Methylmercapto-4-acetylamino- stilbenes as Products of the Reaction of N-Acetoxy-4-acetylamino- stilbene with Methionine and as Degradation Products of Liver Protein from Rats Given N-Hydroxy-4-acetylamino- stilbene

Elizabeth C. Miller, Barbara W. Butler, T. Lloyd Fletcher, and James A. Miller

McArdle Laboratory for Cancer Research, University of Wisconsin Medical Center, Madison, Wisconsin 53706 [E. C. M., B. W. B., J. A. M.], and The Fred Hutchinson Cancer Research Center, Seattle, Washington 98104 [T. L. F.]

SUMMARY

The reaction of the carcinogenic electrophile N-acetoxy-4-acetylamino- stilbene with methionine at neutral pH yielded β-methylmercapto-4-acetylamino- stilbene as the major product and 3-methylmercapto-4-acetylamino- stilbene as a minor product. Two other products were also formed. One of these had the apparent molecular weight (283) of a methylmercapto-4-acetylamino- stilbene; it was not identical with 2-, 2', 3', or 4'-methylmercapto-4-acetylamino- stilbene. The second product had an apparent molecular weight of 301 and appeared to be a methylmercapto derivative. It was formed in larger amounts at slightly acidic pH's, and its yield was inversely related to that of β-methylmercapto-4-acetylamino- stilbene. 3- and β-methylmercapto-4-acetylamino- stilbene were also obtained on alkaline degradation of the liver protein from rats that had received i.p. injections of N-hydroxy-4-acetylamino- stilbene. These data provide evidence that an ester of N-hydroxy-4-acetylamino- stilbene or a derivative with similar electrophilic reactivity is formed from N-hydroxy-4-acetylamino- stilbene in the rat liver.

INTRODUCTION

N-Hydroxy-AAS is a proximate carcinogenic metabolite of the potent carcinogenic amide AAS (1-3). Thus, a conjugate (presumably a glucuronide) of the hydroxamic acid has been identified as a urinary metabolite in rats given AAS (1, 2). Further, the hydroxamic acid is more carcinogenic than the parent amide at sites of injection and in the mammary gland, forestomach, and ear duct gland (1-3). Some tumors also develop in the liver and in the epithelium of the small intestine on p.o. administration. The greater carcinogenic activity of N-acetoxy-AAS, as compared to that of N-hydroxy-AAS, in the s.c. tissue of rats and the electrophilic reactivity of this ester (27, 31) suggested that esters of N-hydroxy-AAS might be ultimate carcinogenic metabolites in vivo.

The idea that esters of N-hydroxy-AAS might be ultimate carcinogenic derivatives was also based on studies that implicated the sulfuric acid ester of N-hydroxy-2-acetylaminofluorene as a major ultimate reactive and carcinogenic metabolite of this hydroxamic acid in the rat liver (8, 9, 11, 14-18, 24, 27, 35, 37). In these studies 1- and 3-methylmercapto-2-acetylaminofluorene and N(guan-8-yl)-2-acetylaminofluorene derivatives were identified as degradation products of the liver protein and nucleic acids, respectively, from rats administered N-hydroxy-2-acetylaminofluorene. These products are readily formed in nonenzymatic reactions at pH 7 of electrophilic esters of N-hydroxy-2-acetylaminofluorene such as 2-acetylaminofluorene-N-sulfate or N-acetoxy-2-acetylaminofluorene with methionine, guanosine, or deoxyguanosine.

Data from this laboratory have shown that N-acetoxy-AAS is also an electrophilic reactant. Thus, like N-acetoxy-2-acetylaminofluorene, N-acetoxy-AAS-acetoxy-14C released acetate-14C readily in aqueous solution and reacted with methionine and guanosine at neutral pH (27, 31). In the present study 3 products formed in the reaction of N-acetoxy-AAS with methionine were identified and were shown to be identical with products obtained on degradation of the liver protein from rats given N-hydroxy-AAS. These products have been identified as 3-methylmercapto-AAS and the cis and trans isomers of β-methylmercapto-AAS. Two other products, which also appear to be methylmercapto derivatives, were also formed in the in vitro reaction.

MATERIALS AND METHODS

Chemicals. N-Hydroxy-AAS (1), N-acetoxy-AAS (1), and 2-, 3-, 2', 3', 4', and β-methylmercapto-AAS (7, 10) were synthesized as described previously. The stereochemistry of N-hydroxy-AAS and N-acetoxy-AAS has not been examined in detail. However, from our earlier work on the decrease in the extinction coefficient of ethanolic solutions of N-hydroxy-AAS on storage (1) and from studies by others on related 4-aminostilbene derivatives (12, 28), the freshly prepared solutions probably contain mainly the trans isomers.

Methionine-methyl-3H was obtained from Schwarz/Mann, Orangeburg, N. Y. All other chemicals were of reagent grade or were of the best grade available. All solvents were redistilled from glasc.
Instrumentation and Chromatography. UV spectra were determined with a Beckman DB spectrophotometer equipped with a Sargent SR recorder. Mass spectra were determined at 70 eV with a Varian CH-7 mass spectrometer equipped with a mass marker. The mass spectrometer was used with a direct insertion probe (Variset Co., Madison, Wis.) or was set up in series with a Varian Aerograph No. 1700 gas-liquid chromatograph. The column (0.25 inch x 3 feet, stainless steel; see below for packings for Column 1), detector, and injector were maintained at 305°, 260°, and 315°, respectively. Helium was used as the carrier gas at a flow rate of 45 to 55 ml/min. The connector, separator, and probe of the mass spectrometer were set at 280°, 280°, and 230°, respectively. The ions were scanned from 50 to 330 mass units at a rate of 70 mass units/sec.

Routine gas-liquid chromatography was carried out on a Barber-Colman Model 10 chromatograph, which was equipped with a hydrogen ion flame detector. For the early studies the chromatograph was equipped with a 0.7-x 60-cm glass column (Column 1) packed with Gas Chrom S, 80 to 100 mesh, containing 11% silicone rubber gum SE-30 (Applied Science Laboratory, State College, Pa). Nitrogen, at a pressure of 7 to 8 psi, was the carrier gas, and the column, detector cell, and flash heater were maintained at 238°, 260°, and 288°, respectively. For the later studies a 0.7-x 120-cm glass column (Column 2) packed with Gas Chrom Q, 60 to 80 mesh, containing 5% OV-1 (Anspec Co., Ann Arbor, Mich.) was used. This column was was used with nitrogen at a pressure of 7 to 8 psi and with column, detector cell, and flash heater temperatures of 219°, 322°, and 299°, respectively. With each of these conditions 3-methylmercapto-AAS had a retention time of approximately 10 min; this compound was used routinely to standardize the chromatograph. In specific instances the chromatograph was also standardized with the other methylmercapto-AAS isomers.

The Barber-Colman gas-liquid chromatograph was also used for the collection of products from the in vitro or in vivo reactions. In these cases the instrument was standardized with 3-methylmercapto-AAS and 3-methylmercapto-AAS in the usual manner. The column exit tube was then disconnected from the detector, and the product mixture or eluates from thin-layer plates were injected into the chromatograph. Approximately 1 min before the peak elution time of the desired product, a 10-cm Teflon tube (1 mm diameter) was attached to the elution tube; it was disconnected about 1 min after the peak elution time. Repeated injections (up to 10) could be made with the same Teflon tubes for collection of the products, after which the product was washed from the Teflon tubing with ethyl acetate. Recoveries of 3-methylmercapto-AAS averaged 60%, and recoveries of the in vitro products, with the exception of Product 5, were in the same range. Product 5 apparently decomposed under the chromatography conditions; the recovery of material with the original retention time was no more than 10%.

The products were chromatographed on silica gel thin-layer plates with benzene:acetone:n-hexane (Skelly Solve B) (35:50:85, v/v/v). For samples to be examined by UV or mass spectrometry, 0.25-mm thin layers of silica (HF-254, Merck, Darmstadt, Germany) on glass plates were used. These thin-layer plates were prewashed by development with the chromatography solvent and were dried. In other cases silica gel thin layers on plastic plates (Eastman 6060 silica gel) were used. For collection of the products the silica gel from appropriate areas was transferred to small columns with sintered glass frits and eluted with methanol. 3- and 3-Methylmercapto-AAS were chromatographed on each plate as standards, and the R_p's of the products were calculated relative to that of 3-methylmercapto-AAS.

Reaction of N-Acetoxy-AAS with Methionine. The standard reaction system for isolation of nonradioactive products contained (per ml) 66.7 µmoles of DL-methionine, 2.7 µmoles of N-acetoxy-AAS, 67 µmoles of Tris-HCl buffer (pH 7.0, 37°), and 0.33 ml of acetone. After incubation for 20 hr at 37°, the reaction mixture was extracted 3 times with 4-ml aliquots of benzene. The combined benzene extracts were washed once with 5 ml of water, dried with anhydrous MgSO_4, and evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate for gas-liquid chromatography or in methanol or acetone for thin-layer chromatography. The products on the thin-layer plates were located by brief observation under UV (254 nm).

In a few cases sodium phosphate buffer was substituted for the Tris-HCl buffer or the pH of the reaction mixture was altered. For some experiments methionine-methyl-3H, which had been purified by chromatography on cellulose thin layers (Cel 300 UV254, Brinkman Instruments, Inc., Westbury, N. Y.) with n-butyl alcohol:glacial acetic acid:water (100:22:50, v/v/v) was added to the reaction mixture. In the latter cases the benzene extract of the reaction mixture was washed 3 times to ensure the absence of unreacted methionine-methyl-3H. Aliquots of the benzene extract or of the products separated by chromatography were added to a toluene-based scintillator (RPI scintillator, Research Products International Corp., Elk Grove, Ill.) for determination of 3H in Packard scintillation counters.

To obtain material for characterization the products from the in vitro reaction were isolated by thin-layer chromatography on silica (prewashed plates) and subsequent collection from the effluent stream of the gas-liquid chromatograph. Product 5 appeared not to be stable under the conditions of gas-liquid chromatography and was therefore purified only by repeated thin-layer chromatography. Except for Product 5, the products analyzed by UV absorption and mass spectrometry showed only a single spot on thin-layer chromatography and a single peak on gas-liquid chromatography. On gas-liquid chromatography Product 5 showed 2 minor impurities with short retention times.

Isolation of Methylmercapto-AAS Isomers from Liver Protein. Male animals received i.p. injections of 8 mg of N-hydroxy-AAS per 100 g body weight per 0.5 ml of dimethyl sulfoxide:corn oil emulsion. The N-hydroxy-AAS was dissolved in dimethyl sulfoxide, which was then diluted with 5 volumes of corn oil and emulsified by rapid stirring with a magnetic stirrer. The animals were decapitated 16 hr later, and the proteins from 50% homogenates of liver were prepared and treated in alkaline ethanol as described previously (8), except that the extractions were made with benzene rather than with benzene:hexane. The extracts containing the methylmercapto-AAS...
Thus, 3 UV-absorbing areas that were not seen in control was separated into 2 components by gas-liquid chromatography. Likewise, 1 fraction obtained on gas-liquid chromatography of the extract of the reaction mixture. Further analysis revealed that peaks were also detected on gas-liquid chromatography of the extract of the complete reaction mixture (Table 1). Three observed in the upper half of thin-layer chromatographs of mixtures that lacked methionine or N-acetoxy-AAS were prominent in the lower half of the chromatograms and were probably due to various stilbene derivatives. Likewise, a number of components with relatively short retention times, probably decomposition products of N-acetoxy-AAS, were detected on gas-liquid chromatography of the extracts of the reaction mixtures of N-acetoxy-AAS alone or with methionine.

In experiments in which methionine-methyl-3H was added to the reaction mixture (molar ratio, methionine to N-acetoxy-AAS = 25:1), approximately 0.2% of the 3H was converted to a benzene-soluble form in 8 hr and 0.3% was

**RESULTS**

The Formation of Products 1 to 5 from the Reaction of N-Acetoxy-AAS with Methionine

Preliminary studies showed that the reaction of N-acetoxy-AAS with methionine at neutrality yielded several products. Thus, 3 UV-absorbing areas that were not seen in control mixtures that lacked methionine or N-acetoxy-AAS were observed in the upper half of thin-layer chromatograms of extracts of the complete reaction mixture (Table 1). Three peaks were also detected on gas-liquid chromatography of the extract of the reaction mixture. Further analysis revealed that 1 of the materials eluted from the thin-layer chromatograms was separated into 2 components by gas-liquid chromatography. Likewise, 1 fraction obtained on gas-liquid chromatography was separated into 2 components by thin-layer chromatography. These data indicated the formation of 4 reaction products, which were designated as Products 1 to 4 (Table 1).

Interpretation of the lower portions of the thin-layer chromatograms was more difficult. There were 9 UV-absorbing areas with Rf's of 0.1 to 0.9 on the chromatograms of extracts of incubation mixtures that contained N-acetoxy-AAS but no methionine. The UV-absorbing areas were most prominent in the lower half of the chromatograms and were probably due to various stilbene derivatives. Likewise, a number of components with relatively short retention times, probably decomposition products of N-acetoxy-AAS, were detected on gas-liquid chromatography of the extracts of the reaction mixtures of N-acetoxy-AAS alone or with methionine.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics of the products from the in vitro reaction of N-acetoxy-AAS, of the products isolated from the liver protein of rats given N-hydroxy-AAS, and of the synthetic methylmercapto-AAS's</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Product</th>
<th>Gas chromatography relative retention time</th>
<th>Thin-layer chromatography (relative Rf)</th>
<th>Mass spectrometry (m/e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>From reaction</td>
<td>Column 1</td>
<td>Column 2</td>
<td></td>
</tr>
<tr>
<td>Product 1</td>
<td>in vitro</td>
<td>1.03</td>
<td>1.00</td>
</tr>
<tr>
<td>Product A</td>
<td>in vivo</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>3-Methylmercapto-AAS</td>
<td>(1.00)</td>
<td>(1.00)</td>
<td>(1.00)</td>
</tr>
<tr>
<td>Product 2</td>
<td>in vitro</td>
<td>0.70</td>
<td>0.70</td>
</tr>
<tr>
<td>Product 3</td>
<td>in vitro</td>
<td>0.75</td>
<td>0.76</td>
</tr>
<tr>
<td>Product B</td>
<td>in vivo</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>β-Methylmercapto-AAS (cis?)</td>
<td></td>
<td>0.74</td>
<td>0.75</td>
</tr>
<tr>
<td>Product 4</td>
<td>in vitro</td>
<td>1.01</td>
<td>1.04</td>
</tr>
<tr>
<td>Product C</td>
<td>in vivo</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>β-Methylmercapto-AAS (trans?)</td>
<td></td>
<td>1.01</td>
<td>1.03</td>
</tr>
<tr>
<td>Product 5</td>
<td>in vitro</td>
<td>1.00</td>
<td>1.01</td>
</tr>
<tr>
<td>2-Methylmercapto-AAS</td>
<td></td>
<td>1.40</td>
<td>1.48</td>
</tr>
<tr>
<td>2'-Methylmercapto-AAS</td>
<td></td>
<td>1.59</td>
<td>1.68</td>
</tr>
<tr>
<td>3'-Methylmercapto-AAS</td>
<td></td>
<td>1.99</td>
<td>2.07</td>
</tr>
<tr>
<td>4'-Methylmercapto-AAS</td>
<td></td>
<td>2.17</td>
<td>2.39</td>
</tr>
</tbody>
</table>

---

a The column packings and chromatography conditions are described in "Materials and Methods." The relative retention times were determined with 3-methylmercapto-AAS as the standard; its retention time averaged 9.8 min for Column 1 and 9.7 min for Column 2. There were minor variations from day to day depending on the gas pressure and the condition of the columns.
b The relative Rf's were determined with 3-methylmercapto-AAS as the standard. The Rf of 3-methylmercapto-AAS averaged 0.76 ± 10%. The silica plates were developed with benzene:acetone:n-hexane, 35:50:85, by volume.
c The mass spectra for the synthetic compounds and Product 5 were obtained with the direct insertion probe. The other peaks listed were 10% or more of the height of the molecular ion peak. For 2-methylmercapto-AAS the highest peak was at m/e = 226, and the molecular ion peak was the second highest. Data are presented only for m/e > 200.
d Detection of Product 5 appeared to be satisfactory by gas-liquid chromatography, although the compound was apparently decomposed under the chromatography conditions. Thus, attempts to collect the effluent from the column yielded only a small amount of product.
e Detection of Product 5 could not be interpreted at m/e < 253, since the contaminant(s) in control samples (incubation of N-acetoxy-AAS in the absence of methionine) showed fragments in this region.
consistent by 24 hr. Thus, the percentage of reaction based on the limiting reactant (N-acetoxy-AAS) was about 7.5% by 24 hr. Chromatography of the reaction mixtures on silica gel thin layers showed 4 bands of radioactivity. These were observed at the $R_F$'s at which Products 1 + 2, 3, and 4 had been detected by their UV absorption and at a lower $R_F$ ($R_F$ relative to 3-methylmercapto-AAS = approximately 0.35). This latter radioactive area coincided with a broad UV-absorbing band that partially overlapped a UV-absorbing area in control samples from which the methionine was omitted. Analysis by gas-liquid chromatography of eluates of this radioactive area revealed a component (Product 5) with the same retention time as that of 3-methylmercapto-AAS. Although the overall extent of reaction (total benzene-extractable $^3$H) was not appreciably influenced by variations in $pH$, the yield of Product 5 increased markedly as the acidity of the reaction mixture was increased (Table 2). The amounts of products at the approximate $R_F$'s of the combined Products 2, 3, and 4 decreased as the amount of Product 5 increased. The reduction in yield of these combined products was apparently due primarily to a reduction in the amounts of Products 3 and/or 4, since Product 2 contributed only about 10% of the total benzene-extractable products formed at $pH$ 7. Independent studies showed that a mixture of Products 3 and 4 and Product 5 were stable under incubation conditions. In this $pH$ range 3 to 6% of the radioactivity was at the $R_F$ of 3-methylmercapto-AAS.

**Initial Studies on Products A, B, and C Released from the Liver Proteins of Rats after i.p. Injection of N-Hydroxy-AAS**

Gas-liquid chromatography of the benzene extracts of alkaline digests of the liver proteins from rats that received i.p. injections of N-hydroxy-AAS showed components with the same retention times as Product 3 and Products 1, 4, and 5 [all of which cochromatographed (Table 1)]. These 2 gas-liquid chromatographic fractions were collected and thin-layered on silica plates. The more slowly moving fraction from the gas-liquid chromatography was resolved into 2 components, A and C, with $R_F$'s similar to those of Products 1 and 4, respectively. No component with the $R_F$ of Product 5 was detected on the thin layers. The component from liver that had the same retention time on thin-layer chromatography as Product 3 was designated Product B.

**Characterization of the in Vitro and in Vivo Products**

β-Methylmercapto-AAS and each of the ring-substituted methylmercapto derivatives of AAS were synthesized to facilitate the characterizations of the products formed by reaction of N-acetoxy-AAS and methionine and the products isolated from the liver proteins of rats administered N-hydroxy-AAS (7, 10). Attempts to synthesize α-methylmercapto-AAS have not been successful so far. The data that follow establish the identities of the in vitro Products 1, 3, and 4 with hepatic Products A, B, and C, respectively. They further establish that Products A and 1 are 3-methylmercapto-AAS, while Products B and 3, and C and 4 are β-methylmercapto-AAS (presumably the cis and trans forms, respectively).

**Comparisons of the Synthetic Methylmercapto-AAS's and the in Vitro and in Vivo Products by Gas-Liquid and Thin-Layer Chromatography.** Comparisons of the retention times on gas-liquid chromatography and of the $R_F$'s on thin-layer chromatography showed that none of the products from the reaction of N-acetoxy-AAS and methionine could be 2-, 2', 3', or 4'-methylmercapto-AAS (Table 1). The same comparisons suggested the identity of Products 1 and A with 3-methylmercapto-AAS and of Products 3, 4, B, and C with β-methylmercapto-AAS. The latter compound was resolved by gas-liquid chromatography into 2 forms that were interconvertible upon standing in light (see below). None of the synthetic products available, which included all of the possible isomers except α- and N-methylmercapto-AAS, had the gas-liquid and thin-layer chromatographic properties of Product 2 or Product 5.

**UV Spectra of the Synthetic Methylmercapto-AAS’s and the Products Formed in Vitro and in Vivo.** Comparisons of the UV spectra of the in vitro and in vivo products with those of the synthetic methylmercapto-AAS's showed that the spectra of Product 1, Product A, and 3-methylmercapto-AAS are essentially identical (Chart 1). Likewise, the spectra of Product 3, Product B, and the component of β-methylmercapto-AAS that eluted first from the gas-liquid chromatogram are essentially identical. Similarly, Product 4, Product C, and the component of β-methylmercapto-AAS with the longer retention time on the gas-liquid chromatograph have very similar absorption spectra.

Apparent cis-trans Isomerism of β-Methylmercapto-AAS. Since the 2 isomers of β-methylmercapto-AAS were formed from each other in light, they were collected separately from the effluent of the gas-liquid chromatograph and immediately dissolved in ethyl acetate or methanol. The samples were protected from light. Rechromatography of each isomer on the gas-liquid chromatograph immediately after collection showed only traces of the other isomer (Chart 2). Further, as shown in Chart 1, the spectra of the 2 isomers differed significantly. However, if the solutions were allowed to stand in the light, each solution became a mixture of the 2 isomers (Chart 2). This change was particularly evident on analysis by gas-liquid chromatography and was also apparent on close inspection of the UV spectra. Equilibration of the 2 isomers occurred very slowly if the solutions were stored in the dark. The higher wavelength for the maximum absorption of the late-eluting as compared to the more rapidly eluting form suggests that the late-eluting form is the trans and the early-eluting form is the cis isomer (12, 28).

No evidence was obtained for cis-trans isomerism of the synthetic ring methylmercapto-AAS compounds. Each of these compounds showed only 1 retention time on gas-liquid chromatography. When solutions of the ring methylmercapto-AAS's were exposed to room light for several hr to days, each compound yielded a new derivative. Each of these products had a retention time approximately one-half of that of the parent compound on gas-liquid chromatography, had an $R_F$ on silica that was 5 to 20% greater than that of the parent compound, and had a UV absorption spectrum in which the maximum occurred at a lower wavelength (usually about 30.
The spectra of Products 1, 3, and 4 isolated from the reaction of methylmercapto-AAS were obtained for cis- and mms-0-methylmercapto-AAS. Molar absorbances were not shown by the arrows. The molar absorbances of the synthetic compounds. These spectra were matched at the positions shown by the arrows. The molar absorbances of the synthetic compounds are as follows: 2-methylmercapto-AAS, 31,700 (308 nm); 3-methylmercapto-AAS, 40,600 (309 nm); 2'-methylmercapto-AAS, 29,400 (308 nm); 3'-methylmercapto-AAS, 40,700 (322 nm); 4'-methylmercapto-AAS, 52,600 (337 nm). Molar absorbances were not obtained for cis- and trans-β-methylmercapto-AAS.

Chart 1. The UV spectra of methanolic solutions of the isomeric methylmercapto-AAS's and of Product 2. All synthetic compounds, except β-methylmercapto-AAS, were analyzed at a concentration of 5 µg/ml. The spectra of Products 1, 3, and 4 isolated from the reaction of methionine and N-acetoxy-AAS and of Products A, B, and C isolated from the degradation of the liver protein from rats given injections of N-hydroxy-AAS are compared with those of the corresponding synthetic compounds. These spectra were matched at the positions shown by the arrows. The molar absorbances of the synthetic compounds are as follows: 2-methylmercapto-AAS, 31,700 (308 nm); 3-methylmercapto-AAS 40,600 (309 nm); 2'-methylmercapto-AAS, 29,400 (308 nm); 3'-methylmercapto-AAS, 40,700 (322 nm); 4'-methylmercapto-AAS, 52,600 (337 nm). Molar absorbances were not obtained for cis- and trans-β-methylmercapto-AAS.

Also showed m* + 2 peaks characteristic of sulfur-containing compounds and fragment ions consistent with the loss of a methyl group (m/e = 268), ketene (m/e = 241), or both of these fragments (m/e = 226). The spectra for Products 1 and A were qualitatively similar to that for 3-methylmercapto-AAS. The spectra for Products 3 and B as well as for 4 and C were similar to those for the 2 isomers of β-methylmercapto-AAS. The finding of apparent molecular ions at m/e = 283 for the products formed on irradiation of 2- and 4'-methylmercapto-AAS is puzzling, although it is possible that N- or S-oxides or epoxides would lose oxygen so readily that the apparent molecular ion would really be the m* - 16 ion. Product 2 displayed a mass fragment at m/e = 236 (m* - CH3S); this fragment ion was not detected or was less than 10% of the molecular ion peak in the spectra of the synthetic methylmercapto-AAS compounds. Product 5 showed an apparent molecular ion at m/e = 301 and fragments at m/e = 283 and 284 (m* - 17 or 18, OH or H2O)(Table 1). Because there was some impurity in even the best preparations of Product 5 and because a comparable isolation from a control incubation (N-acetoxy-AAS in the absence of methionine) showed peaks at m/e < 253, the mass spectrum could not be further analyzed.

The Possible Identities of Products 2 and 5. The UV spectrum of Product 2 shows increasing absorption at short wavelengths (Chart 1). This suggests the presence of some impurity although none was detected by our thin-layer and liquid chromatography techniques. Nevertheless, the UV absorption maxima and the mass spectrum of Product 2 are consistent with a provisional characterization as a methylmer-
capto-AAS. By elimination of the known methylmercapto-AAS's Product 2 could be either α- or N-methylmercapto-AAS.

The apparent molecular weight of 301 for Product 5 suggests that it, at least formally, the product of the addition of water to a methylmercapto-AAS. The inverse relationship between the amount of Product 5 and the amount of β-methylmercapto-AAS as a function of the pH of the incubation mixture (Table 2) suggested a structural relationship to this isomer. Since β-methylmercapto-AAS was stable under the acidic incubation conditions that yielded the largest amounts of Product 5, the latter product could not have been formed by simple addition of water to β-methylmercapto-AAS; addition of water at the sulfonium stage is a possibility. The UV absorption spectrum of Product 5, although the product was apparently contaminated to some extent by degradation product(s) of N-acetoxy-AAS, was consistent with that of a bibenzyl derivative since there was no appreciable absorption above 280 nm. Several chemical degradations of Product 5 were attempted on the basis of its possible identity with β-hydroxy-β-methylmercapto-4-acetylaminobenzyl, but none was successful.

Attempts to Assay Methylmercapto-AAS Isomers from Rodent Livers

Efforts to obtain quantitative data on the amounts of 3- and β-methylmercapto-AAS recovered from the liver protein of rodents given injections of N-hydroxy-AAS were unsuccessful. However, on the basis of the amounts isolated from the liver and from attempts to quantitate the amounts in extracts from the alkaline digests of the liver protein by gas-liquid chromatography, the amounts of these 2 isomers were estimated to be of the order of 1 to a few µg/g of rat liver. The apparent amounts obtained from mouse and hamster liver protein were less than those from rat liver.

DISCUSSION

The identification of 3- and β-methylmercapto-AAS as reaction products of N-acetoxy-AAS and methionine is consistent with and an extension of earlier observations on the formation of 1- and 3-methylmercapto-2-acetylaminofluorene, 3-methylmercapto-4-acetylaminobiphenyl, 3-methylmercapto-N-methyl-4-aminooazobenzene, 1-methylmercapto-2-acetylaminophenanthrene, and 8-methylmercaptoxanthine on reaction of similar esters with methionine (8, 20–26, 30, 32, 36). In each case the reaction presumably proceeds through a sulfonium intermediate that decomposes with the formation of the methylmercapto derivative and homoserine lactone (Chart 3). This mechanism was established in detail for the reaction of N-acetoxy-2-acetylaminofluorene with methionine (23). The finding of β-methylmercapto-AAS as a major reaction product in the present study is unusual in that, except for the formation of 8-methylmercaptoxanthine, only ortho-substituted derivatives have been detected in the other reactions. The β-position is a logical site of substitution in view of the extension of the conjugation from the ortho position through the benzene ring to the β-carbon of the ethylenic bridge. Likewise, the as yet uncharacterized Products 2 and 5 may also represent new sites of substitution by the methylmercapto group of methionine.

The cis-trans isomerization of stilbene derivatives is a complication in the study of these compounds. Thus, the finding of 2 isomers of β-methylmercapto-AAS both in the in vitro reaction and on isolation of products from the liver protein is presumably a reflection of the fact that each isomer is converted to the other in solution. It is not possible to determine from these data whether only 1 isomer of the methion-S-yl bound form existed in the liver protein or whether both the cis and trans isomers occurred in vivo. No evidence was obtained for the occurrence of more than 1 isomer of the other reaction products or of the synthetic methylmercapto derivatives.

In parallel to studies on N-hydroxy-2-acetylaminofluorene and structurally related carcinogenic aromatic hydroxamic acids and hydroxylamines (as reviewed in Refs. 25 and 26), a variety of data point to the formation of esters of

---

**Table 2**

Distribution of 3H among the reaction products of N-acetoxy-AAS and methionine-methyl-3H as a function of the pH of the reaction mixture

<table>
<thead>
<tr>
<th>pH</th>
<th>% of the benzene-soluble 3H at the Rf of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Product 1</td>
</tr>
<tr>
<td>6.3</td>
<td>6</td>
</tr>
<tr>
<td>7.1</td>
<td>5</td>
</tr>
<tr>
<td>7.5</td>
<td>3</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Estimated together, since Products 3 and 4 co-chromatograph, and the relatively minor Product 2 is difficult to separate quantitatively from Products 3 and 4 under these conditions.*

---

**Chart 3**

The reaction of N-acetoxy-AAS with methionine to yield 3- and β-methylmercapto-AAS.
N-hydroxy-AAS in vivo. Thus, characterization of 3-methylmercapto-AAS and β-methylmercapto-AAS as degradation products of the liver protein from rats given N-hydroxy-AAS and a recent preliminary report (29) on the release of 3-methylmercapto-AAS from the liver protein of rats given 4-dimethylaminostilbene indicate that esters of N-hydroxy-AAS or similar electrophilic reactants are ultimate reactive metabolites of these aminostilbene derivatives. Likewise, rat liver preparations contain sulfotransferase activity for N-hydroxy-AAS (8, 19), and the sulfuric acid ester is thus presumably formed in vivo. N-Hydroxy-AAS is also subject to 1-electron oxidation to a nitrooxide free radical that dismutates to 2-nitrosostilbene and N-acetoxy-AAS (6). Evidence for catalysis of this oxidation with rodent systems is not available, but it might be expected by analogy to the 1-electron oxidation of N-hydroxy-2-acetylaminoarene by various peroxidases (5, 6, 13) and of 4-aminoarene by rat liver preparations (33, 34). Electrophilic esters of N-hydroxy-4-aminoarene may also be formed in vivo; thus, rat liver preparations can catalyze deacetylation and transacetylation reactions that generate N-acetoxy-4-aminoarene from N-hydroxy-AAS (4). This electrophile could insert aminostilbene residues at nucleophilic sites in cellular macromolecules.

The studies on in vivo-bound AAS derivatives were restricted in this report to hepatic tissue. The rat liver is a site of tumor induction by AAS (1). Nevertheless, Zymbal's gland (an ear duct sebaceous gland) of both sexes and the mammary glands of female rats are more susceptible than is the liver to carcinogenesis by AAS (1–3). Sarcomas are readily induced in the s.c. tissue by N-hydroxy-AAS and N-acetoxy-AAS (27). Thus, analyses for the protein-bound as well as the nucleic acid-bound adducts of AAS and 4-aminoarene must be extended to these tissues.

The multiplicity of reactive forms of chemical carcinogens that may be formed in vivo and the multiplicity of nucleophilic sites on the macromolecules subject to their attack raise important questions as to the molecular nature of the critical interactions that lead to neoplasia. Much further study of these reactions in vivo and their correlations with carcinogenic activity will be needed to approach an answer to this problem. It is perhaps significant that the reactions of esters of N-hydroxy-AAS, which is one of the most carcinogenic hydroxamic acids for the s.c. tissue of the rat, involve, in addition to the ortho-carbon atoms, the β carbon atom. Further investigation is needed to determine whether or not this reaction at the β carbon atom has any special importance in carcinogenesis by AAS or N-hydroxy-AAS.

ACKNOWLEDGMENTS

We are indebted to David H. Swenson for the mass spectral analyses.

REFERENCES


Methylmercapto-AAS


Methylmercapto-4-acetylamino stilbenes as Products of the Reaction of $N$-Acetoxy-4-acetylamino stilbene with Methionine and as Degradation Products of Liver Protein from Rats Given $N$-Hydroxy-4-acetylamino stilbene

Elizabeth C. Miller, Barbara W. Butler, T. Lloyd Fletcher, et al.

*Cancer Res* 1974;34:2232-2239.