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Design and Synthesis of Lignostilbene-α,β-dioxygenase Inhibitors

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Abstract—Lignostilbene-α,β-dioxygenase cleaves the olefinic double bond of phenolic stilbenes by a mechanism similar to that of 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis. Several analogues of stilbene were designed and synthesized, and their efficacy as inhibitors of lignostilbene-α,β-dioxygenase was examined. The compound (*Z*)-1-(4-hydroxyphenyl)-1-fluoro-2-phenylethene (**2**) was found to be a potent inhibitor of this enzyme with an IC_{50} of 3 μM.

Lignostilbene-α,β-dioxygenase (LSD, EC 1.13.11.43) is an enzyme of functional interest from *Pseudomonas paucimobilis* TMY 1009.[1,2] LSD is involved in oxidative cleavage of the central double bond of stilbene-type intermediates arising from degradation of dimeric lignin model compounds. LSD catalyzes the reaction of lignostilbene and molecular oxygen to form the corresponding aldehydes, as illustrated in Figure 1. This reaction is closely analogous to the carotenoid cleavage reaction catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) in biosynthesis of the plant hormone abscisic acid (ABA), which performs several specific functions in plant growth and development. The LSD and NCED reactions require molecular oxygen and an Fe cofactor, and they each yield two molecules of aldehyde from one substrate molecule.[3] In addition, the protein sequences of NCED and LSD are similar. Thus, the LSD and NCED are thought to comprise a novel class of dioxygenases that catalyze similar double-bond cleavage reactions.[3,4] Drought-induced *NCED* genes encoding NCED-like enzymes were subsequently isolated from in tomato, Arabidopsis, bean, cowpea, and avocado. Furthermore, these studies have provided the hypothesis that the cleavage of 9-*cis*-epoxycarotenoids by NCED is a key regulatory step in ABA biosynthesis.[5]

Organisms possessing mutations in biosynthetic pathways are useful in investigations of the physiological functions of endogenous substances. Inhibitors specific for the biosynthetic pathway of interest also have been used for this purpose. Likewise, inhibitors of LSD or NCED would be valuable for investigating the physiological functions of these enzymes. The design of specific enzyme inhibitors is greatly aided by prior knowledge of the enzyme reaction mechanism and the structure(s) of the substrate(s), but the structures of double-bond cleaving dioxygenase active sites are not known, and no specific inhibitors have been found. Therefore, we used the structure of the stilbene substrate of LSD as a starting point for the design of LSD inhibitors. We selected LSD as our target because

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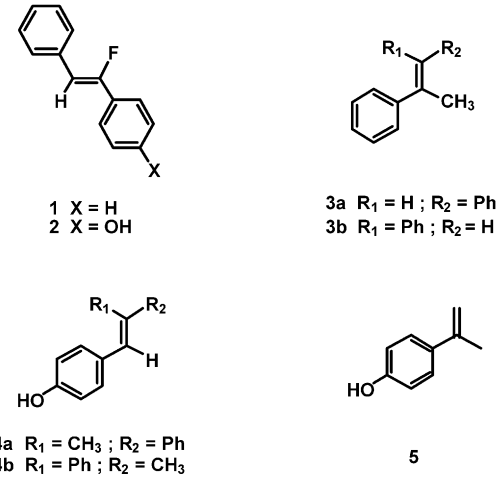
(1) the simple structure of the LSD substrate suggested that the design and synthesis of LSD inhibitors would be relatively straightforward, and (2) the reaction catalyzed by LSD is analogous to that catalyzed by NCED, so information gleaned from LSD inhibitors is likely to be useful in design of NCED inhibitors. In this report, we describe the synthesis of various stilbene analogues and their inhibitory activity against LSD.

Lignostilbene-α,β-dioxygenase was obtained by a method previously described.[1] Transformed *Escherichia coli* MV1184 cells were grown aerobically at 26.5 °C in 500 mL of YT medium (8 g/L Bacto tryptone, 5 g/L Bacto yeast extract, 5 g/L NaCl, pH 7.6) containing 150 μg/mL ampicillin in a 3-L flask. After 12 h of cultivation, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM to induce expression. Cells were shaken for an additional 6 h at 37 °C and then harvested by centrifugation. The cell pellet was resuspended in 20 mL of 20 mM sodium phosphate buffer (pH 7.5), disrupted by ultrasonication, and centrifuged at 20,000 *g* for 30 min. The resulting supernatant fraction was used in enzyme assays. Protein concentration was determined by the method of Lowry, using bovine serum albumin as the standard. LSD activity was assayed at 30 °C in 2 mL of 50 mM Tris–HCl buffer (pH 8.5) containing a suitable amount of enzyme solution (4 μg/mL = 1 U) and various concentrations of inhibitor. Reactions were started by addition of the substrate 4-*trans*-hydroxystilbene to the indicated concentration. Enzymatic activity was estimated by detection of the 4-hydroxybenzaldehyde product at 280 nm by HPLC on a Shiseido Capcell Pak C18 column (4.6×250 mm), using a solvent system of acetonitrile/water (80:20, v/v) at a flow rate of 0.7 mL/min. IC_{50} is the concentration of inhibitor that causes 50% inhibition of the reaction.

Stilbene analogues were synthesized according to the methods described below and evaluated for their inhibitory activity against LSD (Table 1). The fluoro olefin

Table 1. Inhibition of lignostilbene-α,β-dioxygenase with stilbene analogues



Compd	IC_{50} (μM)[a]
1	>100
2	3
3a	>100
3b	22
4a	15
4b	7
5	12

[a]LSD activity was assayed at 30 °C in 2 mL of 50 mM Tris–HCl buffer (pH 8.5) containing a suitable amount of enzyme solution (4 μg/mL = 1U). Reactions were started by addition of the substrate 4-*trans*-hydroxystilbene to a final concentration of 10 μM. IC_{50} values were determined by adding the enzyme to mixtures of substrate and inhibitor.

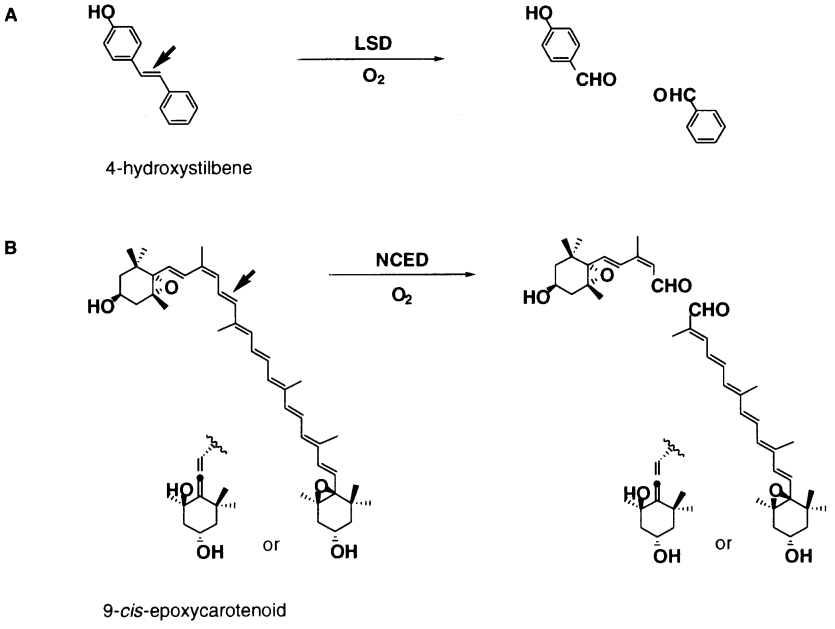


Figure 1. Oxidative cleavage of central double bonds by dioxygenases. Arrows indicate the cleaved bonds: (a) lignostilbene-α,β-dioxygenase (LSD);[1] (b) 9-*cis*-epoxycarotenoid dioxygenase (NCED).[4]

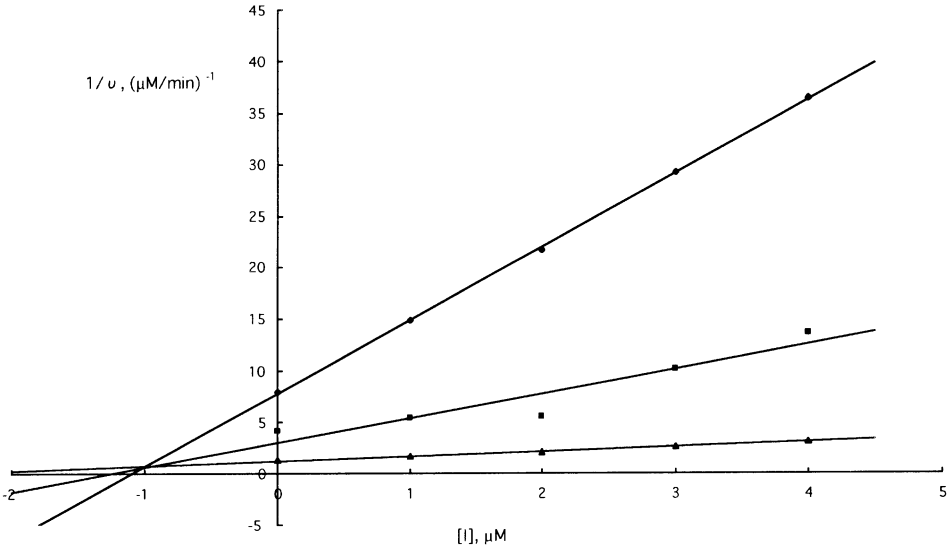


Figure 2. Kinetic analysis of the inhibition of lignostilbene-α,β-dioxygenase by (*Z*)-1-(4-hydroxyphenyl)-1-fluoro-2-phenylethene (**2**). Lignostilbene-α,β-dioxygenase activity was measured in the presence of 1.25 μM (◆), 5 μM (■), and 20 μM (▲) 4-hydroxystilbene using the indicated concentrations of (*Z*)-1-(4-hydroxyphenyl)-1-fluoro-2-phenylethene (**2**). The inhibition constant (K_i) was determined to be 0.98 μM from a Dixon plot.

group has useful functionality in designing mechanism-based enzyme inhibitors since the fluorine can mimic a hydrogen atom while creating a different electronic environment.[6–8] Thus, in this research, the fluoro olefins **1** and **2** were designed as inhibitors of LSD (Table 1). In the synthesis of (*Z*)-1-fluoro-1, 2-diphenylethenes (**1**, **2**), the first step is a fluoro Pummerer reaction. Fluoromethyl phenyl sulfide was prepared and then readily oxidized to sulfone with 3-chloroperbenzoic acid at room temperature. 1-Fluorovinyl phenyl sulfone was synthesized by condensation of the fluoromethyl phenyl sulfone with benzaldehyde. Reaction of the 1-fluorovinyl phenyl sulfone with 2 equivalents of tributyltin hydride in refluxing benzene afforded the required 1-fluorostannane. Coupling of 1-fluorovinylstannane to iodobenzene under standard conditions gave the expected cross-coupled product (*Z*)-1-fluoro-1, 2-diphenylethenes (**1**, **2**). The *Z* conformation of fluorostilbene was established on the basis of the $^3J_{H-F}$ coupling constant of about 39 Hz for *trans* coupling by proton NMR.[9–13] Fluoro olefin **2** is a potent inhibitor ($IC_{50} = 3$ μM), whereas **1** is substantially less active ($IC_{50} > 100$ μM). At least a 33-fold increase in potency was observed when the 4-hydrogen atom on the phenyl ring was replaced with a hydroxy group. 4-Hydroxystilbene was cleaved faster than *trans*-stilbene as a substrate of LSD (data not shown), suggesting that the 4-hydroxy group should be involved in specific favorable interaction(s) between the substrate and the enzyme active site. In this context, the 4-hydroxy group in the phenyl ring of the inhibitor was expected to play a role in enhancing specific interaction(s) with the enzyme active site. Bonding to a sp^2-carbon reduces the electron density at the carbon of a C–F bond but increases the electron density at the β-carbon. The electronic effects of fluorine bonded to a sp^2-carbon were identified by the chemical shift of the carbon in the C–F bond (δ 159.0, d, $J = 255.0$ Hz) and at the β-carbon (δ 104.3, d, $J = 9.9$ Hz) by ^{13}C NMR.[9] Thus, the polarization of the double bond by fluorine explains its effect on the interaction of the inhibitor with the enzyme.

Next, we replaced the hydrogen on the double bond between the two phenyl rings with a methyl group and evaluated these *cis*- and *trans*-stilbene derivatives as LSD inhibitors. 1,2-Diphenylpropene (**3**) was prepared by the reaction of diethyl benzylphosphate with acetophenone in the presence of sodium hydride.[14] 4-(2-Phenyl-1-propenyl)-phenol (**4**) was synthesized by palladium-catalyzed coupling of α-methylstyrene with 4-iodophenol.[15] The *E* and *Z* isomers were separated by HPLC on a Chiralpak AD column (*n*-hexane/isopropanol = 19:1). The (*Z*)-isomers **3b** and **4b** were 4.5- and 2-fold more potent than the corresponding (*E*)-isomers **3a** and **4a**, respectively. Furthermore, when a hydrogen at the 4-position of the aromatic ring was replaced with a hydroxy group, the inhibitory potency of the resulting **4a** and **4b** was increased by up to 6.7- and 3-fold compared with **3a** and **3b**, respectively. This result is similar to that observed in the case of fluorinated olefins **1** and **2**. The difference in activity of geometrical isomers of **3** and **4** suggests that the geometry of the olefin or phenyl ring moiety plays an important role in LSD inhibition. α-Methyl-4-methoxystyrene (**5**) was also active as an LSD inhibitor ($IC_{50} = 12$ μM).[16] These results suggest that the 4-hydroxystyrene group has important functionality in inhibition of LSD.

The K_m value of LSD was determined to be 8.3 μM by varying the substrate concentration from 1.25 to 20 μM. The data in Figure 2 demonstrate that the inhibition of LSD by (*Z*)-1-(4-hydroxyphenyl)-1-fluoro-2-phenylethene (**2**) was competitive with 4-hydroxystilbene, with a K_i of 0.98 μM. In summary, we prepared stilbene analogues, tested them for inhibition of LSD, and found **2** to be a potent inhibitor of LSD. Effective inhibition of LSD requires not only polarization of the double bond but also the *Z* configuration, which limits the delocali-

zation of π-electrons. In addition, the presence of the hydroxy group at the 4-position of the phenyl group is crucial for potent LSD inhibition.

To examine whether **2** could inhibit NCED, we performed an enzymatic assay of NCED as described. [17] As a result, **2** was not effective in inhibiting NCED activity (data not shown). The lack of the inhibitory activity of this LSD inhibitor seems to be due to the lack of the affinity to NCED enzyme. The information provided in this study may allow us to define the specific binding characteristics of the LSD-substrate and LSD-inhibitor complexes, and may be useful in revealing mechanistic details for this intriguing enzyme. In addition, fluorination or methylation of the 9- and/or 10-positions of 9-cis epoxycarotenoid may be important in the design of NCED inhibitors, which could then be developed as specific inhibitors of ABA biosynthesis.

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References and Notes

1. Kamoda, S.; Saburi, Y. *Biosci. Biotech. Biochem.* **1993**, *57*, 926.
2. Habu, N.; Samejima, M.; Yoshimoto, T. *Mokuzai Gakkaishi* **1988**, *34*, 1026.
3. Tan, B. C.; Schwartz, S. H.; Zeevaart, J. A. D.; McCarty, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12235.
4. Schwartz, S. H.; Tan, B. C.; Gage, D. A.; Zeevaart, J. A. D.; McCarty, D. R. *Science* **1997**, *276*, 1872.
5. (a) Burbidge, A.; Grieve, T.; Jackson, A.; Thompson, A.; Taylor, I. *J. Exp. Bot.* **1997**, *48*, 2111. (b) Neill, S. J.; Burnett, E. C.; Desikan, R.; Hancock, J. T. *J. Exp. Bot.* **1998**, *49*, 1893. (c) Qin, X.; Zeevaart, J. A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 15354. (d) Iuchi, S.; Kobayashi, M.; Yamaguchi-Shinozaki, K.; Shinozaki, K. *Plant Physiol.* **2000**, *123*, 553. (e) Chernys, J. T.; Zeevaart, J. A. D. *Plant Physiol.* **2000**, *124*, 343.
6. Moore, W. R.; Schatzman, G. L.; Jarvi, E. T.; Gross, R. S.; McCarthyl, J. R. *J. Am. Chem. Soc.* **1992**, *114*, 360.
7. McCarthy, J. R.; Matthews, D. P.; Stemerick, D. M.; Huber, E. W.; Bey, P.; Lippert, B. J.; Snyder, R. D.; Sunkara, P. S. *J. Am. Chem. Soc.* **1991**, *113*, 7439.
8. Ling, T.-S.; Shiu, S.; Yang, D.-Y. *Bioorg. Med. Chem.* **1999**, *7*, 1459.
9. (*Z*)-1-(4-Hydroxyphenyl)-1-fluoro-2-phenylethene (2); white solid, mp 168–169 °C, ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.64 (2H, d, J = 7.3 Hz), 7.57 (2H, d, J = 8.8 Hz), 7.40 (2H, dd, J = 7.8, 7.4 Hz), 7.27 (1H, m), 6.89 (2H, d, J = 8.8 Hz), 6.20 (1H, d, J = 39.8 Hz), 5.08 (1H, s, OH). ^{13}C NMR (CD_3OD, 100 MHz) δ 104.3 (d, J = 9.9 Hz), 125.4 (d, J = 28.0 Hz), 126.9 (d, J = 8.2 Hz), 127.8 (d, J = 3.3 Hz), 129.5, 129.6, 129.7, 135.6 (d, J = 3.3 Hz), 159.0 (d, J = 255.0 Hz), 159.8. HRMS (EI) m/z (M^+): Calc for $C_{14}H_{11}FO$: 214.0794. Found: 214.0753.
10. McCarthy, J. R.; Peet, N. P.; LeTourneau, M. E.; Inbasekaran, M. *J. Am. Chem. Soc.* **1985**, *107*, 735.
11. McCarthy, J. R.; Matthews, D. P.; Edwards, M. L.; Stemerick, D. M.; Jarvi, E. T. *Tetrahedron Lett.* **1990**, *31*, 5449.
12. Chen, C.; Wilcoxen, K.; Kim, K.-I.; McCarthy, J. R. *Tetrahedron Lett.* **1997**, *38*, 7677.
13. Chen, C.; Wilcoxen, K.; Zhu, Y.-F.; Kim, K.-I.; McCarthy, J. R. *J. Org. Chem.* **1999**, *64*, 3476.
14. Wadsworth, W. S., Jr.; Emmons, W. D. *J. Am. Chem. Soc.* **1961**, *83*, 1733.
15. Jeffery, T. *Tetrahedron* **1996**, *52*, 10113.
16. Stevenes, R. V.; Albizati, K. F. *J. Org. Chem.* **1985**, *50*, 632.
17. Iuchi, S.; Kobayashi, M.; Taji, T.; Naramoto, M.; Seki, M.; Kato, T.; Tabata, S.; Kakubari, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K. *Plant J.* **2001**, *27*, 325.