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THE ENZYMATIC FORMATION OF δ-CADINENE FROM FARNESYL DIPHOSPHATE IN EXTRACTS OF COTTON

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Key Word Index—*Gossypium barbadense*; Malvaceae; sesquiterpenoid phytoalexins; farnesyl diphosphate; δ-cadinene.

Abstract—Incubation of extracts of cotton stele tissue which had been infected with *Verticillium dahliae* led to the enzymatic incorporation of [1-^{3}H]-farnesyl diphosphate into hydrocarbons soluble in hexane–ethyl acetate. The mixture of [^{3}H]-hydrocarbons was separated into components by HPLC. The hydrocarbon with a retention time of 17.5–18.0 min, biosynthetically prepared from (1*RS*)-[1-^{2}H]-(*E*,*E*)-farnesyl diphosphate, was analysed by GC-MS. The data not only agree with the reported mass spectrum of δ-cadinene but also support the proposed hydride shift in the biosynthesis of δ-cadinene from farnesyl diphosphate.

INTRODUCTION

It has been suggested that the cyclization of farnesyl diphosphate (FDP) to a cadinene was the initial step in the formation of the sesquiterpenoid 2,7-dihydroxycadalene induced in cotton leaves by *Xanthomonas campestris* pv. *malvacearum* [1]. δ-Cadinene from *Pinus silvestris* has been analysed by mass fragmentography [2] and the synthesis of (±)-δ-cadinene from germacrene D has been achieved [3].

In this paper we report the enzymatic formation of δ-cadinene from FDP in extracts of cotton stems infected with *Verticillium dahliae*, which induces the formation of the sesquiterpenoid phytoalexins, desoxyhemigossypol and hemigossypol [4]. δ-Cadinene cyclase in the cotton stele tissue is induced by *V. dahliae* and may be the first enzymatic step in the formation of desoxyhemigossypol and hemigossypol.

RESULTS AND DISCUSSION

The [^{3}H]-hydrocarbons enzymatically synthesized from [1-^{3}H]-FDP in soluble preparations of cotton stele tissue were extracted with hexane–ethyl acetate and separated by HPLC as shown in Fig. 1a. The [^{3}H]-hydrocarbons are not synthesized in significant amounts in reaction mixtures containing boiled extract (Fig. 1b). Separate experiments demonstrated that the enzymatic activity for the incorporation of [1-^{3}H]-FDP into the hydrocarbon with a retention time of 17.5–18.0 min was induced in the cotton stele tissue by *Verticillium dahliae* and the activity peaked 2–3 days following the inoculation of the tissue. To identify the hydrocarbon with a retention time of 17.5–18.0 min on the HPLC column, it was enzymatically labelled for 15 hr in extracts of cotton stele tissue from (1*RS*)-[1-^{2}H]-(*E*, *E*)-FDP. The deuterated hydrocarbon was extracted into hexane–ethyl acetate, collected from the HPLC column between 17 and 18 min and analysed by GC-MS. The GC separation resulted in one significant peak with a retention time of 57.50 min. The major ions and relative intensities from the mass spectrum of this hydrocarbon are given in the Experimental. The mass spectrum agreed with that reported for δ-cadinene [2]. The identity of the other [^{3}H]-hydrocarbons formed in the complete reaction mixture (Fig. 1a) is currently being investigated.

In order to account for the major ions in the mass spectrum of the deuterated hydrocarbon formed enzymatically from deuterated FDP we present a proposed pathway for the formation of deuterated δ-cadinenes from (1-*RS*)-[1-^{2}H]-(*E*,*E*)-FDP (Fig. 2) and their fragmentation scheme (Fig. 3). The proposed pathway for the formation of δ-cadinenes (Fig. 2) includes a nerolidyl diphosphate intermediate and a 1,3-hydride shift. Cane [5] has established nerolidyl diphosphate as an intermediate in the cyclization of (*E*,*E*)-FDP to many sesquiterpene precursors and Arigoni [6], Masciadri *et al.*, [7], and Davis *et al.* [8] have established a similar 1,3-hydride shift in the formation of cadalenes from (*E*,*E*)-FDP. As shown in the formation of δ-cadinene from (1*RS*)-[1-^{2}H]-(*E*,*E*)-FDP, one expects deuterium atoms to be located at

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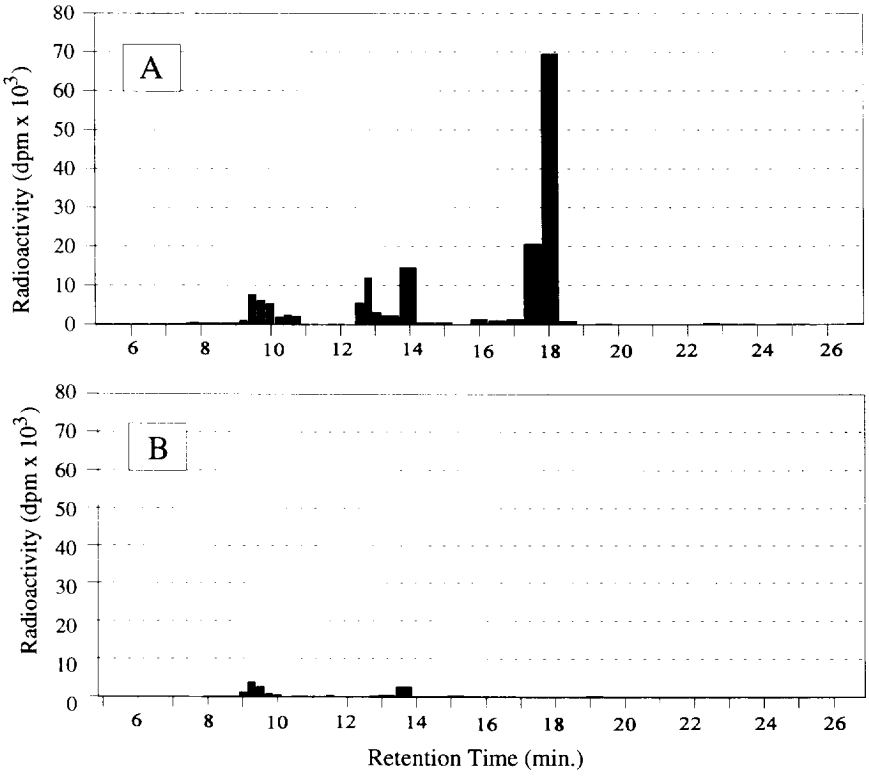

Fig. 1. The HPLC profile of [^{3}H]-hydrocarbons formed from [1-^{3}H]-FDP in extracts of cotton stele tissue. (a) Complete reaction mixture (see Experimental). (b) The same reaction mixture as in (a) except the stele extract was boiled for 10 min at 100° before adding it to the reaction mixture.

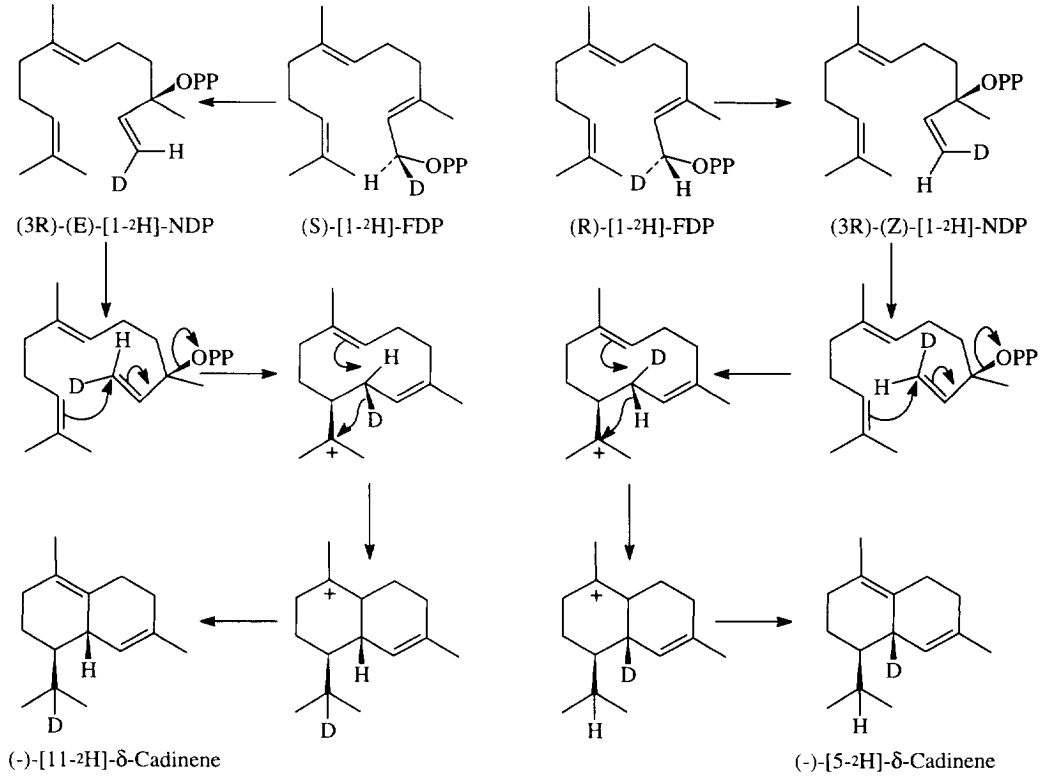

Fig. 2. Proposed reaction scheme for the biosynthesis of [5-^{2}H]-and [11-^{2}H]-δ-cadinene from (1*RS*)-[1-^{2}H]-(*E*, *E*)-FDP in cotton stele extracts.

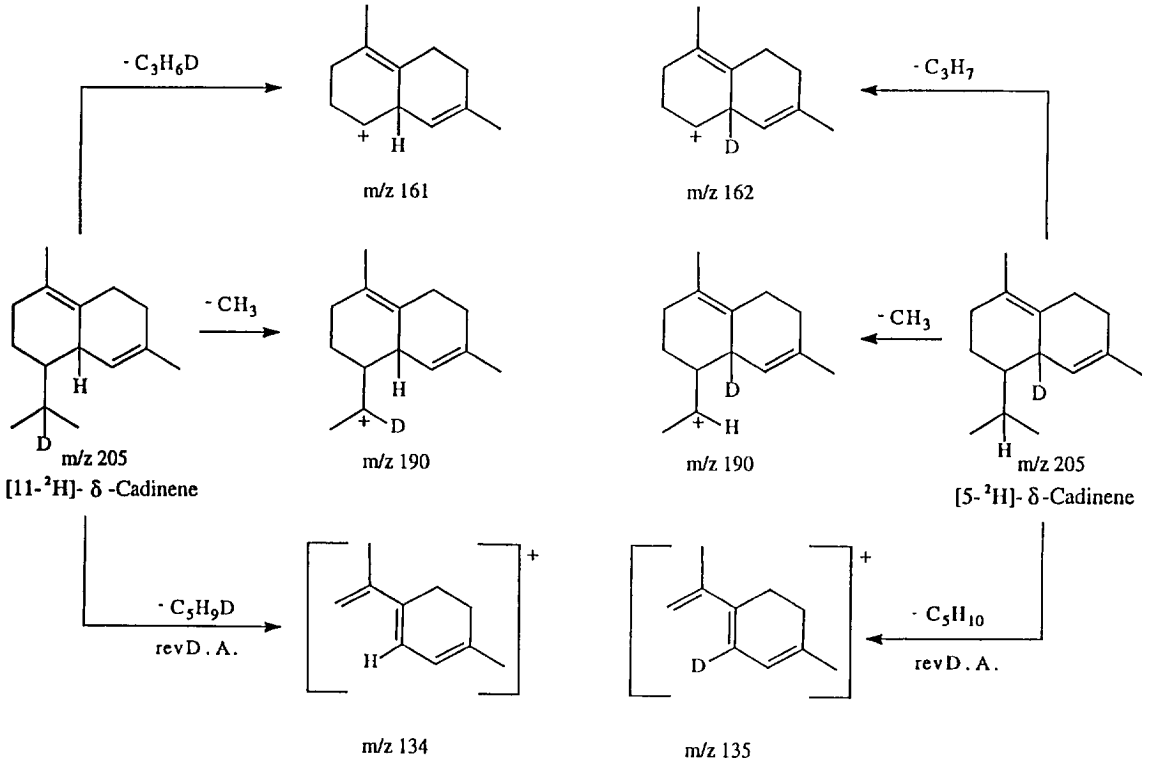

Fig. 3. Proposed fragmentation scheme of [5-^{2}H]- and [11-^{2}H]- and δ-cadinene.

C-11 of δ-cadinene derived from (1*S*)-[1-^{2}H]-FDP and at C-5 of δ-cadinene derived from (1*R*)-[1-^{2}H]-FDP.

A proposed fragmentation scheme which accounts for the major peaks in the mass spectrum of the deuterated δ-cadinenes is shown in Fig. 3. A single ion at *m/z* 205 accounts for the [11-^{2}H]- and [5-^{2}H]-δ-cadinenes. From a mixture of [11-^{2}H]- and [5-^{2}H]-δ-cadinene the loss of a methyl group from the isopropyl side chain produces a single ion at *m/z* 190 since no deuterated species is involved in this transformation. The peaks at *m/z* 161 and 162 result from the loss of the isopropyl group. The isopropyl group containing the deuterium atom produces the peak at *m/z* 161, while loss of a completely protonated isopropyl group in which the deuterium is retained at C-5 produces the peak at *m/z* 162. Similarly, a reverse Diels-Alder reaction provides two peaks at *m/z* 134 and 135. A significant peak at *m/z* 134 has been used to distinguish δ-cadinene from other cadinenes [2]. The loss of a methyl group from *m/z* 134 and 135 provides the *m/z* 119 and 120 peaks (not shown in Fig. 3). The peaks at *m/z* 105 and 106 result from a further fragmentation due to the loss of a methylene group from *m/z* 119 and 120. This fragmentation pattern supports the conclusion that the deuterated hydrocarbon is δ-cadinene.

(−)-δ-Cadinene has previously been isolated from cotton [9]. The identity of the GC-peak eluting at 57.50 min was further confirmed by comparison of its GC-MS with that of an extract from cotton leaves. (−)-δ-Cadinene isolated from cotton leaves eluted at 57.51 min from GC columns and its identity was confirmed by mass spectrometry (see Experimental). The quantity of δ-cadinene isolated in the enzyme reaction mixtures from the cotton stele tissue was too small to measure its optical rotation.

EXPERIMENTAL

Radiochemical. [1-^{3}H]-Farnesyl diphosphate (FDP, 832.5 GBq mmol^{-1}) was purchased from New England Nuclear.

Instrumentation. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Brüker ARX-300 instrument in the specified deuterated solvents with $CDCl_3$ as int. standard unless specified otherwise.

Plant material. Cotton (*Gossypium barbadense* cv, Seabrook Sea Island 12B2) seed weed pre-germinated in paper rolls at 30° for 48 hr and then transferred to 16-ounce plastic cups. The seedlings were greenhouse grown to the 6–8 true leaf stage, transplanted to gallon containers and placed in an environmental growth chamber programmed for a 14-hr day at 28° and a 10-hr night at 22°. The plants were equilibrated in the growth chamber for 1 week prior to inoculation with *Verticillium dahlia.*

Inoculum prepn. Verticillium dahliae defoliating strain V-76 was isolated from cotton plants grown in Sonora, Mexico and grown on potato dextrose agar plates at

room temp. (*ca* 22°). The agar plates were flood inoculated with 10^8 conidia ml^{-1} and the fungus was allowed to grow for 3 to 4 days before conidia were washed from the plates with sterile H_2O. Conidia were diluted to a concn of 2 to 5×10^7 cells ml^{-1} and used as inoculum.

Inoculation of the plants. A 20-μl droplet of inoculum was placed at each of 3 locations equally spaced around the stem 1/4″ below the cotyledons. A puncture wound was made through the droplets with a 23-gauge needle so that the inoculum was taken up by the xylem vessels.

Prepn of stele extracts. Three days after inoculation, the plants were removed from the environmental chambers and the first internode was excised. The bark was removed and 1 g of the stele tissue was ground to a powder in a mortar in liquid N_2. The powder was further ground in 8 ml of 0.1 M Tris–HCl buffer (pH 7.5) containing 5 mM GSH, 0.1% Triton X-100 detergent and 5% insoluble PVP. The homogenate was centrifuged in a Beckman L8-55M Ultracentrifuge at 100 000 *g* for 60 min. The supernatant fr. was used as an enzyme source.

Enzymatic utilization of [1-^{3}H]-*FDP.* To determine the enzymatic incorporation of [1-^{3}H]-FDP into hydrocarbon compounds, the reaction mixt. contained: 200 μl of the supernatant fr. from the above 100 000 *g* centrifugation, 20 μl 0.1 M GSH (pH 7.5), 40 μl 0.2 M KF, 20 μl 0.1 M $MgCl_2$, and 0.48 nmol [1-^{3}H]-FDP containing 22.2×10^6 dpm of radioactivity. The reaction mixts were incubated for 1 hr at 30°. The reactions were stopped with the addition of 1 ml of hexane–ethyl acetate (3:1). The aqueous layer was extracted 5 more times with 1 ml of hexane–ethyl acetate. The organic phase was removed and the extraction of the aqueous phase was repeated 2 more times with 1 ml of hexane–ethyl acetate. The organic solubles were combined, evapd to dryness under N_2. The resulting [^{3}H]-hydrocarbons were next dissolved in 20 μl ethyl acetate and 60 μl acetone–H_2O (9:1) containing 0.1% ascorbic acid. A 25 μl sample of the [^{3}H]-hydrocarbons was sepd on a 250 × 4.6 mm Scientific Glass Engineering MOS-Hypersil-1 C-8 column (5μ) at a column temp. of 40° and a flow rate of 1.25 ml min^{-1} using a Hewlett Packard 1090 HPLC equipped with a diode array detector. A linear MeOH–H_2O gradient protocol containing 0.07% phosphoric acid was used with an initial ratio of 2.8 progressing to 7:3 over 7 min, to 8:2 over the next 5 min and to 9:1 over the next 7 min and to 100% MeOH over the last 4 min. The column eluate was collected in 30 sec intervals between 2 and 9 min and 13 to 28 min and at 15 sec intervals between 9 and 13 min. The frs were assayed in a Beckman Liquid Scintillation Spectrometer and background subtracted. The [^{3}H]-hydrocarbon eluting from the column between 17.5 and 18.0 min was volatile and about a 50% loss in radioactivity was experienced following evapn of the hexane–ethyl acetate solvent with N_2. The rate of the reaction was approximately 528.0 pmol hydrocarbon formed g^{-1} stele tissue.

(E,E)-3,7,11-*Trimethyl*-2,6,10-*dodecatrien*-1-*al* (E,E-*farnesal*). *E,E*-Farnesol was oxidized with active manganese dioxide [10] to give the *E,E*-farnesal as an oil in 94% yield. It was unstable at 0° under nitrogen and was freshly prepd before use or stored at −78° under N_2.

(1RS)-[1-^{2}H]-(E,E)-3,7,11-*Trimethyl*-2,6,10-*dodecatrien*-1-*ol* ((1RS)-[1-^{2}H]-(E,E)-*farnesol*) [11, 12]. Farnesal was reduced with sodium borodeuteride (98 atom%D). The crude product was purified by silica gel (50 g) CC using toluene–EtOAc gradient elution (starting, 2% EtOAc (v/v); 4% EtOAc increment for every 100 ml solvent mixt.). The effluent was collected in 30 ml frs. The desired material eluted between fr. 10 and fr. 14 (TLC, silica gel, toluene–EtOAc, 9:1, R_f 0.31). These frs were combined to yield 1.185 g (83%) of the title compound as an oil.

(1RS)-[1-^{2}H]-(E,E)-3,7,11-*Trimethyl*-1-*chloro*-2,6,10-*dodecatrien* ((1RS)-[1-^{2}H]-(E,E)-*farnesyl chloride*). (1*RS*)-[1-^{2}H]-(*E,E*)-Farnesol was converted to the chloride by treatment with *N*-chlorosuccinimide as previously reported [13, 14].

(1RS)-[1-^{2}H]-(E,E)-3,7,11-*Trimethyl*-2,6,10-*dodecatrienyl triammonium diphosphate* ((1RS)-[1-^{2}H]-(E,E)-*farnesyl diphosphate*). (1*RS*)-[1-^{2}H]-(*E,E*)-Farnesyl diphosphate was prepd from the chloride as previously reported [13, 14]. ^{1}H NMR (D_2O–ND_4OD, pH 8, DOH signal is taken as 4.84 ppm.), δ 5.42 (1H, *d*, $J_{1,2} = 6.3$ Hz, H2), 5.13 (1H, *t*, $J_{5,6} = 6.6$ Hz, H6), 5.09 (1H, *t*, $J_{9,10} = 5.8$ Hz, H10), 4.43 (1H, *br dd*, $J_{1,2} = 6.3$ Hz, $^3J_{1,P} = 6.7$ Hz, H1(*RS*)), 1.93 ~ 2.13 (8H, *m*, H4, H5, H8 and H9), 1.70 (3H, *s*, H13), 1.64 (3H, *s*, Me), 1.58 (3H, *s*, Me), 1.56 (3H, *s*, Me); ^{13}C NMR (D_2O–ND_4OD, pH 8, the upmost field signal is set to 18.20 ppm [5]). δ145.1 (C-3), 138.0 (C-7), 133.9 (C-11), 127.1 (C-6), 126.7 (C-10), 122.2 (*d*, $^3J_{C,P} = 8.3$ Hz, C-2), 65.0 (*br dt*, $^1J_{C,D} = 19.5$ Hz, $^2J_{C,P}$: not measurable, C-1), 42.1 (C-4), 42.0 (C-8), 29.1 (C-9), 28.9 (C-5), 27.9 (C-12), 19.9 (C-15), 18.5 (C-13), 18.2 (C-14).

Enzymatic utilization of (1RS)-[1-^{2}H]-(E,E)-*farnesyl diphosphate.* To determine the enzymatic incorporation of [1-^{2}H]-FDP into deuterated isoprenoids the reaction mixts contained: 200 μl of the supernatant fr., from the above 100 000 *g* centrifugation, 20 μl 0.1 M GSH (pH 7.5), 40 μl 0.2 M KF, 20 μl 0.1 M $MgCl_2$ and 50 nmol (1*RS*)-[1-^{2}H]-(*E,E*)-FDP. Ten individual reaction mixts were incubated overnight (15 hr) at 30°. The reactions were stopped with the addition of 1 ml hexane–EtOAc (3:1), and the aqueous layer was extracted with hexane–EtOAc (6 × 1 ml). The organic solubles from the individual reaction tubes were evapd to dryness with N_2. The [^{2}H]-hydrocarbons were then dissolved in 28 μl hexane–EtOAc (3:1) and subsequently sepd by HPLC as previously described. The [^{2}H]-hydrocarbon eluting between 17.0 and 18.0 min was collected and evapd to dryness under N_2. The residue was dissolved in 10 μl hexane–EtOAc (3:1) and subjected to GC-MS analysis.

The [^{2}H]-hydrocarbon was identified using a Hewlett-Packard 5971A GC-Mass Spectrometer equipped with a split/splitless injector and a DB-1 fused silica capillary column (60 m × 0.32 mm; 0.25 μm film thickness). The linear velocity was 30 cm sec^{-1} at 150°. The sample (3 μl) was injected manually. The following temp. program

was followed: initial temp. 40°, hold 5 min; to 100° at 3.5° min^{-1}, hold 5 min; to 150° at 2.0° min^{-1}, hold 5 min; to 250° at 5.0° min^{-1}, hold 15 min (total run time 110.47 min). Other than background peaks only one significant peak was observed with a retention time of 57.50 min. The mass spectrum of the [^{2}H]-hydrocarbon shows ions at *m/z* (%) 206 (15), 205 (84), 190 (29), 163 (21), 162 (100), 161 (90), 160 (10), 159 (12), 135 (58), 134 (58), 133 (15), 131 (11), 129 (11), 120 (48), 119 (51), 118 (13), 117 (13), 115 (12), 106 (31), 105 (52). Natural abundance δ-cadinene isolated from fresh cotton leaves by pentane extraction had a retention time under identical GC conditions of 57.51 min. MS *m/z* (%) 205 (13), 204 (51), 189 (20), 162 (27), 161 (100), 157 (14), 145 (11), 134 (59), 133 (16), 129 (10), 128 (11), 121 (11), 120 (10), 119 (55), 115 (13), 105 (49).

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