Prodrugs of Nitroxyl as Potential Aldehyde Dehydrogenase Inhibitors vis-à-vis Vascular Smooth Muscle Relaxants

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The synthesis and the chemical/biological properties of *N*-hydroxysaccharin (**1**) (2-hydroxy-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide), a nitroxyl prodrug, are described. When treated with 0.1 M aqueous NaOH, **1** liberated nitroxyl (HN=O), a known inhibitor of aldehyde dehydrogenase (AlDH), in a time-dependent manner. Nitroxyl was measured gas chromatographically as its dimerization/dehydration product N_2O. Under these conditions, Piloty's acid (benzenesulfohydroxamic acid) also gave rise to HNO. However, whereas Piloty's acid liberated finite quantities of nitroxyl when incubated in physiological phosphate buffer, pH 7.4, formation of nitroxyl from **1** was minimal. This was reflected in the differential inhibition of yeast AlDH (IC_{50} = 48 and >1000 μM) and the differential relaxation of preconstricted rabbit aortic rings in vitro (EC_{50} = 1.03 and 14.0 μM) by Piloty's acid and **1**, respectively. The *O*-acetyl derivative of **1**, *viz.*, *N*-acetoxysaccharin (**13a**), was much less active in both assays. It is concluded that *N*-hydroxysaccharin (**1**) is relatively stable at physiological pH and liberates nitroxyl appreciably only at elevated pH's. As a consequence, neither **1** nor its *O*-methyl (**8a**) and *O*-benzyl (**8b**) derivatives were effective AlDH inhibitors in vivo when administered to rats at 1.0 mmol/kg.

Nitroxyl (HN=O, nitrosyl hydride), which is produced metabolically by the action of catalase/H_2O_2 on the alcohol deterrent agent cyanamide,[1] has been shown to be the putative aldehyde dehydrogenase (AlDH) inhibitor of cyanamide in vivo.[2] Nitroxyl has also been postulated—but not yet proved—to be one of the intermediates in the five-electron oxidation of the amino acid L-arginine to L-citrulline and nitric oxide (NO•) catalyzed by the enzyme nitric oxide synthase.[3] Contemporary interest in nitric oxide, believed to be the endothelium-derived relaxing factor (EDRF)[4] and a brain neurotransmitter,[5] has directed attention to its one-electron reduction product nitroxyl, a possible biological precursor of nitric oxide. Indeed, nitroxyl donors such as bioactivated cyanamide (*viz.*, *N*-hydroxycyanamide), Angeli's salt ($Na_2N_2O_3$, sodium trioxodinitrate), *N,O*-dibenzoyl-*N*-hydroxycyanamide, and the *N,O*-diacylated derivatives of 4-chlorobenzenesulfohydroxamate (*N,O*-diacylated Piloty's acid derivatives) have been shown not only to be good inhibitors of AlDH[6] but also potent vasodilators as evidenced by their dose-dependent effect on the relaxation of preconstricted rabbit aortic rings in vitro.[3,7] Thus, all of these nitroxyl donors appeared to mimic the action of EDRF, this effect being magnified in the presence of superoxide dismutase (SOD). It has been demonstrated[8] that nitroxyl can be reversibly oxidized to nitric oxide by SOD, the reverse reaction being less favorable.

These considerations prompted the synthesis of other potential nitroxyl donors (prodrugs of nitroxyl) that could generate nitroxyl spontaneously or by enzymatic action in order to study their biological properties as inhibitors of AlDH and as vascular smooth muscle relaxants. We had previously shown that Piloty's acid derivatives of the general structure **I** (Scheme 1) that are *N*-acylated *and* alkylated or acylated on the hydroxyl moiety serve as prodrugs of nitroxyl and release nitroxyl either by the cytochrome P-450-catalyzed dealkylation of the *O*-alkyl group or by esterase-mediated deacylation of the *O*-acyl moiety (Scheme 1).[6d,9]

N-Hydroxysaccharin (**1**) (2-hydroxy-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide) represents a fused ring analog of the (unstable) *N*-acylated arylenesulfohydroxamate intermediate depicted [bracketed] in Scheme 1, and it was of considerable interest to us to ascertain (a) whether **1** would release nitroxyl spontaneously and (b) whether its *O*-alkylated and *O*-acylated derivatives of the general structure II would give rise to **1** and therefore nitroxyl on enzymatic *O*-dealkylation or *O*-deacylation (Scheme 2). The fact that **1** has not previously been reported in the literature suggested that it might have limited stability, possibly due to decomposition along the pathway depicted in Scheme 2. The *O*-alkylated and *O*-acylated **1** would then represent stable derivatives that could be bioactivated to nitroxyl via **1**. Accordingly, we undertook their syntheses.

Chemistry and Biochemical Pharmacology

N-Methoxysaccharin (**8a**) has been prepared[10] starting from *O*-sulfobenzoic anhydride (**2**) via the bis-acid chloride **3** by reaction of the latter with methoxylamine followed by a light-catalyzed thermal rearrangement of the isoimide **4** to **8a** (Scheme 3). However, this second step could not be duplicated in good yields in our hands, and an alternative route for the preparation of **8a,b** was devised (Scheme 3, paths a–d). Thus, **5** was converted to the synthon *O*-carbethoxybenzenesulfonyl chloride (**6**), which was condensed with the corresponding *O*-alkylhydroxylamines to give the intermediates **7**. Thermal cyclization, either neat or in refluxing acetic acid, afforded the target compounds **8** in overall yields of 48–56% from **6**.

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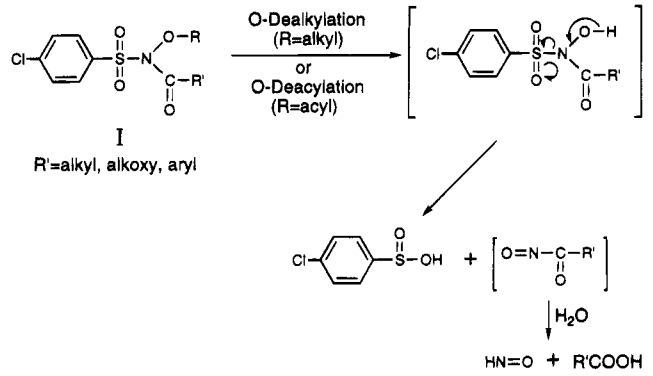
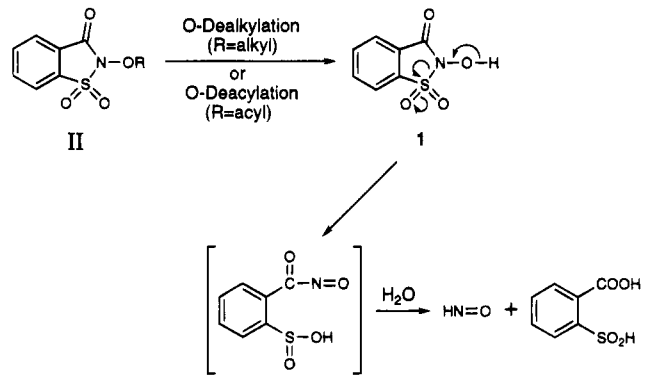
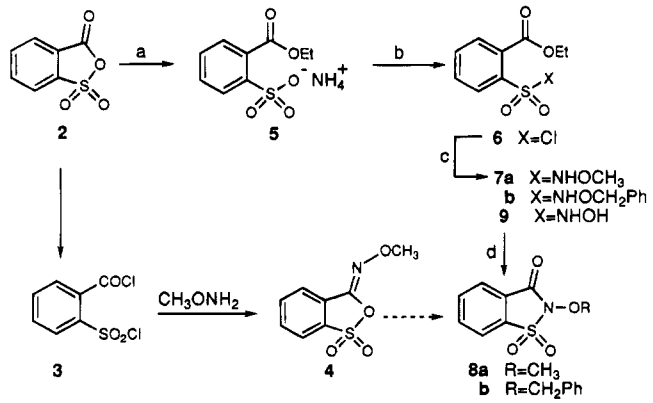
Scheme 1



Scheme 2



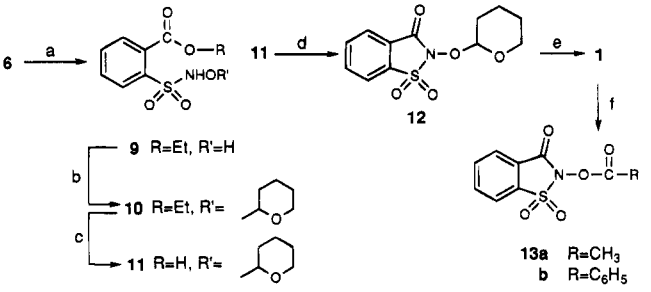
Scheme 3[a]



[a] (a) EtOH, then NH_3; (b) $SOCl_2$/DMF; (c) $RONH_2$·HCl/NaOH; (d) 170 °C, neat (**7a**); HOAc, reflux (**7b**).

Administration of **8a** or **8b**, as well as the isoimide **4** (1.0 mmol/kg, ip), to rats followed by an ethanol challenge (2.0 g/kg, ip) 1 h later failed to elicit any rise in ethanol-derived blood acetaldehyde (AcH) levels when measured 1 h postethanol (data not presented). This lack of elevation of blood AcH following ethanol administration, however, does not of itself indicate a total absence of inhibition of the hepatic AlDH, since blood AcH is not significantly elevated until >70% of the mitochondrial enzyme is inhibited.[11] On the other hand, an elevation in blood AcH would suggest a profound inhibition of hepatic AlDH. Phenobarbital pretreatment of the rats to induce the hepatic microsomal *O*-demethylating enzyme(s) likewise had essentially no effect in raising blood AcH levels by **8a** following ethanol administration.[12] These results suggested that either (a) *O*-dealkylation of **8** was not taking place in vivo or (b) the expected dealkylated product, *i.e.*, *N*-hydroxysaccharin (**1**), was stable under these conditions and did not produce nitroxyl in the manner depicted in Scheme 2. In order to test this second premise, it was necessary to synthesize **1** itself.

Scheme 4[a]



[a] (a) H_2NOH·HCl/5% $NaHCO_3$; (b) dihydropyran, TsOH/THF; (c) 6 N NaOH/dioxane, Δ, then H^+; (d) *i*-BuOCOCl/Et_3N; (e) CF_3COOH; (f) Ac_2O (for **13a**) or $C_6H_5COOCOOEt$ (for **13b**).

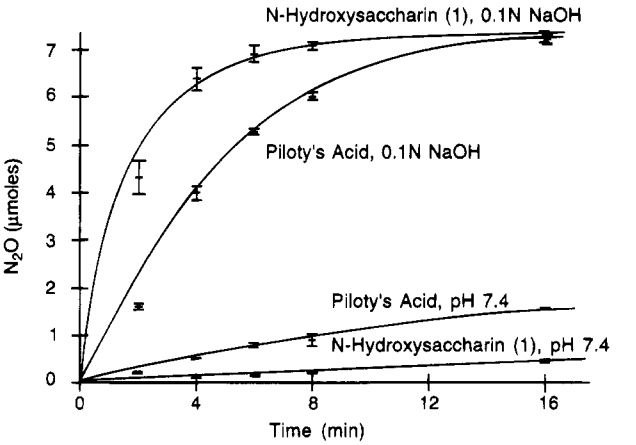


Figure 1. Comparison of the rate of nitroxyl generation (measured as N_2O) from *N*-hydroxysaccharin (**1**) and Piloty's acid in 0.1 N aqueous NaOH and in phosphate buffer, pH 7.4.

Attempts to prepare **1** by thermal cyclization of **9** (Scheme 3), or by debenzylation of **8b** in 50% HBr/HOAc, failed to produce **1** in any isolable quantities. The synthesis of **1** was, however, successfully accomplished according to Scheme 4. Protection of the *N*-hydroxy group of **9** was effected with dihydropyran to give **10**, which on alkaline hydrolysis of the ester group gave the free carboxylic acid **11**. Cyclization of **11** to **12** was effected smoothly with isobutyl chloroformate and Et_3N. Finally, acid (CF_3COOH)-catalyzed removal of the tetrahydropyranyl blocking group on **12** afforded **1** as a crystalline solid in a (recrystallized) yield of 61%. The *O*-acetylated (**13a**) and *O*-benzoylated (**13b**) derivatives of **1** were prepared by treating **1** with acetic anhydride and benzoic ethoxyformic anyhydride, respectively, the latter, the mixed anhydride, isolated from the reaction of benzoic acid and ethyl chloroformate.

N-Hydroxysaccharin (**1**), when incubated in 0.1 M aqueous NaOH, rapidly liberated nitroxyl as evidenced by the formation of gaseous N_2O, the dimerization/dehydration product of nitroxyl (Figure 1). The initial rate of nitroxyl formation from **1** in base was greater than that for Piloty's acid itself. However, in phosphate buffer at pH 7.4, **1** solvolyzed only slowly. This is contrasted to Piloty's acid whose initial rate of N_2O production in phosphate buffer was measurably faster than that of **1** (Figure 1).

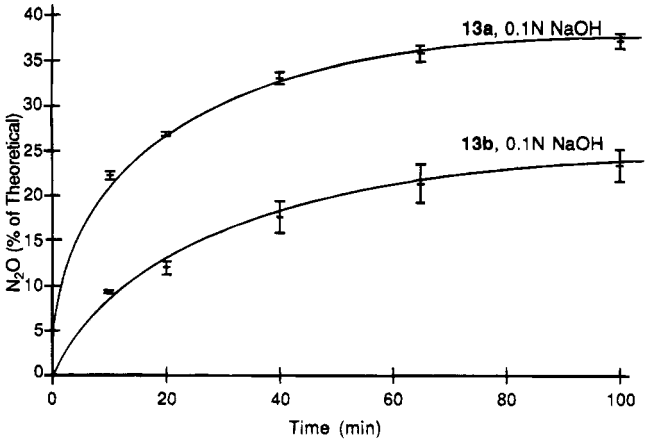


Figure 2. Relative rates of nitroxyl generation (measured as N_2O) from *N*-acetoxysaccharin (**13a**) and *N*-(benzoyloxy)-saccharin (**13b**) in 0.1 N aqueous NaOH.

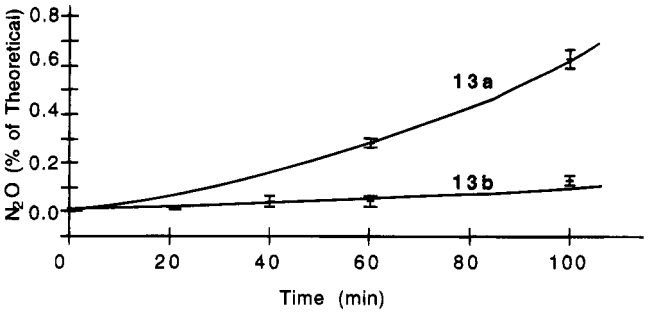


Figure 3. Action of rat plasma in releasing nitroxyl (measured as N_2O) from *N*-acetoxysaccharin (**13a**) and from *N*-(benzoyloxy)saccharin (**13b**). See the Experimental Section for further details.

The relative rates of nitroxyl generation (as N_2O) from *N*-acetoxysaccharin (**13a**) and *N*-(benzoyloxy)saccharin (**13b**) in 0.1 M NaOH and by the action of rat plasma are shown in Figures 2 and 3. It can be seen that the benzoate **13b** was less susceptible to hydrolysis by base; similarly, **13b** did not appreciably liberate nitroxyl by the action of rat plasma over 100 min (Figure 3). Therefore, **13b** was not further tested.

We had already alluded to the fact that **8a,b**, the *O*-alkylated derivatives of **1**, whose bioactivation depends on *O*-dealkylation by the microsomal cytochrome P-450 enzymes as the first step, were essentially ineffective in maximally inhibiting the hepatic AlDH of rats in vivo. Although AlDH cannot dealkylate xenobiotic substances, carboxylic esters are readily hydrolyzed by this enzyme due to an esterase activity intrinsic to this enzyme,[13] in part due to the presence of a sulfhydryl group at the active site.[14] Thus, it was possible to evaluate the activity of **13a** directly against yeast AlDH in vitro, as was done previously for the *N,O*-diacylated Piloty's acid derivatives **I**.[6d]

N-Acetoxysaccharin (**13a**), when tested against yeast AlDH, was moderately active in inhibiting this enzyme (Table 1), while **1** inhibited this enzyme only slightly even at a concentration of 1.0 mM, a somewhat perplexing result that may, however, be rationalized on the basis that nitroxyl may have been released proximal to the active site, however slightly, by **13a**. Under the same conditions, Piloty's acid was more active by 1 order of magnitude (Figure 4; Table 1). Similarly, **1** and **13a** were only moderately active in relaxing precontracted rabbit aortic rings in vitro (Table 1), while Piloty's acid

Table 1. Inhibition of Yeast AlDH and Relaxation of Precontracted Rabbit Aortic Rings in Vitro by *N*-Hydroxysaccharin (**1**) and Related Nitroxyl Donors

compound	inhibition yeast AlDH IC_{50} (μM) ($n = 3$)	relaxation of rabbit aortic rings EC_{50} (μM) (n)
1	>1000[a]	14.40 ± 1.7 (6)
13a	240	52.20 ± 5.0 (7)
Piloty's acid	48	1.03 ± 0.44 (9)
4-chlorobenzenesulfohydroxamic acid		1.36 ± 0.17 (6)

[a] The enzyme was inhibited only 21% compared to controls at a concentration of 1.0 mM.

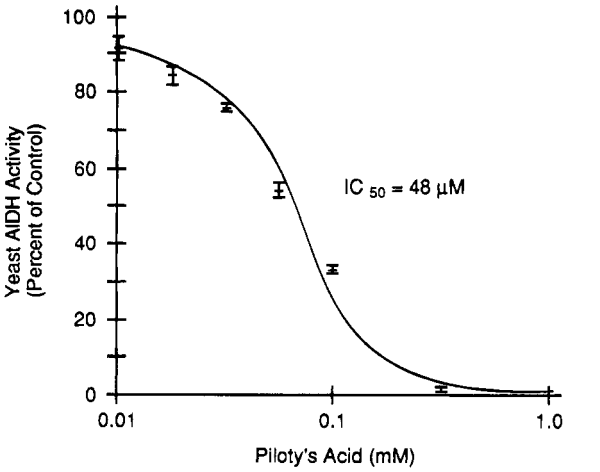


Figure 4. log dose vs response curve for the inhibition of yeast AlDH by Piloty's acid. The concentration of Piloty's acid that elicited half-maximal inhibition (IC_{50}) was as indicated.

was highly potent in keeping with the latter's greater propensity to release nitroxyl at physiological pH (Figure 1). Addition of SOD (100 units/mL) lowered the EC_{50} of Piloty's acid approximately 4-fold. 4-Chlorobenzenesulfohydroxamic acid had similar activity as Piloty's acid as a vasorelaxer.

Conclusions

The synthesis of *N*-hydroxysaccharin (**1**) has now been described for the first time. As predicted, **1** readily solvolyzed in dilute aqueous NaOH solution to generate nitroxyl (measured as N_2O). However, at physiological pH, **1** was much more stable than Piloty's acid and liberated nitroxyl only marginally over time. As a consequence, **1** inhibited yeast AlDH in vitro only slightly at a concentration of 1.0 mM and did not inhibit hepatic AlDH sufficiently in vivo to elicit a rise in ethanol-derived blood AcH when administered to rats at a dose of 1.0 mmol/kg, ip (data not shown). Nevertheless, **1** was moderately active in mediating the relaxation of rabbit aortic rings in vitro (Table 1), its activity being less than that of Piloty's acid but greater than that for cyanamide in the presence of catalase/H_2O_2 ($EC_{50} = 2.6 \pm 0.1 \times 10^{-4}$M).[7b]

In summary, our hypothesis that *N*-acylated arenesulfohydroxamic acids, whether in open-chain or cyclic forms, would disproportionate and liberate nitroxyl following nonenzymatic solvolysis[9] has now been experimentally verified. However, the cyclic *N*-hydroxysaccharin (**1**) only slowly liberated nitroxyl at pH 7.4 and, therefore, did not exhibit maximal inhibitory effect

on AlDH or maximal vasorelaxation properties at this physiological pH. Extrapolation to other systems to provide further structure–activity relationships among various other prodrug forms of nitroxyl is in progress. The multiple biological actions of nitroxyl suggest that compounds that release nitroxyl in vivo, *i.e.*, prodrugs of nitroxyl, may have profound pharmacological/physiological ramifications not unlike that exhibited by nitric oxide itself.[15]

Experimental Section

Melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, and were within 0.4% of the theoretical values. IR spectra were obtained on a Perkin-Elmer 281 infrared spectrometer, and peak positions are expressed in cm^{-1}. NMR spectra were recorded on GE-300 MHz and Bruker AC-200 MHz spectrometers, and chemical shifts are reported as δ values (ppm). Mass spectra (FAB using thioglycerol or glycerol as matrix, EI or CI) were obtained on a Kratos MS 25 instrument. For TLC, Analtech silica gel GF plates were used. Column chromatography was carried out using EM Science Kieselgel 60 (230–400 mesh silica gel) as adsorbant. Volatile solvents were all evaporated and condensed using a rotary evaporator. 2-Sulfobenzoic anhydride was obtained from Fluka Chemical Corp. and Piloty's acid (benzenesulfohydroxamic acid) was purchased from Aldrich Chemical Co. All other reagent chemicals and solvents were purchased from commercial vendors. Unless indicated, these were used without further purification.

Ammonium 2-(Ethoxycarbonyl)benzenesulfonate (5). 2-Sulfobenzoic anhydride (**2**; 25.0 g, 0.136 mol) in 80 mL of absolute ethanol was stirred overnight. After the addition of 30 mL of methanolic ammonia, the mixture was stirred further for 2 h and the resulting thick white reaction mixture was clarified by addition of 80 mL of methanol. The solution was diluted with ether (550 mL) until no more solids precipitated, and the product was collected and air-dried overnight to give 29.1 g (86.7% yield) of **5**: ^1H NMR (D_2O) δ 1.35 (t, 3H, CH_3), 4.40 (q, 2H, CH_2), 7.56 (m, 3H, Ar-*H*), 7.90 (m, 1H, Ar-*H*); MS (FAB) *m*/*z* 248 (MH^+). This product was used in the next step without further purification.

Ethyl 2-(Chlorosulfonyl)benzoate (6). Compound **5** obtained above (15.0 g, 0.061 mol) was dissolved in dimethyl formamide (10 mL), and thionyl chloride (80 mL) was added. The reaction mixture was heated under reflux overnight and then cooled in an ice bath and carefully added to crushed ice (about 1000 mL). The cold mixture was immediately extracted with dichloromethane (3 × 100 mL). The organic layer was washed with 5% $NaHCO_3$ (1 × 150 mL) and then water (1 × 150 mL) and dried (Na_2SO_4). Evaporation of the solvent gave a light greenish-yellow oil (14.7 g). The oil was purified by passing it through 250 g of Kieselgel 60 using EtOAc:hexane (1:3) as the eluting solvent. A colorless oil was obtained (14.1 g, 93.5% yield): ^1H NMR ($CDCl_3$) δ 1.42 (t, 3H, CH_3), 4.48 (q, 2H, CH_2), 7.75 (m, 3H, Ar-*H*), 8.17 (m, 1H, Ar-*H*); IR (neat, cm^{-1}) 3100 (wk, ArH), 1736 (ester), 1377 and 1181 (SO_2); MS (FAB) *m*/*z* 250 (MH^+); MS (EI) *m*/*z* 249 ($MH^{\bullet+}$). Anal. ($C_9H_9ClO_4S$) C, H.

Ethyl 2-[(*N*-Methoxyamino)sulfonyl]benzoate (7a). A solution of methoxylamine hydrochloride (3.10 g, 0.037 mol) and 8% NaOH (2 M NaOH, 56 mL, 0.112 mol) was cooled in an ice bath, and to this was added dropwise a solution of **6** (7.0 g, 0.028 mol) in tetrahydrofuran (15 mL). The cooled reaction mixture was stirred for 3.5 h, and the pH was adjusted to 2 using 6 N HCl. The acidified solution was extracted with EtOAc (3 × 100 mL). The combined organic extract was washed with water (1 × 100 mL), dried (Na_2SO_4), and evaporated to give an oil which solidified on refrigeration. Recrystallization from EtOAc/hexane gave 3.80 g of **7a** as colorless crystals (52.1% yield): mp 96–99 °C; ^1H NMR ($CDCl_3$) δ 1.41 (t, 3H, CH_3), 3.83 (s, 3H, OCH_3), 4.45 (q, 2H, CH_2), 8.18, 7.85 and 7.69 (m, 4H, Ar-*H*), 8.97 (s, 1H, N*H*); IR (neat, cm^{-1}) 3213 (wk, NH), 1708 (C=O), 1286 and 1173 (SO_2); MS (FAB) 260 (MH^+). Anal. ($C_{10}H_{13}NO_5S$) C, H, N.

Ethyl 2-[[*N*-(Benzyloxy)amino]sulfonyl]benzoate (7b). This compound was prepared in the same manner as for **7a** above using benzyloxy amine hydrochloride (1.14 g, 7.16 mmol), 8% NaOH (2 M NaOH, 15 mL, 30 mmol), and **6** (1.27 g, 5.11 mmol) to give 1.09 g of crude oil. The oil was purified by passing it through a column of Kieselgel 60 using EtOAc:hexane (1:3) as the eluting solvent. The product was an oil which solidified on cooling. The solid was recrystallized from EtOAc/hexane to give **7b** (0.41 g, 24.0% yield): mp 73–76 °C; ^1H NMR ($CDCl_3$) δ 1.39 (t, 3H, CH_3), 4.39 (q, 2H, CH_2), 5.04 (s, 2H, benzyl CH_2), 7.38 (s, 5H, benzyl Ar-H), 8.22, 7.81 and 7.69 (m, 4H, Ar-H), 8.88 (s, 1H, NH); IR (neat, cm^{-1}) 3213 (NH), 3100 (Ar-H), 1708 (C=O), 1286 and 1173 (SO_2); MS (FAB) *m*/*z* 336 (MH^+). Anal. ($C_{16}H_{17}NO_5S$) C, H, N.

2-Methoxy-1,2-benzisothiazol-3(2*H*)-one 1,1-Dioxide (*N*-Methoxysaccharin, 8a). Compound **7a** (1.5 g, 5.79 mmol) was heated neat in an oil bath to 170 °C for about 7.5 h. To the hot reaction mixture was slowly and carefully added EtOAc (20 mL). The cloudy mixture was washed with water (2 × 10 mL). The organic layer was then dried (Na_2SO_4) and evaporated to give a light brown oil. Purification by column chromatography using 35 g of Kieselgel 60 as the adsorbant and EtOAc:hexane (1:3) as the eluting solvent gave starting material (0.3 g) and product (oil) which solidified on cooling. The latter was recrystallized from EtOAc/hexane to give 0.5 g of **8a** (50.7% yield based on recovered starting material): mp 96–97 °C; ^1H NMR ($CDCl_3$) δ 4.23 (s, 3H, OCH_3), 7.91 and 8.07 (m, 4H, Ar-*H*); IR (neat, cm^{-1}) 3100 (wk, aromatic CH), 1750 (C=O), 1349 and 1188 (SO_2); MS (FAB; CI) *m*/*z* 214 (MH^+); MS (EI) *m*/*z* 213 ($MH^{\bullet+}$). Anal. ($C_8H_7NO_4S$) C, H, N.

2-(Benzyloxy)-1,2-benzisothiazol-3(2*H*)-one 1,1-Dioxide [*N*-(Benzyloxy)saccharin, 8b]. Method 1: Compound **7b** (0.400 g, 1.20 mmol) was heated in an oil bath to about 170 °C for about 3 h. The product was isolated as a white solid by column chromatography using Kieselgel 60 as the stationary phase and EtOAc:hexane (1:3) as the eluting solvent. The solid was recrystallized from EtOAc/hexane to give 0.185 g of *N*-(benzyloxy)saccharin (**8b**; 52.9% yield): mp 105–109 °C; ^1H NMR ($CDCl_3$) δ 5.34 (s, 2H, CH_2), 7.41 and 7.53 (m, 5H, benzyl Ar-*H*), 7.89 and 8.05 (m, 4H, Ar-*H*); IR (neat, cm^{-1}) 3093 (wk, aromatic CH), 1750 (C=O), 1349 and 1195 (SO_2); MS (FAB; CI) *m*/*z* 290 (MH^+). Anal. ($C_{14}H_{11}NO_4S$) C, H, N.

Method 2: Compound **7b** (1.50 g, 4.48 mmol) was dissolved in glacial acetic acid (40 mL), and the solution was heated under reflux for about 75 h. The reaction mixture was cooled to room temperature and evaporated to give a brown oil. The oil solidified after seeding and refrigeration. The product was purified by passing it through 35 g of Kieselgel 60 using EtOAc:hexane (1:3) as the eluting solvent. Some starting material was recovered (0.5 g). The product was obtained as a white solid which was recrystallized from EtOAc/hexane to give 0.52 g of crystalline **8b** (60.3% yield after accounting for the recovered starting material): mp 108–111 °C. The IR and NMR spectra were identical to the above.

Ethyl 2-[(*N*-Hydroxyamino)sulfonyl]benzoate (9). To a solution of ethyl 2-(chlorosulfonyl)benzoate (**6**; 13.61 g, 0.055 mol) in tetrahydrofuran (180 mL) was added a solution of hydroxylamine hydrochloride (7.61 g, 0.109 mol) in water (50 mL). The mixture was cooled in an ice–salt bath, and 10% $NaHCO_3$ (185 mL, 18.48 g, 0.220 mol) was added dropwise with stirring over a period of 1 h. After stirring for another 1 h two layers separated. The aqueous bottom layer was extracted two times with dichloromethane (1 × 100 and 1 × 50 mL), and the combined organic extract was added to the THF top layer. The combined solution (cloudy) was washed with water (1 × 100 mL), and the organic layer was dried (Na_2SO_4) and evaporated to give a thick colorless oil (9.46 g). The oil crystallized from EtOAc (50 mL) and hexane (100 mL) in three crops of 4.49 g (mp 112–116 °C), 1.50 g (mp 111–115 °C), and 0.54 g (mp 112–115 °C). Total yield = 6.53 g (48.7%): ^1H NMR ($CDCl_3$) δ 1.42 (t, 3H, CH_3), 4.43 (q, 2H,

CH_2), 5.90 (d, 1H, O*H*), 7.70, 7.85 and 8.19 (m, 4H, Ar-*H*), 8.65 (d, 1H, N*H*); ^{1}H NMR ($CDCl_3$ with a drop of D_2O) δ 1.43 (t, 3H, CH_3), 4.45 (q, 2H, CH_2), 7.70, 7.87 and 8.21 (m, 4H, Ar-*H*); IR (neat, cm^{-1}) 3388 (OH), 3227 (NH), 1708 (C=O), 1370 and 1173 (SO_2); MS (FAB) m/z 246 $(MH)^+$. Anal. ($C_9H_{11}NO_5S$) C, H, N.

Ethyl 2-[[*N*-(Tetrahydropyranyloxy)amino]sulfonyl]-benzoate (10). To a solution of **9** (6.40 g, 26.1 mmol) in dichloromethane (125 mL) was added dihydropyran (4.77 mL, 4.39 g, 52.3 mmol) and *p*-toluenesulfonic acid monohydrate (0.1 g). The reaction mixture was stirred at room temperature for 1 h, and the dark reaction mixture was evaporated to give a dark colored oil. The oil was purified by passing it through 200 g of Kieselgel 60 using EtOAc:hexane (1:5) as the eluting solvent to give 7.24 g of **10** as a light yellow oil (84.2% yield): ^{1}H NMR ($CDCl_3$) δ 1.36–1.89 (m, 9H, -CH_2- of THP and -CH_3), 3.51–3.87 (m, 2H, O-CH_2- of THP), 4.36–4.51 (m, 2H, O-CH_2), 5.16–5.17 (m, 1H, O-C*H*-O of THP), 7.42–8.16 (m, 4H, Ar-*H*), 8.85 (s, 1H, N*H*); IR (neat) 3220 (NH), 2945 (aliphatic CH), 1708 (O-C=O), 1286 and 1173 (SO_2) cm^{-1}; MS (FAB) m/z 330 (MH^+). This product was used in the next step without further purification.

2-[[*N*-(Tetrahydropyranyloxy)amino]sulfonyl]benzoic Acid (11). **10** above (6.75 g, 20.5 mmol) in dioxane (61 mL) was saponified by addition of 6 M NaOH (95 mL) and water (50 mL) and heating the mixture under reflux for 2 h. The reaction mixture was cooled and then extracted with EtOAc (2 × 50 mL). The combined EtOAc extract was washed with water (100 mL), and the washing was added to the original aqueous solution. The aqueous solution was overlaid with EtOAc (300 mL) and acidified with 6 N HCl (100 mL). The phases were separated, and the aqueous phase was again extracted with EtOAc (100 mL). The combined extracts were washed with water (2 × 200 mL), dried (Na_2SO_4), and evaporated to give 14.7 g of a thick white paste. This was redissolved in EtOAc (75 mL) by warming on a steam bath, and hexane (5 mL) was added. The solution was seeded and placed in the refrigerator to give 2.80 g of product (crop 1), mp 107 °C with browning at 103 °C. The second crop of 0.93 g was obtained as white crystals, mp 105 °C with browning at 100 °C (3.73 g, 65.5% yield after taking into account the 0.53 g of recovered starting material): ^{1}H NMR ($CDCl_3$ with 2 drops of DMSO) δ 1.23 (m, 6H, CH_2 of THP), 3.52–3.32 (m, 2H, O-CH_2 of THP), 4.82 (s, 1H, O-C*H*-O of THP), 7.78–7.41 (m, 4H, Ar-*H*), 8.89 (s, 1H, N*H*); IR (neat) 3213 (NH), 3009 (bd, COOH), 2952 (wk, CH stretch), 1715 (C=O), 1342, 1272 and 1173 (SO_2) cm^{-1}. Anal. ($C_{12}H_{15}NO_6S$) C, H, N.

2-(Tetrahydropyranyloxy)-1,2-benzisothiazol-3(2*H*)-one 1,1-Dioxide [*N*-(Tetrahydropyranyloxy)saccharin, 12]. A stirred solution of **11** (3.50 g, 11.7 mmol) in dry distilled tetrahydrofuran (50 mL), under nitrogen, was cooled to about −10 to −15° C in an 2-propanol–dry ice bath, and isobutyl chloroformate (2.11 mL, 2.20 g, 16.2 mmol) was added followed by triethylamine (2.26 mL, 1.64 g, 16.2 mmol). After 5 min the dry ice bath was removed, and when room temperature was reached, the precipitate of triethylamine hydrochloride was collected by filtration and the filtrate evaporated to give 4.49 g of a white solid. The product was recrystallized from EtOAc (30 mL) to give 2.49 g of **12** as colorless crystals, mp 133–138 °C. A second crop (0.44 g) was also obtained: mp 135–138 °C (total yield = 2.93 g, 88.8%); ^{1}H NMR ($CDCl_3$) δ 1.70–2.17 (m, 6H, -CH_2- of THP), 3.80–4.39 (m, 2H, O-CH_2- of THP), 5.45 (s, 1H, O-C*H*-O of THP), 7.83–8.09 (m, 4H, Ar-*H*); IR (neat) 3093 (wk, aromatic CH), 2952 (aliphatic CH), 1757 (N-C=O), 1356 and 1195 (SO_2) cm^{-1}; MS (FAB; CI) m/z 284 (MH^+). Anal. ($C_{12}H_{13}NO_5S$) C, H, N.

2-Hydroxy-1,2-benzisothiazol-3(2*H*)-one 1,1-Dioxide (*N*-Hydroxysaccharin, 1). Compound **12** (2.75 g, 9.72 mmol) was dissolved in tetrahydrofuran (20 mL) by warming in a water bath at 45 °C, and water (5 mL) was added followed by trifluoroacetic acid (0.15 mL, 0.22 g, 1.9 mmol). The solution was stirred at 45–50 °C for 7.5 h, and after addition of water (10 mL), the mixture was evaporated to a small volume. The solid suspension was collected and washed with water and then with cold ethyl ether. After air-drying the solid was recrystallized from EtOAc/hexane to give 0.82 g of crystalline **1**. A second crop of 0.35 g was also obtained (total yield = 1.17 g, 60.6%): mp 189-190 °C dec; ^{1}H NMR ($CDCl_3$ with 1 drop of DMSO) δ 7.72–7.93 (m, 4H, Ar-*H*), 11.00 (b, 1H, N-O*H*); IR (KBr) 3274 (bd, -OH), 1748 and 1727 (C=O), 1348 and 1194 (SO_2) cm^{-1}; MS (FAB; CI) m/z 200 (MH^+). Anal. ($C_7H_5NO_4S$) C, H, N.

2-Acetoxy-1,2-benzisothiazol-3(2*H*)-one 1,1-Dioxide (*N*-acetoxysaccharin, 13a). Compound **1** (0.16 g, 0.804 mmol) in tetrahydrofuran (10 mL) was acetylated with 2.0 mL of acetic anhydride by stirring for about 1 h. The solvent was removed under a stream of nitrogen gas, and the reaction mixture was further concentrated to give a solid which was recrystallized from ether–petroleum ether (30–60 °C) to give 0.53 g of **13a** (54.7% yield): mp 137–138 °C; ^{1}H NMR ($CDCl_3$) δ 2.42 (s, 3H, CH_3), 7.91–8.14 (m, 4H, Ar-*H*); IR (neat) 3093 (wk, aromatic CH), 1820 (O-C=O), 1757 (N-C=O), 1356, 1195 and 1152 (SO_2) cm^{-1}; MS (FAB; CI) m/z 242 (MH^+); MS (EI) m/z 241 ($M^{\bullet+}$). Anal. ($C_9H_7NO_5S$) C, H, N.

2-(Benzoyloxy)-1,2-benzisothiazol-3(2*H*)-one 1,1-Dioxide [*N*-(Benzoyloxy)saccharin, 13b]. Benzoic ethoxyformic anhydride was prepared from benzoic acid (5.00 g, 41.0 mmol), ethyl chloroformate (4.34 mL, 4.93 g, 45.4 mmol), and triethylamine (6.30 mL, 4.60 g, 45.5 mmol) in dry distilled tetrahydrofuran (100 mL), under nitrogen, at about −10 to −15 °C in an 2-propanol–dry ice bath. After 2 h, the precipitate of triethylamine hydrochloride was removed and the filtrate evaporated to give 8.60 g of **25** as a light yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.41 (t, 3H, CH_3), 4.42 (q, 2H, CH_2), 7.13–8.10 (m, 5H, Ar-*H*); IR (neat) 3065 (wk, aromatic CH), 1806 (O-COO), 1743 (O-C=O) cm^{-1}.

This product (0.15 g, 0.77 mmol) was used to benzoylate *N*-hydroxysaccharin (**1**) (0.10 g, 0.50 mmol) in dry distilled tetrahydrofuran (15 mL), under nitrogen. The reaction mixture was stirred at room temperature for 30 min and the solvent removed under a stream of nitrogen gas. To the thick oil was added ice–water (20 mL), and the mixture was stirred for 5 min. The cloudy aqueous solution was extracted with dichloromethane (2 × 10 mL), and the organic phase was dried (Na_2SO_4) and evaporated to give 0.17 g of white solid. The product was recrystallized from ether–petroleum ether (30–60 °C) to give 0.04 g of white crystalline **13b** (26.3% yield): mp 141–143 °C; ^{1}H NMR ($CDCl_3$) δ 7.54–7.71 (m, 3H, 3′, 4′, 5′ benzoyl Ar-*H*), 7.96–7.99 (m, 3H, 4, 5, 6 Ar-*H*), 8.15–8.22 (m, 3H, 3, 2′, 6′ Ar-*H*); IR (neat) 1785 (O-C=O), 1757 (N-C=O), 1356, 1237 and 1195 (SO_2) cm^{-1}; MS (FAB; CI) m/z 304 (MH^+); MS (CI) m/z 303 ($M^{\bullet+}$). Anal. ($C_{14}H_9NO_5S$) C, H, N.

Pharmacological Evaluation. Drug Administration Protocol. Male Sprague–Dawley rats (150–180 g; Harlan Sprague–Dawley Inc., Indianapolis, IN) were divided into groups of three and fasted overnight. All test compounds were suspended in 2% aqueous carboxymethyl cellulose (CMC). Each animal was administered CMC or drug suspension (1.0 mmol/kg, ip) followed by ethanol (2.0 g/kg, ip) 1 h later. The animals were sacrificed 1 h after ethanol administration for measurement of blood acetaldehyde (AcH) and ethanol. N^1-Methoxychlorpropamide (N^1-OMe-CP)-treated animals served as positive controls. The control group received vehicle (CMC) alone followed by ethanol.

Blood AcH Assay. Blood AcH levels were measured 1 h after ethanol administration. Blood was collected, and the samples were prepared for analysis as previously described.[16] The vials were heated at 55 °C in a shaking water bath for 10 min, and an aliquot (2.0 mL) of the headspace gas was removed using a gas-tight syringe. The samples were assayed using an HP 5890 Series II gas chromatograph equipped with dual fid detectors. Chromatographic conditions were glass column (6 ft × 2 mm, i.d.) packed with Poropak Q, 80–100 mesh; oven, 140 °C; injection port, 155 °C; detector, 200 °C; flow rate, 25–28 mL/min with nitrogen as the carrier gas. Blood AcH levels as well as ethanol levels were measured on duplicate samples and quantitated by reference to a standard curve based on known concentrations of AcH. The lower limit of detection of AcH was 5 μM.

Headspace Gas Chromatographic Analysis of N_2O. (a) N_2O Formation from Nitroxyl Prodrugs in Aqueous

Base.[6d] GC vials (25 mL) containing 1.0 mL of 100 mM potassium phosphate buffer (pH 7.4) and 0.2 mL of 1 N NaOH were prepared in triplicate for each sample. Water was added before addition of the test compound to give a final reaction mixture volume of 2.0 mL. Test compounds (10 mM) were made up in THF. An aliquot of the stock solution was added to give a final concentration of 20 μmol of the test compound in the reaction mixture. The vials were each immediately capped with a rubber septum and incubated at 37 °C in a shaking water bath. Aliquots (0.60 mL) of the headspace gas were removed at 2, 4, 6, 8, and 16 min for the determination of N_2O. The samples were assayed for N_2O on a HP 5880 gas chromatograph equipped with a thermal conductivity detector. The chromatographic conditions were glass column (6 ft × 2 mm, i.d.) packed with Poropak Q, 80–100 mesh; oven, 30 °C; injection port, 100 °C; detector, 100 °C, flow rate, 80–85 mL/min with helium as the carrier gas. Standard curve was obtained by mixing in a GC vial (25 mL) a known quantity of hydroxylamine hydrochloride solution with a known quantity of sodium nitrite solution and water to a final volume of 2 mL to generate N_2O. This time course was extended in a similar experiment where aliquots (0.6 mL) of the headspace gas were also removed at longer intervals of 10, 20, 40, 65, and 100 min. Blanks contained 1.0 mL of 100 mM potassium phosphate buffer (pH 7.4) and 1.0 mL of water.

(b) N_2O Formation in Aqueous Phosphate Buffer, pH 7.4. The procedure was identical to procedure a above except that NaOH was omitted. Aliquots (0.60 mL) of the headspace gas were removed at 2, 4, 6, 8, and 16 min for the determination of N_2O. The gas chromatographic conditions were as described above under procedure a. The time course was extended in a similar experiment where aliquots of the headspace gas were also removed at longer intervals of 10, 20, 40, 65, and 100 min. Blanks were as above.

(c) N_2O Formation on Incubation with Rat Plasma. GC vials (25 mL) containing 1.0 mL of 100 mM potassium phosphate buffer (pH 8.0) and 0.2 mL of rat plasma were prepared in quadruplicate for each compound. Aliquots of the test compound in THF were added to give a final concentration of 10 μmol of the test compound in the reaction mixture. The chromatographic conditions were as described above under procedure a. N_2O was quantitated using certified N_2O gas standards of known concentrations.

Isolation of Rat Plasma. Blood was withdrawn from the abdominal aorta of Metofane-anesthetized rats (Harlan Sprague–Dawley Inc., Indianapolis, IN) using a 23 gauge needle. The blood was aspirated into ice-cold tubes containing 3–4 drops of heparin and centrifuged at 1500 rpm for 10 min at 5 °C in a Sorvall RC-3C refrigerated centrifuge to sediment the red cells. The plasma was removed, divided into four portions, and kept frozen at −20 °C until used.

Inhibition of Yeast AlDH. The compounds were tested for inhibition of yeast AlDH activity using a two-step assay system.[17] The primary reaction mixture contained 1.0 mM of the test compound (dissolved at a concentration of 20 mM in DMSO, and therefore the primary reaction mixture contained 5% DMSO), approximately 0.10 IU of yeast AlDH, and 100 mM potassium phosphate buffer (pH 7.5) in a total volume of 0.1 mL. The primary mixture was preincubated at 37 °C for 5 min. The reaction was initiated by addition of the test compound 30 s before the addition of yeast AlDH. Ten minutes after incubation of the mixture at 37 °C under aerobic conditions, an aliquot (20 μL) of the primary reaction mixture was removed and added directly to a secondary reaction mixture containing 0.5 mM NAD^+, 1.0 mM EDTA, 30% glycerol, and 90 mM potassium phosphate buffer (pH 8.0) in a total volume of 1.0 mL. The reaction was initiated by the addition of benzaldehyde (0.6 μmol), and the remaining yeast AlDH activity in this secondary mixture was determined spectrophotometrically by following the increase in absorbance at 340 nm over time. Individual values represent the mean ± SEM of three to four determinations.

Vasorelaxation by Nitroxyl Prodrugs. The relaxation of precontracted rabbit aortic rings by these nitroxyl prodrugs was measured in vitro as described previously.[7a]

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