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N-Benzylideneaniline and *N*-Benzylaniline are Potent Inhibitors of Lignostilbene-α,β-dioxygenase, a Key Enzyme in Oxidative Cleavage of the Central Double Bond of Lignostilbene

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Lignostilbene-α,β-dioxygenase (LSD, EC 1.13.11.43) is involved in oxidative cleavage of the central double bond of lignostilbene to form the corresponding aldehydes by a mechanism similar to those of 9-*cis*-epoxycarotenoid dioxygenase and β-carotene 15,15′-dioxygenase, key enzymes in abscisic acid biosynthesis and vitamin A biosynthesis, respectively. In this study, several *N*-benzylideneanilines and amine were synthesized and examined for their efficacy as inhibitors of LSD. *N*-(4-Hydroxybenzylidene)-3-methoxyaniline was found to be a potent inhibitor with IC_{50} = 0.3 μM and *N*-(4-hydroxybenzyl)-3-methoxyaniline was also active with IC_{50} = 10 μM. The information obtained from the structure-activity relationships study here can aid in discovering inhibitors of both abscisic acid and vitamin A biosynthesis.

Keywords: 9-*cis*-epoxycarotenoid dioxygenase; Lignostilbene-α, β-dioxygenase; Lignostilbene

INTRODUCTION

Lignostilbene-α,β-dioxygenase (LSD, EC 1.13.11.43) catalyzes the oxidative cleavage reaction of the central double bond of stilbene-type intermediates arising from degradation of dimeric lignin model compounds.[1,2] The LSD reaction requires molecular oxygen and a Fe cofactor and it yields two molecules of the corresponding aldehydes from one molecule of the substrate, as illustrated in Figure 1. The protein sequence and the reaction mechanism of the LSD are closely similar to those of 9-*cis*-epoxycarotenoid dioxygenase (NCED) existing in the biosynthesis pathway of the plant hormone abscisic acid (ABA), which performs several specific functions in plant growth and development. Thus, 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 tomato, Arabidopsis, bean, cowpea, and avocado. These studies have provided the hypothesis that the cleavage of 9-*cis*-epoxycarotenoids by NCED is a key regulatory step in ABA biosynthesis.[5–9] While mouse β-carotene 15, 15 β-dioxgenase (β-CD), which catalyzes the cleavage of β-carotene to yield a vitamin A precursor retinal and retinoic acid, is reported to belong to an extended family of dioxygenases including bacterial LSD and plant NCED.[10] Vitamin A is also essential for embryonic development,[11] pattern formation[12,13] and vision.[14]

There are no reports of the mechanism and the structural characterization of the active sites of these

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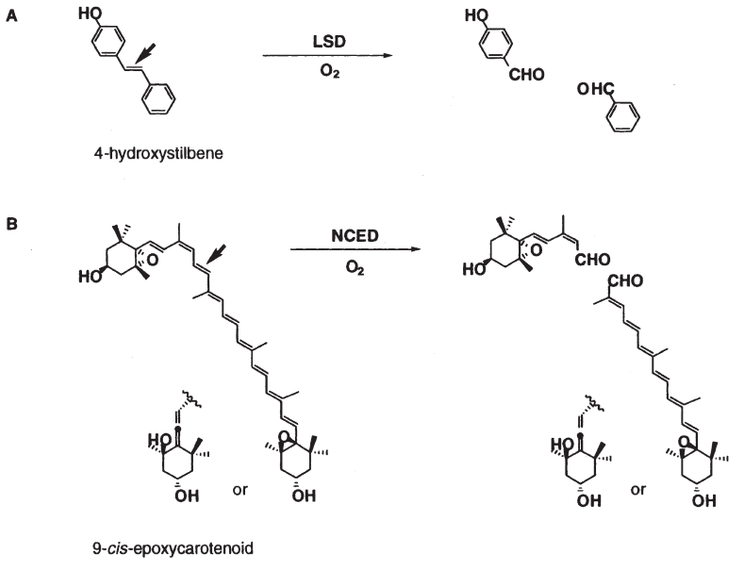


FIGURE 1 Oxidative cleavage of central double bonds by dioxygenases. Arrows indicate the cleaved bonds. A: Lignostilbene-α, β-dioxygenase (LSD). B: 9-*cis*-Epoxycarotenoid dioxygenase (NCED).

double-bond cleaving dioxygenases. The design of specific enzyme inhibitors has played an essential role in the investigation of the reaction mechanism and the structure of the active site.[15,16] Moreover, biosynthesis inhibitors of active molecules provided a useful way to find mutants in which genes involved in signal transduction of active molecules were altered.[17–20] In this context, both abscisic acid and vitamin A biosynthesis inhibitors can also play a role in shedding light on the signal transduction of abscisic acid and vitamin A, respectively. We initially, selected LSD as our target in order to discover specific inhibitors of LSD, NCED or β-CD because, (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 or β-CD. The information gleaned from LSD inhibitors is likely to be useful in design of NCED or β-CD biosynthesis inhibitors. In this report, the structure of the stilbene substrate of LSD was used as a starting point for the design of LSD inhibitors. The synthesis of *N*-benzylideneanilines and amine as LSD inhibitors is described, and their inhibitory activity against LSD.

MATERIALS AND METHODS

Materials

Chemicals and reagents were purchased from Wako Pure Chem. Ind. Co., LTD. or Kanto Chemical Co., INC. Japan. 4-Hydroxystilbene was purchased from Aldrich Chemical Co., and 9-*cis*-epoxycarotenoid was purified from spinach leaves.[8]

Preparation of *N*-benzylideneanilines and Amine

N-benzylideneanilines were prepared by condensation of the appropriate amines with benzaldehydes using a Dean and Stark apparatus according to methods previously described (Figure 2).[21,22] The residue was recrystallized from methanol and was then used without further purification. Sodium borohydride reduction of *N*-(4-hydroxybenzylidene)-3-methoxyaniline (**7**) in ethanol under reflux provided the desired *N*-(4-hydroxybenzyl)-3-methoxyaniline (**13**) as a white solid after recrystallization from ethanol. All compounds were identified by ^{1}H-NMR and elemental analysis.

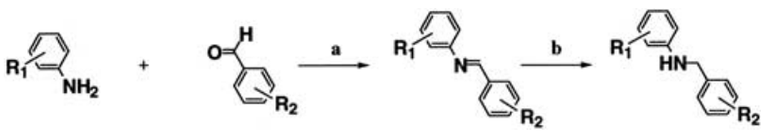


FIGURE 2 (a) Benzene, reflux (4 h); (b) $NaBH_4$, ethanol, reflux (2 h).

N-(4-hydroxybenzylidene)-3-methoxyaniline (7)

Mp: 197–198°C; ^{1}H-NMR: (CD_3OD, 300 MHz) δ 8.44 (s, 1H), 7.80 (d, $J = 8.7$ Hz, 2H), 7.31 (m, 1H), 6.91 (d, $J = 8.7$ Hz, 2H), 6.83–6.78 (m, 3H), 3.84 (s, 3H); Anal. Found: C, 73.86; H, 5.82; N, 6.10. Calcd for $C_{14}H_{13}NO_2$: C, 73.99; H, 5.77; N, 6.16%.

***N-(4-hydroxybenzyl)-3-methoxyaniline* (13)**

Mp: 122–123°C; ^{1}H-NMR (CD_3OD, 300 MHz) δ 7.20 (d, $J = 8.55$ Hz, 2H), 6.99 (m, 1H), 6.76 (d, $J = 8.55$ Hz, 2H), 6.29–6.19 (m, 2H → 3H), 4.18 (s, 2H), 3.71 (s, 3H); Anal. Found: C, 73.13; H, 6.63; N, 6.07. Calcd for $C_{14}H_{15}NO_2$: C, 73.34; H, 6.59; N, 6.11%.

***N-(4-hydroxybenzyl)-3-methoxyaniline* (13)**

It is a novel compound but the others in this report have been reported elsewhere.

Lignostilbene-α,β-dioxygenase Assay

LSD was prepared from transformed *Escherichia coli* MV1184 cells by a method previously described.[1] Protein concentration was determined using a Bio-Rad kit based on the Lowry method. The LSD activity was tested 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 inhibitors. Substrate and inhibitors were dissolved in dimethylsulfoxide (DMSO) and added to the buffer so that the final DMSO concentration was ca. 0.2% (v/v). Reactions were started by addition of the substrate 4-*trans*-hydroxystilbene at the indicated concentration. The reaction mixture was incubated for 10 min and the reaction stopped by the addition of 500 μl 0.1N HCl. Enzymatic activity was estimated by detection of the product, 4-hydroxybenzaldehyde, at 280 nm by HPLC on a Shiseido CAPCELL PAK C18 column (4.6 mm × 250 mm) using a solvent system of acetonitrile:water (80:20, v/v) at a flow rate of 0.6 ml/min. IC_{50} is the concentration of inhibitor that caused 50% inhibition of the reaction.

9-*cis*-Epoxycarotenoid Dioxygenase Assay

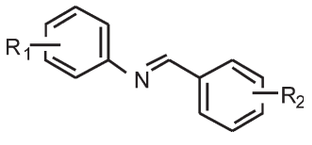
The purification and the assay of the enzymatic activity of NCED were performed as described.[23] Appropriate amounts of protein and substrate were added in a total volume of 100 μl. The predicted C_{25} compound was identified by HPLC on a column of Pegasil-B ODS (150 mm length, 6 mm inner diameter; Senshu Scientific, Tyoko, Japan). The column was eluted with a linear gradient between solvent A (80:20 v/v, methanol:water) and solvent B (1:1 v/v, chloroform:methanol) at a flow rate of 1.5 ml/min. The concentration of solvent B was increased from 10 to 50% in 25 min, and kept at 50% for 5 min. The substrate carotenoid and C_{25} products were monitored with an UV/visible detector at a wavelength of 440 nm.

RESULTS AND DISCUSSION

In our previous report concerning the preparation and activity of stilbene analogues, compound **12** was found to be an inhibitor of LSD.[24] The polarization of the double bond by fluorine could likely explain its effect on the interaction of the inhibitor with the enzyme. The assumption that polarization of the double bond was required for the effective inhibition of LSD has led us to begin a biological and biochemical evaluation of *N*-benzylideneaniline, in which one of the carbon atoms forming the double bond of stilbene was substituted with a nitrogen atom. *N*-benzylideneanilines have been synthesized and studied as stilbene-like species, which have a structural similarity but have a marked dissimilarity with stilbene with regard to electronic properties.[25,26] They have also been synthesized and evaluated as tubulin polymerization inhibitors or human cytochrome P450 1B1 inhibitors.[22,27,28]

N-benzylideneanilines (**1**–**8**) and the amine (**13**) were evaluated for their inhibitory activity against LSD and were found to be active as potent inhibitors of LSD except for *N*-benzylideneaniline (**1**) (Table I).

TABLE I Inhibition of lignostilbene-α,β-dioxygenase with *N*-benzylideneanilines and an amine



Compound	R_1	R_2	IC_{50} (μM)[a]
1	H	H	100
2	4-OH	H	1
3	H	4-OH	0.9
4	4-OH	4-OH	0.7
5	4-OCH_3	4-OH	0.8
6	3-OH	4-OH	0.5
7	3-OCH_3	4-OH	0.3
8	4-OH	3-OCH_3	0.5
9	3-Methoxyaniline		Inactive
10	4-Hydroxybenzaldehyde		Inactive
11	(*Z*)-1-Fluoro-1,2-diphenylethene		>100
12	(*Z*)-1-Fluoro-1-(4-hydroxyphenyl)-2-phenylethene		3
13	*N*-(4-Hydroxybenzyl)-3-methoxyaniline		10

[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 = 1 U). 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. The experiment was repeated three times and the standard error for each value was within ±10% of the mean.

At least a 100-fold increase in potency was observed when the 4-hydrogen atom on the phenyl ring of **1** ($IC_{50} = 100\,\mu M$) was replaced with a hydroxy group (**2** and **3**). This result is similar to that observed in the case of fluorinated olefins (**11** and **12**). As a substrate of LSD, 4-hydroxystilbene was cleaved faster than *trans*-stilbene (data not shown). This result suggested that the 4-hydroxy group could be involved in specific favorable interaction(s) between the substrate and the enzyme active site. In this context, when the hydroxy group at position-3 of **6** was replaced with a methoxy group, the resulting compound **7** was more active as an inhibitor.

N-(4-Hydroxybenzylidene)-3-methoxyaniline (**7**) inhibited LSD with an IC_{50} value of $0.3\,\mu M$ and was 10-fold more active than the fluorinated olefin **12**. At $1\,\mu M$ concentration the enzyme activity was almost abolished (data not shown). We found that **7** was slowly hydrolyzed in a reaction solution, however **9** and **10**, which are the products of hydrolysis, did not inhibit the enzyme activity. When the imine moiety of **7** was reduced, the resulted *N*-(4-hydroxybenzyl)-3-methoxyaniline (**13**) was more stable than **7** but about 30-times less active. Although **13** still showed significant inhibitory activity, this decrease in activity could be ascribed to the distortion of its *E* conformation about the double bond, its molecular planarity or a change in electronic properties around the α–β bond.

In this report, when the ethylene bridge in the substrate was replaced with an imine linkage, the resultant imines possessed significant inhibitory activity. This result suggests that the imine linkage plays an important role in enhancing inhibitory activity. It was recently reported that the stable chelates of *N*-benzylideneaniline with metal ions were stronger inhibitors of bovine β-trypsin.[29] Therefore, one possible mechanism for how imines and amines inhibit the enzyme was expected to be due to the binding of the compound to the essential iron of the enzyme.

The K_m value of 4-hydroxystibene with LSD was found to be $8.3\,\mu M$ by varying the substrate concentration from $1.25–20\,\mu M$. The data in Figure 3 demonstrate that the inhibition of LSD by *N*-(4-hydroxybenzyl)-3-methoxyaniline (**13**) was competitive with a K_i value of $5.04\,\mu M$ from a Dixon plot.

To examine whether **7** and **13** could inhibit NCED, a key enzyme in abscisic acid (ABA) biosynthesis in plants, we performed assays of the enzymatic activity of NCED as described.[23] However, **7** and **13** they did not show any appreciable inhibitory effect on NCED activity. The lack of inhibitory activity of these LSD inhibitors seems to be due to the lack of affinity for NCED because the design of LSD inhibitors was based on the structure of LSD substrate.

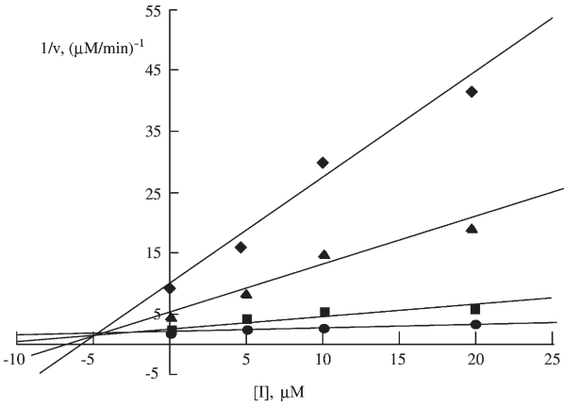


FIGURE 3 Kinetic analysis of the inhibition of lignostilbene-α, β-dioxygenase. Lignostilbene-α, β-dioxygenase activity was assayed in the presence of $1.25\,\mu M$ (◆), $5\,\mu M$ (▲), $10\,\mu M$ (■), and $20\,\mu M$ (●) 4-hydroxystilbene with the indicated concentrations of *N*-(4-hydroxybenzyl)-3-methoxyaniline (**13**). The inhibition constant (K_i) was determined as $5.04\,\mu M$ from the Dixon plot.

In summary, we prepared *N*-benzylideneanilines and an amine, tested them for inhibition of LSD, and found **7** to be a potent inhibitor of LSD. The reduced compound (**13**) of **7** was also active as an LSD inhibitor ($IC_{50} = 10\,\mu M$). The presence of the C = N bond or C–N bond instead of the C = C bond plays an important role in influencing inhibitory activity. 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. Furthermore, the design of inhibitors based on the structure of the cleavage products of 9-*cis*-epoxycarotenoid or β-carotene may provide a way to develop specific NCED or β-CD inhibitors of ABA or vitamin A biosynthesis, respectively.

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