Prodrugs of Nitroxyl and Nitrosobenzene as Cascade Latentiated Inhibitors of Aldehyde Dehydrogenase

Terry T. Conway,[†] Eugene G. DeMaster,[†] Melinda J. C. Lee,[‡] and Herbert T. Nagasawa*[,†,§]

Minneapolis VA Medical Center; Department of Chemistry, St. Cloud State University; and the Department of Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota 55417

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The prototypic aromatic *C*-nitroso compound, nitrosobenzene (NB), was shown previously to mimic the effect of nitroxyl (HN═O), the putative active metabolite of cyanamide, in inhibiting aldehyde dehydrogenase (AlDH). To minimize the toxicity of NB in vivo, pro-prodrug forms of NB, which were designed to be bioactivated either by an esterase intrinsic to AlDH or the mixed function oxidase enzymes of liver microsomes, were prepared. Accordingly, the prodrug *N*-benzenesulfonyl-*N*-phenylhydroxylamine (**3**) was further latentiated by conversion to its *O*-acetyl (**1a**), *O*-methoxycarbonyl (**1b**), *O*-ethoxycarbonyl (**1c**), and *O*-methyl (**2**) derivatives. Similarly, pro-prodrug forms of nitroxyl were prepared by derivatization of the hydroxylamino moiety of methanesulfohydroxamic acid with *N*,*O*-bis-acetyl (**7a**), *N*,*O*-bis-methoxycarbonyl (**7b**), *N*,*O*-bis-ethoxycarbonyl (**7c**), and *N*-methoxycarbonyl-*O*-methyl (**7d**) groups. It was expected that the bioactivation of these prodrugs would initiate a cascade of nonenzymatic reactions leading to the ultimate liberation of NB or nitroxyl, thereby inhibiting AlDH. Indeed, the ester pro-prodrugs of both series were highly active in inhibiting yeast AlDH in vitro with IC_{50} values ranging from 21 to 64 μM. However, only **7d** significantly raised ethanol-derived blood acetaldehyde levels when administered to rats, a reflection of the inhibition of hepatic mitochondrial AlDH-2.

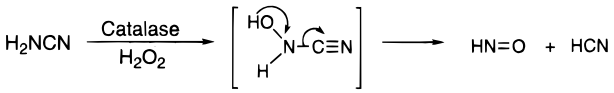
Introduction

The inhibition of hepatic mitochondrial aldehyde dehydrogenase (AlDH-2, EC 1.2.1.3) in vivo by the clinically used alcohol deterrent agent, cyanamide, has been postulated to be mediated by nitroxyl (HN═O, nitrosyl hydride).[1] Nitroxyl is produced by metabolic *N*-hydroxylation of cyanamide catalyzed by catalase/H_2O_2,[2] followed by spontaneous, nonenzymatic decomposition of the intermediate *N*-hydroxycyanamide (Scheme 1).

Since nitroxyl is a highly reactive species that interacts readily with sulfhydryl groups[3] and/or dimerizes to hyponitrous acid which in turn dehydrates to H_2O and nitrous oxide (N_2O),[4] we postulated that *N*-aryl- or *N*-alkyl-substituted nitroxyls, viz., *C-nitroso compounds*, might represent stable forms that would inhibit AlDH directly without the need for bioactivation. Indeed, nitrosobenzene (NB) and 1-nitrosoadamantane (NA) were found to inhibit yeast AlDH with IC_{50} values of 2.5 μM and 8.6 μM, respectively.[5] The more sterically hindered 2-methyl-2-nitrosopropane (MNP) was several orders of magnitude less inhibitory (IC_{50} = 150 μM), but none of these *C*-nitroso compounds required prior bioactivation to manifest AlDH inhibition.

Although NA exhibited high in vitro activity, it was totally inactive when administered to rats, and this was suggested as being due to lack of site-specific delivery of the *monomeric* form[6] of NA to the liver.[5] In contrast, NB, which is mostly monomeric, inhibited AlDH when administered to rats at one-fourth the molar dose of NA,

Scheme 1



as indicated by the dramatic increase in ethanol-derived blood acetaldehyde (AcH) levels, a reflection of the inhibition of hepatic mitochondrial AlDH-2. However, NB exhibited systemic toxicity even at this lower dose.[5]

In an effort to minimize the toxicity of NB, we sought to develop prodrug forms of this prototypic aromatic nitroso compound that would undergo site-specific bioactivation in the liver—as model *C*-nitroso inhibitors of AlDH. The drug design principle was based on our successful development of prodrug forms of nitroxyl as cascade-latentiated inhibitors of AlDH.[7] Unlike cyanamide (Scheme 1), these prodrugs liberate nitroxyl but *not* cyanide on bioactivation, and effectively utilizes the intrinsic esterase activity of liver AlDH-2 or the hepatic microsomal cytochrome P-450 enzymes for bioactivation, followed by a cascade of sequential nonenzymatic disproportionation and solvolysis reactions leading to the ultimate liberation of nitroxyl, the putative inhibitor of AlDH.[7]

Based on this drug design, the immediate precursor of NB (the *prodrug* of NB) would be the *N*-benzenesulfonylated phenylhydroxylamine **3** (Scheme 2), which would be expected to dissociate nonenzymatically at physiological pH to NB and the sulfinic acid **4**. In the "pro" form of this prodrug (i.e., the *pro-prodrug* of NB), the hydroxyl group of **3** is masked as a carboxylic ester (as in **1a**), as carbonate esters (as in **1b** and **1c**), or as an ether functionality (as in **2**), and the bioactivation cascade is initiated by (a) esterase action on the esters

[†] VA Medical Center.
[‡] St. Cloud State University.
[§] University of Minnesota.

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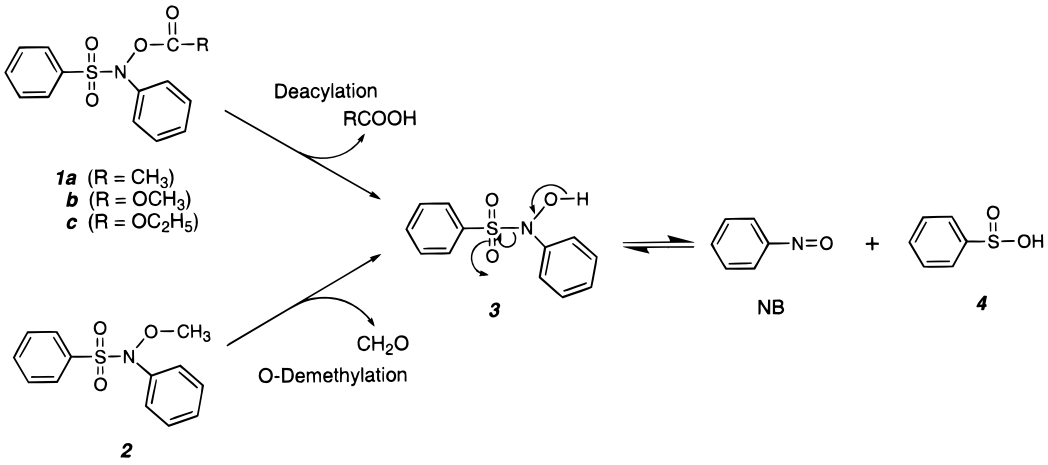
Scheme 2



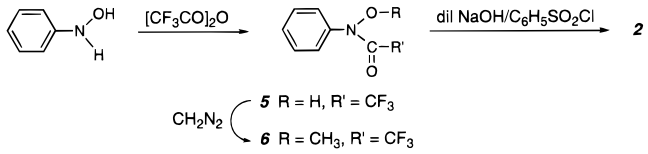
1, or (b) oxidative *O*-demethylation of the ether **2** mediated by the mixed function oxidases of liver microsomes (Scheme 2).

In earlier studies we were unable to demonstrate in vivo activity with the ester type pro-prodrugs of nitroxyl derived from aromatic sulfohydroxamates (i.e., from Piloty's acid derivatives).[7] We will now describe an ether prodrug of nitroxyl derived from methanesulfohydroxamic acid that does in fact manifest in vivo AlDH inhibitory activity by raising ethanol-derived blood AcH levels when administered to rats, a consequence of the >70% inhibition[8] of hepatic mitochondrial AlDH-2. We will also present evidence that the ester type prodrugs of NB and nitroxyl are highly potent inhibitors of yeast AlDH in vitro.

Results

Prodrug and Pro-prodrug Forms of NB. Phenylhydroxylamine, prepared by the reduction of nitrobenzene with Zn/NH_4Cl,[9] was *N*-benzenesulfonylated[10] to give the prodrug **3**. Although prodrug **3** can theoretically be prepared by the addition of benzenesulfinic acid to NB itself,[11] this reaction was not preparative in our hands. *O*-Acylation of **3** with Ac_2O, MeOCOCl, and EtOCOCl gave the *O*-acetyl- (**1a**), *O*-methoxycarbonyl (**1b**), and *O*-ethoxycarbonyl (**1c**) derivatives, respectively, the carboxylic ester and carbonate ester pro-prodrugs of NB. However, direct *O*-methylation of **3** with dimethyl sulfate, methyl iodide, trimethyloxonium fluoroborate, or "magic methyl" (methyl trifluoromethanesulfonate) in the presence of a suitable organic base did not proceed as expected, presumably because of the facile dissociation of **3** to NB in base. Methylation of **3** with excess diazomethane over 24 h produced **2** in extremely poor yield. A presumed nitrone byproduct[12] was also formed in this reaction, but was not isolated. Addition of BF_3 to catalyze this methylation reaction[13] resulted in accelerated decomposition of the diazomethane. By using silica gel as catalyst,[14] the reaction was accelerated somewhat, but the nitrone was the major ether-soluble product, along with unchanged **3** and a small amount of **2**. Compound **2** was, therefore, prepared by an indirect route (Scheme 3) as follows: Phenylhydroxylamine was N-trifluoroacetylated and this product (**5**) O-methylated with diazomethane[15] to give **6**. Hydrolysis of the trifluoroacetyl group and

Scheme 3



sequential benzenesulfonylation of the deblocked O-methylphenylhydroxylamine (structure not shown) in situ gave **2**, but, again, in low yield. A considerable amount of azobenzene, a byproduct derived from the decomposition of *O*-methylphenylhydroxylamine,[15a] was also produced in this reaction.

Compounds **1b** and **1c** are expected not only to release NB by the esterase action of AlDH on the ester linkages but also to carbomethoxylate (methoxycarbonylate) and carbethoxylate, respectively, the active-site Cys-302 residue of the enzyme, thereby serving as "double-barreled" inhibitors of AlDH.[16] On the other hand, compound **2** should not inhibit AlDH in vitro in the absence of *O*-demethylation, but would be expected to have finite activity in vivo following catalysis by the cytochrome P-450 enzymes.

Incubation of prodrug **3** with yeast AlDH resulted in a time-dependent, log-linear reduction in the ability of this enzyme to oxidize benzaldehyde (Figure 1) and this inhibition was clearly dose-dependent (Figure 2). Similarly, the ester pro-prodrug of NB, viz., compound **1a**, inhibited AlDH in a time-dependent, essentially identical manner in the presence of or in the absence of NAD (data not shown). From the dose−response relationship, the IC_{50} was calculated to be 36.0 μM. The IC_{50} values for the *O*-methoxycarbonyl (**1b**) and the *O*-ethoxycarbonyl (**1c**) analogues were 38.3 μM and 64.3 μM, respectively (Table 1), i.e., considerably higher than NB itself (IC_{50} = 2.5 μM),[5] in keeping with their pro-prodrug relationship to NB. Moreover, the time course for inhibition of AlDH by compound **1b** was not log-linear, but appeared to have an initial lag phase followed by acceleration over time (Figure 1). This pattern held for **1c** as well (data not shown), further confirming our bioactivation cascade mechanism (Scheme 2) for these compounds. It is also significant that the IC_{50} of prodrug **3** (1.66 μM, Figure 2) was approximately of the same order of magnitude as NB, suggesting the ready bioavailability of NB from **3** once released from its

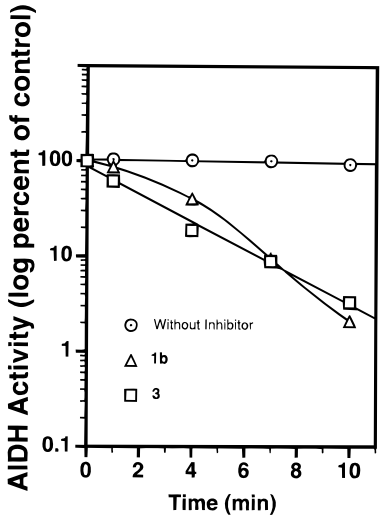


Figure 1. Inhibition of yeast AlDH by prodrugs **1b** and **3** over the initial 10 min of incubation. Each point represents the average of triplicate determinations.

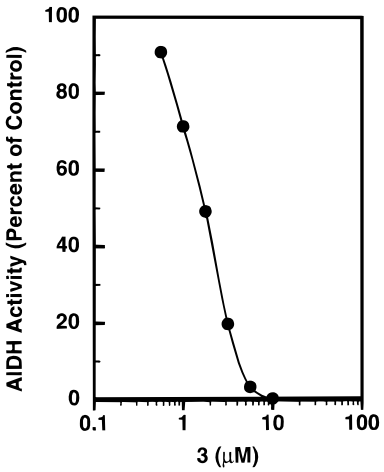


Figure 2. Dose vs remaining AlDH activity after incubation of prodrug **3** with yeast AlDH. The IC_{50} was calculated to be 1.66 μM.

Table 1. Inhibition of Yeast AlDH by Prodrug and Pro-Prodrug Forms of NB and Nitroxyl

inhibitor (compound no.)	IC_{50} (μM)
1a	36.0
1b	38.3
1c	64.3
2	>1000.0
3	1.66
5	>1000.0
NB	2.5[a]
7a[b]	~ 1000.0
7b	20.9
7c	35.6
7d	>1000.0
CH_3SO_2NHOH	180.0

[a] From ref 7. [b] Although all stock solutions were freshly prepared, this compound may have hydrolyzed prematurely.

respective pro-prodrug forms, i.e., from **1a**−**c** and/or **2**. Moreover, compound **5**, the *N*-trifluoroacetyl derivative of phenylhydroxylamine which cannot dissociate to NB in the manner of prodrug **3**, was totally inactive (Table 1). Compound **2** which is dependent on the hepatic cytochrome P-450 enzymes for bioactivation was, as expected, inactive in vitro. However, site-specific delivery of the ester prodrugs of NB appears not to have been achieved in vivo, since compounds **1a**, **1b**, and, especially, **3** still exhibited toxicity at 1.0 mmol/kg (Table 2, Supporting Information). Even with lower doses which ensured survival of the animals, blood AcH levels were not significantly elevated, although blood ethanol levels were comparable to that of controls. The ether prodrug **2**, however, appeared to show finite, if weak, in vivo activity at doses of 0.32 and 0.56 mmol/kg (to avoid toxicity), but the data were not statistically significant (Figure 3).

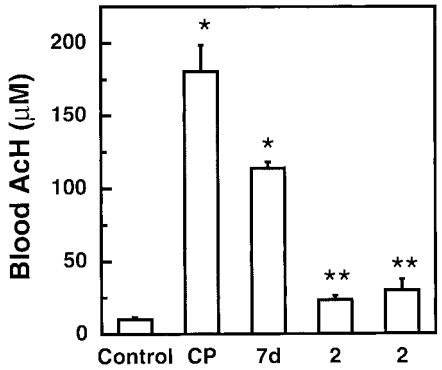
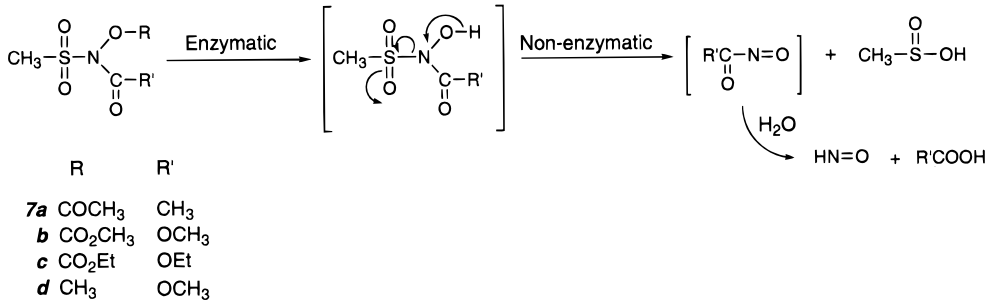


Figure 3. Blood AcH concentrations in rats pretreated with chlorpropamide (CP) or the ether prodrugs **7d** or **2**, followed by ethanol. The doses for CP and **7d** were 1.0 mmol/kg, while the doses for **2** were 0.32 (left) and 0.56 (right) mmol/kg. (*) $p < 0.01$; (**) not significant. The details of this experiment are described in the Experimental Section.

Nitroxyl Prodrugs Based on Methanesulfohydroxamic Acid. As alluded to above, esterase-bioactivated nitroxyl prodrugs based on aromatic sulfohydroxamates, while displaying good inhibitory activity against AlDH in vitro, were uniformly inactive when administered to rats. This was ascribed as being due to absence of targeted delivery of nitroxyl to the liver, possibly by premature hydrolysis of these ester prodrugs in plasma or in the peritoneal cavity following administration. The water insolubility of these compounds may also have contributed to this inactivity.

To circumvent these problems, the much smaller methanesulfohydroxamic acid derivatives with identical or similar functional groups on the hydroxylamine moiety were prepared (compounds **7**, Scheme 4). The syntheses of these compounds paralleled those used for the corresponding aromatic sulfohydroxamic acid derivatives[7,16] and will not be further elaborated except in the Experimental Section. The mechanism of nitroxyl liberation from these prodrugs by the bioactivation cascade is depicted in Scheme 4. That nitroxyl is, in fact, liberated by esterase action on the ester prodrugs **7a**, **7b**, and **7c** was shown by the time-dependent formation of N_2O, the stable end-product of nitroxyl dimerization/dehydration,[4] when these compounds were incubated with commercially available porcine liver esterase (Figure 4). As shown in Table 1, these ester prodrugs were good inhibitors of yeast AlDH in vitro and, indeed, were better than methanesulfohydroxamic acid itself from which they were derived. However, none of the ester prodrugs tested was active when administered to rats, i.e., these compounds did not raise blood AcH levels statistically above control levels (Table 2, Supporting Information). On the other hand, the ether

Scheme 4



	R	R'
7a	$COCH_3$	CH_3
b	CO_2CH_3	OCH_3
c	CO_2Et	OEt
d	CH_3	OCH_3

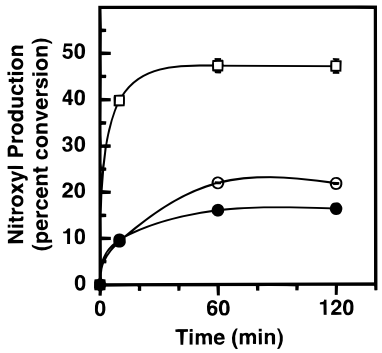


Figure 4. The generation of nitroxyl (measured as N_2O) by the action of porcine liver esterase on the ester prodrugs **7a** (G), **7b** (E), and **7c** (J). Each point represents the average of triplicate determinations. (Error bars were not added in the interest of clarity.) The details of this experiment and the determination of N_2O by headspace gas chromatography are described in the Experimental Section.

prodrug **7d**, which was inactive in vitro, was active in inhibiting hepatic AlDH when administered to rats (Figure 3).

Discussion and Conclusions

Compounds that are biologically active in vitro frequently do not achieve their desired pharmacological effects in vivo because (a) their lack of lipophilicity prevents their passage through cell membranes, (b) they are intrinsically toxic or are metabolized to inactive forms, or (c) they are readily eliminated.[17] The use of stable derivatives of such agents that are designed to be inactive until released by enzymatic or nonenzymatic action in vivo, i.e., prodrug forms, has been successful in resolving these problems.[18]

We have shown that the model *aromatic C*-nitroso compound, NB, as well as nitroxyl itself, can be latentiated to prodrug forms that are highly effective in inhibiting yeast AlDH in vitro. However, this prodrug design, where the compounds are latentiated with selected functional groups in order to render the highly reactive nitroxyl and the potentially toxic NB to be more target organ specific in vivo, has only been partially successful. Prodrug forms of nitroxyl and NB that utilize enzymes peculiar to the liver for bioactivation should, theoretically, have rendered these compounds more liver specific and much less toxic. Although compounds **1a** and **7a** with labile ester groups[19] may be less specific due to their high reactivity, the less labile carbonate esters (**1b**, **1c**, **7b**, **7c**) should have been more liver specific due to their relative chemical stability and the intrinsic esterase activity of liver mitochondrial AlDH-2 as well as the presence of carboxy esterase isozymes in liver microsomes.[20] That esterase action is required to initiate the bioactivation cascade was supported by (a) the detection of nitroxyl (as N_2O) when the ester prodrugs **7a**, **7b**, and **7c** were subjected to esterase action (Figure 4), and (b) the lack of inhibitory activity of **1a** (1.0 mM) against glyceraldehyde-3-phosphate dehydrogenase (which has no esterase activity), whereas the chemical nitroxyl donors Piloty's acid (1.0 mM) and Angeli's salt ($Na_2N_2O_3$, 0.10 mM) inhibited this enzyme by 97% and >99%, respectively, while NB itself (1.0 mM) inhibited the enzyme by 47% (Table 3, Supporting Information).

Since the carbonate esters of both series were *all* good inhibitors of yeast AlDH (Table 1), the differences in their specificities for rat liver AlDH-2 in vivo vs the yeast enzyme in vitro must be rationalized. Devaraj et al.[21] have reported that the apparent K_i's of some ester type nitroxyl prodrugs for yeast AlDH and for recombinant *human liver* AlDH-2 were different. However, other factors must also be considered since we had shown earlier that the *N*,*O*-bis-carbethoxylated derivative of 4-chlorobenzenesulfohydroxamic acid corresponding to **7c**, although inactive in vivo, was a good inhibitor of AlDH-2 in isolated rat liver mitochondria.[16] In the absence of pharmacokinetic data, we are unable to explain the differential in vitro vs in vivo activities of these ester prodrugs of NB and of nitroxyl.

This prodrug design should also be directly applicable for the latentiation and potential liver targeted delivery of the monomeric form of the *aliphatic C*-nitroso compound, NA. These studies are being actively pursued.

Experimental Section

Chemistry. Melting points were taken on a Fischer-Johns hot-stage melting point apparatus and are uncorrected. IR spectra were recorded on a Nicollet FT-IR spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz NMR spectrometer. Chemical shifts (δ) are in parts per million (ppm) relative to $Si(CH_3)_4$ and coupling constants (J) are in hertz. EI-MS were recorded on a Kratos MS 25 mass spectrometer. Microanalyses were performed by M-H-W Laboratories, Phoenix, AZ. Thin-layer chromatography (TLC) was performed using Analtech silica gel GF Uniplates, and the products were visualized either by fluorescence quenching observed under UV light or by exposure to iodine vapor in an iodine chamber. Thick layer chromatography was performed using Analtech 1000 μm preadsorbent silica gel GF Uniplates. Organic solvent extracts were dried over Na_2SO_4 for 15−20 min before filtration.

Reagents and Chemicals. Hydroxylamine hydrochloride, benzenesulfonyl chloride, methanesulfonyl chloride, Diazald, methoxylamine hydrochloride, and sodium hydride were purchased from the Aldrich Chemical Co. (Milwaukee, WI). *N*-Phenylhydroxylamine[9], methanesulfohydroxamic acid,[22] *N*-benzenesulfonyl *N*-phenylhydroxylamine (**3**),[10] *N*-phenyltri-

fluoroacetohydroxamic acid (**5**),[15] methyl *N*-phenyltrifluoroacetohydroxamate (**6**),[15] and *N*-benzenesulfonyl-*O*-acetyl-*N*-phenylhydroxylamine (**1a**)[23] were prepared according to the methods described in the literature as referenced and were stored under a nitrogen atmosphere at −20 °C when not in use. Yeast AlDH, NAD^+, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO).

***N*-Benzenesulfonyl-*O*-carbethoxyphenylhydroxylamine (1c).** Ethyl chloroformate (108 mg, 1.00 mmol) was added to an ice-cold solution of **3** (249 mg, 1.00 mmol) in 10 mL of methylene chloride. Triethylamine (101 mg, 1.00 mmol) was added, and the solution was stirred for 10 min and then for 1 h at ambient temperature. TLC (benzene) showed the reaction to be complete after 1 h (**3**, R_f 0.23; **1c**, R_f 0.68) The light-blue reaction mixture was washed with water (2 × 5 mL), dried, and evaporated to dryness in vacuo to give 250 mg of a light-yellow solid. Recrystallization from benzene−hexane afforded 200 mg (62% yield) of **1c** as colorless crystals, mp 106.5−108.5 °C. NMR ($CDCl_3$): 1.35 (3H, J = 7.14, CH_3), 4.32 (2H, J = 7.14, CH_2), 7.12−7.66 (10H, *aromatic H*); IR (KBr): 1796 cm^{-1} (C═O). Anal. ($C_{15}H_{15}NO_5S$) C, H, N.

***N*-Benzenesulfonyl-*O*-carbomethoxyphenylhydroxylamine (1b).** This compound was prepared by an analogous procedure as above, except using methyl chloroformate (61% yield). Colorless crystals from benzene−hexane, mp 94.5−95 °C, R_f 0.49 (benzene). NMR ($CDCl_3$): 7.12−7.66 (m, 10H, *aromatic H*), 3.95 (s, 3H, OCH_3); IR (KBr): 1802 cm^{-1} (C═O). Anal. ($C_{14}H_{15}NO_5S$) C, H, N, S.

***N*-Benzenesulfonyl-*N*-methoxyaniline (2). Method A:** (*Caution*: *Diazomethane is toxic as well as an explosion hazard. The initial steps of this synthesis should be carried out behind a safety shield in a fume hood*.) Solid **3** (661 mg, 2.65 mmol) was added, in portions, to an ice-cold, freshly prepared solution of diazomethane (582 mg, 13.9 mmol, from Diazald) in 50 mL of ether. The reaction was allowed to stand at room-temperature overnight. (Addition of acetic acid to an aliquot of the reaction mixture showed that, at this point, there was no more diazomethane present since nitrogen was not evolved.) TLC (5% hexane−benzene) showed the presence of **2** at R_f 0.62, a trace of starting material (R_f 0.21), and two unidentified, strongly yellow-colored spots at R_f 0.05 and 0.32. The reaction mixture was filtered to remove some flocculent material, and the filtrate was evaporated to dryness in vacuo at 40 °C to give 870 mg of a reddish-yellow oil. Ether (4 mL) was added, and the flask was cooled and maintained at 5 °C for 30 min. The resultant suspension of yellow solids were collected and washed with 1 mL of petroleum ether−ether (1:1). The combined filtrate and washings were evaporated to dryness, and the resultant yellow oil was subjected to flash chromatography on SiO_2 using benzene as eluent. The first fraction (everything up to the first translucent band) was collected (500 mL) and evaporated to dryness in vacuo at 45 °C to give 240 mg of a yellow oil. This oil was purified by preparative TLC using 5% hexane−benzene. $CHCl_3$ elution of the R_f 0.30−0.45 zone afforded 128 mg (18.3% yield) of **2** as a pale-yellow oil, TLC = single spot at R_f 0.62 (5% hexane−benzene) and R_f 0.45 (EtOAc−hexane 1:20). NMR ($CDCl_3$): 3.89 (s, 3 H, OCH_3), 7.12−7.52 (m, 10 H, *aromatic H*). IR (thin film): 1182, 1363 cm^{-1} (S═O). CI-MS: 264 (M + 1), 232, 168, 136. Anal. ($C_{13}H_{13}NO_3S$). C, H, N, S.

Method B: Compound **6** (148 mg; 0.675 mmol) was dissolved in a mixture of 8 mL of THF and 4.5 mL of water. $KHCO_3$ (135 mg; 1.35 mmol) was added, and the reaction was stirred at 45 °C for 7 h. At that point, TLC (20% hexane−benzene) indicated that **6** (R_f 0.65) had completely hydrolyzed. The mixture was allowed to cool to room temperature, benzenesulfonyl chloride (87 μL; 0.68 mmol) was added, and the reaction was allowed to proceed with stirring at room temperature overnight. The orange reaction mixture was diluted with 3 volumes of H_2O and extracted with ether (2 × 20 mL). After drying, the ether extract was evaporated to dryness in vacuo to give 0.30 g of a dark brown liquid. This material was purified by preparative TLC with 5% hexane−benzene to afford 16.1 mg (18% yield) of **2** as a pale yellow oil with identical physical characteristics to the material prepared by method A above. A less polar zone from the preparative TLC afforded 31.5 mg (51.2% yield) of azobenzene, R_f 0.90 (10% hexane−benzene), mp 68−69 °C. Mixture mp with an authentic sample was not depressed.

***O*-Acetyl-*N*-methanesulfonylacetohydroxamic Acid (7a).** A mixture of methanesulfohydroxamic acid (1.11 g; 10.0 mmol) and acetic anhydride (4.26 mL; 45.3 mmol) was allowed to stand at room-temperature overnight. The mixture was then heated on a steam bath for 1 h. Excess acetic anhydride was removed by evaporation in vacuo at 55 °C. The residual acetic acid was removed as an azeotrope by repeated addition of toluene (6 × 10 mL) followed by evaporation in vacuo. This afforded 1.95 g of a clear colorless oil. Crystallization from ethyl acetate−hexane gave 395 mg (20% yield) of **7a** as large white prisms, mp 49−50 °C. An analytical sample was recrystallized twice from ethyl acetate−hexane, mp 53−54 °C. *This compound is moisture sensitive.* R_f 0.95 (EtOAc); NMR ($CDCl_3$): 3.43 (s, 3H, SO_2CH_3), 2.33 (s, 3H, COCH_3), 2.24 (s, 3H, COCH_3); IR (KBr): 1803, 1726 cm^{-1} (C═O's). Anal. ($C_5H_9NO_5S$) C, H, N.

***N*,*O*-Bis(carbomethoxy)methanesulfohydroxamic Acid (7b).** Triethylamine (949 mg: 9.38 mmol) was added to a cooled mixture of methanesulfohydroxamic acid (474 mg; 4.26 mmol) and methyl chloroformate (887 mg; 9.38 mmol) in 4 mL of THF. After stirring for 1 h, TLC (EtOAc/hexane; 1:2) showed that the starting material (R_f 0.35) had disappeared. The precipitate of $Et_3N{\cdot}HCl$ was removed by filtration and washed with ether (2 × 5 mL). Evaporation of the combined filtrate and washings in vacuo gave a cream-colored solid, which was recrystallized from EtOAc−petroleum ether to give 0.93 g (96% yield) of **7b** as off-white crystals, mp 93−94 °C. NMR ($CDCl_3$): 3.80 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3,42 (s, 3H, O_2SCH_3). IR (KBr): 1807, 1745 cm^{-1} (C═O's). Anal. ($C_5H_9NO_7S$) C, H, N.

***N*,*O*-Bis(carboethoxy)methanesulfohydroxamic Acid (7c).** This compound was prepared by an analogous procedure as above except using ethyl chloroformate. **7c**, a clear colorless oil, was isolated in 94% yield after SiO_2 flash chromatography with methylene chloride as eluent; R_f 0.92 (methanol:chloroform 5:95). NMR ($CDCl_3$): 3.45 (s, 3H, O_2SCH_3), 4.43 (q, 4H, J = 7.5, 2 × CH_2), 1.40 (doublet of triplets, J = 7.5, 6H, 2 × CH_2CH_3); IR (KBr): 1804, 1737 cm^{-1} (C═O's). Anal. ($C_7H_{13}NO_7S$) C, H, N, S.

***N*-Carbomethoxy-*O*-methylmethanesulfohydroxamic Acid (7d).** A solution of **8** (119 mg, 0.95 mmol) in 2 mL of dry THF was added to NaH (24 mg, 0.95 mmol) in a dried flask equipped with a N_2 inlet, bubbler guard, and an ice−water bath for cooling. When H_2 evolution had ceased, methyl chloroformate (77.3 μL, 1.0 mmol) was added. The resultant suspension was stirred at room temperature for 2 h at which time another 77.3 μL of methyl chloroformate and 3 mL of dry *N*,*N*-dimethylacetamide were added. After stirring for 24 h at room temperature the mixture was filtered to remove insoluble inorganic material. The filtrate was diluted with two volumes of water and extracted with ether (4 × 10 mL). The combined ether extracts were washed with water, dried, and evaporated to dryness in vacuo to give 0.29 g of an oil. This oil was dissolved in CH_2Cl_2 and the solution dried over Na_2SO_4 overnight. Evaporation of the solution to dryness in vacuo gave 150 mg (86% crude yield) of a clear colorless glass. This glass was subjected to the action of a vacuum pump at 45 °C for 30 min and then cooled in the freezer compartment of a refrigerator whereupon it crystallized. Further purification by trituration with petroleum ether containing a little ethyl acetate afforded **7d** as a crystalline white solid, mp 77−78 °C. NMR (CDCl3): 3.96 (s, 3H, CO_2CH_3), 3.92 (s, 3H, OCH_3), 3.18 (S, 3H, O_2SCH_3); IR (thin film): 1730 cm^{-1} (C═O). Anal. ($C_4H_9NO_5S$) C, H, N, S.

***N*-(Methoxy)methanesulfonamide (8).** Methanesulfonyl chloride (2.87 g, 25.0 mmol) was added to an ice-cold stirring solution of methoxylamine hydrochloride (2.0 g; 25.0 mmol) in a mixture of methanol (15 mL) and H_2O (5 mL), followed

by the dropwise addition of a solution of K_2CO_3 (3.45 g, 25 mmol) in 4 mL of water. After 15 min the ice-bath was removed, and the reaction mixture was stirred for another 20 min. The precipitate was removed by filtration and washed with methanol (2 × 1 mL). The combined filtrates were cooled (5 °C) for 15 min and refiltered. Concentration of the solvent in vacuo (<30 °C) to approximately 2 mL, and cooling the residue gave crude **8** as white crystals. Recrystallization from a small volume of methanol gave 0.80 g (26% yield) of **8**, mp 94.5−98 °C; R_f 0.32 (EtOAc−hexane 1:2). NMR ($CDCl_3$):3.73 (s, 3H, O*CH*$_3$), 2.97 (s, 3H, O_2S*CH*$_3$), 3.56 (s, 1H, *NH*). Anal. ($C_2H_7NO_3S$) C, H, N, S.

Inhibition of Yeast AlDH. The test compounds were evaluated for inhibitory activity against yeast AlDH as previously described.[24]

Inhibition of Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH). The primary reaction mix containing 20 mM sodium arsenate (pH 7.4), 1.0 mM EDTA, and 0.5 mM NAD^+ was preincubated for 5 min at 37 °C followed by the addition of GAPDH (10 μL) and inhibitor in 5 μL of DMSO for a total of 0.1 mL. In the case of Angeli's salt, 2 μL of 5 mM solution (in 20 mM KOH) was added where indicated. After 10 min, a 20 μL aliquot of the primary mix was added to a cuvette containing 0.5 mM NAD^+, 1.0 mM EDTA, 20 mM sodium arsenate, 50 mM Tris-Cl (pH 8.6) and 1.0 mM glyceraldehyde-3-phosphate. This secondary reaction was initiated by the addition of 20 μL (giving a final volume of 1.0 mL) of the primary reaction mix containing GAPDH. Enzyme activity was determined spectrophotometrically by following the increase in absorbance at 340 nm with time. The data presented (Table 3, Supporting Information) represent triplicate incubations.

Analysis of N_2O following Action of Porcine Liver Esterase on 7a, 7b, and 7c. Reaction vessels (25 mL) containing 1.25 mL of 80 mM potassium phosphate buffer (pH 8.0) and 0.525 mL of H_2O 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 μL) was added, followed by the addition of 25 μL of porcine liver esterase (19 mg protein/mL; 335 units/mg protein) to initiate the reaction. The vessels were immediately stoppered and placed in a 37 °C shaking water bath for 10 min before sampling for N_2O determination by headspace gas chromatography.[1c] Controls contained 25 μL of H_2O in place of porcine liver esterase.

Pharmacological Evaluations in Vivo. These studies were performed in adherance with guidelines established in the *Guide for the Care and Use of Laboratory Animals* published by the U.S. Department of Health and Human Resources (NIH Publication 85-23, revised 1985). Animals were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and the research protocol was approved by the subcommittee on Animal Studies of the Minneapolis VA Medical Center. This committee is vigorous in enforcing its charge of minimizing the use of animals in research.

Drug Administration Protocol. Sprague−Dawley male rats (Biolab Corp., St. Paul, MN) weighing 170−220 g were fasted ~24 h prior to the time of sacrifice. All drugs were dissolved or suspended in 2% carboxymethylcellulose (CMC). Doses of 0.5 mmol/kg were given ip as 1.0 mL/100 g body weight except where noted. Ethanol was administered 2 h later and was given as a 20% (w/v) solution, 1.0 mL/100 g of body weight. The animals were sacrificed 1 h following ethanol administration, and blood was collected for AcH and ethanol measurements.

Measurement of Blood AcH and Ethanol Levels. Blood AcH and ethanol levels were measured as previously described.[25]

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Supporting Information Available: Table 2, Blood AcH and Ethanol Levels in the Rat after Pretreatment with Prodrugs of NB and Nitroxyl, and Table 3, Inhibition of Glyceraldehyde-3-phosphate Dehydrogenase (GADPH) by NB, Cpd **1a**, Piloty's Acid and Angeli's Salt (2 pages). Ordering information is given on any current masthead page.

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