

## Prodrugs of Nitroxyl as Inhibitors of Aldehyde Dehydrogenase

Melinda J. C. Lee,<sup>†§</sup> Herbert T. Nagasawa,<sup>\*†‡</sup> James A. Elberling,<sup>‡</sup> and Eugene G. DeMaster<sup>‡</sup>

Department of Medicinal Chemistry, University of Minnesota, and Medical Research Laboratories, VA Medical Center, Minneapolis, Minnesota 55417

Received December 20, 1991

In the preceding paper, analogs of chlorpropamide with an OMe substituent on the sulfonamide nitrogen were shown to inhibit aldehyde dehydrogenase (AIDH), and it was postulated that these compounds were bioactivated by O-demethylation to release nitroxyl (HN=O, nitrosyl hydride), which is an inhibitor of AIDH. Further evidence for the production of nitroxyl from compounds with *O*-acyl instead of OMe on the sulfonamide nitrogen is now presented. Thus, nitrous oxide (N<sub>2</sub>O), the end product of nitroxyl dimerization and disproportionation, was found to be generated on alkaline or enzymatic hydrolysis of *N,O*-diacylated *N*-hydroxyarylsulfonamides. Since the latter compounds strongly inhibit yeast AIDH *in vitro* after bioactivation by an esterase intrinsic to this enzyme, nitroxyl generated from these compounds must be the common intermediate that inhibits AIDH.

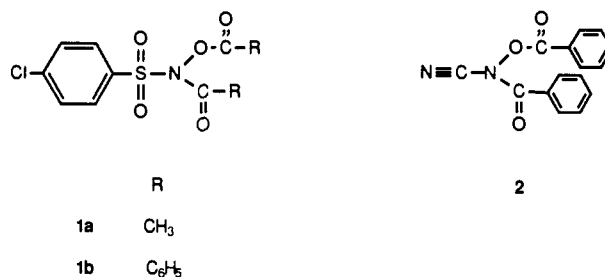
In the preceding paper,<sup>1</sup> it was shown that (arylsulfonyl)ureas and (arylsulfonyl)carbamates with an OMe substituent on the sulfonamide nitrogen exemplified by the general structure I, inhibited aldehyde dehydrogenase (EC 1.2.1.3; AIDH) when administered to rats, as evidenced by the quantum elevation of blood acetaldehyde (AcH) levels when ethanol was given subsequent to I. It was postulated that compounds such as I undergo metabolic O-demethylation *in vivo* to give compounds represented by II, which disproportionate nonenzymatically to products that solvolyzed to nitroxyl (nitrosyl hydride, HN=O), the putative inhibitor of AIDH (Scheme I). Since nitroxyl has also been shown to be the AIDH inhibitor produced by metabolic activation of cyanamide,<sup>2</sup> a clinically prescribed alcohol deterrent agent, this hypothesis that unifies the mechanism of action of two structurally dissimilar classes of compounds that inhibit AIDH *in vivo* by bioactivation to *identical* molecular species should be deserving of corroborative experimental evidence. Such evidence, albeit deductive, will be presented herewith.

It was shown in the preceding paper that rat liver microsomes can O-demethylate compounds such as I to II *in vitro*, although II was not isolated and this reaction was not extensive relative to *N*-demethylation of the aminopyrine used as standard. Since compounds of structure II have not been described in the literature—perhaps because of their instability—and O-demethylation was at best slow *in vitro*, we sought to block this *N*-hydroxy group in II as the acetate or benzoate ester. This would result in compounds such as III which could liberate II *in vivo* by esterase rather than oxidative action. We intended to compare the AIDH-inhibitory activities in rats of compounds having structures I and III to gain

further insights into the mechanism of bioactivation of these compounds.

### Results and Discussion

Attempted selective O-acetylation of the synthon, *N*-hydroxy-4-chlorobenzenesulfonamide, with Ac<sub>2</sub>O or AcCl,<sup>3</sup> or selective O-benzylation with C<sub>6</sub>H<sub>5</sub>COCl under a variety of conditions gave the *N,O*-bis-acylated derivatives, 1a and 1b, as the only isolable products. Compound 1b was of special interest because it bore a structural resemblance to the *N,O*-dibenzyloated derivative 2 of *N*-hydroxycyanamide, the latter the postulated, unstable metabolic oxidation product of cyanamide that decomposes to nitroxyl.<sup>2b</sup> This compound 2 was shown previously to be a good inhibitor of yeast AIDH *in vitro*,<sup>4</sup> following debenzylation of the *O*-benzoyl moiety by esterase action intrinsic to this enzyme.<sup>5</sup>



An alternative reaction sequence (Scheme II) was used to prepare compounds similar to the general structure III (X = O); viz., 5a and 5b. Thus, *N*-(*tert*-butyloxycarbonyl)hydroxylamine (3) was O-acetylated, and the sodium salt of this product 4a was arenesulfonylated with 4-chlorobenzenesulfonyl chloride to give 5a. The O-benzyloated 5b was prepared in the same manner by initial O-benzylation of 3, followed by arenesulfonylation of the product, 4b.

(3) Smith, P. A. S.; Hein, G. E. The alleged role of nitroxyl in certain reactions of aldehydes and alkyl halides. *J. Am. Chem. Soc.* 1960, 82, 5731-5740.

(4) Nagasawa, H. T.; Lee, M. J. C.; Kwon, C.-H.; DeMaster, E. G.; Shirota, F. N. An *N*-hydroxylated derivative of cyanamide that inhibits yeast aldehyde dehydrogenase. *Alcohol* 1992, in press.

(5) Dickinson, F. M.; Haywood, G. W. The role of the metal ion in the mechanism of the K<sup>+</sup>-activated aldehyde dehydrogenase of *Saccharomyces cerevisiae*. *Biochem. J.* 1987, 247, 377-384.

<sup>†</sup> University of Minnesota.

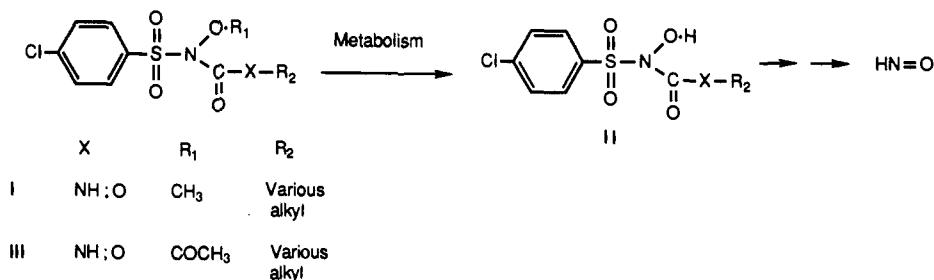
<sup>‡</sup> VA Medical Center.

<sup>§</sup> Present address: Chemistry Department, St. Cloud University, St. Cloud, MN 56301.

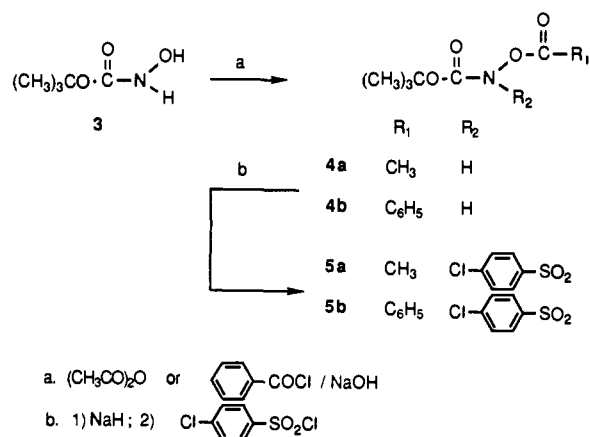
(1) Lee, M. J. C.; Elberling, J. A.; Nagasawa, H. T. *N*-Hydroxylated derivatives of chlorpropamide and its analogs as inhibitors of aldehyde dehydrogenase *in vivo*. *J. Med. Chem.*, preceding paper in this issue.

(2) (a) Shirota, F. N.; DeMaster, E. G.; Nagasawa, H. T. Cyanide is a product of the catalase-mediated oxidation of the alcohol deterrent agent, cyanamide. *Toxicol. Lett.* 1987, 37, 7-12. (b) Nagasawa, H. T.; DeMaster, E. G.; Redfern, B.; Shirota, F. N.; Goon, D. J. W. Evidence for nitroxyl in the catalase-mediated bioactivation of the alcohol deterrent agent cyanamide. *J. Med. Chem.* 1990, 33, 3120-3122.

## Scheme I



## Scheme II



Because **2**, the *N,O*-dibenzoylated derivative of *N*-hydroxycyanamide, was active in inhibiting yeast AIDH *in vitro* (vide supra), we had anticipated that **1b** might also inhibit this enzyme, since *O*-debenzoylation of this compound by the esterase action of yeast AIDH would give an intermediate *N*-hydroxy compound that could eliminate 4-chlorobenzenesulfinic acid, thereby generating nitroxyl. The insolubility of **1b** in our enzymatic assay medium presented a severe obstacle to such *in vitro* experiments, and we were unable to make a valid comparison. However, the *N,O*-diacetyl derivative **1a** was sufficiently water soluble for evaluation in this system for the inhibition of yeast AIDH. Indeed, **1a** turned out to have comparable potency as **2**, with an  $IC_{50}$  of 0.039 mM (Figure 1) compared to an  $IC_{50}$  of 0.025 mM for **2**.<sup>4</sup>

That nitroxyl formation is the likely mechanism for this inhibitory action on yeast AIDH by **1a** was indicated by the time-dependent formation of N<sub>2</sub>O on incubation of **1a** with yeast AIDH (Figure 2). Similar results were obtained on incubation of **1a**, **5a**, and **5b** with porcine liver esterase. Enzymatic hydrolysis of the acetyl group of **5a** by porcine liver esterase was much more rapid than the hydrolysis of the benzoyl group of **5b**, as reflected by the differential production of N<sub>2</sub>O from these two compounds (Figure 2). Moreover, the rate of deacetylation of **1a** appeared to be much slower with yeast AIDH than with the porcine liver esterase, although the specific esterase activities of the two enzymes were not strictly comparable.

Hydrolysis of the acetyl group of **1a**, **5a**, and **5b** with dilute aqueous NaOH likewise gave rise to time-dependent production of N<sub>2</sub>O (Figure 3). As with the enzymatic reaction, the benzoate group of **5b** appeared to be less readily hydrolyzed in this system, and N<sub>2</sub>O formation over 250 min was minimal.

We envision the following mechanism for the generation of nitroxyl (measured as its end product, N<sub>2</sub>O) from compounds **1a**, **5a**, and **5b** (Scheme 3). Enzymatic

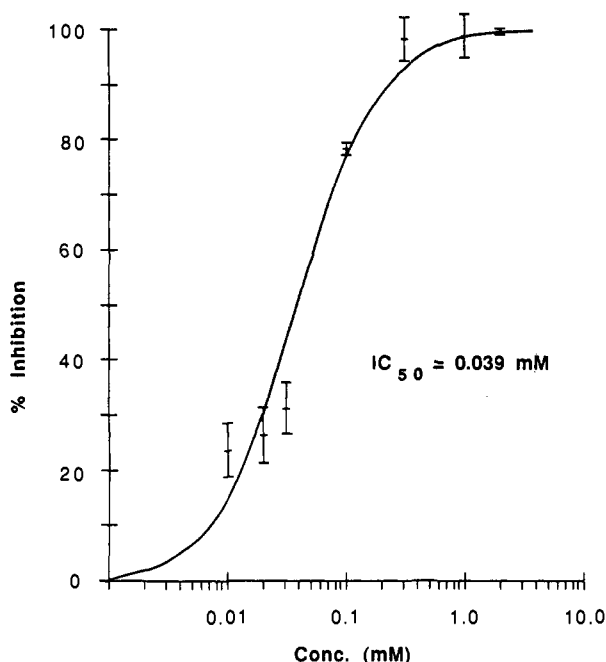


Figure 1. Concentration-dependent inhibition of yeast AIDH *in vitro* by the *N,O*-diacetyl derivative of 4-chloro-*N*-hydroxybenzenesulfonamide (**1a**).  $IC_{50}$  = 50% inhibitory concentration.<sup>12</sup>

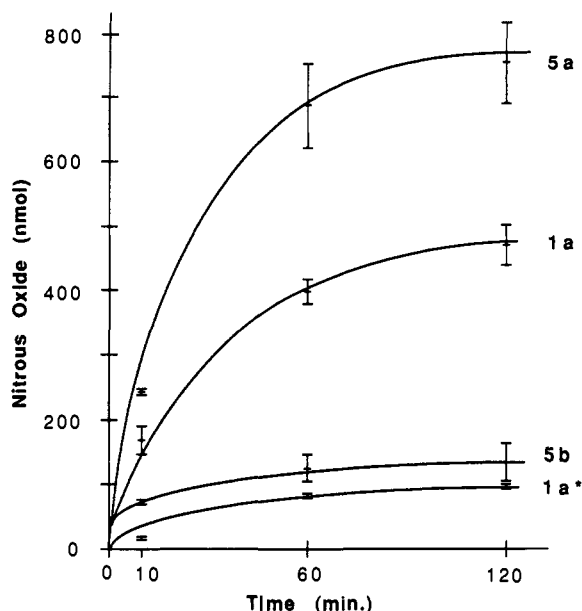
hydrolysis of the acetyl group in **1a** or **5a**, or the benzoyl group in **5b**, would give an intermediate *N*-acylated *N*-arenesulfonylated hydroxylamine **6** which we believe to be unstable and to disproportionate to 4-chlorobenzenesulfinic acid (**7**) and an acylnitroso compound **8**. The latter, **8**, would be expected to solvolyze readily in aqueous physiological systems to nitroxyl and the corresponding carboxylic acid.<sup>6</sup> The fate of nitroxyl ultimately giving N<sub>2</sub>O follows Scheme 3. In support of this mechanism, the action of porcine liver esterase on **5a** was significantly inhibited by bis(*p*-nitrophenyl) phosphate (BNPP), a specific esterase inhibitor<sup>7</sup> (Figure 4).

That acylnitroso compounds such as **8** do in fact solvolyze to nitroxyl was indicated by the rapid concentration dependent production of N<sub>2</sub>O from **8a**, prepared *in situ* by oxidation of *N*-(*tert*-butyloxycarbonyl)hydroxylamine (**3**) with 1,2-diazenedicarboxylic acid bis-(*N,N'*-dimethylpiperazine) diiodide (DIP+2)<sup>8</sup> in aqueous phosphate buffer at pH 7 (Scheme 4). Although quantitative conversion was not achieved, it can be seen that N<sub>2</sub>O production was essentially over by 10 min (Figure 5).

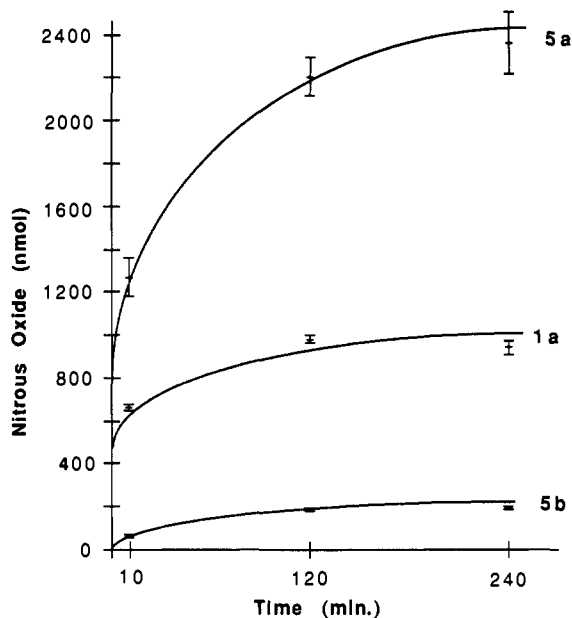
(6) Beckwith, A. L. J.; Evans, G. W. Reactions of alkoxy-radicals. Part III. Formation of esters from alkyl nitrites. *J. Chem. Soc.* 1962, 130-137.

(7) Wick, M. J.; Hanna, P. E. Bioactivation of *N*-arylhydroxamic acids by rat hepatic *N*-acetyltransferase. *Biochem. Pharmacol.* 1990, 991-1003.

(8) Kosower, E. M.; Kanety-Londner, H. Glutathione. 13. Mechanism of thiol oxidation by diazenedicarboxylic acid derivatives. *J. Am. Chem. Soc.* 1976, 98, 3001-3007.



**Figure 2.** Generation of  $N_2O$  by the action of porcine liver esterase on the *N,O*-diacylated *N*-hydroxyarylsulfonamides, **1a**, **5a**, and **5b**. Generation of  $N_2O$  by the action of yeast AIDH on **1a** is indicated by **1a\***. The details of the experiment and the determination of  $N_2O$  by headspace gas chromatography are described in the Experimental Section. The results are given as mean  $\pm$  SEM,  $n = 3$ .



**Figure 3.** Generation of  $N_2O$  by hydrolysis of the *N,O*-diacylated *N*-hydroxyarylsulfonamides **1a**, **5a**, and **5b**, in dilute NaOH. See the Experimental Section for further details.

The relative activities of **1a**, **5a**, and **5b** in inhibiting yeast AIDH *in vitro* are compared in Figure 6. It is noteworthy that **1a** was the best inhibitor of this series, even though  $N_2O$  production by the action of yeast AIDH on **1a** was less than with porcine liver esterase which in turn was less than that from **5a** with the porcine esterase (Figure 2). Thus, the substrate specificity of yeast AIDH relative to its *esterase* activity must be different from that of the porcine enzyme and suggests that such differences might also be observed with the mammalian mitochondrial class II AIDH. Notwithstanding, these results lend support to our hypothesis that compounds represented by the general structure II are unstable and disproportionate to

give 4-chlorobenzenesulfinic acid and acylnitroso intermediates that solvolyze to nitroxyl. Compounds that can release potential nitroxyl by metabolic action are, by definition, prodrugs of nitroxyl, and our observation that **1a**, **5a**, and **5b** inhibit yeast AIDH with intrinsic esterase activity suggests that these compounds are esterase-activated prodrugs of nitroxyl.

Whereas compounds of structure I were good inhibitors of the hepatic low  $K_m$  AIDH when administered to rats *in vivo*, as reflected by the elevation of blood AcH levels following ethanol,<sup>1</sup> in preliminary experiments with a limited number of animals, the ester prodrugs **5a** and **5b** were found to be inactive *in vivo* (data not shown). This lack of *in vivo* activity of these ester prodrugs of nitroxyl is likely due to premature hydrolysis of the ester group, possibly by plasma esterases and lack of targeting to the liver. Nevertheless, the cumulative *in vitro* data presented here clearly implicate nitroxyl, generated by esterase action on these compounds, as the reactive species that inhibits AIDH. By deduction, the hepatic microsomal *O*-demethylase activated compounds that inhibit AIDH in rats and represented by structure I (see preceding paper<sup>1</sup>) must also give rise to nitroxyl *in vivo*. These latter compounds are therefore prodrugs of nitroxyl that are bioactivated by the microsomal *O*-demethylase of liver.

## Experimental Section

The preceding paper<sup>1</sup> should be consulted for the general experimental parameters. Chemical ionization mass spectra were provided by the University of Minnesota Mass Spectrometry Service using a Finnegan 4000 mass spectrometer.

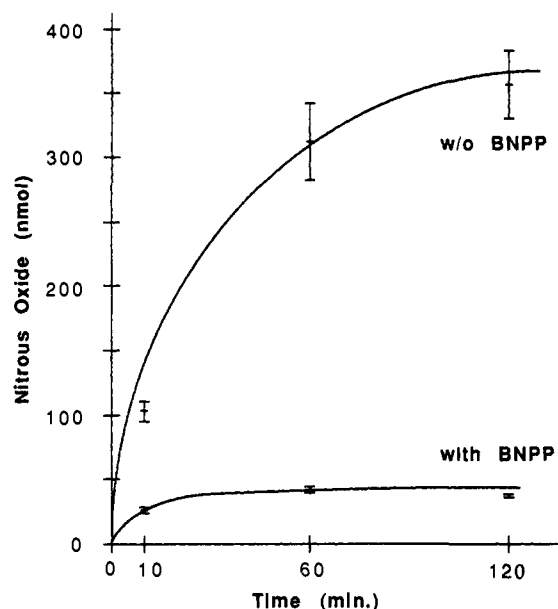
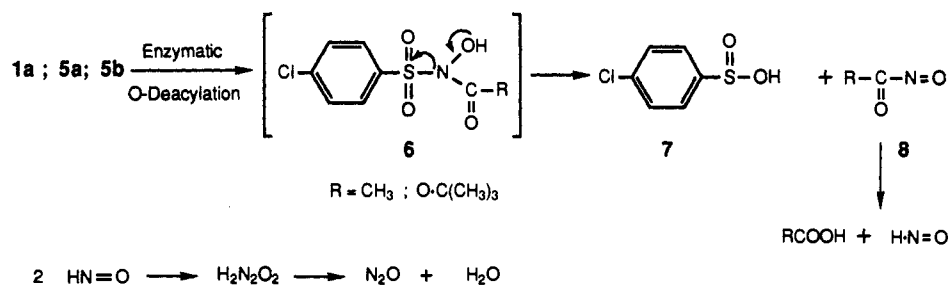
***N*-Acetyl-*N*-(acetyloxy)-4-chlorobenzenesulfonamide (1a).** To a cooled (ice bath) stirred solution of 4-chloro-*N*-hydroxybenzenesulfonamide<sup>9</sup> (1.04 g, 5 mmol) in 10 mL of THF (HPLC grade), acetic anhydride (0.95 g, 0.95 mL, 10 mmol) was added dropwise, followed by pyridine (1.02 g, 0.81 mL, 10 mmol). The reaction mixture was allowed to come to room temperature over 1 h. The THF was then evaporated, and the residue was treated with cold  $H_2O$  (20 mL). The solid which formed was collected and dissolved in EtOAc, and the solvent was dried over  $Na_2SO_4$  and evaporated to dryness. The residue was recrystallized from Et<sub>2</sub>O to give fluffy white crystals: 0.85 g (58% yield); mp 124–125 °C; IR (KBr,  $cm^{-1}$ ) 1806 (O—C=O), 1736 (N—C=O), 1388 and 1175 ( $SO_2$ ); <sup>1</sup>H NMR (200 MHz,  $\delta$ ) 2.18 (s, 3 H, NCOCH<sub>3</sub>), 2.29 (s, 3 H, OCOCH<sub>3</sub>), 7.49–7.99 (q, 4 H, 1,4-disubstituted benzene); <sup>13</sup>C NMR ( $\delta$ ) 17.90 (1 C, OCOCH<sub>3</sub>), 22.51 (1 C, NCOCH<sub>3</sub>), 129.5 and 130.6 (4 C, aromatic), 135.6 and 141.7 (2 C, aromatic substituted), 167.1 (ArCON), 167.6 (ArCOON). Anal. (C<sub>10</sub>H<sub>10</sub>ClNO<sub>5</sub>S) C, H, N.

***N*-Benzoyl-*N*-(benzoyloxy)-4-chlorobenzenesulfonamide (1b).** To a cooled (ice bath) stirred solution of 4-chloro-*N*-hydroxybenzenesulfonamide (1.04 g, 5 mmol) in 10 mL of THF (HPLC grade), benzoyl chloride (1.41 g, 1.16 mL, 10 mmol) was added dropwise, followed by pyridine (0.79 g, 0.81 mL, 10 mmol). The reaction mixture was allowed to come to room temperature, and the precipitate of pyridine hydrochloride was removed by filtration. The filtrate was cooled until white crystals appeared; these were collected and recrystallized from THF, yielding 1.25 g (60% yield) of **1b**: mp 132–134 °C, IR (KBr,  $cm^{-1}$ ) 1775 (C=O), 1698 (N—C=O), 1381 and 1176 ( $SO_2$ ); <sup>1</sup>H NMR (300 MHz,  $\delta$ ) 7.44–8.18 (aromatic H); <sup>13</sup>C NMR (300 MHz,  $\delta$ ) 125.7–141.7 (18 C, aromatic), 163.5 (ArCOON), 168.3 (ArCO); CI-MS (CH<sub>4</sub>) (positive ion) 105 (100) [C<sub>6</sub>H<sub>5</sub>CO]<sup>+</sup>, 77 (8) [C<sub>6</sub>H<sub>5</sub>]<sup>+</sup>; (negative ion) 294 (62) [M - 121]<sup>-</sup>, 240 (69) [C<sub>6</sub>H<sub>5</sub>COONCOC<sub>6</sub>H<sub>5</sub>]<sup>-</sup>, 121 (100) [C<sub>6</sub>H<sub>5</sub>COO]<sup>-</sup>. Anal. (C<sub>20</sub>H<sub>14</sub>ClNO<sub>5</sub>S) C, H, N.

**(Acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic Acid 1,1-Dimethylethyl Ester (5a).** (Acetyloxy)carbamic acid 1,1-

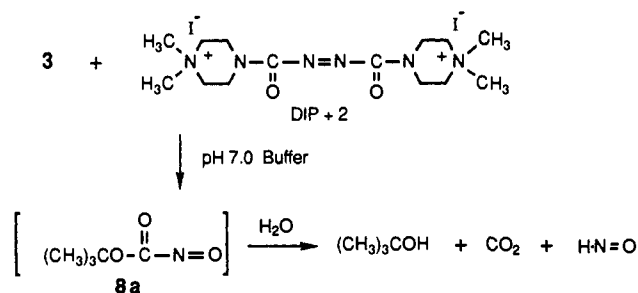
(9) Przybylski, J.; Kupryszewski, G. *O*-[*N*-Acylaminoacyl]-*N*-[aryl-sulfonyl]-hydroxylamines and their application in synthesis of peptide bonds. *Rocz. Chem.* 1975, 49, 529–537.

## Scheme III



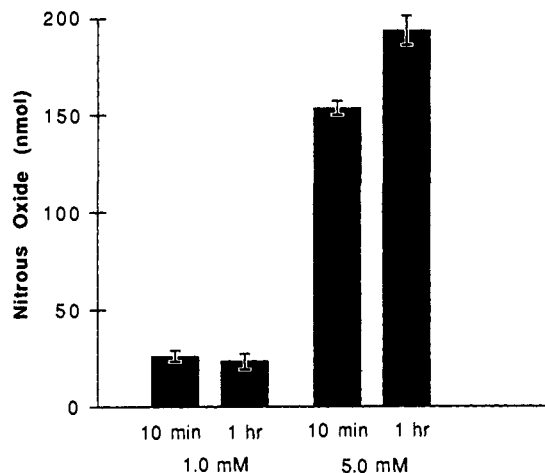
**Figure 4.** Inhibition of the action of porcine liver esterase on **5a** by BNPP. Details of this experiment are described in the Experimental Section.

## Scheme IV

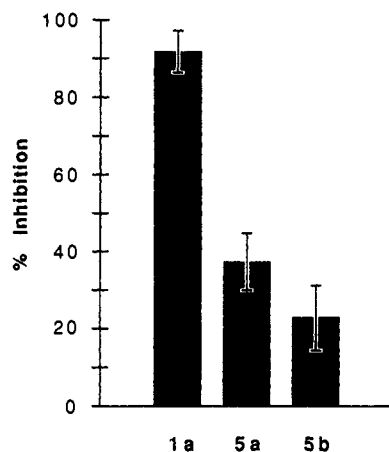


dimethylethyl ester<sup>10</sup> (0.81 g, 4.6 mmol) was dissolved in 5 mL of dimethylacetamide, and NaH (0.22 g of a 50% dispersion, 4.6 mmol) was added. This mixture was stirred for 30 min after which time 4-chlorobenzenesulfonyl chloride (0.97 g, 4.6 mmol) was added and the reaction allowed to proceed at room temperature for 2 h. The mixture was then added to 25 mL of H<sub>2</sub>O and extracted with ether (4 × 15 mL). The ether layer was separated and evaporated on the steam bath, and replaced with CH<sub>2</sub>Cl<sub>2</sub> (30 mL). After drying, the mixture was concentrated to a clear oil, which was applied to a silica gel column and eluted with THF-hexane, 1:5. Fractions 9-13 were collected and the solvent evaporated to give **7a** as a white, fluffy solid: 0.44 g (27% yield); IR (KBr, cm<sup>-1</sup>) 1814 (acetyl carbonyl), 1762 (BOC carbonyl), 1389 and 1191 (SO<sub>2</sub>); <sup>1</sup>H NMR (200 MHz, δ) 1.40 (s, 9 H, (CH<sub>3</sub>)<sub>3</sub>CO), 2.28 (s, 1 H, COCH<sub>3</sub>), 7.51-7.99 (q, 4 H, 1,4-disubstituted benzene); <sup>13</sup>C NMR (δ) 17.7 (1 C, COCH<sub>3</sub>), 27.78

(10) Carpino, L.; Giza, C.; Carpino, B. O-Acylhydroxylamines. I. Synthesis of O-benzoylhydroxylamine. *J. Am. Chem. Soc.* **1959**, *81*, 955-957.



**Figure 5.** Generation of N<sub>2</sub>O by oxidation of **3** with DIP+2. The concentrations of **3** are as indicated. See the Experimental Section for further details.



**Figure 6.** Inhibition of yeast ALDH by the N,O-diacetylated N-hydroxyarylsulfonamides, **1a**, **5a**, and **5b**. Details of this experiment are described in the Experimental Section.

(3 C, (CH<sub>3</sub>)<sub>3</sub>CO), 86.75 ((CH<sub>3</sub>)<sub>3</sub>CO), 129.2 and 130.4 (4 C, aromatic), 136.2 and 141.1 (aromatic, substituted), 148.4 (1 C, carbamate carbonyl), 167.2 (CH<sub>3</sub>CO). Anal. (C<sub>13</sub>H<sub>16</sub>ClN<sub>2</sub>O<sub>6</sub>S) C, H, N.

**(Benzoyloxy)[(4-chlorophenyl)sulfonyl]carbamic Acid 1,1-Dimethylethyl Ester (5b).** (Benzoyloxy)carbamic acid 1,1-dimethylethyl ester<sup>10</sup> (0.47 g, 2.0 mmol) was stirred in 10 mL of N,N-dimethylacetamide, and NaH (0.1 g of a 50% dispersion, 2 mmol) was added. This mixture was stirred for 30 min and 4-chlorobenzenesulfonyl chloride (0.42 g, 2 mmol) was added. The reaction was allowed to proceed at room temperature for 18 h, after which time the mixture was added to 25 mL of H<sub>2</sub>O and extracted with ether (4 × 15 mL). The ether was evaporated on the steam bath and replaced with CH<sub>2</sub>Cl<sub>2</sub> (30 mL). The solvent was dried and concentrated to a clear oil which solidified when placed in the freezer compartment of a refrigerator. The solid was collected, washed with H<sub>2</sub>O, and redissolved in EtOAc. Recrystallization from EtOAc-hexane gave 0.44 g of **7b** (54%

yield): mp 133–138 °C; IR (KBr,  $\text{cm}^{-1}$ ) 1779 (PhC=O), 1757 (BOC C=O), 1387 and 1149 ( $\text{SO}_2$ );  $^1\text{H}$  NMR (200 MHz,  $\delta$ ) 1.41 (s, 9 H,  $(\text{CH}_3)_3\text{CO}$ ), 7.26–8.13 (m, 9 H, aromatic H's);  $^{13}\text{C}$  NMR ( $\delta$ ) 27.62 (3 C,  $(\text{CH}_3)_3\text{CO}$ ), 86.79 ( $(\text{CH}_3)_3\text{CO}$ ), 126.1 (1 C, aromatic), 129.8, 129.2, 130.3, and 130.6 (8 C, aromatic), 134.5, 136.3, and 141.2 (3 C, aromatic, substituted), 148.5 (1 C,  $(\text{CH}_3)_3\text{COCON}$ ), 163.6 (PhCO). Anal. ( $\text{C}_{18}\text{H}_{18}\text{ClNO}_6\text{S}$ ) C, H, N.

**Headspace Gas Chromatographic Analysis of  $\text{N}_2\text{O}$ .** (a) **By Action of Yeast AIDH on 1a.** Gas chromatography bottles (25 mL) containing 0.5 mL of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mL of 0.15 mM KCl, and 0.70 mL of  $\text{H}_2\text{O}$  were prepared in triplicate. Compound 1a was dissolved in DMSO at a concentration of 200 mM, and a 100- $\mu\text{L}$  aliquot was added to the GC bottle, followed by addition of 200  $\mu\text{L}$  of yeast AIDH (Sigma Lot 12H820; 11 mg of protein/mL; 230 units/mg of protein) to initiate the reaction. The bottles were immediately stoppered and placed in a 37 °C shaking water bath and sampled periodically for  $\text{N}_2\text{O}$  determination by gas chromatography as previously described.<sup>2b</sup> Controls contained 200  $\mu\text{L}$  of  $\text{H}_2\text{O}$  in place of yeast AIDH.

(b) **By Base Hydrolysis of 1a, 5a, and 5b.** Gas chromatography bottles (25 mL) containing 1.25 mL of 80 mM potassium phosphate buffer (pH 7.0) and 0.35 mL of  $\text{H}_2\text{O}$  were prepared in triplicate for each sample. The compounds to be tested were dissolved in THF at a concentration of 100 mM, and an aliquot (200  $\mu\text{L}$ ) was added to the GC bottle, followed by 200  $\mu\text{L}$  of 1 N NaOH. Depending on the compound tested, the final concentrations of the sample in the reaction mixture were 1, 5, or 10 mM. The bottles were incubated and the  $\text{N}_2\text{O}$  was determined as above.

(c) **By Action of Porcine Liver Esterase on 1a, 5a, and 5b.** Gas chromatography bottles (25 mL) containing 1.25 mL of 80 mM potassium phosphate buffer (pH 8.0) and 0.525 mL of  $\text{H}_2\text{O}$  were prepared in triplicate for each sample. The compounds to be tested were dissolved in THF at a concentration of 100 mM, and an aliquot (200  $\mu\text{L}$ ) was added to the GC bottle, followed by addition of 25  $\mu\text{L}$  of porcine liver esterase (Sigma Lot 107F-8235; 19 mg of protein/mL; 335 units/mg of protein) to initiate the reaction. The bottles were immediately stoppered and placed in a 37 °C shaking water bath for 10 min before sampling for  $\text{N}_2\text{O}$  determination as above. Controls contained 25  $\mu\text{L}$  of  $\text{H}_2\text{O}$  in place of porcine liver esterase.

**Inhibition of Porcine Liver Esterase on 5a by Bis(*p*-nitrophenyl)phosphate (BNPP).** Gas chromatography bottles (25 mL) containing 1.00 mL of 100 mM potassium phosphate

buffer (pH 8.0) and 0.8 mL of 37.5 mM BNPP were prepared in quadruplicate for each run. A solution of (100  $\mu\text{L}$ ) porcine liver esterase (Sigma Lot 29F-8055; 11 mg of protein/mL; 230 units/mg of protein) was diluted with  $\text{H}_2\text{O}$  (1.53 mL) to give an activity of 100  $\mu\text{mol}/\text{mL}$  per min. A solution of 1a in THF was made at a concentration of 100 mM, and an aliquot (100  $\mu\text{L}$ ) was added to the GC bottle, followed by addition of 100  $\mu\text{L}$  of esterase solution to initiate the reaction. The ratio of BNPP concentration to substrate concentration was 3:1. The bottles were immediately stoppered and placed in a 37 °C shaking water bath. Samples for  $\text{N}_2\text{O}$  determination as described above were taken at 10, 60, and 120 min. The uninhibited reaction contained 0.8 mL  $\text{H}_2\text{O}$  in place of BNPP. Controls contained 100  $\mu\text{L}$  of  $\text{H}_2\text{O}$  in place of porcine liver esterase.

**$\text{N}_2\text{O}$  Formation by the Oxidation of (Acetyloxy)carbamic Acid 1,1-Dimethylethyl Ester (3).** Two reaction mixtures of 3 where the final concentrations were 1.0 and 5.0 mM in a total of 5.0 mL were prepared in phosphate buffer (pH 7.0), and 200  $\mu\text{L}$  of a 100 mM aqueous solution of DIP+2 was added to each sample. The  $\text{N}_2\text{O}$  formed at 10 min and after 1 h of incubation at 55 °C was analyzed in triplicate by gas chromatography as described above.

**Inhibition of Yeast AIDH.** A primary mix containing 50 mM potassium phosphate buffer (pH 7.5), yeast AIDH (20–30 units/mg, 0.07 IU), 1.0 mM  $\text{NAD}^+$ , and 5  $\mu\text{L}$  of the test compound dissolved in DMSO, in a final volume of 0.1 mL, was incubated at 37 °C for 10 min. A 20- $\mu\text{L}$  aliquot was then removed for assay of the remaining AIDH activity as described previously.<sup>11</sup>

**Acknowledgment.** This work was supported in part by the Department of Veterans Affairs and in part by ADAMHA Grant 1R01-AA07317.

**Registry No.** 1a, 142867-52-5; 1b, 142867-53-6; 2, 142867-54-7; 3, 36016-38-3; 4a, 99768-83-9; 4b, 105340-85-0; 5a, 142867-55-8; 5b, 142867-56-9; *p*- $\text{ClC}_6\text{H}_4\text{SO}_2\text{Cl}$ , 98-60-2; *p*- $\text{ClC}_6\text{H}_5\text{O}_2\text{NHOH}$ , 50695-53-9;  $\text{AcCl}$ , 75-36-5;  $\text{PhCOCl}$ , 98-88-4;  $\text{N}_2\text{O}$ , 10024-97-2;  $\text{HNO}$ , 14332-28-6; aldehyde dehydrogenase, 9028-86-8; esterase, 9013-79-0.

(11) Nagasawa, H. T.; Elberling, J. A.; DeMaster, E. G.; Shirota, F. N.  $\text{N}^1$ -Alkyl-substituted derivatives of chlorpropamide as inhibitors of aldehyde dehydrogenase. *J. Med. Chem.* 1989, 32, 1335–1340.

(12) Berkson, J. A statistically precise and relatively simple method of estimating the bioassay with quantal response, based on the logistic function. *Am. Stat. Soc. J.* 1953, 48, 565–599.