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Synthesis and biological properties of pinane-thromboxane A_2, a selective inhibitor of coronary artery constriction, platelet aggregation, and thromboxane formation

(thromboxane A_2/prostaglandin H_2 analogs/thromboxane synthetase)

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ABSTRACT **Pinane-thromboxane A_2 (PTA_2, [1α,2β(Z),3α(1E,3R*),5α]-7-{3-(3-hydroxy-1-octenyl)-6,6-dimethylbicyclo[3.1.1]hept-2-yl}-5-heptenoic acid) has been synthesized and tested for biological activity in systems responsive to thromboxane A_2, stable prostaglandin endoperoxide (PGH_2) analogs, and prostacyclin (PGI_2). At low concentrations, PTA_2 inhibited cat coronary artery constriction induced by stable prostaglandin endoperoxide analogs, and it stabilized liver lysosomes. At slightly higher concentrations, it inhibited platelet aggregation. At still higher concentrations, PTA_2 inhibited thromboxane synthetase, but it had no effect on prostacyclin synthetase. The analog also had no effect on the inhibition of platelet aggregation by PGI_2 or prostaglandin D_2. It is suggested that PTA_2 has a suitable biochemical profile for use as an antithrombotic agent.**

In 1975, Hamberg *et al.* (1) proposed the structure (Fig. 1) for an unstable (half-life of about 30 s at pH 7.4 and 37°C) substance with potent thrombotic and smooth muscle contracting properties that they named thromboxane A_2 (TA_2). The isolation or chemical synthesis of this important member of the arachidonic acid cascade (2) has not yet been achieved. Furthermore, no analogs of this structurally unusual molecule have been reported to date. We now wish to report the synthesis of a thromboxane A_2 analog (structure **8a**) and some of its interesting biological properties.

MATERIALS AND METHODS

Synthesis of Pinane-Thromboxane A_2, [1α,2β(Z),3α-H(1E,3R*),5α]-7-{3-(3-Hydroxy-1-octenyl)-6,6-dimethylbicyclo[3.1.1]hept-2-yl}-5-heptenoic Acid. The thromboxane analog pinane-thromboxane A_2 (PTA_2, **8a**) was synthesized from (−)-myrtenol (**1**) as outlined in Fig. 2. (−)-Myrtenol (**1**) was efficiently (95%) converted (MnO_2/CH_2Cl_2, 25°C, 48 hr) to the α,β-unsaturated aldehyde **2**, which underwent smooth 1,4-addition with the mixed cuprate derived from (±)-*trans*-lithio-1-octen-3-ol *tert*-butyldimethylsilyl ether and 1-pentynylcopper hexamethylphosphorus triamide complex (3) to afford the aldehyde **3** (80% yield).[§,¶] Condensation of **3** with methoxymethylenetriphenylphosphorane (1.5 equivalents) in toluene/tetrahydrofuran solution at 0°C furnished in 94% yield the enol ether **4** (mixture of geometrical isomers), from which the aldehyde **5** was liberated quantitatively [$Hg(OAc)_2$/KI/H_2O/tetrahydrofuran]. The upper side chain was completed by the standard prostaglandin (PG) Wittig reaction employing the sodium salt of 4-carboxybutyltriphenylphosphorane in dimethyl sulfoxide, leading, after diazomethane treatment, to the methyl ester of the protected TA_2 analog **6** (mixture of C-15 epimers, PG numbering) in 80% yield. Deprotection of the hydroxy group (acetic acid/water/tetrahydrofuran, 3:2:2 vol/vol, 45°C) led to the methyl esters **7a** and **7b** (1:1, 100%), which were separated by preparative thin-layer chromatography (silica, ether/petroleum ether, 1:1 vol/vol; **7a**, R_F = 0.53; **7b**, R_F = 0.59). Hydrolysis of the more polar compound **7a** with lithium hydroxide in aqueous tetrahydrofuran at 25°C furnished PTA_2 (**8a**) quantitatively, R_F = 0.66 (silica, ether), whereas similar hydrolysis of the less polar methyl ester **7b** produced the 15-epimer (PG numbering) **8b** (100%), R_F = 0.70 (silica, ether). The methyl ester of PTA_2 (**7a**)[¶] showed the following properties: Infrared spectrum, ν_{max} 3400 (OH), 1742 cm^{-1} ($COOCH_3$). ^1H nuclear magnetic resonance spectrum (220 MHz, C^2HCl_3) τ relative to tetramethylsilane 4.40–4.78 (multiplet, 4 H, olefin), 5.95 (multiplet, 1 H, CH—O), 6.33 (singlet, 3 H, $COOCH_3$), 8.80 and 8.94 (singlets, 3 H each, pinane CH_3), 7.55–9.30 (multiplet, 28 H). ^{13}C nuclear magnetic resonance spectrum (15 MHz, C^2HCl_3) ppm relative to tetramethylsilane 174.0, 139.6, 131.2, 130.2, 128.9, 73.0, 51.4, 48.8, 43.9, 41.7, 39.3, 38.6, 37.4, 34.9, 34.3, 33.5, 32.4, 31.7, 28.2, 26.7, 25.2, 24.9, 23.0, 22.6, 14.0. Mass spectrum, parent ion mass-to-charge ratio 390. $[\alpha]^{22}_D$ +26.40° (methanol). Analysis, calculated for $C_{25}H_{42}O_3$: C 76.86% and H 10.87%; found: C 77.05% and H 11.08%. The ^{13}C NMR spectra of **7a** and **7b** are of crucial value in assigning the stereochemistry of these compounds. In particular, the relatively low chemical shift for C-10 (PG numbering) in the ^{13}C NMR spectra of **7a** and **7b** (δ 33.3 ± 2 ppm) reveals the relative stereochemistry of the upper side chain by comparison to the corresponding model epimeric compounds from the myrtenol family, which exhibit this carbon at δ 33.2 ppm (C *trans* to the side chain) and 24.2 (C *cis* to the side chain) (4). PTA_2 (**8a**) and its 15*R* isomer (**8b**) are indefinitely stable at 25°C in solution or in the pure liquid state.

Biological Methods. PTA_2 (up to 4 μl of a 2.5 mM solution in ethanol) was tested for its effect on cat coronary arteries continuously perfused with 10 ml Krebs–Henseleit solution as described (5). Constriction of the arteries was induced by addition of 15 or 30 nM 9,11-azo-PGH_2 or 1 μM 9,11-methanoepoxy-PGH_2 (U46619). The effect of PTA_2 on the release of cathepsin D from the cat liver large granule fraction was determined as described (6).

Abbreviations: PG, prostaglandin, TA_2, thromboxane A_2, PTA_2, pinane-thromboxane A_2, PGI_2, prostacyclin.

§ All new compounds showed satisfactory infrared, mass, and NMR spectral data and analyzed correctly for carbon and hydrogen.

¶ The designated stereochemistry for this and the following compounds was tentatively assigned and subsequently confirmed on the basis of: (*i*) the presumption that the cuprate reagent would approach the α,β-unsaturated system from the less hindered β face, (*ii*) ^{13}C and ^1H nuclear magnetic resonance spectral data (4), and (*iii*) the potent biological properties of the isomer **8a** (see *Results*).

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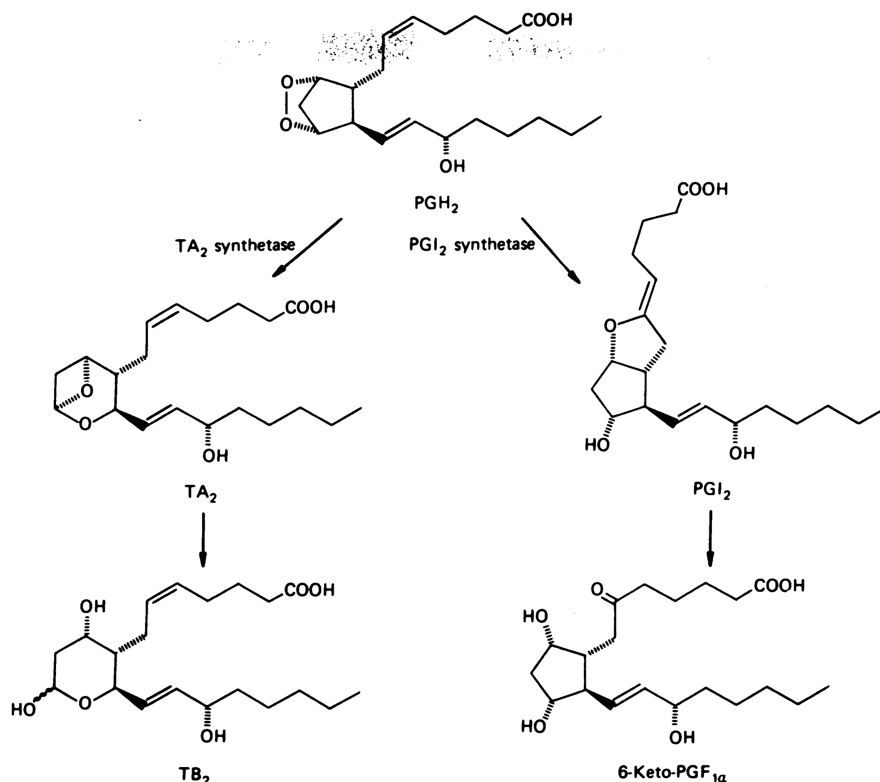


FIG. 1. Biosynthesis and degradation of thromboxane A_2 (TA_2) and prostacyclin (PGI_2). TB_2, thromboxane B_2.

Platelet aggregation was studied in an aggregometer (Chronolog Corporation, Philadelphia, PA) using 0.5 ml of citrated human platelet-rich plasma at 37°C (7). One minute after addition of PTA_2 (up to 2 μl of a 25 mM solution in ethanol), aggregation was initiated by addition of sodium arachidonate (0.3–0.5 mM), ADP (2 μM), collagen (1 μg/ml), epinephrine (50 μM), 9,11-azo-PGH_2, (0.1–0.6 μM), 9,11-methanoepoxy-PGH_2 (U46619, 0.3–0.6 μM) or 9,11-epoxymethano-PGH_2 (U44069, 1–3 μM). The analog was also tested for its effects on the inhibition of ADP-induced aggregation by 2 nM PGI_2 or 20 nM PGD_2.

To study the effects of PTA_2 on thromboxane synthesis, rabbit platelets were washed free of plasma, resuspended in buffered saline solution (8), and incubated at 37°C for 5 min with PTA_2 at either 10 or 100 μM. [1-^{14}C]Arachidonic acid (0.5 μCi, specific activity 50 Ci/mol, New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) was added and incubation was continued for 15 min, then lipids were extracted by the method of Folch *et al.* (9) and subjected to thin-layer chromatography (solvent system C of ref. 10). In additional experiments, human platelet-rich plasma was incubated at 37°C in the aggregometer for 1 min with the analog. Sodium arachidonate (0.4 mM) was added and incubation was continued for 5 min. The samples were decanted into 1/10th vol of 100 mM EDTA and centrifuged for 2 min at 15,000 × *g*, and thromboxane B_2 in the supernatant was determined by radioimmunoassay (11).

The effects of PTA_2 on prostacyclin synthetase were determined by using sheep vesicular glands as the enzyme source and [1-^{14}C]arachidonic acid as substrate essentially as described (12).

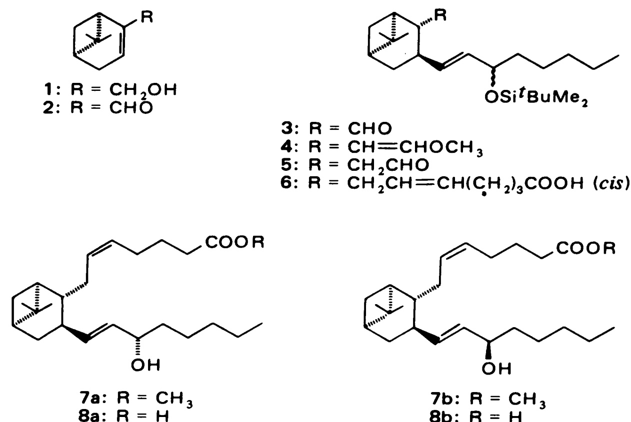


FIG. 2. Synthesis of pinane-thromboxane A_2 (PTA_2).

RESULTS

Coronary Artery Constriction. Incubation of cat coronary arteries with PTA_2 (1 μM) abolished the constriction induced by 30 nM 9,11-azo-PGH_2 (Fig. 3). Mean constrictions equivalent to 32 and 53 mm Hg (1 mm Hg = 133 Pa) were observed in three experiments at 15 nM and 30 nM 9,11-azo-PGH_2, respectively. At a concentration of 100 nM, PTA_2 reduced these constrictions by 73% and 50%, respectively. Similar results were obtained in one experiment using 9,11-methanoepoxy-PGH_2 as the agonist.

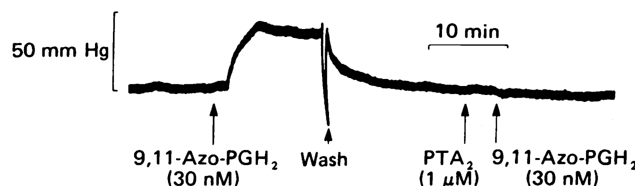


FIG. 3. Constriction of a cat coronary artery induced by 9,11-azo-PGH_2 (30 nM) and antagonism of this constriction by PTA_2 (1 μM).

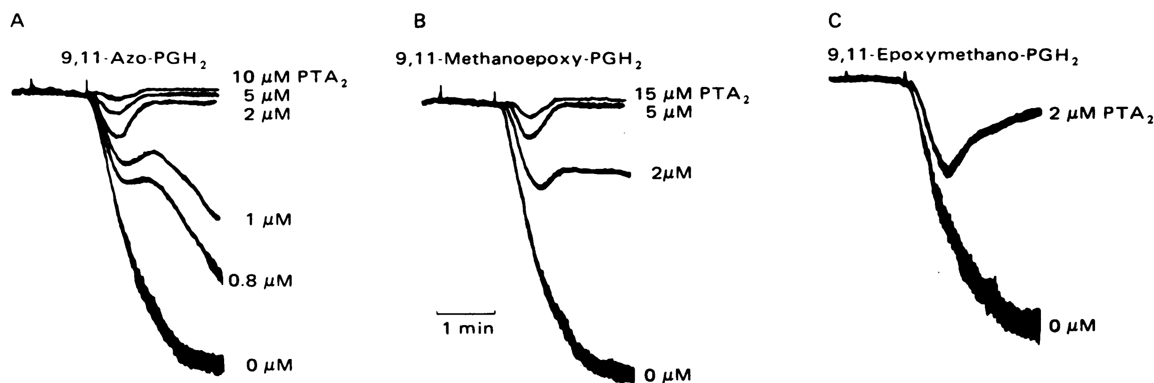


FIG. 4. Effect of PTA_2, at different concentrations, on the aggregation of human platelets induced by: (*A*) 0.18 μM 9,11-azo-PGH_2; (*B*) 0.29 μM 9,11-methanoepoxy-PGH_2; or (*C*) 1.43 μM 9,11-epoxymethano-PGH_2. Downward displacement of the curves indicates aggregation (decreased optical density).

At a final concentration of 1 μM, PTA_2 caused a significant ($P < 0.005$) inhibition of the release of cathepsin D from cat liver lysosomes (four experiments).

Platelet Aggregation. PTA_2 in intermediate concentrations (1–15 μM) inhibited the aggregation of human platelets induced by 9,11-azo-PGH_2, 9,11-epoxymethano-PGH_2 (U44069) or 9,11-methanoepoxy-PGH_2 (U46619) in a dose-dependent fashion (Fig. 4). PTA_2 at 10 μM[‖] abolished platelet aggregation induced by arachidonic acid, abolished the second wave of aggregation induced by ADP or epinephrine, and partially inhibited platelet aggregation induced by collagen. At this concentration, PTA_2 did not inhibit primary aggregation induced by ADP or epinephrine, nor did it affect the inhibition of aggregation by PGI_2 or PGD_2. The 15*R*-epimer (PG numbering) of PTA_2 (**8b**) was approximately 1/10th as effective as PTA_2 as a prostaglandin endoperoxide antagonist.

Thromboxane Synthetase. At 10 μM, PTA_2 inhibited the formation of thromboxane B_2 from [1-^{14}C]arachidonic acid by washed rabbit platelets by 23%. At 100 μM, 83% inhibition of thromboxane B_2 formation was observed. This was associated with corresponding increases in radioactive PGE_2, PGD_2, and $PGF_{2\alpha}$, indicating that the effect of the analog was on thromboxane synthetase and not on cyclooxygenase. While the analog, at 50 μM, abolished arachidonic acid-induced aggregation in human platelet-rich plasma, thromboxane B_2 formation was reduced by only 50%.

Prostacyclin Synthetase. When incubated with ram seminal vesicle microsomes, 62% of arachidonic acid was converted into 6-keto-$PGF_{1\alpha}$, the stable end-product of PGI_2 (Fig. 1). This conversion was unaffected by the analog at 100 μM.

DISCUSSION

The isolation (10, 13) of the prostaglandin endoperoxides PGH_2 and PGG_2 not only confirmed their intermediary role in the biosynthesis of prostaglandins but also led to the discovery of two pathways of metabolism of arachidonic acid (Fig. 1). The thromboxane pathway was discovered by Hamberg and associates (1), who demonstrated that the platelet-aggregating and rabbit aorta-contracting activities of prostaglandin endoperoxides were significantly enhanced after they had been transformed by platelets into the highly unstable thromboxane A_2 (TA_2). Subsequently, it was demonstrated that the release of TA_2 from platelets induced by thrombin was associated with the development of activity that markedly contracted coronary arteries, and it was suggested that TA_2 may be the cause of the angina associated with arterial thrombosis (14). The second pathway, described by Moncada and associates (15), is now known to involve endothelial cells that transform prostaglandin endoperoxides into prostacyclin (PGI_2), a potent inhibitor of platelet aggregation and a vasodilator (Fig. 1). Thus, two unstable agents, TA_2 and PGI_2, with directly opposing effects, either promoting or inhibiting thrombosis, can be formed from prostaglandin endoperoxides.

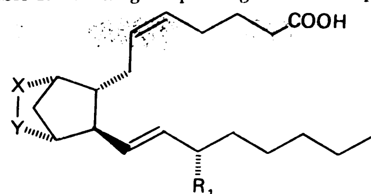
Progress in understanding the actions of prostaglandin endoperoxides has been achieved by chemical synthesis of stable analogs. Several analogs of PGH_2 possessing a 15*S*-hydroxyl group have been synthesized (e.g., compounds **I–V**, Table 1) and have been found to induce platelet aggregation and cause contraction of the rabbit aorta. The potency of 9,11-azo-PGH_2 is comparable to that of TA_2 in both of these systems (16, 24). Because these compounds are stable and mimic the agonistic effects of the naturally occurring but unstable PGG_2, PGH_2, and TA_2, we used them to evaluate the effects of PTA_2 in our studies.

Two stable analogs of PGH_2 lacking the 15*S*-hydroxyl group have been found to inhibit platelet aggregation (compounds **VI** and **VII**, Table 1). The 15-deoxy derivative of 9,11-azo-PGH_2 was found to be a potent inhibitor of thromboxane synthetase. Because this compound inhibited platelet aggregation induced by arachidonic acid or PGH_2 but not by 9,11-epoxymethano-PGH_2, it was suggested that this proved that the conversion of PGH_2 into TA_2 was obligatory for platelet aggregation. However, this suggestion is open to question, because it was shown subsequently that imidazole, another inhibitor of thromboxane synthetase, has no effect on platelet aggregation induced by PGH_2 (25). Subsequently, 15-deoxy-9,11-azo-PGH_2 was found to be a potent inhibitor of prostacyclin synthetase (26). Recently, 15-deoxy-9,11-epoxyimino-PGH_2 was reported to be a TA_2 receptor antagonist because it inhibited platelet aggregation induced by arachidonic acid, TA_2, PGH_2, and PGH_2 analogs. This compound did not inhibit thromboxane synthetase. However, it did inhibit prostacyclin synthetase and mimicked the effects of TA_2 on rabbit aorta, producing vasoconstriction.

Pinane-thromboxane A_2 (PTA_2) has properties that clearly distinguish it from prostaglandin endoperoxide analogs (Table 2). First, in low concentrations PTA_2 inhibited the constriction of cat coronary arteries induced by the PGH_2 analogs that mimic TA_2. This must be a direct antagonistic effect and not inhibition of enzyme activity, because the stable analogs cannot be converted to TA_2 by thromboxane synthetase, and, besides, cat coronary arteries are incapable of thromboxane synthesis

‖ At concentrations above 10 μM, PTA_2 itself induced platelet shape change and, at 50 μM, a very small reversible aggregation was observed, indicating partial agonistic activity. At these concentrations, subsequent responses to prostaglandin endoperoxide analogs were abolished.

Table 1. Analogs of prostaglandin endoperoxides



PGH$_2$: X—Y = O—O, R$_1$ = OH; PGG$_2$: X—Y = O—O, R$_1$ = OOH

Analog	Name	X—Y	R$_1$	Platelet-aggregating activity, % of PGG$_2$	Aorta-contracting activity, % of PGH$_2$	Refs.
I	9,11-Azo-PGH$_2$	N=N	OH	790	700	16
II	9,11-Methanoepoxy-PGH$_2$ (U46619)	CH$_2$—O	OH	370	620	17, 18, 19
III	9,11-Epoxymethano-PGH$_2$ (U44069)	O—CH$_2$	OH	63	360	17, 18, 19
IV	9,11-Etheno-PGH$_2$	CH=CH	OH	10	10	3
V	9,11-Dithia-PGH$_2$	S—S	OH	100	2400	20, 21
VI	15-Deoxy-9,11-azo-PGH$_2$	N=N	H	Inhibits	?	22
VII	15-Deoxy-9,11-epoxyimino-PGH$_2$	O—NH	H	Inhibits	Contracts	23

(unpublished observations). Similarly, PTA$_2$ antagonized the effects of the PGH$_2$ analogs on platelet aggregation. The selectivity of this antagonism for prostaglandin-induced aggregation was evidenced by the finding that PTA$_2$ had no effect on primary aggregation induced by ADP or epinephrine. On the other hand, PTA$_2$ did inhibit secondary aggregation induced by these agents, and it abolished aggregation induced by arachidonic acid. This indicates that PTA$_2$ can antagonize the effects of TA$_2$ formed endogenously by platelets.

PTA$_2$ at higher concentrations also inhibited thromboxane synthetase. However, it was less potent as an enzyme inhibitor than as an antagonist. The abolition of arachidonic acid-induced aggregation by PTA$_2$ is probably mainly due to direct antagonism of thromboxane effects because, even at 50 μM, PTA$_2$ inhibited thromboxane B$_2$ formation by only 50%. Most significant was the finding that PTA$_2$ has no effect on prostacyclin synthesis or on the inhibition of platelet aggregation produced by PGI$_2$ or PGD$_2$.

Increased TA$_2$ formation has been implicated in the vasospasm associated with coronary artery disease and angina (19). PTA$_2$ would seem to possess an ideal biochemical profile for use as an antithrombotic agent (Table 2). The primary effect of PTA$_2$ would be to prevent vasoconstriction of coronary arteries due to excess thromboxane formation. Additionally, PTA$_2$ would reduce platelet aggregation and the synthesis of thromboxanes. Because these effects occur only at higher concentrations, and PTA$_2$ has no effect on primary aggregation induced by ADP, PTA$_2$ should have little influence on hemostasis. The lysosomal stabilizing property of PTA$_2$ suggests that it may have a beneficial effect on the ischemic myocardium. Unlike aspirin, which inhibits cyclooxygenase, or the 15-deoxy-PGH$_2$ analogs (compounds **VI** and **VII**, Table 1) that inhibit prostacyclin synthetase, PTA$_2$ does not compromise PGI$_2$ formation or effects.

Table 2. Properties of PTA$_2$

1. Inhibits coronary artery contraction by prostaglandin endoperoxide analogs, ID$_{50}$ 0.1 μM
2. Stabilizes lysosomes at 1 μM
3. Inhibits aggregation by prostaglandin endoperoxide analogs, ID$_{50}$ 2.0 μM
4. Inhibits thromboxane synthetase, ID$_{50}$ 50 μM
5. Has no effect on prostacyclin synthetase at 100 μM
6. Has no effect on the inhibiton of platelet aggregation by PGD$_2$ or PGI$_2$

ID$_{50}$, dose that causes 50% inhibition of activity.

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