tallized and was collected and dried to give 11.8 g of **8d**, mp 183–184 °C.

Ethyl 2-Cyano-3-[[2-(4-pyridinyl)-4-pyrimidinyl]amino]-2-propenoate (8f). A mixture of 10 g (0.058 mol) of **4a**, 13 g of ethyl 2-cyano-3-ethoxy-2-propenoate, and 700 mL of xylene was refluxed with stirring for 72 h, treated with charcoal, and concentrated to a yellow solid residue, which was recrystallized from EtOH to give 12.4 g of **8f**, mp 194–196 °C.

Ethyl 3-Oxo-2-[[[2-(4-pyridinyl)-4-pyrimidinyl]amino]-methylene]butanoate (8i). A mixture of 8.6 g (0.05 mol) of **4a**, 13 g of ethyl acetoacetate, 15 g of triethyl orthoformate, 100 mg of *p*-toluenesulfonic acid, and 700 mL of xylene was refluxed with stirring for 92 h, treated with charcoal, and evaporated to dryness. The yellow residue was recrystallized from EtOH to yield 7.2 g of **8i**, mp 198–200 °C.

Preparation of Amides and Carbamates (9). 2-Methyl-*N*-[2-(4-pyridinyl)-4-pyrimidinyl]propionamide (9b). After a mixture of 8.6 g (0.05 mol) of **4a**, 10 mL of isobutyryl chloride, and 50 mL of pyridine was left at room temperature overnight, it was poured into ice-cold H_2O. The solid was collected and recrystallized from EtOH to yield 7.9 g of white crystals of **9b**, mp 215–217 °C.

Butyl *N*-[2-(4-Pyridinyl)-4-pyrimidinyl]carbamate (9g). A mixture of 9 g (0.052 mol) of **4a**, 10 mL of butyl chloroformate, and 50 mL of pyridine was stirred in an ice bath for 1 h and then left at room temperature overnight; this was poured into ice-cold H_2O, and the precipitate was collected and recrystallized from CH_3CN to give 11.2 g of **9g**, mp 180–182 °C.

Preparation of Ureas 11. Scheme IIA. *N*-(1,1-Dimethylethyl)-*N'*-methyl-*N'*-[2-(4-pyridinyl)-4-pyrimidinyl]urea (11t). After a mixture of 8.5 g (0.045 mol) of **4f**, 5 mL (0.044 mol) of *tert*-butyl isocyanate, 2.4 g of 50% NaH/oil, and 75 mL of DMF had been stirred for 2 h, it was treated with 5 mL of glacial AcOH and then the solvent was removed under reduced pressure. The residue was diluted with H_2O, and a solid was precipitated, which after washing with hexane was recrystalized from *i*-PrOH to give 7.4 g of **11t** as white needles, mp 152–154 °C.

***N*-(1-Methylethyl)-*N'*-[2-(3-pyridinyl)-4-pyrimidinyl]urea (11b).** A mixture of 17.2 g (0.1 mol) of **4b**, 4.8 g of 50% NaH/oil, and 100 mL of Me_2SO was stirred for 25 min and then 10 mL (0.1 mol) of isopropyl isocyanate was added slowly. The resulting mixture was further stirred for 5 h, acidified with AcOH, and poured into ice-cold H_2O. The precipitate was collected, washed with hexane, and recrystallized from EtOH to afford 17.9 g of **11b** as a white solid, mp 224–225 °C.

Scheme IIB. *N*-(2,2-Dimethylpropyl)-*N'*-[2-(4-pyridinyl)-4-pyrimidinyl]urea (11q). A slurry of 8.6 g (0.05 mol) of **4a**, 2.4 g (0.05 mol) of 50% NaH/oil, and 50 mL of DMF was stirred until the evolution of H_2 had ceased (20 min), and then the resulting solution was slowly stirred into a solution of 8.2 g (0.05 mol) of 1,1'-carbonyldiimidazole and 50 mL of DMF over a 15-min period. This was followed by the addition of 4.4 g (0.05 mol) of neopentylamine. The resulting mixture was allowed to stand at room temperature overnight and then treated with 6 mL of glacial AcOH. The solvent was removed under reduced pressure, and the residual semisolid material was treated with H_2O. The solid was filtered, washed with H_2O and then hexane (to remove oil), and recrystallized from *i*-PrOH–$CHCl_3$ to give 7.4 g of **11q** as white needles, mp > 250 °C dec.

Preparation of Pyridine *N*-Oxide Derivatives is Illustrated by the Following Examples: *N*-(1,1-Dimethylethyl)-*N'*-[2-(4-pyridinyl)-4-pyrimidinyl]urea *N*(py)-Oxide (11n). A mixture of 15 g (0.055 mol) of **1m**, 15 g of 85% *m*-chloroperbenzoic acid, and 500 mL of $CHCl_3$ was stirred at room temperature for 2 h and then concentrated. The residue was stirred in 200 mL of 10% aqueous K_2CO_3 solution. The resulting yellow solid was collected, washed with EtOH, and dried to give 13.1 g of **11n**, mp >300 °C dec.

4-Amino-2-(4-pyridinyl)pyrimidine *N*(py)-Oxide (10). A mixture of 8 g (0.03 mol) of butyl *N*-[2-(4-pyridinyl)-4-pyrimidinyl]carbamate (**9g**), 6.5 g of 85% *m*-chloroperbenzoic acid, and 100 mL $CHCl_3$ was stirred overnight and then extracted with aqueous K_2CO_3 solution. The $CHCl_3$ extract was concentrated. The residue (5.8 g) thus obtained was dissolved in 50 mL of EtOH and 10 mL of 35% aqueous NaOH solution and then heated under reflux of 4 h; the solid that separated was collected, washed with H_2O and then EtOH, and dried to give 4.8 g of **10**, mp >310 °C.

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Arylhydroxamic Acid Bioactivation via Acyl Group Transfer. Structural Requirements for Transacylating and Electrophile-Generating Activity of *N*-(2-Fluorenyl)hydroxamic Acids and Related Compounds

Heui-mei Yeh and Patrick E. Hanna*

Departments of Medicinal Chemistry and Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455.
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The synthesis of a series of 12 *N*-(2-fluorenyl)hydroxamic acids, *N*-(2-fluorenyl)-*N*-hydroxyureas, and *N*-(2-fluorenyl)-*N*-hydroxycarbamates is reported. The compounds were evaluated for their ability to serve as substrates for a partially purified hamster hepatic arylhydroxamic acid *N,O*-acyltransferase preparation. Transacylating activity was measured spectrophotometrically with 4-aminoazobenzene as the acyl group acceptor, and electrophile-generating activity was quantified by the *N*-acetylmethionine trapping assay. Only the *N*-acetyl, *N*-propionyl, and *N*-methoxyacetyl derivatives exhibited relatively high levels of activity as measured by either of the assay methods. These results are generally consistent with previously reported conclusions regarding the steric and electronic characteristics of acyl groups that are required for activation by this enzyme system. *N,O*-Acyltransferase inactivation by *N*-hydroxy-2-acetamidofluorene depressed the bioactivation of the *N*-acetyl compound to a greater extent than either the *N*-propionyl or *N*-methyloxyacetyl derivative.

N-(2-Fluorenyl)acetohydroxamic acid (1; Table I) is an *N*-arylhydroxamic acid which, upon metabolic activation, is converted to electrophilic reactants capable of covalent binding to nucleophilic sites on biological macromolecules. The latter process is believed to be responsible for the toxic and carcinogenic activity of **1** and related *N*-arylhydroxamic acids.[1] *N*-Arylhydroxamic acid *N,O*-acyltransferase (AHAT) is a widely distributed mammalian enzyme system that is capable of converting certain *N*-arylhydroxamic acids into electrophilic intermediates that react with biological nucleophiles, including those present on AHAT itself.[2-5]

(1) Miller, E. C. *Cancer Res.* **1978**, *38*, 2479–96.

(2) Bartsh, H.; Dworkin, M., Miller, J. A.; Miller, E. C. *Biochim. Biophys. Acta* **1972**, *286*, 272–98.

(3) King, C. M. *Cancer Res.* **1974**, *34*, 1503–15.

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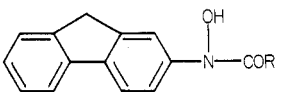
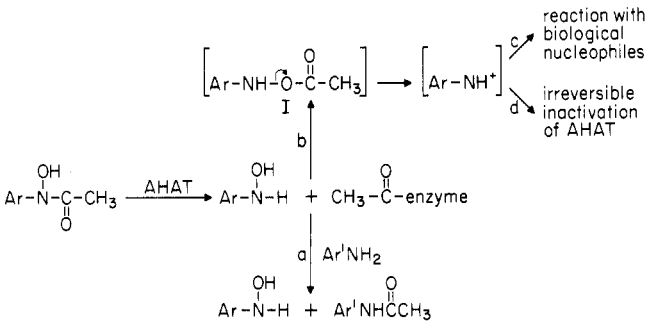
Table I. Physical Properties of *N*-(2-Fluorenyl)hydroxamic Acids, *N*-Hydroxy-*N*-(2-fluorenyl)ureas, and *N*-Hydroxy-*N*-(2-fluorenyl)carbamates



no.	R	mp,[a] °C	yield,[b] %	formula[c]
1	CH_3	149	17	$C_{15}H_{13}NO_2$ [d]
2	CH_2CH_3	149	29	$C_{16}H_{15}N_2O$ [e]
3	$(CH_2)_2CH_3$	145	28	$C_{17}H_{17}NO_2$
4	$(CH_2)_3CH_3$	147	28	$C_{18}H_{19}NO_2$
5	C_6H_5	170	12	$C_{20}H_{15}NO_3$
6	$CH_2C_6H_5$	162	26	$C_{21}H_{17}NO_2$
7	CH_2OCH_3	144	22	$C_{16}H_{15}NO_2$
8	c-C_4H_7	161	24	$C_{18}H_{17}NO_2$
9	NH_2	170	1	$C_{14}H_{12}N_2O_2$
10	$NHCH_3$	159	16	$C_{15}H_{14}N_2O_2$
11	OCH_3	150	29	$C_{15}H_{13}NO_3$
12	OC_6H_5	163	16	$C_{20}H_{15}NO_3$

[a] Compounds 5, 11, and 12 were recrystallized from benzene. Compounds 1-4 and 6-10 were recrystallized from benzene-petroleum ether (60-70 °C). [b] Yields are based on 2-nitrofluorene. [c] All compounds were analyzed for C, H, and N, and the results are within 0.4% of the theoretical values. [d] Lotlikar, P. D.; Miller, E. C.; Miller, J. A.; Margreth, A. *Cancer Res.* **1965**, *25*, 1743. [e] Literature[3] mp 151 °C.

Scheme I



The biochemical process by which AHAT catalyzes the activation of *N*-arylhydroxamic acids is believed to involve a two-step mechanism that includes an acylenzyme intermediate that leads to the formation of an unstable *N*-(acyloxy)arylamine (I; Scheme I).[3] The latter substance undergoes heterolytic cleavage of the N–O bond to produce a resonance-stabilized arylnitrenium ion that reacts with biological nucleophiles. Thus, the adduct formed between the AHAT-generated electrophile and a biological nucleophile does not retain the acyl group that was originally present in the *N*-arylhydroxamic acid.[2,5] As shown in Scheme I (pathway a), AHAT also is capable of catalyzing the transfer of acyl groups from *N*-arylhydroxamic acids to arylamines, such as 4-aminoazobenzene.[6] The proposed involvement of an acylenzyme intermediate in both the formation of the reactive *N*-(acyloxy)arylamines and in the transacylation process is consistent with the finding that rabbit hepatic AHAT appears to be identical with the acetylcoenzyme A dependent *N*-acetyltransferase (EC 2.3.1.5) that is responsible for the metabolism of isoniazid, sulfamethazine, and related compounds.[7]

Table II. *N*-Arylhydroxamic Acid *N,O*-Acyltransferase Catalyzed Transacylating and Electrophile-Generating Activities

no.	transacylation rate[a]	n[b]	methylthio adduct formation[c]	n[b]
1	73.2 ± 2.4	3	22.8 ± 3.4	8
2	3.9 ± 1.2	5	10.2 ± 1.0	3
3	0	2	1.5 ± 0.2	4
4	0.5 ± 0.5	3	0.7	2
5	0.9	2	2.2 ± 0.9	4
6	1.8	2	0.4 ± 0.2	3
7	5.2 ± 0.9	5	8.6 ± 0.8	3
8	0.7	2	1.6 ± 0.2	3
9	1.3	2	3.0 ± 1.4	4
10	0	2	1.4 ± 0.8	5
11	0	3	3.2 ± 0.6	6
12	0	2	0.4 ± 0.2	3

[a] The transacylation rate is expressed as nanomoles of AAB acylated per milligram of protein per 5 min plus or minus SD for three or more experiments or as the mean value of two experiments. Each experiment was done in triplicate. The procedure is described under Experimental Section. [b] n = number of experiments. [c] The methylthio adduct formation rate is expressed as nanomoles of methylthio adduct formed per milligram of protein per 30 min plus or minus SD for three or more experiments or as the mean value of two experiments. Each experiment was done in duplicate. The procedure is described under Experimental Section.

Because of the potential toxicological significance of the AHAT-catalyzed activation of *N*-arylhydroxamic acids, it is of interest to determine the structural characteristics of molecules that influence the probability that they will be transformed into electrophilic reactants by this enzyme system. Although previous studies of the effect of acyl group structure on the activation of *N*-(2-fluorenyl)-hydroxamic acids by liver AHAT preparations from various species have been reported, only the *N*-formyl, *N*-acetyl, *N*-propionyl, *N*-chloroacetyl, and *N*-trifluoroacetyl analogues appear to have been investigated.[8-11] In this paper we describe the synthesis of 12 *N*-(2-fluorenyl)-hydroxamic acids and structurally related molecules, as well as the results of their evaluation as substrates for a partially purified hamster hepatic AHAT preparation. A number of the acyl groups were selected on the basis of the results of a study of the structural requirements for mutagenic activity of 2-(acylamino)fluorenes.[12]

Synthesis. The hydroxamic acids 1–8 (Table I) were prepared from 2-nitrofluorene by a previously described modification of the method of Smissman and Corbett.[13,14]

(4) (a) Banks, R. B.; Hanna, P. E. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 1423-29. (b) Hanna, P. E.; Banks, R. B.; Marhevka, V. C. *Mol. Pharmacol.* **1982**, *21*, 159-65.
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(6) Booth, J. *Biochem. J.* **1966**, *100*, 745-53.
(7) Glowinski, I. B.; Weber, W. W.; Fysh, J. M.; King, C. M. *J. Biol. Chem.* **1980**, *255*, 7883-90.
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This procedure involves reduction of the nitro compound to the hydroxylamine in the presence of zinc and ammonium chloride, followed by acylation of the unpurified hydroxylamine with an acyl chloride. A representative procedure for the synthesis of **7** is described under Experimental Section.

N-Hydroxy-*N*-(2-fluorenyl)urea (**9**) was prepared from nitrourea and *N*-hydroxy-2-aminofluorene according to the procedure described by Shillington and co-workers for the synthesis of *N*-arylureas.[15] The yield of **9** was very low because of the apparent instability of the hydroxylamine under the reaction conditions. The synthesis of *N*-hydroxy-*N*-(2-fluorenyl)-*N'*-methylurea (**10**) was accomplished by the reaction of *N*-hydroxy-2-aminofluorene with methyl isocyanate.

The *N*-hydroxycarbamates **11** and **12** were prepared according to the general method described by Boyland and Nery involving the reaction of the hydroxylamine with the appropriate chloroformate.[16]

Transacylation Assay Results. As illustrated in pathway a of Scheme I, AHAT mediates the transfer of acyl groups from *N*-arylhydroxamic acids to arylamines. The relative abilities of compounds **1–12** to function as acyl donors in the AHAT-catalyzed transacylation of 4-aminoazobenzene (AAB) was determined by the method of Booth with a partially purified hamster liver preparation.[6] The results shown in Table II indicate that only the propionyl analogue **2** and the methoxyacetyl analogue **7** have as much as 5% of the transacylating activity of *N*-hydroxy-2-acetamidofluorene (**1**). The low transacylation activity of the propionyl analogue **2** is similar to the result reported by King who found no evidence that this hydroxamic acid could function as an acyl donor.[3]

Compounds **1–12** were evaluated for their abilities to act as acyl donors in the chemical transacylation of AAB by incubation in the presence of heat-denatured enzyme under the experimental conditions used for the enzyme-catalyzed reactions. Although compounds **1–11** exhibited rates of only 0–3 nmol of AAB acylated (mg of protein)$^{-1}$ 5 min^{-1} under these conditions, the phenyl carbamate derivative **12** acylated AAB nonenzymatically at a rate of 23.0 ± 2.8 nmol mg^{-1} 5 min^{-1} (mean ± range for two experiments).

Electrophile Generation Assay Results. Compounds **1–12** were evaluated for their ability to undergo AHAT-catalyzed conversion to electrophilic species by the *N*-acetylmethionine trapping assay of Bartsch et al.[2,17] The basis for this assay involves the reaction of the positively charged species (Scheme I) with the sulfur atom of *N*-acetylmethionine to yield a sulfonium intermediate. The sulfonium compound is decomposed thermally to produce a methylthio adduct that is quantified by liquid scintillation counting.[17] Bartsch and co-workers have shown that a mixture of *o*-methylthio adducts are formed when fluorenylhydroxamic acids are used as substrates in this AHAT-catalyzed process. Thus, the sulfur atom of *N*-acetylmethionine reacts with a positive center at either the 1- or 3-position of fluorene. These carbocations arise from the delocalization of the positive charge of the nitrenium ion that forms from heterolytic cleavage of the *N*-acetoxy intermediate (I; Scheme I).

Table III. Inactivation of AHAT-Catalyzed Bioactivation Activity by *N*-(2-Fluorenyl)acetohydroxamic Acid (**1**)

	activity[a]		
no.	control	after preincubation with **1**	% inhibn
1	32.3 ± 1.6	10.2 ± 1.3	69
2	17.2 ± 1.2	12.5 ± 1.4	27
7	13.5 ± 2.1	10.1 ± 1.1	25

[a] Activities are expressed as nanomoles of methylthio adduct formed per milligram of protein per 30 min plus or minus range for two experiments, each of which was done in duplicate. The procedure is described under Experimental Section.

The results shown in Table II exhibit a structure–activity trend that is similar to the one found for the transacylation rates. The fact that all of the compounds exhibited a measurable rate of methylthio adduct formation whereas several of them were inactive in the transacylation assay may be at least partially a reflection of the difference in the sensitivities of the spectrophotometric and radioisotope methods used to quantify the two processes. The two analogues which displayed the greatest rates of methylthio adduct formation were the propionyl derivative **2**, which was 45% as active as the acetyl compound **1**, and the methoxyacetyl derivative **7**, which was 38% as active as **1**. The methyl carbamate analogue **11** was 14% as active as **1**, and the urea derivative **9** exhibited a rate of adduct formation that was 13% of that of **1**. In general, those compounds that contain relatively small acyl groups are the best substrates for the AHAT-catalyzed conversion to reactive electrophiles. A possible exception to this structure–activity trend is the benzoylhydroxamic acid **5**, which is 10% as active as the acetohydroxamic acid **1**.

As noted above, the phenyl carbamate derivative **12** exhibited a high rate of transacylation of AAB in the presence of heat-denatured enzyme. Compound **12** did not, however, exhibit any significant amount of methylthio adduct formation in the presence of heat-denatured enzyme.

Inactivation of AHAT. It was recently reported from this laboratory that certain *N*-arylhydroxamic acids, including **1**, function as suicide substrates for hamster hepatic AHAT.[4b] Additionally, it was found that **1** selectively and irreversibly inactivates certain transacetylase activities but has little effect on others.[4a,b] In order to gain some insight in regards to whether compounds **2** and **7** are activated by the same enzyme systems that are responsible for the conversion of the prototype compound **1** into an electrophilic reactant, we preincubated the partially purified AHAT preparation with 0.1 mM **1**, dialyzed it to remove unreacted **1** and any other small organic molecules that might be present, and assayed it for the ability to convert **1**, **2**, and **7** to electrophilic species that form methylthio adducts. The results are shown in Table III. The preincubation with **1** resulted in a 69% decrease in the ability of AHAT to bioactivate **1**, whereas the bioactivation of **2** and **7** was depressed by only 27 and 25%, respectively.

Discussion

The data reported herein are in general agreement with the results of related studies with AHAT derived from species other than hamster.[18] Beland and co-workers investigated the ability of partially purified AHAT obtained from rat, guinea pig, monkey, baboon, pig, and human

(14) Smissman, E. E.; Corbett, M. D. *J. Org. Chem.* **1972**, *37*, 1847.
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livers to catalyze the nucleic acid binding of the *N*-formyl, *N*-acetyl, and *N*-propionyl derivatives of *N*-hydroxy-2-aminofluorene.[5] Human and pig enzymes catalyzed binding in the order formyl ≫ acetyl > propionyl, whereas the order of activity was acetyl > propionyl > formyl for enzyme preparations obtained from the other species. It was proposed that the low activity of the *N*-acetyl compound (1) in the human liver incubation may have been the result of degradation of the acetyl-specific AHAT during the 4–18 h postmortem period. King and co-workers had previously reported that incubation of 1 with tRNA and the 105000*g* supernatants of homogenates of human liver resulted in the formation of arylamine-substituted nucleic acid adducts in a manner consistent with AHAT-catalyzed bioactivation.[19]

Weeks and co-workers synthesized the *N*-formyl, *N*-acetyl, *N*-propionyl, *N*-chloroacetyl, and *N*-trifluoroacetyl derivatives of *N*-hydroxy-2-aminofluorene and evaluated them as substrates for a partially purified AHAT preparation from rat liver.[8] The order of AHAT activity, as determined by quantification of the incorporation of arylamine residues into tRNA, was acetyl > propionyl > formyl ≫ chloroacetyl ≃ trifluoroacetyl derivative. Both the chloroacetyl and trifluoroacetyl analogues exhibited a significant amount of tRNA adduct formation in the absence of added AHAT, and little increase in adduct formation occurred with these two compounds when partially purified AHAT was present in the incubation mixtures. Additionally, the AHAT-dependent mutagenicities of the compounds in *Salmonella typhimurium* strain TA 1538 did not correlate with their activities in the tRNA adduct formation assay.[8]

Weeks and co-workers concluded from their study of the *N*-formyl, *N*-acetyl, *N*-propionyl, *N*-chloroacetyl, and *N*-trifluoroacetyl analogues that there are strict steric and electronic requirements for the rat hepatic AHAT-mediated activation of *N*-acyl derivatives of *N*-hydroxy-2-aminofluorene.[8] The results obtained in the present study with hamster hepatic AHAT and a much broader range of types of *N*-acyl groups support that conclusion. The most effective substrates in both the AAB transacylation assay and the methionine trapping assay were the acetyl (1), propionyl (2), and methoxyacetyl (7) compounds, whereas all of the other N-hydroxylated compounds were much less effective (Table II). Linear regression analysis revealed a correlation coefficient of 0.91 for the transacylation and methylthio adduct formation activities. The contention that electronic factors, as well as steric factors, may be important is reinforced by the finding that the *N*-hydroxyureas **9** and **10** and the *N*-hydroxycarbamate **11**, which are similar in steric bulk to the more effective substrates, exhibit very little activity.

The finding that *N*-(2-fluorenyl)acetohydroxamic acid (1) is an effective suicide substrate for certain species of hamster hepatic transacetylases renders it a useful tool for determining whether the bioactivation of various *N*-arylhydroxamic acids by partially purified AHAT preparations is catalyzed by similar enzyme systems or by systems that have different substrate specificities.[4] When AHAT was partially inactivated by preincubation with **1**, followed by measurement of the residual activity for conversion of **1**, **2**, and **7** to reactive electrophiles, the absolute amount of methylthio adduct formed by each compound was the same, but the bioactivation of **1** was depressed to a much greater extent than was that of **2** or **7** (Table III). Although it is possible that this result may indicate that a different species of AHAT than that which is responsible for the transformation of **1** into an electrophilic intermediate participates in the bioactivation of **2** and **7**, the present results do not permit firm conclusions regarding the participation of more than one enzyme in the bioactivation of **1**, **2**, and **7**.

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Experimental Section

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were obtained with a Beckman 33 or a Perkin-Elmer 281 recording spectrophotometer. NMR spectra were obtained with a Varian T-60 spectrometer; the samples were dissolved in Me_2SO-d_6 with tetramethylsilane as internal reference standard. Mass spectra were obtained with an Associated Electronic Industries (AEI) MS 30 mass spectrometer in the University of Minnesota Department of Chemistry Mass Spectrometry Laboratory; samples were introduced by direct inlet, and spectra were run in the electron-impact mode. For each compound, the infrared, NMR, and mass spectrum were consistent with the assigned structure. Analytical TLC was carried out with either plastic-backed plates (Eastman 13181 silica gel with fluorescent indicator, no. 6060) or glass-backed plates (EM, 250 μm, silica gel 60 F-254). Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, IN. The yields, physical properties, and recrystallization solvents for compounds **1**–**12** are listed in Table I.

***N*-(2-Fluorenyl)methoxyacetohydroxamic Acid (7).** 2-Nitrofluorene (3.97 g, 0.018 mol) was dissolved in 110 mL of DMF and 110 mL of 95% ethanol. Ammonium chloride (4.03 g, 0.072 mol) in 33 mL of water and zinc dust (4.92 g, 0.07 mol) were added to the rapidly stirred solution. The mixture was stirred for 3 h at room temperature and was filtered into 300 mL of water. The resulting aqueous suspension (filtrate) was extracted with two 250-mL portions of ether, which were combined, washed with water and saturated NaCl, and dried ($MgSO_4$). After the $MgSO_4$ was removed, the ether solution was cooled in an ice bath, and $NaHCO_3$ (1.60 g, 0.018 mol) in 1.2 mL of water was added. Methoxyacetyl chloride (1.86 g, 0.019 mol) in 25 mL of ether was added dropwise over 30 min to the well-stirred mixture. The mixture was allowed to stir overnight at room temperature. The ether was removed under vacuum, and the yellow residue was triturated with saturated $NaHCO_3$ and collected by filtration. The yellow solid was suspended in 400 mL of ether and extracted with three 100-mL portions of 0.5 N NaOH. The combined basic extracts were acidified with concentrated HCl, and the acidic mixture was extracted with three 150-mL portions of ether, which were combined, dried ($MgSO_4$), and evaporated to yield 2.20 g of crude **7**, which was purified by recrystallization (Table I).

***N*-Hydroxy-*N*-(2-fluorenyl)urea (9).** A 2.67-g sample of crude *N*-hydroxy-2-aminofluorene that had been prepared from 2-nitrofluorene (2.48 g, 0.012 mol) in the manner described above was ground to a fine powder, together with nitrourea (1.45 g, 0.012 mol). The mixture was transferred to an Erlenmeyer flask, and 70 mL of water was added. The mixture was heated on a steam bath for 3 h and allowed to stand in the cold for several hours. The solid was collected by filtration (1.85 g) and extracted with ether and 0.5 N NaOH. The basic extract was acidified with concentrated HCl. The white precipitate that formed was extracted with ether, and the ether extract was washed with water, dried ($MgSO_4$), and evaporated to yield 0.31 g of a residue, which was purified by recrystallization (Table I): EIMS (40 eV), *m/e* (relative intensity) 240 (M^+, 12.42); IR (KBr) 1670, 3455 cm^{-1}.

***N*-Hydroxy-*N*-(2-fluorenyl)-*N'*-methylurea (10).** A benzene solution of unpurified *N*-hydroxy-2-aminofluorene was prepared from 2-nitrofluorene (9.14 g, 0.04 mol) as described above for compound **7**. Methyl isocyanate (2.51 g, 0.04 mol) was dissolved in 5 mL of benzene and added dropwise to the solution of the hydroxylamine. The reaction mixture was allowed to stir overnight at room temperature. The precipitate that formed was collected by filtration to afford 2.32 g of **10**, which was purified by recrystallization (Table I).

Methyl *N*-Hydroxy-*N*-(2-fluorenyl)carbamate (11). An ether solution of unpurified *N*-hydroxy-2-aminofluorene was prepared from 2-nitrofluorene (3.69 g, 0.018 mol) as described

above for compound **7**. Methyl chloroformate (0.74 g, 0.008 mol) was dissolved in 10 mL of anhydrous ether and added dropwise to the solution of the hydroxylamine. The reaction mixture was allowed to stir overnight at room temperature. A precipitate was removed by filtration and washed with several portions of ether. The ether washings were combined with the filtrate, washed with water, and dried ($MgSO_4$). Evaporation of the ether afforded crude **11** (2.08 g), which was purified by recrystallization (Table I).

Phenyl *N*-Hydroxy-*N*-(2-fluorenyl)carbamate (12). An ether solution of unpurified *N*-hydroxy-2-aminofluorene was prepared from 2-nitrofluorene (6.48 g, 0.03 mol) as described above for compound **7**. To this solution was added dropwise phenyl chloroformate (2.21 g, 0.014 mol) in 35 mL of ether. The white precipitate that formed was collected by filtration and washed with several portions of ether. The ether washings were combined with the filtrate, washed with water and saturated NaCl, and dried ($MgSO_4$). Evaporation of the ether afforded crude **12**, which was purified by recrystallization (Table I).

Enzymatic Studies. Male golden Syrian hamsters were purchased from Charles River (Wilmington, MA). Ultracentrifugation was performed on a Beckman L5-65 ultracentrifuge, and low-spin centrifugation was performed on a Beckman J-21B or a J2-21 centrifuge. A Beckman 24 or 24/25 spectrophotometer was used. Incubations were performed in a Dubnoff shaker bath. Cellulose dialysis sacks (10 × 0.62 in., washed in H_2O and packed in 0.2% benzoic acid), dithiothreitol (DTT), methionine, and grade III NAD^+ were obtained from Sigma Chemical Co. *N*-Acetyl-L-[*methyl*-^{14}C]methionine (0.2-0.3 mCi/mmol) was prepared from L-[*methyl*-^{14}C]methionine (New England Nuclear Corp.) and acetic anhydride according to the procedure of Wheeler and Ingersoll.[20]

Tissue Preparation. Animals were lightly etherized before decapitation. Livers were excised and placed in cold 0.05 M sodium pyrophosphate buffer (pH 7.0) containing 1 mM DTT. Livers were blotted dry, weighed, minced, and homogenized with 1 mL of cold buffer per gram of liver in a Potter-type homogenizer with a motor-driven pestle. This 50% homogenate was centrifuged at 105000*g* for 60 min in a refrigerated Beckman preparative ultracentrifuge. The resultant supernatant was diluted with an equal volume of cold pyrophosphate buffer.

Enzyme Preparation. Arylhydroxamic acid *N*,*O*-acyltransferase (AHAT) was partially purified (2- to 3-fold) from hamster liver cytosol (25% solution of 105000*g* supernatant) by ammonium sulfate fractionation as described by King.[3]

The enzyme pellets obtained from the fractionation procedure were stored at −70 °C and were reconstituted in enough 0.05 M sodium phrophosphate buffer (pH 7.0, 1 mM DTT) to give approximately 30 mg/mL of protein. Protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard.[21]

AAB Transacylation Assay. Incubation flasks (25 mL) contained 0.05–0.15 mL of enzyme preparation (2.5 mg of protein from partially purified enzyme), sodium pyrophosphate buffer (50 μmol, pH 7.0), dithiothreitol (1.0 μmol), an *N*-arylhydroxamic acid (2.5 μmol), 4-aminoazobenzene (0.375 μmol), and sufficient 1.15% KCl to give a final volume of 2.5 mL. The *N*-arylhydroxamic acid was omitted from incubations used for reference standards. Reactions were started by addition of substrates (*N*-arylhydroxamic acid/4-aminoazobenzene) dissolved in 0.1 mL of Me_2SO–95% ethanol (1:4) and were carried out at 37 °C in air for 5 min. After termination of the reaction by addition of 2.5 mL of cold 20% trichloroacetic acid (in ethanol–H_2O, 1:1), the transacylation of 4-aminoazobenzene was assayed spectrophotometrically by the method of Booth.[7] Control experiments were carried out with heat-denatured enzyme; all results were adjusted for any detectable nonenzymatic transacylation activity.

Electrophile Generation Assay. The production of electrophiles was measured by the procedure of Bartsch et al.[2] Incubation flasks contained sodium phosphate buffer (41 μmol, pH 6.8), 0.8 μmol of NAD^+, 10 μmol of *N*-acetyl-L-[*methyl*-^{14}C]-methionine, 1 mg of partially purified enzyme, substrate [1 μmol in 0.05 mL of Me_2SO–95% ethanol, (1:4)], and sufficient water to give a final volume of 1.0 mL. It was determined that the inclusion of 0.1 μmol of arylhydroxylamine in the incubation mixtures did not significantly affect the results of these experiments. The inclusion of NAD^+, however, did result in an approximately 50% enhancement of adduct formation.[2]

Reaction was initiated by the addition of substrate. Incubations were carried out at 37 °C in air for 30 min. At the end of the incubation period, the flasks were placed on ice, and 2.5 mL of ether was added. The contents of the flasks were transferred to test tubes and mixed thoroughly with a vortex mixer. The test tubes were then immersed in a dry ice–acetone bath until the aqueous layer was frozen. After the ether layer was decanted, the test tube was heated in a water bath (90 °C) for 2 h. Benzene–petroleum ether (60–70 °C) (5 mL, 3:7) was added, and the two phases were mixed thoroughly. The organic layer was separated, washed with 2 mL of water, and dried ($MgSO_4$). One milliliter of the organic extract was dissolved in 10 mL of Econoflour scintillation fluid. The distintegrations per minute of ^{14}C present in each sample were determined with a Beckman LS-100 liquid scintillation counter. Control experiments were carried out with heat-denatured enzyme, and all results were adjusted for any nonenzymatic adduct formation.

Inactivation of AHAT Bioactivation Activity by *N*-(2-Fluorenyl)acetohydroxamic Acid (1). Preincubation mixtures contained the following final concentrations of reagents: compound **1** (0.5 μmol dissolved in 0.05 mL of 95% ethanol) or an equal volume of 95% ethanol (controls), sodium pyrophosphate–NaCl buffer (250 μmol, pH 7), dithiothreitol (5 μmol), and 1.4 mL of partially purified hamster hepatic AHAT (25 mg of protein). Total volume of the preincubation mixture was 5 mL. Reactions were initiated by the addition of **1** and were carried out in air at 37 °C for 15 min. At the end of the 15-min period, the flasks were placed on ice, and the contents were transferred to dialysis sacks and dialyzed (4 h, 0–4 °C) against four portions (500 mL) of 0.05 M sodium pyrophosphate–NaCl buffer (pH 7.0) that contained 1 mM dithiothreitol and 1% ethanol. The buffer was purged with nitrogen throughout the dialysis period. Samples of the dialyzed enzyme were used to determine the rate of AHAT-catalyzed methylthio adduct formation with compounds **1**, **2**, and **7** as described above under "electrophile generation assay". In all experiments, parallel incubations were carried out with heat-denatured enzyme, and all results were corrected for any nonenzymatic product formation. The control values shown in Table III were obtained from reaction mixtures that were assayed after preincubation for 15 min (without the addition of **1**), followed by a 4-h dialysis under the above described conditions.

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