Cytotoxic Diterpenoids from the Stem Bark of *Annona squamosa* L.

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Three new *ent*-kaurane diterpenoids, (4α)-19-nor-*ent*-kaurane-4,16,17-triol (**1**), (4α,16α)-17-(acetyloxy)-19-nor-*ent*-kaurane-4,16-diol (**2**), and 17-hydroxy-*ent*-kaur-15-en-19-al (**3**), together with 11 known compounds, were isolated from the stem bark of *Annona squamosa* L. The structures of **1**–**3** were identified by analysis of their spectroscopic data. All compounds were evaluated for cytotoxic activity against human lung cancer (95-D) and ovarian cancer (A2780) cell lines, and compounds **3**, **5**, **7**, **11**–**14** exhibited promising antiproliferative activities with IC_{50} values ranging from 0.38 to 34.66 μM.

Introduction. – *Annona squamosa* L. (Annonaceae) is a small tree native to Central America. It is now cultivated throughout tropical areas of China for its fruit known as custard apple. Pharmacological studies on *A. squamosa* have demonstrated analgesic, anti-inflammatory [1], anti-ulcer [2], antidiabetic [3], antimicrobial [4], anti-ovulatory [5], antifertility [6], and most importantly, antitumor activities [7][8]. Previous phytochemical investigations have resulted in the isolation of acetogenins [9][10], *ent*-kaurane diterpenoids [11][12], flavonoids [3], lignans [13], alkaloids [14], and cyclopeptides [15], among which the acetogenins have been considered as promising antitumor candidates for future clinical application [16]. On the other hand, over twenty-five diterpenoids have been discovered from *A. squamosa*, and some of them also showed potential antitumor activities [17]. In the current study, fourteen *ent*-kaurane diterpenoids, including three new ones (see **1**–**3**[1]), *Fig. 1*), were isolated from the stem bark of *A. squamosa*. All compounds were evaluated for cytotoxicity against human lung cancer (95-D) and ovarian cancer (A2780) cell lines by using the sulforhodamine B (SRB) assay, and the primary structure–activity relationships of these diterpenoids are discussed briefly.

Results and Discussion. – Compound **1** was obtained as a white amorphous powder. Its molecular formula was deduced as $C_{19}H_{32}O_3$ from the HR-ESI-MS (*m*/*z* 331.2237 ($[M+Na]^+$)). The IR absorption band at 3365 cm^{-1} implied the presence of OH

[1]) Trivial atom numbering; for systematic names, see *Exper. Part*.

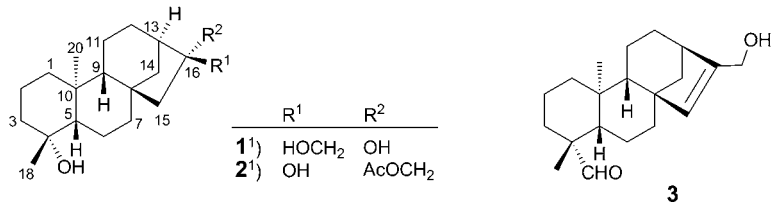

Fig. 1. *Compounds* **1**–**3**, *isolated from* Annona squamosa

groups. The ^{1}H-NMR spectrum of **1** (*Table 1*) displayed two characteristic Me *s* of an equatorial Me(18) and an axial Me(20) group at δ(H) 1.00 and 0.94 for an *ent*-kaurane diterpene [11]. A pair of intercoupling *d*s at δ(H) 3.62 and 3.72 ($J = 11.4$ Hz, each 1 H) indicated the presence of an oxygenated methylene group. The ^{13}C-NMR spectrum (*Table 1*) exhibited 19 C-atom signals. An oxygenated quaternary C-atom signal at δ(C) 82.8, together with the oxygenated methylene C-atom at δ(C) 66.8, indicated that compound **1** probably possessed a 16α,17-dihydroxy-substituted *ent*-kaurane skeleton, similar to that of 16,17-dihydroxy-*ent*-kauran-19-oic acid (OH–C(16) α-oriented; **6**) [11][12]. Comparison of the ^{13}C-NMR spectra of both compounds showed that the C=O group at δ(C) 180.1 of the *ent*-kauran-19-oic acid was absent, and an additional oxygenated quaternary C-atom signal at δ(C) 73.1 was present in **1**, which suggested the presence of an OH group at C(4). The above deduction was further confirmed by HSQC, HMBC, and NOESY experiments. The 16α,17-dihydroxy moiety was confirmed by the HMBCs CH_2(17)/C(13), C(15), and C(16) (*Fig. 2*). The correlations Me(18)/C(3), C(4), and C(5) located the OH–C(4) group properly. The NOESY correlations CH_2(17)/H_β–C(15), H_β–C(15)/H–C(9), H–C(9)/H_β–C(1), H_β–C(1)/H–C(5), H_β–C(1)/H_β–C(3), and H_β–C(3)/Me(18) suggested their relative β-orientation. The α-orientation of H_α–C(1), Me(20), H_α–C(14), H–C(13), and H_α–C(12) was then confirmed by the corresponding NOESY correlaions (*Fig. 3*). Therefore, the structure of compound **1** was identified as (4α)-19-nor-*ent*-kaurane-4,16,17-triol[1]).

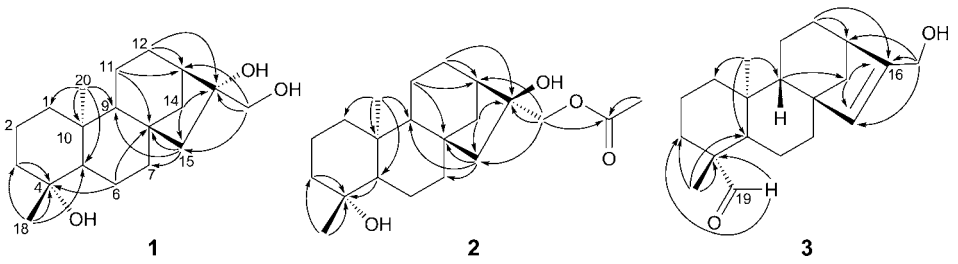

Fig. 2. *Selected HMBCs of compounds* **1**–**3**[1])

Compound **2** showed a HR-ESI-MS with a quasi-molecular-ion peak at *m*/*z* 373.2349 ([M + Na]$^+$), which suggested a molecular formula $C_{21}H_{34}O_4$. The IR spectrum revealed the presence of an ester carbonyl group at 1710 cm^{-1}. In the ^{1}H-NMR spectrum (*Table 1*), besides two Me *s* at δ(H) 1.04 and 1.32 familiar to these

Table 1. *^{1}H- and ^{13}C-NMR Data* (400 and 100 MHz, resp.) *of Compounds* **1**–**3**. δ in ppm, J in Hz.

Position[1])	**1**[a])		**2**[b])		**3**[c])	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
CH_2(1)	0.72 (*td*, J = 12.1, 4.2), 1.64–1.66 (*m*)	40.8	0.79 (*td*, J = 12.6, 4.3), 1.76 (overlapped)	40.4	0.80 (*td*, J = 13.5, 3.6), 1.83 (overlapped)	39.9
CH_2(2)	1.45 (overlapped)	20.6	1.56 (overlapped)	20.5	1.45 (overlapped)	18.5
CH_2(3)	1.22–1.26, 1.62–1.64 (2*m*)	43.6	1.68, 1.98 (both overlapped)	44.1	1.03 (overlapped), 2.13 (br. *d*, J = 13.5)	34.4
C(4)		73.1		71.4		48.6
H–C(5)	1.04 (overlapped)	58.3	1.39 (overlapped)	58.4	1.16 (br. *d*, J = 10.2)	56.5
CH_2(6)	1.19–1.22, 1.69–1.71 (2*m*)	20.3	1.40, 2.27 (both overlapped)	19.8	1.68, 1.90 (both overlapped)	18.9
CH_2(7)	1.44, 1.52 (both overlapped)	42.3	1.51, 1.55 (both overlapped)	42.3	1.63, 1.66 (both overlapped)	39.4
C(8)		45.7		44.5		48.9
H–C(9)	1.00 (overlapped)	58.1	1.24 (overlapped)	57.7	1.04 (br. *d*, J = 9.0)	47.2
C(10)		41.1		40.5		39.6
CH_2(11)	1.55 (overlapped)	19.5	1.69 (overlapped), 2.42–2.45 (*m*)	19.7	1.86 (overlapped)	18.9
CH_2(12)	1.42, 1.56 (both overlapped)	27.3	1.59, 2.23 (both overlapped)	27.7	1.47 (overlapped)	25.5
H–C(13)	1.95 (br. *s*)	46.3	2.23 (overlapped)	42.7	2.57 (overlapped)	41.2
CH_2(14)	1.54 (overlapped), 1.85 (*d*, J = 12.5)	38.3	1.23 (overlapped), 2.06 (*d*, J = 12.1)	38.9	1.44 (overlapped), 2.08 (br. *d*, J = 10.3)	44.2
CH_2 or H–C(15)	1.34 (*d*, J = 14.4), 1.53 (overlapped)	53.9	1.70, 1.78 (both overlapped)	53.9	5.38 (*s*)	135.3
C(16)		82.8		78.2		146.5
CH_2(17)	3.62, 3.72 (2*d*, J = 11.4)	66.8	4.31, 4.33 (2*d*, J = 11.4)	72.4	4.20 (*s*)	61.4
Me(18)	1.00 (*s*)	22.7	1.32 (*s*)	23.8	1.00 (*s*)	24.4
H–C(19)					9.73 (*s*)	206.1
Me(20)	0.94 (*s*)	17.6	1.04 (*s*)	17.7	0.90 (*s*)	16.4
AcO			1.95 (*s*)	21.2, 171.5		

[a]) In CD_3OD. [b]) In C_5D_5N. [c]) In $CDCl_3$.

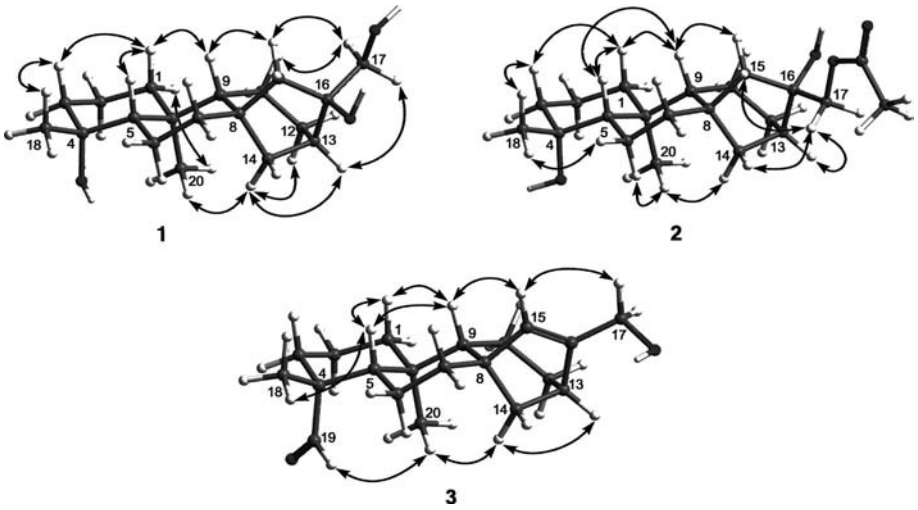

Fig. 3. *Key NOESY correlations of compounds* **1** – **3**[1])

diterpenoids, a Me *s* at δ(H) 1.95 and two deshielded oxygenated methylene H-atoms assignable to CH_2(17) at δ(H) 4.31 and 4.33 (each *d*, $J = 11.2$ Hz) indicated the presence of an Ac group at C(17). The ^{13}C-NMR data (*Table 1*) revealed 21 C-atoms, including two C-atom signals at δ(C) 171.6 and 21.2 attributable to the Ac group. The above data suggested an acetylated 19-nor-*ent*-kaurane diterpene derivative for **2**, which was further confirmed by the HMBC experiment (*Fig. 2*). The HMBCs CH_2(17)/C(13), C(15), C(16), and the acetyl C=O group confirmed their location and linkage. The presence of OH–C(4) group was assigned by HMBC, from H–C(5), Me(18), and CH_2(3) to C(4). The relative configuration of **2** was further confirmed by NOESY data (*Fig. 3*), in which the key correlations CH_2(17)/H–C(14) and H–C(14)/Me(20) showed the α-orientation of CH_2(17) and β-orientation of OH–C(16). Therefore, compound **2** was characterized as (4α,16α)-17-(acetyloxy)-19-nor-*ent*-kaurane-4,16-diol[1]).

Compound **3** was isolated as a white amorphous powder, and its molecular formula was determined as $C_{20}H_{30}O_2$ by HR-ESI-MS, requiring 6 degrees of unsaturation. The IR spectrum revealed the presence of a carbonyl group (1718 cm^{-1}) and a C=C bond (1598 cm^{-1}). The ^{1}H-NMR spectrum (*Table 1*) displayed two Me *s*s at δ(H) 0.90 and 1.00, an oxygenated methylene group at δ(H) 4.20 (*s*), and an aldehyde H-atom at δ(H) 9.73 (*s*). The ^{13}C-NMR data (*Table 1*) indicated 20 C-atom signals, similar to that of 17-hydroxy-*ent*-kaur-15-en-19-oic acid [18]. The C-atom signals at δ(C) 48.6 (C(4)) and 206.1 (C(19)) confirmed the presence of an aldehyde group in **3** instead of the carboxy group in 17-hydroxy-*ent*-kaur-15-en-19-oic acid. In the HMBC spectrum, the correlations H–C(19)/C(4), C(3), and C(18) established the location of the CH(19)=O group. The cross-peaks CH_2(14)/C(15) and C(16), and CH_2(17)/C(13), C(15), and C(16) revealed the CH=C–CH_2OH moiety. The α-orientation of the aldehyde group was assigned by the key NOESY correlations H–C(19)/Me(20), Me(20)/H–C(14), and H–C(14)/H–C(13). Thus, compound **3** was identified as 17-hydroxy-*ent*-kaur-15-en-19-al[1]).

By comparison of their spectroscopic data with those reported in the literature, the structures of the known compounds were determined as annosquamosin C (=(4α)-19-nor-*ent*-kauran-4,17-diol; **4**) [12], (15α)-15,16-epoxy-17-hydroxy-*ent*-kauran-19-oic acid (**5**) [19], 16,17-dihydroxy-*ent*-kauran-19-oic acid (**6**) [11], *ent*-kaur-16-en-19-oic acid (**7**) [11], (4α)-4-hydroxy-19-nor-*ent*-kauran-17-oic acid (**8**) [20], 16-hydroxy-*ent*-kauran-19-oic acid (**9**) [20], (15β)-15-hydroxy-*ent*-kaur-16-en-19-oic acid (**10**) [21], 16,17-dihydroxy-*ent*-kauran-19-al (**11**) [12], annosquamosin B (=(4α,16α)-19-nor-*ent*-kaurane-4,16,17-triol; OH–C(16) β-oriented; **12**) [11], (16α)-16,17-dihydroxy-*ent*-kauran-19-al (OH–C(16) β-oriented; **13**) [11], and 16,17-dihydroxy-*ent*-kauran-19-oic acid methyl ester (**14**) [22].

All compounds were evaluated against human lung-cancer (95-D) and ovarian cancer (A2780) cell lines for their cytotoxicity. As shown in *Table 2*, nine compounds, *i.e.*, **3**–**6**, **7**, and **11**–**14**, exhibited cytotoxic effects against the 95-D and A2780 cell lines with IC_{50} values ranging from 0.38 to 39.83 μM, of which compound **14** inhibited both cell lines with an IC_{50} below 4 μM. From these results, some primary structure–activity relationships can be deduced: Activities related to the C(4) substitutions were in the order aldehyde > carboxy > OH (**11** *vs.* **6** *vs.* **1** and **13** *vs.* **12**), and esterification of the carboxy group at C(4) enhanced the activity (**14** *vs.* **6**). In the case of β-oriented substituents at C(16), the CH_2OH group showed more potent cytotoxic activity than the COOH group (**4** *vs.* **8**), whereas in the case of α-oriented substituents at C(16), a H-atom was more active than an OH group (**4** *vs.* **1**), and acetylation of the CH_2OH group caused the loss of activity (**2** *vs.* **12**). Compound **5** containing a 15α,16α-epoxy moiety had better cytotoxity against both cell lines than compound **6**. Hydroxylation at C(15) caused the loss of activity (**10** *vs.* **7**).

Table 2. *Cytotoxic Activities with* IC_{50} *Values* [μM] *of Compounds* **1**–**14**

Compound	95-D	A2780	Compound	95-D	A2780
1	> 50	> 50	**8**	> 50	> 50
2	> 50	> 50	**9**	> 50	> 50
3	25.10	7.23	**10**	> 50	> 50
4	25.68	> 50	**11**	19.38	0.38
5	34.66	0.89	**12**	28.20	3.10
6	39.83	> 50	**13**	10.69	5.69
7	7.78	14.52	**14**	1.63	3.12
Taxol (ng/ml)	6.57	0.94			

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Experimental Part

General. TLC: precoated silica gel plates (SiO_2 *HSGF 254*; *Yan Tai Jiang You Silica Gel Development Co., Ltd.*). Column chromatography (CC): commercial silica gel for TLC (SiO_2; *Qing Dao Hai Yang Chemical Group Co., Ltd.*); *C18* column (*Phenomenex 00G-4324-N0*; 10 μm, 10 (i.d.) × 250 mm); *MCI* gel (*Mitsubishi*, Japan); *chiral CD-Ph* column (*Shiseido*, Japan). HPLC: *Agilent-1100*

system, Englewood, U.S. Optical rotations: *Jasco*-P-1010 polarimeter. UV Spectra: *Beckman-DU-600* spectrometer; λ_{max} (log ε) in nm. IR Spectra: *Bruker-Vector-22* spectrophotometer; KBr pellets; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Varian-Unity-Inova-400/54* spectrometers; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *Micromass-Quattro* triple-quadrupole mass spectrometer equipped with an ESI source (*Micromass*, Manchester, UK); in *m/z* (rel. %).

Plant Material. The stem barks of *A. squamosa* were collected from Hainan Province, P. R. China, in April 2010, and authenticated by Prof. *Shi-Man Huang*, Department of Biology, Hainan University. A voucher specimen (AS-2009-I) was deposited with the Institute of Modern Chinese Medicine, Zhejiang University.

Extraction and Isolation. The air-dried powder of the stem bark (4.7 kg) of *A. squamosa* was extracted by maceration with 95% EtOH (3 × 20 l, 7 d each time) at r.t. to afford 816 g of crude extract. The extract was suspended in H_2O (1.5 l), then partitioned successively with petroleum ether, AcOEt, and BuOH. The BuOH fraction (122 g) was applied to CC(*MCI* gel, 30% → 70% H_2O/EtOH: *Fractions A–C*. *Fr. A* (1.2 g) was subjected to CC (SiO_2, CH_2Cl_2/MeOH 70:1): **1** (19 mg). *Fr. B* (2.0 g) was subjected to CC (SiO_2, CH_2Cl_2/MeOH 100:1 → 40:1): *Fr. B1–B3*. *Fr. B1* was further purified by CC (*Sephadex LH-20*, CH_2Cl_2/MeOH, 1:1, then *RP18*, EtOH/H_2O 45:55): **2** (14 mg) and **4** (22 mg). Compounds **5** (38.9 mg) and **6** (23.9 mg) were isolated from *Fr. B2* and *Fr. B3*, resp., by the same protocol as that applied to *Fr. B1*. *Fr. C* (2.5 g) was purified by CC (SiO_2, petroleum ether/AcOEt 25:1; then *RP18*, EtOH/H_2O 45:55): **3** (7 mg). The AcOEt fraction (222 g) was subjected to CC (SiO_2, petroleum ether/acetone 100:1 → 20:1): *Frs. D–6*. Recrystallization of *Fr. D* yielded compound **7** (5 g). *Fr. E* was further separated CC (SiO_2, cyclohexane/AcOEt 25:1): **8** (337 mg), **9** (480 mg), and **10** (50 mg). *Fr. F* was separated by CC (SiO_2, cyclohexane/AcOEt 20:1): **11** (295 mg) and **12** (440 mg), and another 2 subfractions, *Fr. F1* and *Fr. F2*). *Fr. F1* was purified by CC (SiO_2, petroleum ether/CH_2Cl_2/MeOH 180:5:1): **13** (42 mg). *Fr. F2* was further subjected to CC (*RP18*, EtOH/H_2O 50:50): **14** (120 mg).

(4α)-19-Nor-ent-*kaurane-4,16,17-triol* (= rel-*(*2R,*4a*R,*4b*S,*8*R,*8a*S,*10a*S,*12*S*)-Dodecahydro-12-(hydroxymethyl)-4b,8-dimehyl-1*H-*2,10a-ethanophenanthrene-8,12-diol*; **1**): White amorphous powder. $[\alpha]_D^{20} = -33.3$ ($c = 0.50$, MeOH). IR (KBr): 3395, 2991, 2926, 2865, 1459, 1385, 1123, 1102, 1060. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS: 331 ($[M+Na]^+$), 639 ($[2M+Na]^+$). HR-ESI-MS: 331.2237 ($[M+Na]^+$, $C_{19}H_{32}NaO_3^+$; calc. 331.2249).

(4α,16α)-17-(Acetyloxy)-19-nor-ent-*kaurane-4,16-diol* (= rel-*(*2R,*4a*R,*4b*S,*8*R,*8a*S,*10a*S,*12*R*)-12-[(Acetyloxy)methyl]dodecahydro-4b,8-dimehyl-1*H-*2,10a-ethanophenanthrene-8,12-diol*; **2**): White amorphous powder. $[\alpha]_D^{20} = -51.9$ ($c = 0.70$, $CHCl_3$). IR (KBr): 3460, 3408, 2931, 2870, 1698, 1465, 1272, 1100, 1041. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS: 373 ($[M+Na]^+$). HR-ESI-MS: 373.2349 ($[M+Na]^+$, $C_{21}H_{34}NaO_4^+$; calc. 373.2355).

17-Hydroxy-ent-*kaur-15-en-19-al* (= rel-*(*2R,*4a*R,*4b*S,*8*R,*8a*S,*10a*R*)-Dodecahydro-12-(hydroxymethyl)-4b,8-dimehyl-1*H-*2,10a-ethanophenanthrene-8-carboxaldehyde*; **3**): White amorphous powder. $[\alpha]_D^{20} = -3.3$ ($c = 0.33$, $CHCl_3$). IR (KBr): 3419, 2918, 2850, 1598, 1463, 1365, 1111. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS: 303 ($[M+H]^+$). HR-ESI-MS: 303.2313 ($[M+H]^+$, $C_{20}H_{31}O_2^+$; calc. 303.2324).

Assay of Cytotoxic Activities. Suspended human lung tumor 95-D and ovarian tumor A2780 cells were cultured in *RPMI 1640* and *Dulbecco's* modified *Eagle's* (*DME*) medium (*Gibco*, Grand Island, N.Y.), resp., and supplemented with 10% fetal bovine serum (*Hangzhou Sijiqing*, P. R. China), L-glutamine (2 nmol/l), penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37° in a humidified atmosphere with 5% CO_2. The logarithmic phase cells (100 μl) were seeded onto 96-well plates at the concentration of $5 \cdot 10^3$ cells per well. After 24 h, different concentrations of the sample, dissolved in DMSO, were added at 10 μl/well and 3 parallel wells for each concentration were tested. Control cells were treated with DMSO alone and positive controls with taxol. The cells were cultivated for 72 h and then fixed with 10% tricholroacetic acid for 1 h and washed with dist. H_2O. Sulforhodamine B (SRB) was dissolved at 4 mg/ml in phosphate-buffered saline (PBS). To each well, 100 μl of this soln. were added and the cells were stained for 20 min. The supernatant was then removed, and 100 μl of *Tris* buffer (10 mM) was added into each well. The absorbance (*A* value) at 515 nm was measured with a microplate reader (*Thermo*). The inhibition rates were calculated by using *OD* mean values, from inhibition rate = $(OD_{control} - OD_{sample})/OD_{control}$. The IC_{50} value was determined using the *Bliss* method.

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