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Hydroxamic Acids As a Novel Family of Serine Racemase Inhibitors: Mechanistic Analysis Reveals Different Modes of Interaction with the Pyridoxal-5′-phosphate Cofactor

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Mammalian serine racemase (SR) is a pyridoxal-5′-phosphate (PLP) dependent enzyme responsible for the biosynthesis of the neurotransmitter D-serine, which activates *N*-methyl-D-aspartate (NMDA) receptors in the CNS. Aberrant regulation of NMDA receptor signaling has been implicated in a variety of neuropathologies, and inhibitors of SR would therefore be a worthwhile tool for further investigation or treatment of such conditions. Here, we identify a series of small aliphatic hydroxamic acids (HAs) that act as potent SR inhibitors. However, specificity studies showed that some of these HAs can act as nonspecific inhibitors of PLP-dependent enzymes. We employed NMR, MS, and UV/vis spectroscopic techniques to reveal that the nonspecific effect is likely due to irreversible interaction of the HA moiety with PLP to form aldoxime species. We also characterize L-aspartic acid β-hydroxamate as a competitive and selective SR inhibitor that could be used as a scaffold for further inhibitor development.

Introduction

Mammalian serine racemase (SR[a]) is a unique pyridoxal-5′-phosphate (PLP)-dependent enzyme responsible for the biosynthesis of D-serine in the central nervous system. D-Serine acts as a neurotransmitter and coagonist of *N*-methyl-D-aspartate (NMDA) receptors,[1] ionotropic glutamate receptors that are found throughout the brain but predominantly within the forebrain.[2] NMDA-receptor-mediated glutamate excitotoxicity has been implicated in a plethora of neuropathological conditions, but treatment of patients with high-affinity NMDA receptor blockers often results in undesirable side effects, such as hallucinations. Thus, alternative methods of decreasing NMDA receptor overactivation are highly sought after. Excitotoxic D-serine levels have been implicated in Alzheimer's disease[3] and amyotrophic lateral sclerosis (Lou Gehrig's disease),[4] while low D-serine levels result in NMDA receptor hypofunction, which may lead to symptoms of schizophrenia.[5] SR inhibitors therefore offer a novel and potentially highly specific approach for attenuation of NMDA-receptor-mediated excitotoxicity and for further study of the pathway.

Since almost all PLP-dependent enzymes share a common transition state, the external aldimine, identification of active-site directed inhibitors with high specificity is a particular challenge.[6] Nevertheless, several PLP-dependent enzymes have been recognized as pharmaceutical targets, as recently reviewed by Amadasi et al.[7] In the case of SR, however, very few potent and specific inhibitors have been identified to date; the two most potent competitive inhibitors, malonic acid and L-*erythro*-3-hydroxyaspartic acid,[8] exhibit inhibitory constants (K_i) in the micromolar range.

With the aim of identifying more effective SR inhibitors, we screened a panel of structurally diverse small molecules for the inhibition of recombinant mouse SR (mSR). As a result of the screening, we identified a series of hydroxamic acids (HAs) with potent inhibitory activity. Since their discovery in 1869,[9] the HAs have remained an intriguing family of organic bioligands.[10] Their bioactivity is usually associated with their metal-binding properties [e.g., natural or artificial siderophores for Fe(III) sequestration and strong chelators of Zn-(II)], but recent interest has focused on the use of HAs as specific enzyme inhibitors and potential pharmacophores. HAs have been shown to inhibit a number of enzyme targets including metalloproteases, hydrolases, ureases, lipoxygenases, cyclooxygenases, and peptide deformylases (for a recent review, see refs 11 and 12). Several of the HAs have been shown to be cell-permeable, and in at least one case, they have been shown to cross the blood−brain barrier.[13]

In the present work, we identify several HAs, including a series of dihydroxamic acids (DHAs), with inhibitory activity toward SR. In fact, one of them, succinodihydroxamic acid (**3**, Chart 1), appears to be the most potent competitive SR inhibitor identified to date. However, screening the HAs for inhibition of a small panel of structurally and functionally diverse PLP-dependent enzymes revealed that the DHAs capable of inhibiting SR also inhibit all the other

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[a]Abbreviations: AR, alanine racemase; ATP, adenosine triphosphate; DHA, dihydroxamic acid; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FDAA, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; HA, hydroxamic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl-β-D-1-thiogalactopyranoside; NMDA, *N*-methyl-D-aspartate; OD, optical density; PLP, pyridoxal-5′-phosphate; SDH, serine dehydratase; SR, serine racemase (mSR, mouse SR; hSR, human SR); TA, transaminase.

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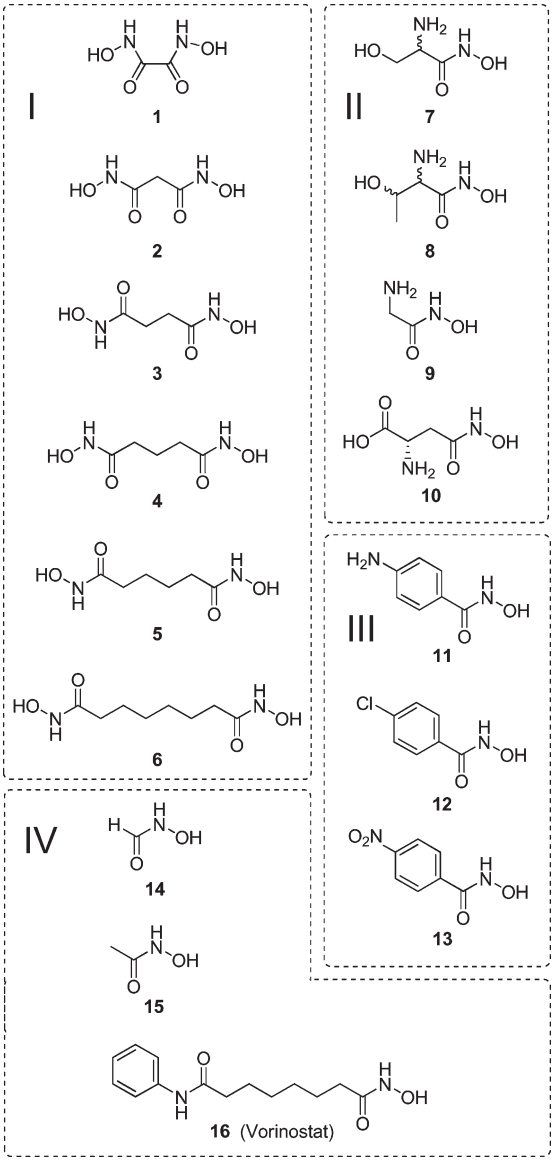
Chart 1. Structures of the Hydroxamic Acids Studied Split into Four Structurally Diverse Groups[a]



[a] The groups are (I) dihydroxamic acids (DHAs) and (II) hydroxamic derivatives of amino acids (compound **8** is a mixture of two isomers: the D-isomer with 2*R*,3*S* configuration and the L-isomer with 2*S*,3*R* configuration), (III) three representatives of benzohydroxamic acids, and (IV) formohydroxamic acid and the clinical compounds acetohydroxamic acid (Lithostat) and vorinostat (Zolinza).

enzymes tested. Although the DHAs are effective SR inhibitors, their compromised specificity renders them unattractive candidates for further drug development. We employ NMR, MS, and UV/vis spectrophotometry to show that the DHA inhibitors act as covalent modifiers of PLP, forming an aldoxime species and in effect depleting the available pool of cofactor.

Notably, not all of the inhibitory HAs identified by our screening experiments are able to act nonspecifically via PLP-aldoxime formation. L-Aspartic acid β-hydroxamate (**10**, Chart 1) acts as a competitive SR inhibitor and forms an aldimine species with PLP that may act as a transition state mimetic. This compound is selective for SR and closely related enzymes and could be used as a lead compound in further inhibitor development studies.

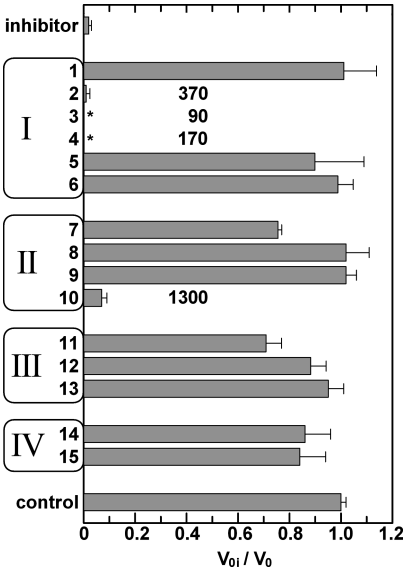


Figure 1. Graph of relative remaining SR activity in the presence of selected hydroxamic acids compared to the noninhibited reaction. Latin numerals indicate the structurally diverse groups of hydroxamic acids described in Chart 1. Activity was assayed at pH 8.0 in the presence of 10 μM PLP (see Experimental Section for details) with both the substrate (L-serine) and the inhibitor at 5 mM concentration. A known potent inhibitor, L-*erythro*-3-hydroxyaspartic acid,[8] was used as the positive inhibition control (inhibitor). Control represents the noninhibited reaction. The data are averages of duplicate or triplicate measurements; the error bars show the standard deviation from the mean. For the effective compounds, IC_{50} values (μM) are shown. IC_{50} values were calculated from two independent data sets. The standard deviation is within 25%, except in the case of **4** (within 60%). *No remaining activity was observed.

Results

Selected Hydroxamic Acids Potently Inhibit Mouse Serine Racemase. A panel of hydroxamic acid analogues of carboxylic and amino acids was analyzed for the inhibition of mouse serine racemase (mSR). We have recently shown that mSR and its human orthologue (hSR) exhibit comparable inhibitor sensitivity.[14] Therefore, we used mSR for the initial screening to facilitate comparison of the results with our previously published mSR data.[8] Compounds were screened for inhibition of mSR-catalyzed L-serine racemization. L-Serine and the test compound were present at equimolar concentration (5 mM);[9] this setup was chosen to reveal ligands of comparable or higher affinity toward serine racemase than the substrate L-serine. The inhibition screening data is summarized in Figure 1.

The first group of test compounds (I) comprised two to six and eight carbon chain dihydroxamic acids (Chart 1). Because of the unavailability of its synthetic precursor, the seven carbon chain dihydroxamic acid was not included. The three, four, and five carbon chain DHAs inhibit racemization significantly and with comparable IC_{50} values: malonodihydroxamic acid (**2**), 370 $\pm$ 30 μM; succinodihydroxamic acid (**3**), 90 $\pm$ 20 μM; and glutarodihydroxamic acid (**4**), 170 $\pm$ 110 μM. The two, six, and eight carbon chain dihydroxamic acids (**1**, **5**, and **6**, respectively) do not affect enzyme activity. The second group of compounds (II) included hydroxamic acid derivatives of the amino acid substrates serine and threonine and the inhibitors glycine (K_i = 1640 μM[8]) and L-aspartic acid (K_i = 1900 μM[15]). Neither the

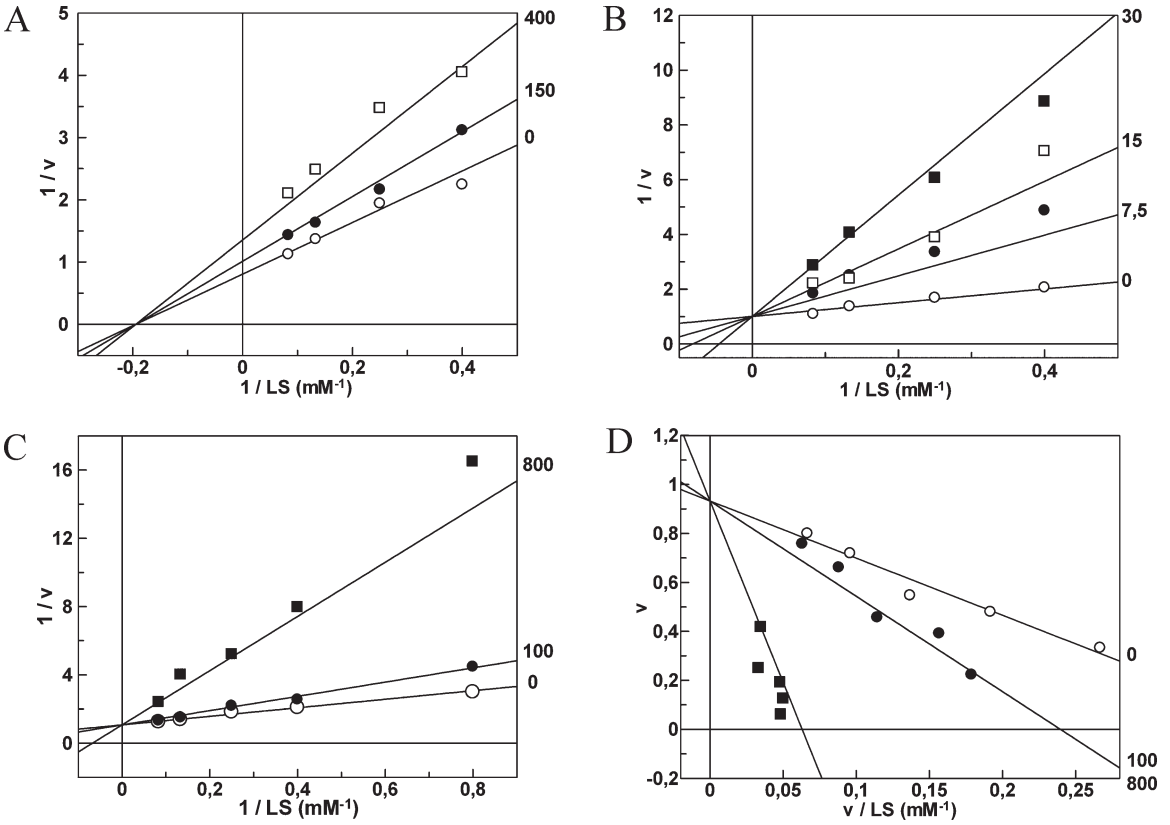


Figure 2. Mechanism of action of **2** (A), **3** (B), and **10** (C,D) depicted as Lineweaver−Burk (A−C) and Eadie−Hofstee (D) plots. Data for the plots were analyzed using global analysis nonlinear fit followed by the linear transformation provided by GraFit 5 software.[19] Kinetic data were recorded using an HPLC-based end point assay as described in Experimental Section. The substrate L-serine is abbreviated as LS. Inhibitor concentrations (μM) are indicted on the right *y*-axes. Reaction velocity is in arbitrary units of product (D-serine) peak area relative to the internal standard (glycine) peak in time. The data points represent triplicate experiments with an average standard deviation of 9%.

substrate (**7** and **8**) nor the glycine (**9**) α-hydroxamic acid derivatives showed significant inhibition. However, L-aspartic acid β-hydroxamate (**10**) inhibited serine racemase with an IC_{50} of 1300 ± 200 μM.

Additionally, we screened several other structurally diverse hydroxamic acids for SR inhibition (III and IV), including a panel of benzohydroxamic acids, exemplified by **11**, **12**, and **13**. None of these compounds significantly affected serine racemization.

Hydroxamic acids often act by chelating functionally important metal ions, and divalent cations are important serine racemase activators.[16,17] Since the screening reactions were performed in the presence of 1 mM $MgCl_2$, we were concerned that the observed SR inhibition might be an effect of metal chelation. Therefore, we tested SR inhibition by **2** (as a representative inhibitory compound) in the presence of varying concentrations of $MgCl_2$. The inhibition of SR by **2** remained unaffected even in the presence of a 5-fold molar excess of Mg^{2+} over the inhibitor (data not shown).

Mechanism of Serine Racemase Inhibition by HAs. We selected **2**, **3**, and **10**, hydroxamic acid analogues of known competitive inhibitors,[8,15] as representative compounds for further analysis. Compound **4** was also an effective inhibitor, but since our synthetic preparation was less than 95% pure, we excluded it from the detailed kinetic analyses. We analyzed the mechanisms of action of **2**, **3**, and **10** by determination of mSR racemization kinetics at various substrate/inhibitor concentrations (see Experimental Section for details). The data were processed with DynaFit4 software, which uses least-squares nonlinear regression analysis to fit the data to defined kinetic models (in this case, models for competitive, mixed-type, uncompetitive, or noncompetitive inhibition).[18] DynaFit4 accomplishes precise numerical and graphical tests to choose the best fitting model. The best fitting model for **2** is noncompetitive inhibition with a K_i value of 417 ± 52 μM. Data for **3** best match the competitive model. The K_i value calculated for **3** is 3.6 ± 0.6 μM, indicating that **3** is the most potent competitive SR inhibitor characterized to date. Compound **10** is also a competitive inhibitor with a K_i of 97.5 ± 23.7 μM. The results of the mechanistic analysis are presented in Figure 2 as Lineweaver−Burk or Eadie−Hofstee plots prepared with GraFit5 software.[19]

Specificity of the HAs. In order to test the specificity of the HA inhibitors for serine racemase, we screened **2**, **3**, and **10** for inhibition of a small panel of PLP-dependent enzymes. The noninhibitory compound **1** was included in the screen as a control, as was the previously identified SR inhibitor malonic acid.[8] The enzyme panel comprised mouse SR, human SR, rat liver serine dehydratase (SDH),[20] alanine racemase from *Bacillus stearothermophilus* (AR), and bacterial transaminase (TA).

The results of the specificity screen are shown in Figure 3. All enzymatic reactions were performed at pH 8.0 in the presence of 10 μM PLP using 5 mM compound (see Experimental Section for details). Compounds **2** and **3** showed an inhibitory effect on all enzymes tested. Interestingly, malonic acid, the dicarboxylic acid analogue of **2**, is specific for mouse and human SR. Compound **1**, which did not inhibit mSR, did not inhibit any of the other enzymes tested. Alone among

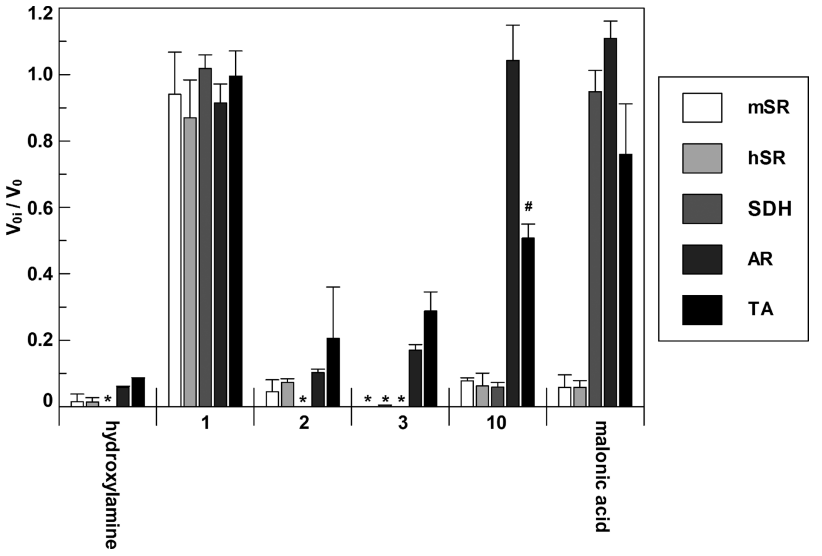


Figure 3. Inhibition of a panel of PLP-dependent enzymes by selected hydroxamic acids. Inhibition was assayed at pH 8.0 in the presence of 10 μM PLP. The inhibitor concentration was kept at 5 mM, while the substrate concentration was chosen to be near the K_M of the enzyme (see Experimental Section for details). *No remaining activity was observed. #The tested compound acts as a substrate.

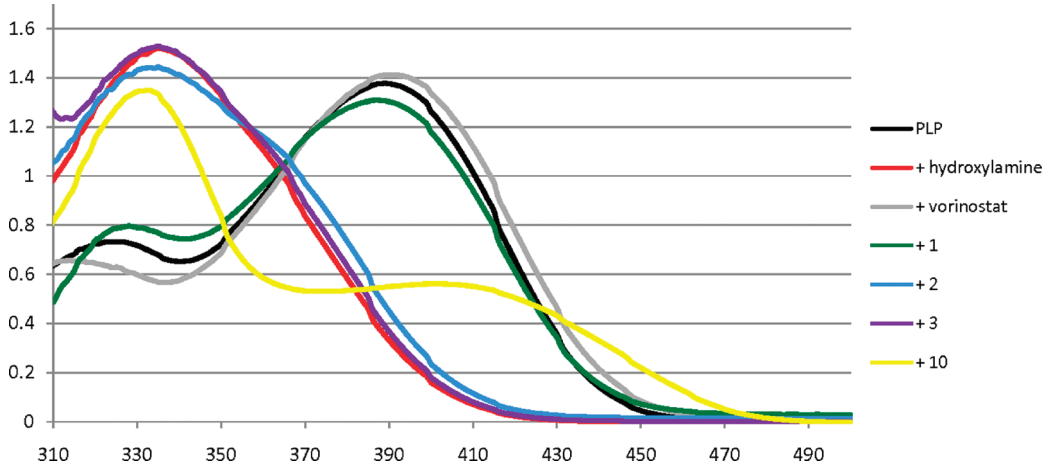


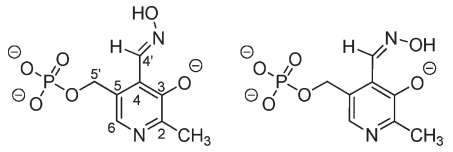
Figure 4. Absorbance spectra of PLP and selected compounds recorded at pH 8.0. The final concentration of the test compound was 5 mM, and the PLP concentration was 0.3 mM.

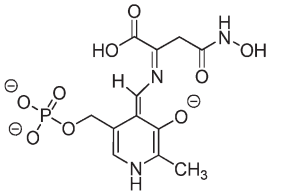
the hydroxamic acids tested, the L-aspartic acid analogue **10** exhibits some selectivity: it inhibits mSR, hSR, and their homologue SDH (20% identity). It does not show any significant inhibitory activity toward AR, and it acts as a substrate of TA (the natural substrate of which is L-aspartic acid).

The only common feature of the enzymes included in the panel is the presence of a PLP−Schiff base in the catalytic center. Therefore, we hypothesized that the DHAs might act via modification of PLP, and to test this hypothesis, we analyzed SR inhibition in the presence of various concentrations of PLP. When the concentration of PLP added to the reaction mixture exceeded the concentration of **2** or **3** in molar equivalents, we observed quenching of the inhibition by excess PLP (data not shown), suggesting that certain HAs may inhibit SR and other PLP-dependent enzymes by interacting with the cofactor.

Some Hydroxamic Acids React with PLP in Buffered Aqueous Solution. In order to investigate the putative interaction of HAs with PLP in aqueous solution, we analyzed the reaction of PLP and HAs using UV/vis spectrophotometry. Solutions of PLP and various HAs were prepared in phosphate buffer, pH 8.0, and absorbance spectra were recorded in the 310−500 nm range. Results of the screening are shown in Figure 4 (**1**, **2**, **3**, **10**, and **16**) and Figure 2S (Supporting Information) (**4**, **5**, and **6**). The absorbance spectrum of PLP alone (Figure 4, black curve) exhibits maxima at 325 and 390 nm. The major chromophore at 390 nm corresponds to PLP with an unsubstituted aldehyde moiety, and the minor peak at 325 nm corresponds to PLP-hydrate.[21] As a positive control, we added an excess of hydroxylamine to PLP. Hydroxylamine reacts rapidly with the aldehyde of PLP to form a PLP-aldoxime (see Scheme 1). The absorbance spectrum of this PLP-aldoxime is shown in Figure 4 (red curve); as expected, one major peak is visible at 335 nm, while the peak at 390 nm has disappeared entirely. Addition of the inhibitory DHAs **2**, **3**, or **4** to PLP resulted in similar spectral shifts. Addition of noninhibitory DHAs **1**, **5**, or **6** had no significant effect on the absorbance profile of free PLP, indicating that these compounds do not react with the aldehyde of PLP. Addition of **10**, a selective and competitive HA inhibitor of SR, to PLP resulted in a unique absorbance spectrum (Figure 4, yellow curve). In addition to the formation of a peak at 330 nm (similar to spectra of PLP in the

Scheme 1





Proposed structure of PLP-**10** aldimine
(deprotonized, quinoid form)

presence of **2**, **3**, **4**, and hydroxylamine), a second broad peak centered around 405 nm appears in the spectrum. This second peak could indicate that the inhibitor interacts with PLP via its amino group, as PLP-amino acid Schiff bases are known to exhibit absorbance maxima in this region.[22,23]

Two pharmacologically active compounds containing a hydroxamic acid moiety, acetohydroxamic acid (**15**, Lithostat), an inhibitor of bacterial urease,[24] and suberoylanilide hydroxamic acid (**16**, vorinostat, see Chart 1), a histone deacetylase inhibitor marketed under the brand name Zolinza,[25] were added to the series as controls. Neither 5 mM **16** (data not shown) nor 5 mM **15** (see Figure 1) exhibits inhibitory activity toward SR, and no change in the spectrum of PLP upon the addition of **15** or **16** was observed.

PLP as a Reactive Target for DHAs: Characterization of Reaction Products. In order to analyze the product(s) resulting from reactions of HAs with PLP, we monitored the reactions with ^{1}H NMR spectroscopy (see Supporting Information for background and details). All solutions were prepared in 100 mM phosphate buffer in D_2O, and the pD was kept as close to 8.0 as possible. Upon addition of **2**, **3**, or the noninhibitory compound **1** to PLP, the chemical shift of PLP's aldehydic proton does not change, but the signal intensity decreases until the signal disappears completely (except in the case of **1**). The rate of the decrease in intensity depends on the nature of the DHA and decreases in the order **3** > **2** > **1**. The reaction of **3** with PLP went to completion in less than a minute and resulted in the formation of two products. A thorough structural analysis based on ^{1}H and ^{15}N NMR (see Supporting Information Tables 2S and 3S for a detailed explanation) resulted in the unambiguous identification of the major and minor products as the *syn*- and *anti*-aldoxime, respectively, as shown in Scheme 1. The reaction of PLP and **2** led to the same final products but proceeded more slowly, and an intermediate product could be observed. PLP and **1** underwent a very slow reaction (after 12 days, 20% of the starting material remained), which eventually resulted in the formation of *syn*- and *anti*-PLP-aldoxime via an unidentified intermediate (with chemical shifts different from those of the intermediate observed in the reaction of PLP and **2**). The NMR analysis was also carried out with DHAs **4**−**6**, with spectra recorded immediately and after 20 h. The noninhibitory compounds **5** and **6** did not react with PLP during this time period, while the inhibitory DHA **4** was reactive. After 20 h, the starting material had been completely consumed, and the PLP-aldoxime was formed.

Formation of PLP-aldoximes in mixtures of PLP and DHAs was further confirmed by ESI-MS analysis of the reaction mixtures. A signal of 261.2 *m*/*z*, which corresponds to PLP-aldoxime, was detected in the spectra of the inhibitory DHAs (**2**, **3**, and **4**) mixed with PLP. Additionally, an artificial ionization signal of 243.2 *m*/*z* was observed, which corresponds to the aldoxime with one water molecule removed. These two signals correspond to the major components visible in the spectrum of a mixture of hydroxylamine with PLP, and thus, both signals can be attributed to PLP-aldoxime. Most of the spectra also contained a signal of 246.1 *m*/*z*, which corresponds to unmodified PLP. The aldoxime signals did not appear in the spectra of noninhibitory compounds **1**, **5**, **6**, or **15** mixed with PLP; these spectra contained only the signal corresponding to unmodified PLP.

Interaction of 10 with PLP Occurs by a Different Mechanism. ^{1}H NMR analysis showed that, like the DHAs, **10** reacts with the aldehyde group of PLP, as evidenced by a decrease in the intensity of the CHO signal over time. However, the PLP-aldoximes shown in Scheme 1 are not formed in this case. Rather, the reaction of PLP and **10** results in the appearance of two new broad signals at 8.73 and 5.75 ppm (as well as a variety of minor side-products). A slow subsequent reaction affects the $CH-CH_2$ moiety of **10**. The proton on the α-carbon is abstracted, keeping the two CH_2 protons nonequivalent. Exchange of the α-proton with D can be excluded (no line broadening of the CH_2 signals was observed). These data do not allow for a conclusive determination of the structure of the product(s) formed from the reaction of PLP and **10**. However, on the basis of the known interactions of PLP with amino acids[26−28] and on the available NMR data, we propose that one of the major products of the reaction of **10** with PLP is the aldimine shown in Scheme 1.

MS analysis of the mixture of **10** and PLP offers support for this proposal. At PLP concentrations of 4−5 mM, the major feature of the spectrum is a peak at 246.1 *m*/*z*, corresponding to unmodified PLP, and only trace amounts of the proposed PLP-**10**-aldimine (as a potassium adduct, 416.3 *m*/*z*) can be observed. However, at higher PLP concentration (30 mM), the spectra contained a significant signal corresponding to the PLP-**10**-aldimine. Under these conditions, the corresponding PLP-amino acid-aldimines can be observed in the spectra of mixtures of PLP and **7**, **8**, **9**, L-serine, or glycine. The fact that the α-HAs **7**, **8**, and **9** have no effect on SR activity (see Figure 1) suggests that these compounds probably do not form aldimine species in the active site, perhaps because of steric or electrostatic clash of the α-hydroxamic acid moiety with active site residues. To address this hypothesis, it would be interesting to test the α-hydroxamic acid analogue of **10**; however, since this compound is not commercially available and its synthesis is rather challenging, we did not include it here. In the case of the β-HA **10**, a competitive and selective SR inhibitor, aldimine formation is a probable mechanism of inhibition.

Discussion

Specific and potent inhibitors of mammalian serine racemase would be advantageous tools for further research of the enzyme function *in vivo* and might have therapeutic potential for the treatment of neuropathologies associated with glutamatergic excitotoxicity. Recently published experiments with SR knockout mice support this hypothesis, showing that SR deficiency is correlated with a reduction in neurotoxic effects

caused by overexcitation of NMDA receptors.[29] SR inhibitor development has been addressed by several groups in recent years.[30,31,15,8] The most effective competitive inhibitors identified by these groups are small amino and dicarboxylic acids, with the most potent being L-*erythro*-3-hydroxyaspartic acid (K_i = 43 μM) and malonic acid (K_i = 71 μM).[8] In order to conduct experiments in animal models or tissue cultures, more potent compounds are needed, and identification of novel structures capable of specifically inhibiting SR is therefore very relevant.

During our screening of various structurally diverse small molecules for SR inhibition, we identified a series of hydroxamic acids as potent SR inhibitors. Hydroxamic acids have been shown to inhibit a variety of enzymes, often by chelating functionally important metal ions. We immediately addressed the possibility that the observed inhibition was due to chelation of Mg^{2+}, a potent SR activator present in our screening assay. Gratifyingly, the HAs retained their inhibitory activity even in the presence of a large molar excess of Mg^{2+} over the test compound, indicating that metal chelation does not play a significant role in SR inhibition.

Kinetic analysis of the mechanism of action of inhibitory HAs shows that **10** is a classical competitive inhibitor with a K_i of 97.5 ± 23.7 μM. Compound **10** is therefore a roughly 20-fold more potent mSR inhibitor than its carboxylic acid analogue L-aspartic acid (competitive mechanism, K_i = 1900 μM[15]). There could be several reasons for the increased potency of **10** compared to that of L-aspartic acid. Hydroxamic acids generally have much higher pK_a values than their corresponding carboxylic acids,[32] which could lead to different electrostatic interactions with active site residues depending on the local pH in the active site. Furthermore, the hydroxamic acid moiety has the potential to form more hydrogen bonding interactions with active site residues than its carboxylic acid counterpart. However, since the 3-D structure of SR has not yet been solved, it is difficult to speculate about which possibility is more likely.

Succinodihydroxamic acid (**3**), which behaves as a competitive inhibitor with a K_i of 3.6 ± 0.6 μM, is also more potent than its dicarboxylic acid analogue succinic acid, which only moderately inhibits mSR.[8] In contrast, malonic acid is one of the most potent competitive SR inhibitors identified to date (K_i = 71 μM[8]), while its dihydroxamic acid analogue **2** acts as a noncompetitive inhibitor with a K_i value of 417 ± 52 μM. It is surprising that **2**, which differs from **3** only in the length of the carbon chain, behaves as a noncompetitive inhibitor, while **3** behaves as a competitive inhibitor. It should be noted that the inhibition mechanisms analyzed in the cases of **2** and **3** are very complex. Both compounds are able to irreversibly interact with the PLP cofactor, as our NMR, MS, and UV/vis analyses indicate, and therefore, the kinetic models used may not be entirely suitable to describe the inhibitor−SR interaction. It is noteworthy, however, that in conditions approaching the initial velocity of the enzymatic reaction (for details, see Experimental Section) **3** and **2** behave as potent competitive and noncompetitive inhibitors, respectively.

In addition to the mechanism of action and potency, we also addressed the target specificity of the HAs. The development of specific inhibitors for PLP-dependent enzymes is always a challenging task since the mechanisms of almost all PLP-dependent enzymes, regardless of their substrate or reaction specificity, proceed via condensation of the amino acid substrate with PLP, forming an external aldimine intermediate. We tested the selectivity of **2**, **3**, and **10** for mammalian SR by repeating the screening assay with a panel of PLP-dependent enzymes [mouse SR, human SR, rat serine dehydratase (SDH), bacterial alanine racemase (AR), and bacterial transaminase (TA)]. Mouse and human SR share 89% sequence identity and are predicted to be members of the fold type II subfamily of PLP-dependent enzymes.[33] SDH, which eliminates L-serine to pyruvate in the liver, shares approximately 20% sequence identity with mammalian SR, and its crystal structure reveals that it is fold type II.[34] AR shares no significant sequence homology with mammalian SR and is a member of the fold type III family,[35] a family that is quite structurally distinct from other PLP-dependent enzymes. As its name suggests, AR catalyzes the interconversion of D- and L-alanine. TA produces oxaloacetate and L-glutamate from L-aspartate and α-ketoglutarate and is a member of the fold type I subfamily, also referred to as the asparate aminotransferase family. The enzymes included in the panel are therefore quite structurally and functionally diverse and are representative of the variety of PLP-dependent enzymes. Direct comparison of the potency of the inhibitors toward the different enzymes is not possible under this setup, as potency is affected by the k_{cat}/K_M of the enzymes toward their respective substrates and by the reaction conditions. The results shown in Figure 3 should therefore be interpreted qualitatively rather than quantitatively. However, it can be concluded that the DHA not capable of inhibiting mouse SR (**1**) did not inhibit any of the other enzymes tested, while the most active DHAs (**2** and **3**) significantly inhibit all of the enzymes tested. Strikingly, malonic acid is a specific inhibitor of SR, while its dihydroxamic acid analogue **2** inhibits all of the PLP-containing enzymes tested indiscriminately. Unlike **2** and **3**, the L-aspartic acid analogue **10** is rather selective; it inhibits mouse and human SR as well as their homologue SDH quite potently but does not inhibit the more distantly related AR. Compound **10** acts as an inefficient substrate for TA and competes with the natural substrate L-aspartic acid.

The ability of **2** and **3** to inhibit such a diversity of PLP-dependent enzymes, together with the observed inhibition quenching in the presence of excess PLP, prompted us to analyze the chemical mechanism of the observed enzyme inhibition in more detail. Hydroxylamine, NH_2OH, which is used in the synthesis of hydroxamic acids, is a reagent commonly used to remove PLP from PLP-dependent holoenzymes. The presence of hydroxylamine in the inhibitor preparations could explain their nonspecific interaction with PLP. However, all of the synthetic compounds described in this work were purified as described in Experimental Section, and analysis of both ^{15}N labeled and unlabeled compounds[36] by NMR, mass spectrometry (MS), and elemental analysis revealed that no significant hydroxylamine was present in the final preparations. Furthermore, hydroxamic acids are generally believed to be stable in aqueous solution, although they are known to undergo acid-catalyzed hydrolysis.[37]

We set out to analyze this putative interaction of HAs and PLP by UV/vis spectroscopy, NMR, and mass spectrometry. We introduce a rapid and simple spectroscopic assay that enables facile identification of compounds with the ability to cross-react with PLP's aldehyde moiety. Addition of the inhibitory DHAs (**2**, **3**, and **4**) to PLP caused a spectral shift consistent with the formation of PLP-aldoxime. As controls, we used clinically available compounds containing a hydroxamic acid moiety, acetohydroxamic acid (**15**, Lithostat) and vorinostat (**16**, Zolinza). These compounds do not inhibit SR, and we expected the compounds to be unreactive in the

presence of PLP since there have been no reports of vitamin B6 deficiency in patients treated by these drugs. Indeed, the addition of noninhibitory compounds (**1**, **5**, **6**, **15**, and **16**) had no significant influence on the UV/vis spectrum, suggesting that, as expected, these compounds are stable at physiological pH and do not cross-react with PLP-dependent enzymes. Again, **10** is an outlier. The major feature of the spectrum of the mixture of **10** and PLP is a peak at 330 nm, consistent with PLP-aldoxime or PLP-hydrate formation. A second broad peak centered around 405 nm indicates that **10** might interact with PLP via its amino group, forming the PLP-aldimine species shown in Scheme 1.

In order to analyze the structures of the putative products of the reaction of PLP and HAs, we used ^{1}H NMR and MS. NMR analysis of the interaction of DHAs **1**, **2**, and **3** with PLP allowed us to unambiguously identify the reaction products as the *syn*- and *anti*-PLP-aldoximes shown in Scheme 1. It is interesting to note that the experimentally observed rates of reaction of DHAs with PLP (i.e., **1** ≪ **2** ≪ **3**) agrees with the published order of hydrolytic stability of the DHAs in acidic solution (**1** > **2** > **3**).[37] One could speculate that the aldoximes reported here are in fact products of the reaction of PLP with hydroxylamine liberated upon hydrolysis of DHAs. While HAs are generally known to be stable in aqueous solutions, acid- or base-catalyzed hydrolysis is a possibility. Hydrolysis experiments in 100 mM phosphate buffer at pH 8.0 and pH 7.4 (see Supporting Information) offer support for this proposal, showing that the DHAs that react rapidly with PLP (**2**, **3**, and **4**) are prone to hydrolysis, while nonreactive DHAs (**1**, **5**, and **6**) are hydrolytically stable under these conditions. However, while the UV/vis, MS, and NMR results draw a picture of the interaction of DHAs and PLP in solution, it is important to note that it remains unclear whether the observed enzymatic inhibition by DHAs results from this nonspecific depletion of PLP in solution or from specific interaction of the inhibitor and enzyme followed by cofactor depletion within the active site.

The information gleaned from the NMR analysis further highlights the complexity of interpreting the kinetic data presented in Figure 2A and B. The enzymatic reaction velocities were recorded at a single time point ranging from 8 to 15 min. However, the NMR measurements revealed that hydroxylamine and **3** react with PLP to form PLP-aldoxime within seconds, while the chemical reaction of PLP and **2** takes considerably longer (on the order of minutes or hours). Like **3**, hydroxylamine behaves as a competitive inhibitor in our assay conditions (data not shown), while **2** appears noncompetitive. Adding a time dimension to the enzymatic assay may help to further unravel the differences between **2** and **3**, but such a detailed kinetic analysis is beyond the scope of this work.

Another interesting point raised in this study is the observation that compounds that are closely structurally related (differing only in the length of the carbon chain) can have such different reactivities, both in buffered solution and in the presence of PLP and enzyme. In this case, the intermediate chain-length DHAs **2**, **3**, and **4** proved to be inhibitory and able to react with PLP in solution according to UV/vis, MS, and NMR analysis, while the compounds with very short (**1**) and very long (**5** and **6**) carbon chains were not. One possibility is that some DHAs may undergo intramolecular condensation, resulting in the release of hydroxylamine, a notion that is supported by hydrolysis experiments (see Supporting Information). The ability of analogues of **3** to cyclize to form 5-membered rings at mildly alkaline pH has been demonstrated.[38] Cyclization of **4** would result in a 6-membered ring, which is sterically favored. Noninhibitory compounds **1**, **5**, and **6** would form 3-, 7-, and 9-membered rings, respectively; these conformations are less likely to occur. The only outlier in this scheme is the inhibitory compound **2**. Intramolecular condensation of **2** would result in the formation of a 4-membered ring, which is not a sterically favorable conformation but is also not impossible to imagine. Alternatively, **2** may undergo hydrolysis or interaction with PLP via a different mechanism, perhaps reflected in its different mode of inhibition.

The findings presented here underscore the notion that caution must be taken when analyzing the results of large-scale inhibitor screening involving HAs and their derivatives. Indeed, the limited stability of some HAs under physiological conditions has been reported in other biomedicinal applications. Although HAs have been identified as one of the most powerful inhibitors of Zn^{2+}-containing metalloproteases, incorporation of this pharmacophore into drugs is not common, partly due to the rapid metabolism of the hydroxamic acid moiety.[39,25]

In this work, we identify a series of DHAs capable of nonspecific interaction with PLP-dependent enzymes via modification of the cofactor to form a catalytically inactive aldoxime species. In contrast to the DHAs, one of the newly identified HA inhibitors, **10**, derived from L-aspartic acid, is a potent competitive SR inhibitor and exhibits moderate selectivity for mouse and human SR. Although it also slowly reacts with the cofactor PLP, it does so less efficiently than the DHAs and yields a different mixture of products, in particular an aldimine species that may act as a transition state mimetic. Compound **10** could serve as a lead molecule for the development of the next generation of SR inhibitors, though its cell permeability and ability to cross the blood−brain barrier must be addressed in the future. One can speculate that its similarity to L-aspartic acid would allow it to use the same physiological pathway.

Experimental Section

Materials. Enzymatic reaction mixtures were resolved on a Zorbax Extend C_{18} reversed phase HPLC column (4.6 × 250 mm, particle size 5 μm, Agilent Technologies, USA) mounted on an Alliance 2795 HPLC system (Waters Co., Milford, MA, USA) coupled to a Waters 2487 dual wavelength absorbance detector. For NMR experiments, the pH was measured using a glass electrode (Spintrode, Hamilton) and a PHH2220 pH-meter (Radiometer) calibrated with Fisher Scientific Buffers; the pH values were converted into pD values by adding 0.40 as recommended.[40] The ^{1}H, ^{13}C, and ^{15}N NMR spectra were measured on a Varian INOVA 500 spectrometer. The instrumental setup and experimental parameters have been described.[41] Mass spectral data were recorded using a Q-TOF microspectrometer from Waters Co. (Milford, MA, USA). UV/vis absorbance scans were conducted on a Unicam UV500 UV/vis spectrophotometer.

D,L-Serine hydroxamate (**7**), D,L-threonine hydroxamate (**8**), glycine hydroxamate (**9**), L-aspartic acid β-hydroxamate (**10**), acetohydroxamic acid (**15**), and pyridoxal-5′-phosphate were purchased from Sigma Aldrich and used without further purification. Experimental procedures for the synthesis of **11**−**14** can be found in Supporting Information. The purity and identity of synthetic compounds were accessed by elemental analysis and NMR spectroscopy. All compounds were found to be at least 95% pure unless otherwise indicated.

Synthesis of Dihydroxamic Acids (DHAs; 1−6). Both ^{15}N labeled and nonlabeled DHAs were prepared from dicarboxylic

acid ethylesters by treatment with hydroxylamine.[42] Detailed procedures have been described.[41] Briefly, the esters were converted to sodium dihydroxamates using an excess of hydroxylamine and sodium methoxide in dry methanol. The free DHAs were liberated from aqueous solutions of dihydroxamates on a weakly acidic catex column in H^+-form. Lyophilization of eluates and drying over P_2O_5 provided the final DHAs in good yields (80−90%). The final preparation of compound **4** was roughly 85% pure.

1-Hydroxy-2,5-pyrrolidinedione (*N*-hydroxysuccinimide) was identified as a minor side-product (about 8 molar %) formed during the synthesis of **3**. It was purchased in pure form from Sigma Aldrich. A freshly prepared solution of *N*-hydroxysuccinimide (5 mM) did not significantly inhibit mSR.

Detailed NMR spectral data has been published elsewhere for compounds **1**−**6**.[41,43] NMR parameters for the predominant *Z*-conformers are given here.

Oxalodihydroxamic acid (**1**): ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.47 (bs, 2H), 9.16 (bs, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 157.02; ^{15}N NMR (50 MHz, DMSO-d_6) δ −212.5.

Malonodihydroxamic acid (**2**): ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.39 (bs, 2H), 8.93 (bs, 2H), 2.75 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.65 (CO), 38.46 (CH_2); ^{15}N NMR (50 MHz, DMSO-d_6) δ −213.4.

Succinodihydroxamic acid (**3**): ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.36 (bs, 2H), 8.69 (bs, 2H), 2.17 (s, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.38 (CO), 27.99 (CH_2); ^{15}N NMR (50 MHz, DMSO-d_6) δ −216.1.

Glutarodihydroxamic acid (**4**): ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.36 (bs, 2H), 8.67 (bs, 2H), 1.94 (t, J = 7.2 Hz, 4H), 1.70 (p, J = 7.2 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.88 (CO), 31.92 (CH_2, 2C), 21.58 (CH_2, 1C); ^{15}N NMR (50 MHz, DMSO-d_6) δ −216.1.

Adipodihydroxamic acid (**5**): ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.34 (bs, 2H), 8.68 (bs, 2H), 1.93 (unresolved multiplet, 4H), 1.4 (unresolved multiplet, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.14 (CO), 32.28 (CH2), 25.02 (CH2); ^{15}N NMR (50 MHz, DMSO-d_6) δ −215.0.

Suberodihydroxamic acid (**6**): ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.37 (bs, 2H), 8.71 (bs, 2H), 1.92 (t, J = 7.4 Hz), 1.45 (unresolved multiplet, 4H), 1.19 (unresolved multiplet, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.26 (CO), 32.41, 28.50, 25.21; ^{15}N NMR (50 MHz, DMSO-d_6) δ −215.2.

Mouse Serine Racemase Preparation. Recombinant mouse serine racemase was prepared as previously described.[8] Briefly, calcium chloride competent *E. coli* MC1061 cells were transformed with pKS-mSR.[8] Cell cultures were grown in nutrient-rich medium at 37 °C and 300 rpm. Serine racemase expression was induced by 1 mM L-arabinose (Sigma) at OD_{600} = 0.8. After 5 h of induction, bacteria were harvested by centrifugation (10000*g*, 10 min, 4 °C) and stored at −70 °C.

The purification procedure was carried out in QA buffer [20 mM triethanolamine hydrochloride−NaOH, pH 7.0, 1 mM $MgCl_2$, 20 μM PLP, 0.1 mM D,L-dithiothreitol, and 0.02% (w/v) NaN_3]. Pellets of induced bacteria were suspended in QA buffer supplemented with 1 mM phenylmethanesulfonyl fluoride, 0.2 mg/mL chicken egg lysozyme, and 0.05% (w/v) sodium deoxycholate and homogenized using a glass Dounce homogenizer. After the addition of 10 mM $MgCl_2$ and DNase I (Roche Molecular Biochemicals) to 10 μg/mL, the cell suspension was sonicated and centrifuged at 20000*g* for 30 min at 4 °C.

The supernatant was subjected to the following three chromatographic steps: reverse phase (Phenyl-Sepharose FastFlow, Pharmacia), ion-exchange (Q-Sepharose, FastFlow, Pharmacia), and affinity chromatography (ATP-agarose, Sigma). The resulting 90% pure protein preparation was dialyzed three times against 200 volumes of QA buffer at 4 °C, concentrated to 1 mg/mL (as determined by quantitative amino acid analysis), and stored at −70 °C.

SR Inhibition Screening. Inhibition of mSR was analyzed as described.[8] Briefly, the racemization reactions were performed in a pH 8.0 buffer supplemented with 10 μM PLP, 1 mM ATP, 1 mM $MgCl_2$, and 5 mM DTT. For the initial screening, a 5 mM concentration of both the substrate L-serine and the test compound was used. Reactions proceeded for 30 min at 37 °C and were stopped by the addition of 0.3 M $HClO_4$. After derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent), the substrate L-serine and the product D-serine were resolved and detected by HPLC.

For the determination of IC_{50} values, various inhibitor concentrations were used while the L-serine concentration was kept constant at 5 mM, and the data were analyzed with GraFit 5 software.[19] For the determination of the mechanism of inhibition, 4 or 5 substrate concentrations (from 1.25 to 12 mM L-serine) and a minimum of 2 inhibitor concentrations and a noninhibited reaction set were used. We performed a preliminary experiment with multiple reaction time points in order to choose reaction times that approach the initial velocity of the enzymatic reaction and result in detectable production of D-serine (not shown). The reaction time of the experiments presented here ranged from 8 to 15 min, and the conversion of L-serine to D-serine was 0.5−2%. All reactions were performed in triplicate, and the averages and corresponding standard deviations were analyzed with DynaFit4 software, which calculates the least-squares nonlinear regressions and compares the possible mechanisms of action.[18] The K_i values were calculated with DynaFit4 from one representative independent experiment. The following inhibitor concentrations were used for the determination of inhibitory constants: **2** (0, 150, and 400 μM), **3** (0, 7.5, 10, and 30 μM), and **10** (0, 100, and 800 μM). Lineweaver−Burk and Eadie−Hofstee transformations were performed with GraFit5 software.[19]

Inhibition Screening with Other PLP-Dependent Enzymes. Alanine racemase from *Bacillus stearothermophilus* (AR) and broad-range bacterial transaminase (TA) were purchased from Sigma Aldrich as lyophilized powders.

The plasmid pCW-SDH, which encodes rat liver serine dehydratase (SDH), was a generous gift from H. Ogawa.[20] To express SDH, pCW-SDH was transformed into *E. coli* BL21-RIL cells, and the cells were cultured in 1 L of Luria−Bertani broth at 37 °C. When the culture reached an OD_{600} of approximately 0.5, IPTG was added to a final concentration of 0.5 mM. The cells were induced for 17 h at 37 °C, then harvested at 2000*g* for 15 min at 4 °C. The cell pellet was resuspended in 20 mL of 20 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA and 5 mM DTT. Ten milligrams of lysozyme and one protease inhibitor tablet (Complete Mini EDTA free, Roche) were added, and cells were subjected to freeze/thaw and sonication. The soluble fraction was clarified by centrifugation, and SDS−PAGE analysis of the supernatant revealed that SDH was heavily overexpressed. To partially purify SDH, the lysate was diluted with buffer to a final volume of 25 mL, and 5 g of ammonium sulfate was added. After incubation at 4 °C for 1 h, the SDH-containing precipitate was separated by centrifugation and then redissolved in lysis buffer. This resulted in an SDH preparation of about 50% purity, as judged by SDS−PAGE stained by Coomassie Brilliant Blue G-250.

The preparation of recombinant human serine racemase has been described,[14] and its activity was assayed according to the protocol for mouse SR described above.

AR, SDH, and TA were assayed in 50 mM phosphate buffer, pH 8.0, containing 10 μM PLP. The enzymes were preincubated with 5 mM inhibitor at room temperature for 15−20 min. Reactions were started by the addition of substrate at a concentration close to the K_M of the enzyme (5 mM D-alanine for AR, 50 mM L-serine for SDH, and 5 mM L-aspartic acid and 5 mM α-ketoglutarate for TA). Reactions were allowed to proceed for 10−40 min at 50 °C (AR) or 37 °C (SDH and TA). Reactions were quenched by the addition of TCA to a final

concentration of 5% (w/v), and protein pellets were separated by centrifugation. The supernatants were extracted twice with water-saturated diethyl ether prior to derivatization. SDH reaction mixtures were derivatized with 2,4-dinitrophenylhydrazine (DNPH), and pyruvate formation was monitored on HPLC as previously described.[8,44] AR and TA reaction mixtures were derivatized with FDAA and separated according to the protocol for separation of L- and D-serine described above. AR mixtures were analyzed for the formation of L-alanine, and TA mixtures were analyzed for the formation of L-glutamic acid. Pure L-alanine, D-alanine, L-aspartic acid, and L-glutamic acid were derivatized and used as calibration standards.

NMR Measurements. An approximately 8 mM stock solution of PLP was prepared in 100 mM phosphate buffer in D_2O, pD 8.4. An appropriate amount of hydroxamic acid (chosen to ensure an approximately 1:1 molar ratio between the acid and PLP) was weighed out and mixed into 2 mL of PLP stock solution. The final pD was adjusted to 7.1−8.1 by the addition of NaOH powder. For ^{13}C NMR measurements (see Supporting Information), an approximately 10-fold higher PLP concentration was used.

Mass Spectrometry. A 4, 5, or 30 mM solution of PLP (pH adjusted to 8.5 with NaOH) was mixed with a 10-fold molar excess of the test compound and incubated at room temperature for one day prior to measurement. Mixtures were prepared both with and without a pH control (200 mM ammonium sulfate, pH 8.0). The mixture was diluted in water 40- to 100-fold and subjected to ESI-MS in a negative ion mode.

Spectrophotometric Assay for the Reaction of PLP with Hydroxamic Acids. A 0.6 mM solution of pyridoxal-5′-phosphate and 10 mM solutions of test compounds were prepared in 100 mM phosphate buffer, pH 8.0. The PLP and inhibitor solutions were mixed in a 1:1 ratio so that the final concentration of PLP was 300 μM, and the final concentration of inhibitor was 5 mM. The reaction mixtures were incubated at room temperature for 1 h prior to measurement. Absorbance spectra were collected in the 310−500 nm range.

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Supporting Information Available: Synthetic procedures for compounds **11**−**14** and additional spectroscopic measurements (NMR and UV/vis).

This material is available free of charge via the Internet at http://pubs.acs.org.

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