagenic response was 10 to 100 times greater than the average response of benzo[*a*]pyrene (200–300 revertants/plate at 4 μg and 4% S9) (*23*). A comparison of the structure of these molecules to the aminophenanthrenes reveals a geometric similarity between them (see Figure 4). The 2-, 3-, and 9-aminophenanthrenes demonstrated the highest mutagenicity of the aminophenanthrene isomers (*24*). The 3-aminophenanthrene isomer had the strongest mutagenicity (30 300 rev/μg average response), and this structure is analogous to 2-aminodibenzothiophene which is the most active of the aminodibenzothiophene isomers. For comparison, the 1- and 9-methylphenanthrenes were mutagenic (*25*), but the methyldibenzothiophene isomers did not exhibit any mutagenic activity (*23*).

The results of the UDS experiments confirm the findings of the Ames test; the 2- and 3-aminodibenzothiophenes were significantly genotoxic. These compounds induced unscheduled DNA synthesis in metabolically competent rat hepatocytes. The number of NG and percent of cells in repair are

Table III. Induction of Unscheduled DNA Synthesis in Rat Hepatocytes by Dibenzothiophene and the Four Aminodibenzothiophenes

compound	concn, mM	NG ± SE[a]	% IR ± SE[b]
dibenzothiophene	0.050	−8.0 ± 0.9	2 ± 2
	0.011	−11.9 ± 3.2	0
	0.0050	−11.4 ± 1.0	0
1-aminodibenzothiophene	0.050	−5.7 ± 1.9	10 ± 9
	0.011	−8.6 ± 1.6	2 ± 2
	0.0050	−4.9 ± 0.4	4 ± 8
2-aminodibenzothiophene	0.050	30.8 ± 10.2	90 ± 14
	0.011	6.0 ± 3.5	53 ± 33
	0.0050	−3.7 ± 0.4	3 ± 3
	0.0011	−10.0 ± 2.5	1 ± 2
3-aminodibenzothiophene	0.050	18.2 ± 3.3	87 ± 8
	0.011	10.8 ± 8.6	57 ± 24
	0.0050	−3.4 ± 2.7	18 ± 19
	0.0011	−7.5 ± 1.5	2 ± 2
4-aminodibenzothiophene	0.050	−13.0 ± 3.9	2 ± 2
	0.011	−7.3 ± 0.9	2 ± 4
	0.0050	−9.1 ± 3.4	2 ± 4
media		−9.6 ± 1.0	0
Me_2SO	1%	−11.5 ± 2.2	1 ± 2
DMN	9.5	15.5 ± 1.6	81 ± 10

[a] Standard error (SE) represents slide-to-slide variation for three slides at each concentration, 30 cells counted per slide. [b] The percent in repair (IR) is defined as those cells with ≥5 NG.

given in Table III. The hepatocyte viability for these experiments ranged from 62% to 80% by trypan blue exclusion. Initial experiments carried out to locate the dose response region ranged from 1 mM to 0.000 10 mM. A toxic effect determined by the lack of attachment was found at 1 mM for all aminodibenzothiophenes. All compounds were assayed for a dose response at the same concentrations. As indicated in Table III, 2-aminodibenzothiophene was most genotoxic at 0.050 mM, followed by 3-aminodibenzothiophene. The 1- and 4-aminodibenzothiophenes did not induce a positive number of NG at this concentration, although 10% of the cells exposed to the 1-aminodibenzothiophene were in repair. This also substantiates the weak mutagenic effect of this isomer in the Ames test. Unscheduled DNA repair synthesis has been shown to be a reliable and sensitive indicator of carcinogenic PAH in that it discriminates between carcinogenic and noncarcinogenic PAH analogues (*26*). From these results, it appears that the addition of the amino group to the 2- or 3-position converts nongenotoxic dibenzothiophene to a genotoxic aminodibenzothiophene. In comparison with other analogues such as the addition of a methyl group to the 5-position in chrysene, it follows that the 2- and 3-aminodibenzothiophenes are probably significant carcinogenic components of coal-derived materials.

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Registry No. 1-Aminodibenzothiophene, 29451-76-1; 2-aminodibenzothiophene, 7428-91-3; 3-aminodibenzothiophene, 25288-76-0; 4-aminodibenzothiophene, 72433-66-0.

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