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Lipoxygenase Inhibitory Activity of Octyl Gallate

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Octyl gallate inhibited soybean lipoxygenase-1 (EC 1.13.11.12, type I) with an IC_{50} of 1.3 μM. The inhibition of the enzyme by octyl gallate is a slow and reversible reaction without residual activity. The inhibition kinetics analyzed by Lineweaver−Burk plots indicates that octyl gallate is a competitive inhibitor, and the inhibition constant, K_I, was obtained as 0.54 μM. One molecule of octyl gallate scavenged six molecules of 1,1-diphenyl-2-picrylhydrazyl and inhibited autoxidative lipid peroxidation. In addition, octyl gallate was effective in preventing lipid peroxidation.

KEYWORDS: Soybean lipoxygenase; octyl gallate; competitive inhibitor; Lineweaver−Burk plots; antioxidant

INTRODUCTION

It is well-known that lipid peroxidation is one of the major factors in deterioration during the storage and processing of foods, because it can lead to the development of unpleasant rancid or off-flavors as well as potentially toxic end products (*1*). Lipoxygenases (EC 1.13.11.12) are a family of non-heme iron-containing dioxygenases widely distributed in both the animal and plant kingdoms. They catalyze the regio- and stereospecific oxygenation of polyunsaturated fatty acids, containing (*Z*,*Z*)-1,4-diene moieties, into the corresponding conjugated hydroperoxides. In plants, the main substrates are linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids, and the primary products of lipoxygeneses, 9*S* and 13*S* fatty acid hydroperoxides, are proposed to have regulatory roles in plant and animal metabolism (*2*). Due to their free radical nature, fatty acid hydroperoxides can be quite active by themselves and are capable of producing membrane damage and promoting cell death (*3*).

In food protection, browning is also deleterious to the color quality of plant-derived foods and beverages, and the browning process has two components in most foods: enzymatic and nonenzymatic oxidation. This unfavorable darkening from oxidation generally results in a loss of nutritional as well as market values. The enzymatic oxidation can be prevented by polyphenol oxidase inhibitors, and the nonenzymatic oxidation can be protected by antioxidants. In our continuing search for multifunctional additives for food protection, octyl gallate (**1**, see **Figure 1** for structure) was found to possess a broad antimicrobial spectrum (*4*), and it is currently permitted for use as an antioxidant additive in food (*5*). Hence, octyl gallate was tested for inhibition of soybean lipoxygenese-1.

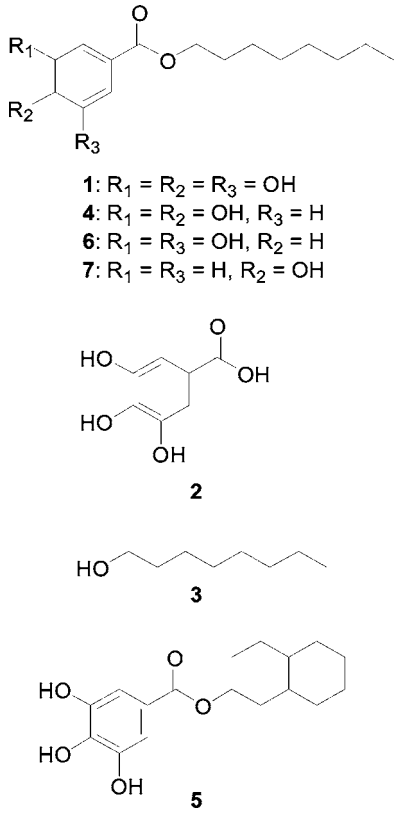

Figure 1. Chemical structures of octyl gallate and related compounds.

MATERIALS AND METHODS

Chemicals. Diisopropyl azodicarboxylate (DIAD), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3,4-dihydroxybenzoic acid, methanol, gallic acid (**2**), octanol (**3**), octyl gallate, triphenylphosphine (TPP), and nordihydroguaiaretic acid (NDGA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Octyl 3,4-dihydroxybenzoate (**4**) and decahydro-2-naphthyl gallate (**5**) were available from our previous work (*6*). Palladium hydroxide (20%) on carbon was purchased from Alfa Aesar (Ward Hill, MA). Linoleic acid (purity >99%) and soybean lipoxygenase (type I-B) were purchased from Sigma Chemical Co. (St.

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Louis, MO). Tris buffer was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Ethanol was purchased from Quantum Chemical Co. (Tuscola, IL).

Synthesis. Octyl 3,5-dihydroxybenzoate (**6**) was synthesized as follows. A mixture of 3,5-dibenzyloxybenzoic acid (200 mg, 0.60 mmol) (*7*), octanol (86 mg, 0.66 mmol), and TPP (190 mg, 0.72 mmol) in THF (4 mL) was cooled to 0 °C and treated with DIAD (146 mg, 0.72 mmol). After 2 h of stirring at room temperature, the solvent was removed in vacuo. The residue was subjected to silica gel chromatography eluted with 1−8% AcOEt/hexane to give an ester as a white solid, which was used in the next step without further purification. The ester was hydrogenated over 20% $Pd(OH)_2$ on carbon (10 mg) in 1% AcOH/AcOEt (4 mL) for 12 h. Filtration through Celite and concentration followed by silica gel chromatography (15−30% AcOEt/hexane) gave 155 mg (92%) of the title compound as a white solid: IR (CCl_4) 3350, 2920, 1680, 1595, 1335, 1240, 1160 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.13 (d, $J = 1.2$ Hz, 2H), 6.58 (t, $J = 1.2$ Hz, 1H), 5.54 (bs, 2H), 4.29 (t, $J = 6.4$ Hz, 2H), 1.74 (quin, $J = 6.4$ Hz, 2H), 1.41 (m, 2H), 1.28 (m, 8H), 0.88 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.1, 156.7, 132.4, 108.9, 107.3, 65.5, 31.8, 29.2, 29.1, 28.6, 26.0, 22.6, 14.1; EI-MS, *m*/*z* 266 (M^+).

According to basically the same procedure, octyl 4-hydroxybenzoate (**7**) was synthesized as a colorless solid (98% yield): IR (CCl_4) 3600, 3360, 2920, 1705, 1675, 1600, 1585, 1505, 1265, 1155, 1100 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.95 (d, $J = 7.2$ Hz, 2H), 6.87 (d, $J = 7.2$ Hz, 1H), 5.83 (bs, 1H), 4.28 (t, $J = 6.8$ Hz, 2H), 1.75 (quin, $J = 6.8$ Hz, 2H), 1.43 (m, 2H), 1.28 (m, 8H), 0.88 (t, $J = 7.2$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.4, 159.5, 131.6, 122.8, 115.0, 64.9, 31.8, 29.22, 29.15, 28.7, 26.0, 22.6, 14.1; EI-MS, *m*/*z* 250 (M^+).

Enzyme Assay. Throughout the experiment, linoleic acid was used as a substrate. In a spectophotomeric experiment, the oxygenase activity of the soybean lipoxygenase was monitored at 25 °C by a Spectra MAX plus spectrophotometer (Molecular Devices, Sunnyvale, CA). The enzyme assay was performed as previously reported (*8*) with slight modification. In general, 5 μL of an ethanolic inhibitor solution was mixed with 54 μL of 1 mM stock solution of linoleic acid and 2.936 mL of 0.1 M Tris-HCl buffer (pH 8.0) in a quartz cuvette. Then, 5 μL of a 0.1 M Tris-HCl buffer solution (pH 8.0) of lipoxygenase (1.02 μM) was added. The resultant solution was mixed well, and the linear increase of absorbance at 234 nm, which expresses the formation of conjugated diene hydroperoxide (13-HPOD, $\epsilon = 25000\ M^{-1}\ cm^{-1}$), was measured continuously. A lag period shown in the lipoxygenase reaction (*9*) was excluded for the determination of initial rates. The stock solution of linoleic acid was prepared with methanol and Tris-HCl buffer at pH 8.0, and, then, total methanol content in the final assay was adjusted below 1.5%. For determining reversible inhibition manner, the enzyme concentration was changed as 0.17, 0.33, 0.50, 0.67, and 0.83 μg/mL with a constant substrate concentration (40 μM). Five concentrations (10, 15, 20, 35, and 40 μM) of linoleic acid were selected for Lineweaver−Burk plots.

Lipoxygenase-dependent O_2 uptake was performed using a Clark-type oxygen electrode (YSI 53, Yellow Springs Instrument Co., Yellow Springs, OH) at 25 °C as essentially the same procedures in the spectophotomeric experiment. For obtaining IC_{50}, the final assay concentrations of the enzyme and the substrate were adjusted to 4.25 nM and 46 μM, respectively. On the other hand, in the study of Lineweaver−Burk plots, the final concentration of the enzyme was fixed at 4.25 nM, but the substrate concentrations selected were 40, 60, 80, and 100 μM. Because a weak substrate inhibition was induced by the high concentration of linoleic acid (*8*, *9*), 100 μM substrate was used as the maximum concentration.

Data Analysis and Curve Fitting. The assay were conducted in triplicate of separate experiments. The data analysis was performed by using Sigma Plot 2000 (SPSS Inc., Chicago, IL). The inhibitory concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to the logistic curve by the equation as follows (*11*):

$$\text{activity (\%)} = 100[1/(1 - ([I]/IC_{50}))]$$

Inhibition mode was analyzed by Enzyme Kinetics Module 1.0 (SPSS Inc.) equipped with Sigma Plot 2000.

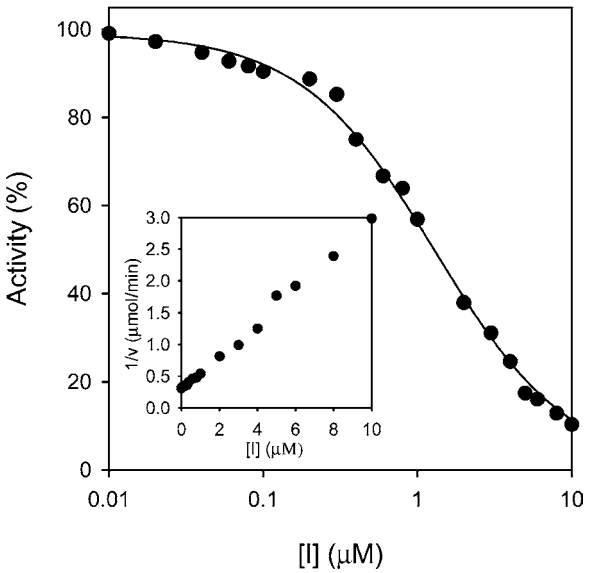

Figure 2. Effects of octyl gallate on the activity of soybean lipoxygenase-1 for the catalysis of linoleic acid at 25 °C. (Inset) Replots of data as 1/*v* versus [I].

RESULTS

The inhibition activity of soybean lipoxygenase-1 was measured by two methods for comparison, because this enzyme seems to be sensitive to assay conditions. Soybean lipoxygenase-1 is known to catalyze the dioxygenation of the (*Z*,*Z*)-diene moiety of linoleic acid. In plants, the primary dioxygenation product is 13*S* linoleic acid hydroperoxide (13-HPOD) (*1*). Hence, the enzyme assay was usually performed using a UV spectrophotometer to detect the increase at 234 nm associated with the (*Z*,*E*)-conjugated double bonds newly formed in the product but not the substrate. In many previous studies, the data were usually obtained at pH 9 because soybean lipoxygenase-1 is reported to have its optimum at pH 9.0 (*12*), but the absorption at 234 nm suffered from unstable baseline activity of unknown origin attributable to the presence of octyl gallate at pH 9.0. This pseudoactivity of the blank control had to be subtracted from activity of the enzyme assay, making precise measurements difficult. Moreover, this basic pH value may not be practical to use for food protection. Because the stable data were obtained at pH 8.0, the evaluation was performed at pH 8.0 (*10*). The data obtained were also compared with those of NDGA used as a reference compound. Its IC_{50} was obtained as 82 μM when the experiment was performed at pH 9.0 but 0.2 μM at pH 8.0. As a result, octyl gallate showed a dose-dependent inhibitory effect on this oxidation as shown in **Figure 2**. As octyl gallate increased, the enzyme activity was rapidly decreased but not completely suppressed. The IC_{50} was estimated to be 1.3 μM. As the need arose, the assay was also monitored by using polarography (oxygen consumption) for comparison. The IC_{50} obtained was 2.1 μM, which is almost comparable with that measured by using the spectrophotometric method.

The oxidation of linoleic acid catalyzed by soybean lipoxygenase-1 follows Michaelis−Menten equations. The plots of the remaining enzyme activity versus the concentrations of enzyme at different concentrations of octyl gallate gave a family of straight lines, which passed through the origin as shown in **Figure 3**. Increasing the inhibitor concentration resulted in descending slopes of the lines, indicating that the inhibition of octyl gallate on the enzyme was reversible. The presence of octyl gallate did not bring down the amount of the efficient

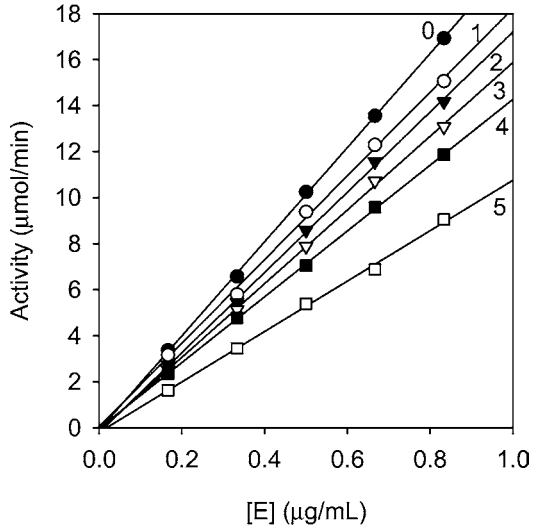

Figure 3. Relationship of catalytic activity of soybean lipoxygenase-1 with the enzyme concentrations at different concentrations of octyl gallate. Concentrations of octyl gallate for curves 0–5 were 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μM, respectively.

Table 1. Kinetics and Inhibition Constants of Octyl Gallate

	detector	
	UV_{235}	O_2
IC_{50}	1.3 μM	2.1 μM
K_m	20.0 μM	38.3 μM
V_m	6.67 μmol/min	8.0 μmol/min
inhibition	reversible	reversible
inhibition type	competitive	competitive
K_I	0.54 μM	0.84 μM

enzyme, but resulted in the inhibition and the descending activity of the enzyme. In addition, a similar result was also obtained by using an O_2-electrode monitoring method.

Subsequently, the inhibition kinetics of soybean lipoxygenase-1 by octyl gallate was investigated. The kinetic behavior of soybean lipoxygenase-1 during the oxidation of linoleic acid was studied first. Under the conditions employed in the present investigation, the oxidation of linoleic acid catalyzed by soybean lipoxygenase-1 follows Michaelis−Menten kinetics. The kinetic parameters for this oxidase obtained from a Lineweaver−Burk plot show that K_m is equal to 20.0 μM and V_m is equal to 6.67 μmol/min. The estimated value of K_m obtained with a spectrophotometric method is in good agreement with the previously reported value (*10*, *13*). The kinetic and inhibition constants obtained are listed in **Table 1**. As illustrated in **Figure 4**, the inhibition kinetics analyzed by Lineweaver−Burk plots show that octyl gallate is a competitive inhibitor because increasing octyl gallate resulted in a family of lines with a common intercept on the $1/v$ axis but with different slopes. This may suggest that octyl gallate displaces linoleic acid from the enzymatic site of oxidation. The equilibrium constant for inhibitor binding, K_I, was obtained from a plot of the apparent Michaelis−Menten constant versus the concentration of octyl gallate, which is linear. The inhibition kinetics analyzed by Dixon plots also confirmed that the octyl gallate is a competitive inhibitor (data not illustrated). In addition, a similar result was also obtained by using an O_2-electrode monitoring method, and the results are listed in **Table 1**. The rate of oxygen consumption was dependent on octyl gallate concentrations. The estimated value of K_m is ~2-fold higher than that obtained with a spectrophotometric method.

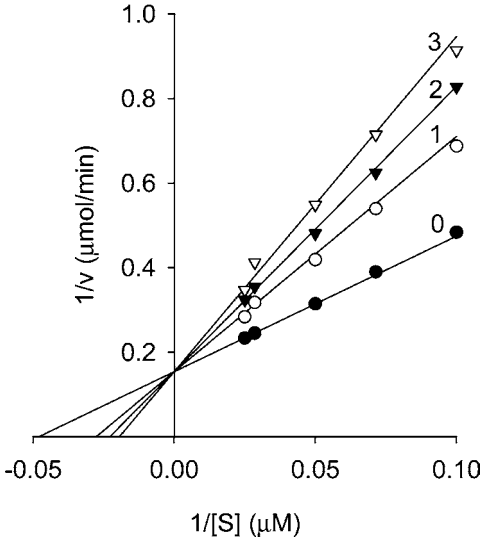

Figure 4. Lineweaver–Burk plots of 13-HPOD generation by soybean lipoxygenase-1 in the presence of octyl gallate at 25 °C, pH 8.0. Concentrations of octyl gallate for curves 0–3 were 0, 0.4, 0.6, and 0.8 μM, respectively.

Neither gallic acid (**2**) nor octanol (**3**) alone inhibited soybean lipoxygenase-1 up to 200 μM. In connection with this, L-ascorbic acid and α-tocopherol (also known as vitamins C and E, respectively) did not inhibit this enzyme up to 300 μM. Octyl gallate inhibited soybean lipoxygenase-1 but is unlikely due to its capability of scavenging linoleic acid derived free radicals. Although intermediate free radicals are formed during the catalytic cycle of lipoxygenases (*9*), they remain tightly bound at the active site, thus not being accessible for free radical scavengers. Moreover, octyl 3,4-dihydroxybenzoate (**4**) inhibited this lipoxygenase, whereas both octyl 3,5-dihydroxybenzoate (**6**) and octyl 4-hydroxybenzoate (**7**) showed little inhibitory activity, indicating that certain head and tail structures are needed to elicit the inhibitory activity. Thus, the catechol moiety seems to be important in eliciting potent soybean lipoxygenase-1 inhibitory activity as a hydrophilic portion (*14*, *15*) but not essential. Moreover, decahydro-2-naphthyl gallate (**5**) also inhibited soybean lipoxygenase-1 with an IC_{50} of 2.1 μM, indicating that the hydrophobic tail portion seems to be flexible.

In most foods, the browning process has two components: enzymatic and nonenzymatic oxidation. This unfavorable darkening from oxidation generally results in a loss of nutritional as well as market values. The enzymatic oxidation can be prevented by tyrosinase inhibitors, and the nonenzymatic oxidation can be protected by antioxidants. Hence, the radical scavenging activity on the DPPH radical was examined first. This activity can be measured as decolorizing activity following the trapping of the unpaired electron of DPPH. One molecule of octyl gallate scavenged six molecules of DPPH. Octyl gallate was also tested for antioxidation activity against autoxidative lipid peroxidation. Unsaturated fatty acids, especially linoleic acid, are the targets of lipid peroxidation in plants (*1*). In the control reaction, the production of lipid peroxide increased almost linearly during 7 days of incubation. α-Tocopherol, one of the most common natural antioxidants, inhibited the linoleic acid peroxidation at 10 μg/mL to almost 50%. Octyl gallate was more effective in preventing the lipid peroxidation. Thus, complete inhibition was observed at 10 μg/mL and 50% inhibition was still observed at 1 μg/mL. Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (*16*, *17*), is a copper-containing enzyme widely distributed in micro-

organisms, animals, and plants. Tyrosinase is responsible for browning in plants and is considered to be deleterious to the color quality of plant-derived foods and beverages. Hence, octyl gallate was tested for its inhibition of the oxidation of L-DOPA catalyzed by tyrosinase without its being oxidized. It was oxidized as a substrate at a slow rate.

DISCUSSION

In our continuing search for multifunctional additives for foods, octyl gallate was found to possess a broad antimicrobial spectrum in addition to its potent antioxidant activity. Both octyl gallate and gallic acid were reported to inhibit squalene epoxidase (*18*). Octyl gallate was reported to inhibit thiyl radical-induced reactions with unsaturated fatty acids (*19*). This gallate is one of three gallates (propyl and dodecyl) that are currently permitted for use as antioxidant additives in food. Safety is a primary consideration for antioxidants in food products. After consumption, octyl gallate is hydrolyzed, to gallic acid and octanol, and both are common components in many edible plants; gallic acid in blackberry bark, henna, tea, mango, and uva ursi and octanol in a large number of essential oils. Because lipid peroxidation is one of the major factors in deterioration during the storage and processing of foods, the lipoxygenase inhibitory activity of octyl gallate is a desirable additional activity for food protection. In the multifunctional concept, we also examined if gallic acid and octanol possess lipoxygenase inhibitory activity, because the arachidonic acid cascade is known to be implicated in the pathogenesis of a variety of human diseases, including cancer, and is now believed to play important roles in tumor promotion, progression, and metastatic diseases (*20*). As aforementioned, we found that neither gallic acid nor octanol inhibited soybean lipoxygenase-1 up to 200 μM. However, octanol was previously reported to inhibit soybean lipoxygenase-1 with much higher concentrations assayed at pH 9.0 (*21*). The freed gallic acid still acts as a potent antioxidant. For example, membrane lipids are particularly susceptible to oxidation not only because of their high polyunsaturated fatty acid content but also because of their association in the cell membrane with enzymic and nonenzymic systems capable of generating free radical species (*22*). Microsomes, especially smooth-surfaced endoplasmic reticulum, easily produce lipid peroxides and are thought to supply the peroxidation products to other tissues (*23*). NADPH-cytochrome P-450 reductase is involved in NADPH-dependent enzymical lipid peroxidation in microsomes. Lipid peroxidation, which can be measured by the thiobarbituric acid (TBA) method, occurs when rat liver microsomes are incubated with Fe(III)-ADP/NADPH (*24*). Gallic acid was reported to inhibit microsomal lipid peroxidation induced by ADP plus NADPH (*25*). In addition, gallic acid and its esters are known to chelate transition metal ions, which are powerful promoters of free radical damage in both the human body (*26*) and foods (*27*). The chelation ability, rendering the metal ions inactive to participate in free radical generating reactions, should be of considerable advantage as an antioxidant. For example, octyl gallate and gallic acid may suppress the superoxide-deriven Fenton reaction (*28*), which is currently believed to be the most important route to active oxygen species. In summary, octyl gallate appears to combine both lipoxygenase inhibitory activity and free radical scavenging property in one agent and thus is an effective antioxidant.

In previous papers, octyl gallate was reported to show a broad antimicrobial spectrum (*4*, *29*, *30*). For example, this gallate is effective against both *Saccharomyces cerevisiae* and *Candida albicans*, with the minimum fungicidal concentration (MFC) of 25 μg/mL (89 μM), and against *Zygosaccharomyces bailii* 50 μg/mL (177 μM). In addition, the fungicidal activity of octyl gallate against these yeasts was not influenced by pH values. The octyl gallate also exhibited fungicidal activity against *Aspergillus niger*, with an MFC of 100 μg/mL (355 μM). In general, no differences in MIC and MFC values were noted, suggesting that the activity of octyl gallate is fungicidal. In the case against bacteria, octyl gallate was noted to show bactericidal activity against all of the Gram-positive bacteria, including two methicillin-resistant *Staphylococcus aureus* (MRSA) strains. *S. aureus* is one of the main bacteria that cause food poisoning. In addition, *Salmonella choleraesuis* was found to be the most susceptible to octyl gallate. Alkyl gallates are usually not effective against Gram-negative bacteria because of their outer membrane barrier (*31*), but octyl gallate was effective against *S. choleraesuis*, with an MBC of 12.5 μg/mL (44 μM).

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