

Carbethoxylating Agents as Inhibitors of Aldehyde Dehydrogenase

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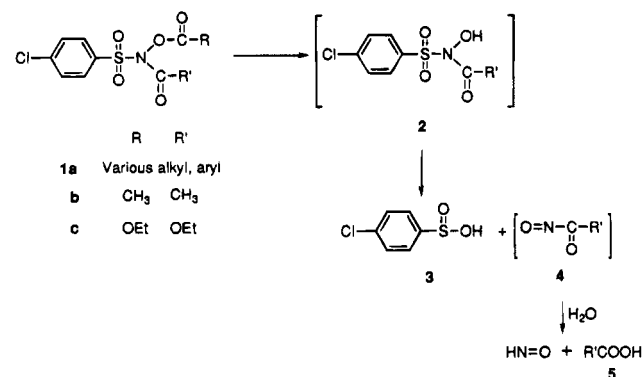
N,O-Dicarbethoxy-4-chlorobenzenesulfohydroxamate (**1c**) and *O*-carbethoxy-*N*-hydroxysaccharin (**6**), both potential carbethoxylating agents, inhibited yeast aldehyde dehydrogenase (ALDH) with IC₅₀'s of 24 and 56 μM, respectively. The esterase activity of the enzyme was commensurably inhibited. ALDH activity was only partially restored on incubation with mercaptoethanol (20 mM) for 1 h. On incubation with rat plasma, **1c** liberated nitroxyl, a potent inhibitor of ALDH. Under the same conditions, nitroxyl generation from **6** was minimal, a result compatible with a previous observation that nitroxyl generation from *N*-hydroxysaccharin (**7**), the product of the hydrolysis of the carbethoxy group of **6**, was minimal at physiological pH. Since chemical carbethoxylating agents represented by the *O*-carbethoxylated *N*-hydroxyphthalimide, 1-hydroxybenzotriazole, and *N*-hydroxysuccinimide (**8**, **9**, and **10**, respectively) likewise inhibited yeast ALDH, albeit with IC₅₀'s 1 order of magnitude higher, we postulate that **1c** and **6** act as irreversible inhibitors of ALDH by carbethoxylating the active site of the enzyme.

The active site of the enzyme aldehyde dehydrogenase (ALDH, E.C.1.2.1.3) has been shown to be Cys 302,¹ a sulfhydryl amino acid which is preserved in all known ALDH's.² ALDH's catalyze the oxidation of alcohols to aldehydes, the most relevant alcohol being ethanol which is converted to acetaldehyde. The enzyme also functions as an esterase³ (possibly aided by a neighboring histidine group⁴), and mechanistic considerations would suggest that this sulfhydryl group at the active site is altered when the enzyme is inactivated.

We have previously reported⁵ that *N,O*-diacylated 4-chlorobenzenesulfohydroxamates **1a** are hydrolyzed at the ester linkage by the intrinsic esterase activity of yeast ALDH to give an *N*-acylated arenesulfohydroxamate (**2**). The latter, being unstable, spontaneously disproportionates to an arenesulfinic acid (**3**) and an acyl nitroso intermediate (**4**) that ultimately solvolyzes to nitroxyl (HN=O) and the corresponding carboxylic acid **5** (Scheme 1). The liberated nitroxyl is a potent inhibitor of ALDH, and **1b** was found to inhibit yeast ALDH with an IC₅₀ of 39 μM.⁵ Nitroxyl has also been shown to be the putative inhibitor of the hepatic mitochondrial class 2 ALDH following the bioactivation of the alcohol deterrent agent cyanamide by catalase/H₂O₂.⁶ The nitroxyl liberated from compounds such as **1b**, or from cyanamide in the presence of catalase/H₂O₂, also elicited the relaxation of precontracted rabbit aortic rings in vitro,⁷ thus mimicking the effect of nitric oxide (NO[•]), the endogenous, endothelium-derived relaxing factor (EDRF).

The ester linkage of **1b** is highly labile, its reactivity resembling that of a carboxylic acid anhydride, and solvolyzes nonenzymatically in aqueous systems. This is reflected by the high carbonyl frequency (~1806 cm⁻¹)⁵ of this ester group in the infrared spectrum indicative of a highly electrophilic carbonyl carbon. In order to stabilize this *O*-acyl group from nonenzymatic solvolysis and yet retain its susceptibility to enzymatic

Scheme 1



cleavage, we replaced this group with a carbethoxy group, rendering it now a carbonate ester rather than a carboxylate ester. The investigation of these compounds as inhibitors of ALDH is the subject of this report.

Results and Discussion

N,O-Dicarbethoxy-4-chlorobenzenesulfohydroxamate (**1c**), prepared by the reaction of 4-chlorobenzenesulfohydroxamic acid with ethyl chloroformate and base, readily hydrolyzed in 0.1 M aqueous NaOH to liberate nitroxyl (measured as N₂O, the dimerization/dehydration product of nitroxyl), whereas in phosphate buffer at pH 7.4, nitroxyl liberation from **1c** was minimal over 100 min (Figure 1). When incubated with rat plasma, nitroxyl formation from **1c** nearly paralleled that from alkaline hydrolysis of **1c**, albeit at somewhat slower initial rate (Figure 1). These data suggested that **1c** should be a good inhibitor of ALDH, and indeed, **1c** inhibited yeast ALDH in vitro with an IC₅₀ of 24 μM (Figure 2).

In order to evaluate the generality of the enzymatic hydrolysis of this carbonate ester group, the *O*-carbethoxy derivative **6** of *N*-hydroxysaccharin (**7**) was prepared. Compound **6** also liberated nitroxyl in dilute aqueous NaOH but appeared not to be appreciably

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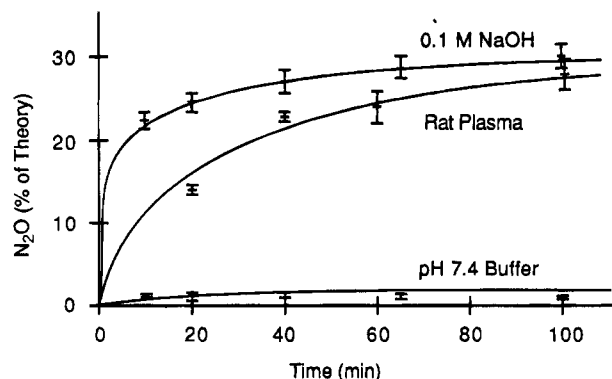


Figure 1. Nitroxyl release (measured as N_2O) from *N,O*-dicarbethoxy-4-chlorobenzenesulfonylhydroxamate (**1c**) in 0.1 M aqueous NaOH and 0.1 M potassium phosphate buffer, pH 7.4, and catalyzed by rat plasma. Each point represents the mean \pm SEM ($n = 3$).

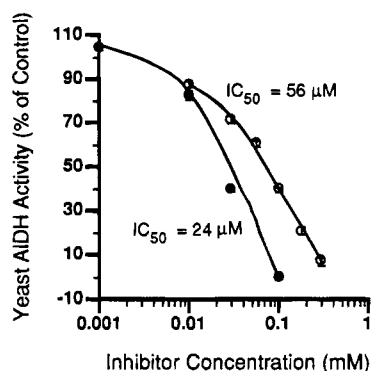


Figure 2. Inhibition of yeast ALDH in vitro by **1c** (●) and *O*-carbethoxy-*N*-hydroxysaccharin (**6**) (○). The calculated IC_{50} 's are as indicated. The details of the experimental procedures are described in the companion paper.⁸

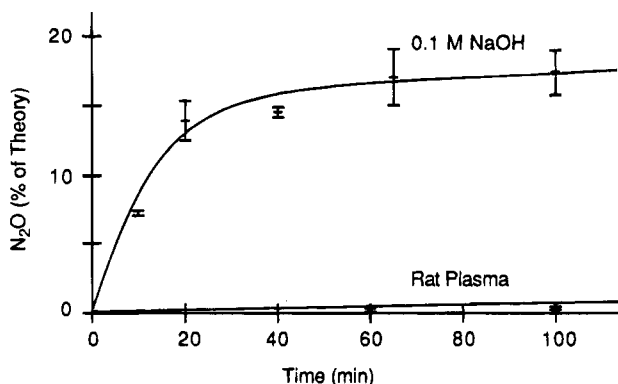


Figure 3. Nitroxyl release (measured as N_2O) from **6** in 0.1 M aqueous NaOH and by rat plasma. Other parameters are as in Figure 1.

hydrolyzed by rat plasma (Figure 3). When tested against yeast ALDH, **6** was highly inhibitory although somewhat less so than **1c**, with an experimentally determined IC_{50} of 56 μM (Figure 2).

The action of esterases present in rat plasma or yeast ALDH on **6** would be expected to give *N*-hydroxysaccharin (**7**). However, we had shown (see preceding paper⁸) that whereas **7** readily solvolyzed in 0.1 M NaOH to give nitroxyl, nitroxyl liberation from **7** was minimal when incubated in phosphate buffer at physiological pH. This appeared to be due to the inherent stability of the 1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide (saccharin) ring system to ring-opening reactions at neutral pH. This

Scheme 2

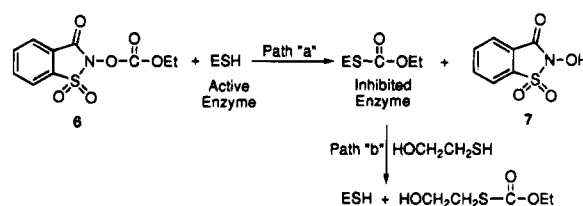


Chart 1

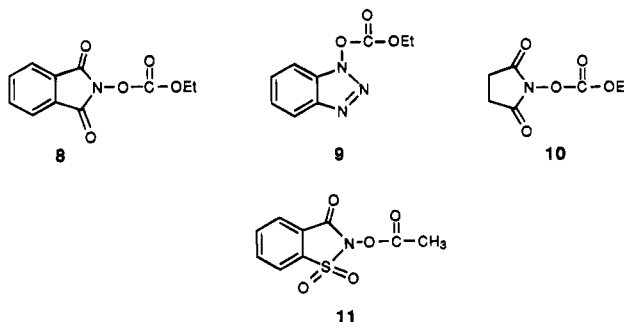


Table 1. Inhibition of Yeast ALDH by Chemical Carbethoxylating Agents

compound tested	IC_{50} (mM)
1c	0.024
6	0.056
8	0.17
9	0.27
10	0.57
diethyl pyrocarbonate (EtOCOOCOEt)	0.74
ethyl formate (1.0 mM)	inactive

raised the question of whether nitroxyl was in fact totally involved in the inhibition of ALDH by the *O*-carbethoxylated *N*-hydroxysaccharin derivative **6**, and an alternative mechanism for the inhibition of ALDH was advanced (Scheme 2, path "a").

We postulate that the active site mercapto group of the enzyme is converted by **6** to a monothiolcarbonate group which would be expected to be much more stable to solvolysis than a thioester group. A thioester is the penultimate chemical form of the dehydrogenase action on aldehydes by ALDH.³ If Scheme 2 were operative, esterase action by the enzyme on **6** would lead to an irreversibly bound *S*-carbethoxy group on the active site Cys 302 leading to enzyme inactivation. Alternatively, a basic, allosteric site of ALDH may be carbethoxylated, since diethyl pyrocarbonate has been shown to carbethoxylate histidine residues of ALDH in a dose-dependent manner resulting in enzyme inactivation.⁴

Such a mechanism (Scheme 2, path "a") may be tested by the use of other chemical carbethoxylating agents that cannot give rise to nitroxyl and determination of their inhibitory activity against ALDH. *O*-Carbethoxy derivatives of well-established activating agents⁹ for peptide coupling of *N*-protected α -amino acids represent such chemical carbethoxylating agents. Accordingly, *O*-carbethoxy-*N*-hydroxyphthalimide (**8**),¹⁰ *O*-carbethoxy-1-hydroxybenzotriazole (**9**), and *O*-carbethoxy-*N*-hydroxysuccinimide (**10**)¹⁰ (Chart 1) were prepared and evaluated as inhibitors of ALDH.

As shown in Table 1, compounds **8–10** were moderately good inhibitors of yeast ALDH with IC_{50} 's of 0.17,

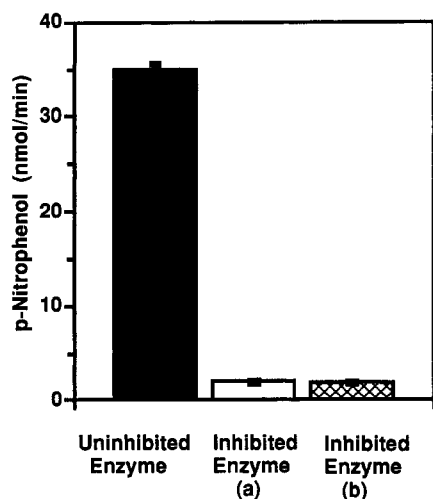


Figure 4. Inhibition of the esterase activity of yeast AldH by the *O*-carbethoxylated *N*-hydroxysaccharin derivative **6**. Comparison of the rate of *p*-nitrophenylacetate hydrolysis (vs the uninhibited enzyme) by the inhibited enzyme before (a) and after (b) dialysis. The activity of the inhibited enzyme was significantly different from that of the uninhibited control ($P < 0.005$) on the basis of statistical analysis using ANOVA and the Neuman–Keuls criteria. Under these conditions, nonenzymatic hydrolysis of the ester was minimal (0.22 nmol/min).

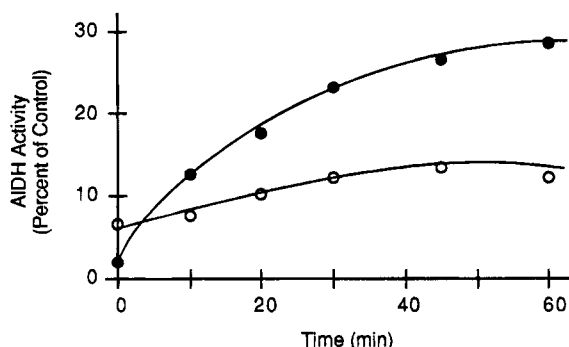


Figure 5. Partial recovery after 60 min of AldH activity (after inhibition by **6**) on incubation of the dialyzed inhibited enzyme with (●) and without (○) 20 mM mercaptoethanol.

0.27, and 0.57 mM, respectively. Diethyl pyrocarbonate also inhibited this enzyme without NAD^+ requirement, but ethyl formate, which is not a carbethoxylating agent under these conditions, was totally inactive at 1.0 mM.

Concomitant to the inhibition of dehydrogenase activity by **6**, the esterase activity of yeast AldH was found to be commensurably inhibited. The latter activity was measured using *p*-nitrophenylacetate as substrate.¹¹ Thus, the dehydrogenase and esterase activities of yeast AldH were both found to be inhibited approximately 95% compared to control values when the enzyme was incubated with 1.0 mM **6** (Figures 2 and 4). Since extensive dialysis did not restore activity (Figure 4), covalent interaction of an inhibitor (or inhibitors) with the enzyme was suggested.

In support of this, incubation of the inhibited (due to **6**), dialyzed enzyme with mercaptoethanol for up to 60 min only partially restored AldH activity, the degree of this restoration being time-dependent (Figure 5). We envision this slow, partial restoration of enzymatic activity by mercaptoethanol as a transcarbethoxylation reaction (Scheme 2, path "b"), suggesting that the monothiolcarbonate group, as postulated, is not readily solvolyzed by water during dialysis and requires a good

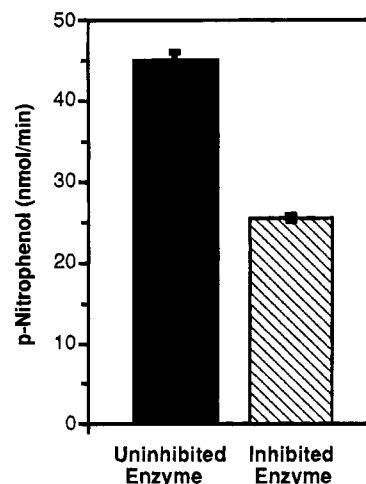


Figure 6. Inhibition of the esterase activity of yeast AldH by *N*-acetoxyaccharin (**11**). See the legend to Figure 4 for details.

nucleophile such as mercaptoethanol to effect ester exchange.

Further evidence that **6** must be carbethoxylating the enzyme is provided by the observation that the esterase activity of yeast AldH after incubation with 1.0 mM *N*-acetoxyaccharin (**11**; Chart 1), a weak inhibitor of this enzyme (IC_{50} 0.24 mM; see preceding paper⁸), and following extensive dialysis, was found to be inhibited only 43% (Figure 6). This partial inhibition of yeast AldH by **11** is likely due to the small amount of nitroxyl released from this compound at the active site (see preceding paper⁸). Although **11**, with an activated acetyl group, can also *acetylate* the enzyme, this should not be inhibitory since the *S*-acetylated enzyme at the active site is the penultimate intermediate in the oxidation of acetaldehyde to acetate,^{3a} and this thioester group readily solvolyzes to acetate.

Conclusions

Chemical carbethoxylating agents represented by compounds such as **8–10**, and by analogy **6** and **1c**, appear to inhibit AldH by carbethoxylating the enzyme. Compounds **1c** and **6** are unique in that they can not only carbethoxylate the enzyme but can also release nitroxyl, a potent inhibitor of AldH, and this is reflected by the observation that the activities of **1c** and **6** were more potent than **8**, **9**, or **10** by 1 order of magnitude.

Although the evidence is still circumstantial and does not contradict the possibility that a participating imidazole group might also be carbethoxylated, we prefer to rationalize this inhibition as due to the carbethoxylation of the sulfhydryl group of the active site Cys 302 residue (Scheme 2, path "a"). This is in harmony with the pioneering studies by Zheng and Weiner¹² who demonstrated that replacement of His 235 (the only His residue preserved in all known AldH's) or His 29 of rat liver mitochondrial AldH with Ala by site-directed mutagenesis did not result in profound alteration of enzyme activity whereas replacement of Cys 302 with Ala 302 completely abolished enzyme activity.^{1b} Although glutamate 268 has also been implicated as being essential for enzymatic activity,¹³ it is unlikely that a carboxylate group would react with a carbonate ester such as found in **1c** or **6**.

In summary, compounds represented by *O*-carbethoxy-*N*-hydroxysaccharin (**6**) and *N,O*-dicarbethoxy-4-chlorobenzenesulfohydroxamate (**1c**) appear to irreversibly inhibit ALDH by carbethoxylating the enzyme. However, these compounds were found not to raise blood AcH levels postethanol (a result reflecting less than maximal inhibition of hepatic ALDH) when administered to rats at a dose of 1.0 mmol/kg (data not shown), perhaps due to premature decomposition and/or failure to reach the liver. In support of this, when tested in vitro against isolated rat liver mitochondria at a concentration of 1.0 mmol, **6** and **1c** inhibited mitochondrial ALDH¹⁴ by 41% and 98%, respectively ($n = 3$). These compounds may therefore find utility in probing the active site of ALDH subsequent to its inactivation in vitro, by identification and characterization of the altered amino acid residue(s) following proteolytic degradation of the enzyme.

It is interesting to note that the active metabolite of the only alcohol deterrent agent in clinical use in the United States, *viz.*, disulfiram, is a carbamoylating agent.¹⁵ This metabolite (methyl *N,N*-diethylthiocarbamate *S*-oxide, DETC-MeSO) presumably inactivates ALDH by carbamoylating the active site sulphydryl group of the enzyme. Biliary excretion of *S*-(*N,N*-diethylcarbamoyl)glutathione after administration of disulfiram¹⁵ to rats suggests that *S*-carbethoxyglutathione may also be found as a biliary conjugate of **1c** or **6**.

Experimental Section

The preceding paper should be consulted for the general experimental parameters used.

2-[(Ethoxycarbonyloxy)-1,2-benzisothiazol-3(2H)-one 1,1-Dioxide, [N-[(Ethoxycarbonyloxy)saccharin] (6**).** A solution of *N*-hydroxysaccharin (**7**)⁸ (0.30 g, 1.5 mmol) in dry distilled THF (30 mL) under nitrogen was cooled to about -10 to -15 °C in an isopropyl alcohol-dry ice bath, and ethyl chloroformate (0.173 mL, 0.197 g, 1.8 mmol) was added followed by 4-(dimethylamino)pyridine (0.22 g, 1.81 mmol). The reaction mixture was stirred with cooling for 10 min. The dry ice bath was then removed and the reaction mixture stirred at room temperature overnight. The precipitated amine salt was removed by filtration and the filtrate evaporated to give a white solid. The solid was dissolved in EtOAc (50 mL), the solution washed successively with 1 N HCl (3 × 25 mL) and water (2 × 50 mL), and the organic layer dried over Na₂SO₄. Evaporation of the solvent gave a white solid which was recrystallized from EtOAc/hexane to give 0.205 g of **6** (50% yield): mp 133–136 °C; ¹H NMR (CHCl₃) δ 1.44 (t, 3H, CH₃), 4.47 (q, 2H, CH₂), 7.9–8.14 (m, 4H, ArH); IR (neat, cm⁻¹) 3100 (wk, aromatic CH), 1813 (-O-C=O), 1764 (N-C=O), 1363, 1230, 1195 (SO₂); MS (CI) m/z 272 (MH⁺). Anal. (C₁₀H₉NO₆S) C, H, N.

4-Chloro-*N*-hydroxybenzenesulfonamide (4-Chlorobenzenesulfohydroxamic acid). To a solution of 4-chlorobenzenesulfonyl chloride (10.0 g, 0.047 mol) in THF (200 mL) was added a solution of hydroxylamine hydrochloride (6.60 g, 0.095 mol) in water (30 mL). The mixture was cooled in an ice-salt bath, and 10% NaHCO₃ (160 mL, 15.96 g, 0.19 mol) was added dropwise over a period of 45 min maintaining the temperature below 10 °C. The mixture was stirred with cooling for over 90 min when the two layers separated. The aqueous layer was extracted with dichloromethane (3 × 75 mL), and the extracts were combined with the THF layer. The cloudy mixture separated into two layers after 30 min. The organic layer was separated, dried over Na₂SO₄, and evaporated to give 11.23 g of colorless oil. The oil was dissolved in ether (80 mL) and the cloudy solution clarified by filtering the mixture through a mat of Celite. The filtrate was evaporated to 50 mL and excess hexane was added. A first crop of 3.23 g of crystalline solid was obtained. A second crop (2.2 g) and a

third crop (0.68 g) were also obtained to give a total yield of 6.11 g (62.1%). All three crops melted at 130–133 °C: ¹H NMR (CD₃OD) δ 7.58–7.90 (m, 4H, Ar-H); MS (CI) m/z 208 (MH⁺). Anal. (C₆H₆NO₃SCl) C, H, N.

***O*-Carbethoxy-*N*-hydroxyphthalimide (**8**), *O*-Carbethoxy-1-hydroxybenzotriazole (**9**), and *O*-Carbethoxy-*N*-hydroxysuccinimide (**10**).** These compounds were prepared according to the general procedure for **8** as follows: To a cooled (ice bath) solution of *N*-hydroxyphthalimide (1.63 g, 0.010 mol) in 30 mL of THF was added with stirring Et₃N (1.53 mL, 1.11 g, 0.011 mol) followed by ethyl chloroformate (1.05 mL, 1.19 g, 0.011 mol) dropwise over <1 min. After 1 h, the precipitate of Et₃NX⁺Cl⁻ was collected and washed successively with THF and Et₂O. The combined filtrates were evaporated on a rotary evaporator, and the residue was recrystallized from EtOAc/hexane to give 1.52 g (64.7% yield) of **8**: mp 94–94.5 °C; IR (KBr, cm⁻¹) 3002, 1818 and 1787 (phthalimide C=O's), 1739 (C=O). Anal. (C₉H₉N₃O₅) C, H, N.

Compound **9** was recrystallized initially from EtOAc/hexane and then from EtOAc: 1.37 g (66.2% yield); mp 144–145 °C; IR (KBr, cm⁻¹) 3112, 2998, 1756 (C=O). Anal. (C₉H₉N₃O₃) C, H, N.

Compound **10** was recrystallized twice from Et₂O/hexane: 1.31 g (70% yield); mp 54–55 °C; IR (KBr, cm⁻¹) 2995, 2957, 1815 and 1786 (succinimide C=O's), 1741 (C=O). Anal. (C₇H₉NO₅) C, H, N.

4-Chloro-*N,O*-bis(ethoxycarbonyl)-*N*-hydroxybenzenesulfonamide (1c**).** The procedure for the preparation of **6** above was followed using 4-chloro-*N*-hydroxybenzenesulfonamide (0.500 g, 2.41 mmol), ethyl chloroformate (0.46 mL, 0.52 g, 4.82 mmol), and triethylamine (0.595 mL, 0.532 g, 4.28 mmol). The reaction was terminated after 5 h at room temperature. The crude product (oil, 0.77 g) was purified by flash chromatography (Kieselgel 60, EtOAc:hexane, 1:3) to give 0.66 g of **1c** as a colorless oil (78% yield): ¹H NMR (CDCl₃) δ 1.26 (t, 3H, NCOOCH₂-CH₃), 1.41 (t, 3H, OCOOCH₂CH₃), 4.23 (q, 2H, NCOOCH₂CH₃), 4.40 (q, 2H, OCOOCH₂CH₃), 7.56 (m, 4H, ArH), 8.01 (s, 1H, NH); IR (neat, cm⁻¹) 3100 (wk, aromatic CH), 1806 (O-C=O), 1764 (N-C=O), 1398, 1230, 1188 (SO₂); MS (CI) m/z 352 (MH⁺). Anal. (C₁₂H₁₄NO₇SCl) C, H, N.

Incubation Mixtures.¹⁶ Commercial yeast ALDH was incubated for 10 min at 37 °C in a primary reaction mixture containing inhibitor (prepared in DMSO and added to mixture in 5 μL), 0.08 IU of yeast ALDH, and 100 mM potassium phosphate, pH 7.4, in a total volume of 0.1 mL. The primary mixture was preincubated for 5 min at 37 °C, and the reaction was initiated by the addition of ALDH followed by inhibitor 5 s later. After 10 min of incubation, an aliquot was removed for the determination of ALDH activity.

In the dialysis experiments, the volume of the primary mixture was increased 8-fold and yeast ALDH 20-fold. After dialysis, a 100 μL aliquot of inhibited or control dialyzed ALDH samples was added to a secondary reaction mixture containing 100 mM potassium phosphate buffer, pH 7.4, and 20 mM mercaptoethanol, where indicated, in a final volume of 0.2 mL. These secondary reaction mixtures were incubated at 37 °C, and 20 μL aliquots were removed at 30 and 60 min for the determination of ALDH activity.

Analysis of the Dehydrogenase Activity of ALDH. Dehydrogenase activity was measured spectrophotometrically using procedures described in the accompanying paper.⁸

Analysis of the Esterase Activity of ALDH. Esterase activity of the dialyzed enzyme was determined spectrophotometrically by following the formation of *p*-nitrophenol from *p*-nitrophenylacetate at 400 nm.¹¹

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