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## Occurrence of Desmosterol and Other Sterols in the Clam, *Tapes philippinarum*

Shin-ichi TESHIMA, Akio KANAZAWA, and Tetsuo ANDO\*

### Abstract

This paper deals with the sterol composition of naturally occurring sterols in the clam, *Tapes philippinarum*. The gas-liquid chromatography on 1.5% OV-17 and 1.5% SE-30 indicated that the clam contain 22-*trans*-24-norcholesta-5,22-dien-3 $\beta$ -ol, 22-dehydrocholesterol, cholesterol, brassicasterol, desmosterol, campesterol, 24-methylenecholesterol, stigmasterol,  $\beta$ -sitosterol, and at least three other sterols. 22-Dehydrocholesterol, desmosterol, and 24-methylenecholesterol were isolated as a pure sterol by a column chromatography on silver nitrate-impregnated silicic acid, and then the identification of them were further confirmed by infrared absorption spectra and mass spectrometry. Moreover, it was found that the sterols isolated from the three samples of clams were composed of relatively similar components.

Recently, the culture of the prawn, *Penaeus japonicus*, has been practiced prosperously in the various districts of Japan. In the culture of this crustacean, the clams, *Mytilus crassitesta* and *Tapes philippinarum*, have been used as a diet. In the previous papers, the authors have reported that the prawn lacks sterol-synthesizing ability from acetate<sup>1)</sup> and requires sterols as a diet.<sup>2)</sup> Furthermore, it has been clarified that the several marine crustaceans are capable of converting some C<sub>28</sub> and C<sub>29</sub>-sterols to cholesterol.<sup>3~5)</sup> The above findings suggest that the prawn may modify some dietary C<sub>28</sub> and C<sub>29</sub>-sterols to the sterols which are appropriate to one. On the other hand, it is a well known fact that marine mollusks contain a variety of sterols in their tissues.<sup>6)</sup> Hence, the authors intended to clarify the sterol composition of the clam, *T. philippinarum*, prior to investigation of sterol metabolism in the prawn, *P. japonicus*.

This paper deals with the sterol composition of naturally occurring sterols in the clam *T. philippinarum*, obtained in the two seasons, spring and late-autumn.

### Materials and Methods

**Clam.** The three samples of the clam, *T. philippinarum*, were obtained alive from the commercial sources in Kagoshima, Japan. The first and second samples were obtained in May and in November, 1970, respectively. The third sample was received in April, 1971. The clams were washed thoroughly with tap water and then stored in a refrigerator (-20°C) until used.

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**Sterols.** Authentic sterols used as a standard in identification of sterols were obtained from the various sources. Cholesterol, desmosterol, campesterol, stigmasterol, and  $\beta$ -sitosterol were purchased from the commercial sources (Nakarai Chemical Co., Tokyo Chemical Co., and Gaschro-Kogyo Co., Japan). 22-*trans*-24-Norcholesta-5,22-dien-3 $\beta$ -ol has been isolated from the clam, *T. philippinarum*.<sup>7)</sup> 22-Dehydrocholesterol, brassicasterol, and fucosterol were obtained from the red alga, *Porphyridum cruentum*, diatom, *Cyclotella nana*, and brown alga, *Sargassum piluliferum*, respectively. Cholestanol, campestanol, and  $\beta$ -sitostanol were prepared from cholesterol, campesterol, and  $\beta$ -sitosterol by the reduction with platinum oxide, respectively. The purity of these sterols was checked by thin-layer chromatography (TLC) on Kieselgel G with ethyl acetate-benzene (1:4) and by gas-liquid chromatography (GLC) on 1.5% OV-17.

**Isolation of the sterols.** From the frozen clam, the shell was removed and the lipids were extracted from the edible parts with chloroform-methanol according to the method of Bligh and Dyer.<sup>8)</sup> The lipids were saponified with 10% of alcoholic potassium hydroxide at 80°C for 2 hours, and then the unsaponifiable materials were extracted with ether in the usual manner. The sterols were isolated from the unsaponifiable materials by a column chromatography on alumina (grade II, Merck) with hexane-benzene.<sup>9)</sup> The sterols so obtained were recrystallized several times from methanol.

**GLC.** The gas-chromatograph used in this study was a dual column Shimadzu model GC-3AF with flame ionization detectors. As a column, the coiled glass (2 m  $\times$  4 mm I. D.) and stainless (3 m  $\times$  4 mm I. D.) columns packed with 1.5% SE-30 on 60-80 mesh Chromosorb W and with 1.5% OV-17 on 80-100 mesh Shimadzu W, respectively, were used. The SE-30 and OV-17 columns were operated at 206°C and 242°C, respectively. The identification of sterols was achieved by comparing the relative retention times (relative to cholesterol) to that of authentic sterols. The carbon numbers of sterols were also determined by the steroid number (S. N.)<sup>10)</sup> in GLC and by the hydrogenation of free sterols.

**Derivative formations.** Steryl acetate was formed by addition of acetic acid anhydride-dry pyridine (1:1) and standing for 48 hours at room temperature. After elimination of reagents by stream of nitrogen, the obtained steryl acetate was purified by recrystallization from methanol. Hydrogenation of sterols was carried out as follows: Thirty mg of sterols was dissolved in 30 ml of acetic acid-ethyl acetate (1:1), and then the container was flushed with nitrogen in order to eliminate oxygen and bubbled with pure hydrogen in the presence of 5 mg of platinum oxide for 3 hours at room temperature. The reaction mixture was filtered through a glass filter and the solvent was removed under reduced pressure. The resultant crystal was recrystallized from methanol.

**Separation of the sterols.** For the purpose of separating the individual sterols, the sterols mixture was acetylated and chromatographed on a mixture of silver nitrate and silicic acid (Mallinckrodt Chemical Works) (1:4, w/w)<sup>11)</sup>. The steryl acetate (1.0 g) was chromatographed on 100 g of the adsorbent. The column was

eluted step-wise with the following solvents; 300 ml hexane, 300 ml hexane-benzene (90:10), 150 ml hexane-benzene (85:15), 550 ml hexane-benzene (80:20), 500 ml hexane-benzene (75:25), 600 ml hexane-benzene (72:28), 500 ml hexane-benzene (70:30), 450 ml hexane-benzene (65:35), and 350 ml hexane-benzene (60:40). The fraction was monitored by TLC and by GLC.

**Spectra analysis.** Infrared absorption spectra were obtained with a Nippon Bunko DS-301 spectrometer in chloroform. Mass spectra were measured on the Hitachi RMU-6D instrument (Chamber voltage, 70 eV).

### Results

The sterol contents of the clam, *T. philippinarum*, examined are given in Table 1. Among the three samples, a remarkable difference was not seen on the sterol contents.

Table 1. The sterol contents of the clam, *T. philippinarum*.

Samples	Date obtained	Fresh weight* (g)	Sterols	
			(g)	(%)**
First	April 12, 1970	7000	5.0	0.07
Second	November 8, 1970	100.0	0.050	0.05
Third	May 1, 1971	20.0	0.012	0.06

\* Weight of the edible parts

\*\* Per fresh weight

**GLC of the sterols.** In GLC on 1.5% OV-17, the sterols isolated from the first sample of the clam were found to contain 12 components as shown in Fig. 1 and Table 2. The peaks a-1, a-3, a-4, a-5, a-6, a-7, a-8, a-9, a-10, and a-11 were identical in the retention times with authentic 22-*trans*-24-norcholesta-5, 22-dien-3 $\beta$ -ol, 22-dehydrocholesterol, cholesterol, brassicasterol, desmosterol, campesterol,

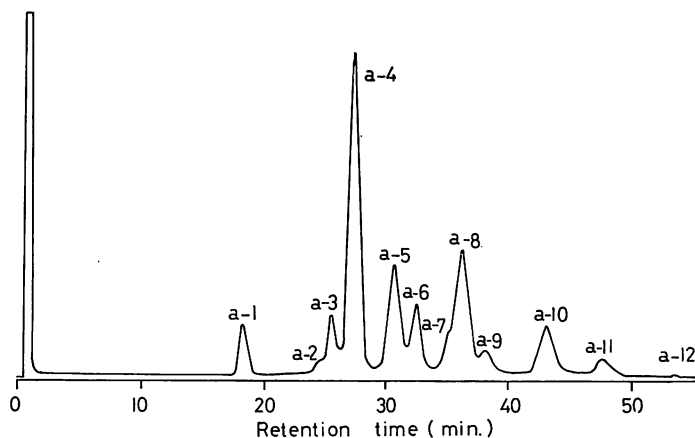


Fig. 1. GLC on 1.5% OV-17 of the sterols isolated from the clam, *T. philippinarum*.

24-methylenecholesterol, stigmasterol,  $\beta$ -sitosterol, and fucosterol, respectively. The peaks a-2 and a-12 gave the different retention times from that of the authentic sterols examined. Considering the relative retention time to cholesterol, the peak a-2 may be assumed to be 22-*cis*-cholesta-5,22-dien-3 $\beta$ -ol<sup>12)</sup> which was isolated recently from the scallop, *Placopecten magellanicus* (Gmelin) by IDLER and WISEMAN.

**Table 2.** The sterol compositions of the clam, *T. philippinarum*, determined by GLC on 1.5% OV-17.

Peaks	Retention times (min.)	Relative retention times	Composition (%)			Identified as
			Samples			
			First	Second	Third	
a - 1	18.6	0.67	4	3	3	22- <i>Trans</i> -24-norcholesta-5, 22-dien-3 $\beta$ -ol*
a - 2	24.7	0.89	1	1	2	Unknown
a - 3	25.8	0.93	6	6	7	22-Dehydrocholesterol
a - 4	27.8	1.00	35	33	35	Cholesterol
a - 5	31.2	1.12	14	11	11	Brassicasterol
a - 6	32.8	1.18	9	6	5	Desmosterol
a - 7	35.5	1.28	trace	6	trace	Campesterol
a - 8	36.8	1.32	17	18	23	24-Methylenecholesterol
a - 9	38.5	1.39	4	2	3	Stigmasterol
a -10	43.5	1.57	8	9	6	$\beta$ -Sitosterol
a -11	48.0	1.73	3	5	4	Fucosterol
a -12	62.6	1.78	trace	-	-	Unknown

\* The peak a-1 was found to contain the two sterols corresponding to the peaks c-1 and c-2 in GLC on 1.5% SE-30.

The GLC on 1.5% OV-17 of the hydrogenate of the first clam sample gave 4 prominent peaks and 2 trace peaks. Compared with the relative compositions of the free sterols and their hydrogenate (see Table 3 and Fig. 2), the peak b-1 was conceivable to be derived from the peak a-1. The peaks b-3, b-5, and b-6 were assigned to be formed from the peaks a-3, a-4, and a-6, the peaks a-5, a-7, a-8, and the peaks a-9, a-10 and a-11, respectively.

Table 4 gave the results of the GLC on 1.5% SE-30 of the first clam sample.

**Table 3.** GLC on 1.5% OV-17 of the hydrogenate of the sterols isolated from the first clam sample.

Peaks	Retention times (min.)	Relative retention times	Compositions (%)	% Compositions of the free sterol*
b - 1	19.2	0.76	6	4 (peak a-1)
b - 2	22.0	0.87	trace	-
b - 3	25.3	0.99	51	50 (peak a-3, a-4, and a-6)
b - 4	29.3	1.15	trace	-
b - 5	32.5	1.28	30	31 (peak a-5, a-7, and a-8)
b - 6	41.8	1.65	15	15 (peak a-9, a-10 and a-11)

\* Calculated from the gas-chromatogram of the free sterols.

In this column the sterols showed the presence of 9 components and the following sterol mixtures could not be separated; the mixtures of 22-*cis*-cholesta-5,22-dien-3 $\beta$ -ol and 22-dehydrocholesterol, desmosterol and brassicasterol, campesterol and 24-methylenecholesterol, and  $\beta$ -sitosterol and fucosterol. However, the unknown sterol (peak c-1) was detected in addition to the sterols found in the GLC on 1.5% OV-17. Considering the S. N. determined by GLC on 1.5% SE-30, the sterol

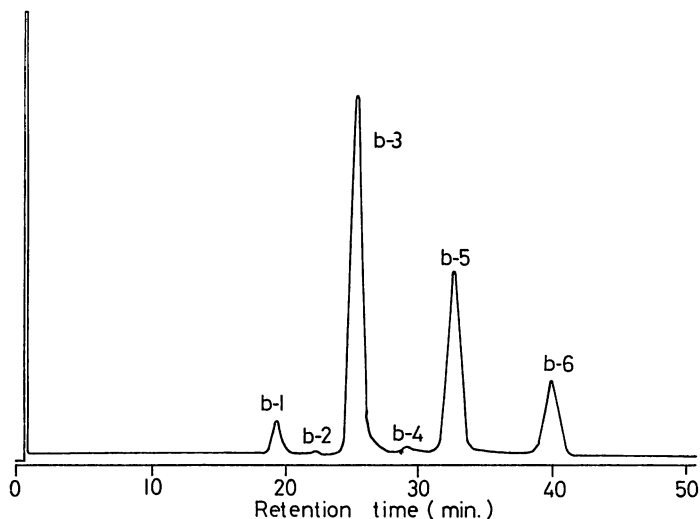


Fig. 2. GLC on 1.5% OV-17 of the hydrogenate of sterols isolated from the clam, *T. philippinarum*.

(S. N., 27.5) was assumed to be the sterol other than  $\Delta^5$ -sterols with the carbon number of  $C_{27}$ ,  $C_{28}$ , and  $C_{29}$  usually found in marine mollusks. In a silver nitrate-impregnated column chromatography, this sterol was eluted with hexane-benzene (72:28) and gave the relative retention time of 0.70 in GLC on 1.5% OV-17.

In the case of the second and third samples of the clam, the GLC analysis showed that the sterols isolated were composed of the essentially same components as that of the first sample. Also, a marked variation in the relative composition of the sterols was not seen among the clam samples. Hence, the sterols (as acetate) isolated from the first sample were chromatographed on a silver nitrate-impregnated silicic acid in order to isolate the individual sterols from

Table 4. GLC on 1.5% SE-30 of the first sample of the clam, *T. philippinarum*.

Peaks	Relative retention times*	Composition (%)
c - 1	0.63	1
c - 2	0.67	3
c - 3	0.91	6
c - 4	1.00	36
c - 5	1.11	23
c - 6	1.27	17
c - 7	1.43	4
c - 8	1.62	11
c - 9	1.85	trace

\* Relative to cholesterol (Retention time, 21.8 min.)

the sterol mixture. As a result, the 5 sterols (S-1, S-2, S-3, S-4, and S-5) were obtained as a pure sterol. These sterols gave the single peaks in the GLC on 1.5 % OV-17 and 1.5 % SE-30. After saponification with 5 % alcoholic potassium hydroxide, the each sterols obtained were subjected to infrared absorption and mass spectral analyses to confirm the identification of them. The sterols S-1 and S-2, eluted with hexane-benzene (72: 28), corresponded to the peaks c-1 and c-2 in GLC on 1.5 % SE-30. The structural elucidation of the above 2 sterols by spectral analyses has been reported in detail elsewhere.<sup>7,13)</sup>

**Identification of 22-dehydrocholesterol.** The sterol S-3, eluted with hexane-benzene (75: 25), corresponded to the peak a-3 in GLC on 1.5 % OV-17. The infrared spectrum of this sterol showed the absorption at  $970\text{ cm}^{-1}$  specific for a trans double bond at C-22 in the side chain of sterols.<sup>16, 17)</sup> As shown in Fig. 4, the mass spectrum of this sterol gave the molecular ion peak at  $m/e$  384 ( $M^+$ ) and other prominent peaks at  $m/e$  369 ( $M^+ - \text{CH}_3$ ), 366 ( $M^+ - \text{HOH}$ ), 299 [ $M^+ - (\text{CH}_3 + \text{C}_{23} - \text{C}_{27}$

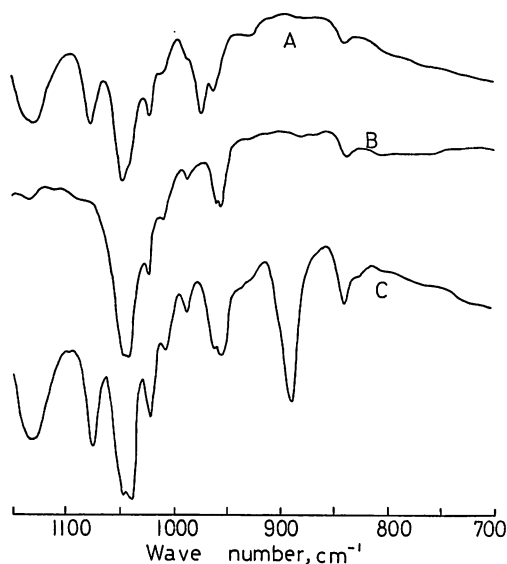


Fig. 3. IR spectra of 22-dehydrocholesterol (A), desmosterol (B), and 24-methylenecholesterol (C) isolated from the clam, *T. philippinarum*.



Fig. 4. The mass spectrum of 22-dehydrocholesterol isolated from the clam, *T. philippinarum*.

+1H)], 273 ( $M^+ - R$ , R=alkyl side chain), 271 [ $M^+ - (R+2H)$ ], 255 [ $M^+ - (R+HOH)$ ], and 213 [ $M^+ - (R+42+HOH)$ ]. These data strongly supported that this sterol is 22-dehydrocholesterol.

**Identification of desmosterol.** The sterol S-4, eluted with hexane-benzene (65:35), corresponded to the peak a-6 in GLC on 1.5% OV-17. The infrared spectrum of this sterol gave no significant absorption at  $890\text{ cm}^{-1}$  as shown in Fig. 3. This rejected the possibility that this sterol may be the 25-dehydroisomer of desmosterol.<sup>14)</sup> In addition, the doublet at  $958$  and  $950\text{ cm}^{-1}$  indicated the presence of a  $\Delta^{5,24}$ -sterol.<sup>15)</sup> The mass spectrum of this sterol is shown in Fig. 5. The mass spectrum gave the molecular ion peak at  $m/e$  384 ( $M^+$ ) and other prominent peaks at  $m/e$  369 ( $M^+ - \text{CH}_3$ ), 366 ( $M^+ - \text{HOH}$ ), 351 [ $M^+ - (\text{CH}_3 + \text{HOH})$ ], 299 [ $M^+ - (\text{CH}_3 + \text{C}_{23} - \text{C}_{27} + 1\text{H})$ ], 271 [ $M^+ - (R+2H)$ , R=alkyl side chain], 255 [ $M^+ - (R+HOH)$ ], 253 [ $M^+ - (R+2H+HOH)$ ], 229 [ $M^+ - (R+27+HOH)$ ], and 213 [ $M^+ - (R+42+HOH)$ ]. The above results confirmed the identity of desmosterol.

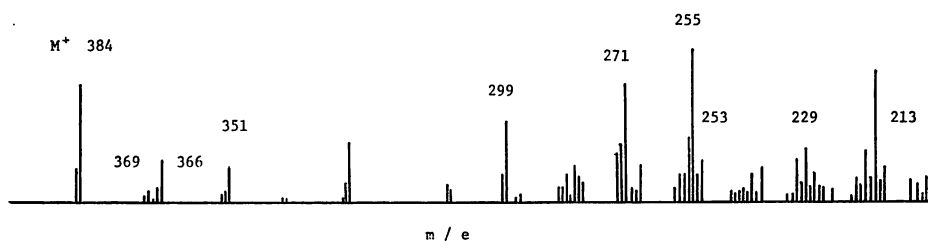


Fig. 5. The mass spectrum of desmosterol isolated from the clam, *T. philippinarum*.

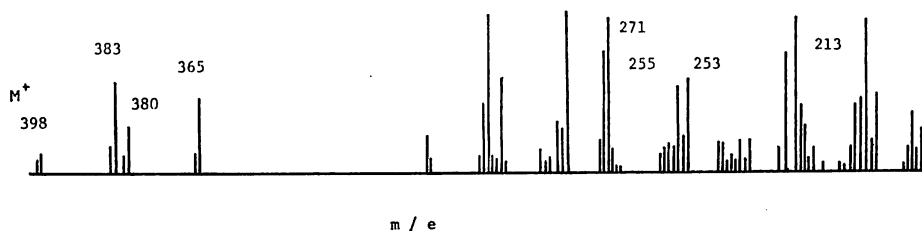


Fig. 6. The mass spectrum of 24-methylenecholesterol isolated from the clam, *T. philippinarum*.

**Identification of 24-methylenecholesterol.** The sterol S-5, eluted with hexane-benzene (60:40), corresponded to the peak a-8 in GLC on 1.5% OV-17. It is generally difficult to separate clearly the mixture of 24-methylenecholesterol and campesterol by the usual GLC. However, the infrared spectrum of this sterol gave the strong absorption at  $890\text{ cm}^{-1}$  indicative of the presence of  $\text{CH}_2 = \text{CRR}'$  in the side chain<sup>14)</sup> and eliminated the possibility of campesterol. Furthermore, the mass spectrum of this sterol showed the molecular ion peak at  $m/e$  398 ( $M^+$ ) and other prominent peaks at  $m/e$  383 ( $M^+ - \text{CH}_3$ ), 380 ( $M^+ - \text{HOH}$ ), 365 [ $M^+ - (\text{CH}_3 + \text{HOH})$ ], 271 [ $M^+ - (R+2H)$ , R=alkyl side chain], 255 [ $M^+ - (R+HOH)$ ], and 253



[ $M^+ - (R + HOH + 2H)$ ]. On the basis of these data, this sterol was identified as 24-methylenecholesterol.

### Discussion

MATSUMOTO and TAMURA have reported the presence of 7-dehydrostigmasterol in the tissues of the clam, *Tapes sp.*<sup>18)</sup> However, the detail composition of the sterols was obscure. Recently YASUDA has demonstrated that the commercial "stripped clam", *Tapes japonica* contained five  $\Delta^5$ -sterols, 22-dehydrocholesterol, cholesterol, brassicasterol, 24-methylenecholesterol, and isofucosterol, and other minor sterols.<sup>19)</sup> The above five  $\Delta^5$ -sterols were identified by TLC, GLC on 1.5 % SE-30, melting points, and infrared absorption spectra. In the present study, it was shown by GLC that the three samples of the clam, *T. philippinarum*, obtained alive in the two different seasons contained at least 11 or 12 components. In addition to the  $\Delta^5$ -sterols except isofucosterol reported on the clam, *T. japonica*, by YASUDA, the presence of 22-*trans*-24-norcholesta-5,22-dien-3  $\beta$ -ol, desmosterol, campesterol, stigmasterol,  $\beta$ -sitosterol, and fucosterol was demonstrated to occur in the clam, *T. philippinarum*. The identification of these sterols was performed by GLC using the both selective (1.5 % OV-17) and non-selective (1.5 % SE-30) columns and by comparing the retention times with authentic sterols. In the case of desmosterol, 22-dehydrocholesterol, and 24-methylenecholesterol, the identities of them were further confirmed by both infrared absorption and mass spectrometry. However, isofucosterol was not detected by GLC. The difference in the sterol composition between the two clams, *T. japonica* and *T. philippinarum*, may be postulated to be due to the discrepancy of the species and/or sampling season etc. 22-Dehydrocholesterol and 24-methylenecholesterol isolated in the present study were found to also occur in the several mollusks. For example, 22-dehydrocholesterol has been detected in the clams, *Crassostrea virginica* (8 %)<sup>20)</sup> and *Venus sp.* (8 %),<sup>21)</sup> the oyster, *Ostrea sp.* (3 %),<sup>21)</sup> and the scallop, *Plocapecten magellanicus* (14 %).<sup>22)</sup> 24-Methylenecholesterol has been demonstrated in the clams, *Saxidomus giganteus* (53 %),<sup>22)</sup> *C. virginica* (11 %),<sup>20)</sup> and *Venus sp.* (20 %),<sup>21)</sup> the oysters, *Ostrea gigas* (36 %),<sup>24)</sup> *O. virginica* (36 %),<sup>22)</sup> and *O. gryphea* (as major sterol),<sup>25)</sup> and the scallop, *P. magellanicus* (17.4 %).<sup>12)</sup>

On the other hand, desmosterol isolated first from the chick embryo<sup>26)</sup> has also been found in the barnacle, *Balanus glandula* (34 %), the Alaskan king crab, *Paralithodes camtschatica* (31.1 %),<sup>27)</sup> the North Atlantic queen crab, *Chionoecetes opilio* (6.1 %),<sup>27)</sup> and the Mysidacea, *Neomysis intermedia* (22 %).<sup>9)</sup> However, as far as the present authors know, this sterol has not been isolated from marine mollusks yet.

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