Clavatadines C−E, Guanidine Alkaloids from the Australian Sponge *Suberea clavata*

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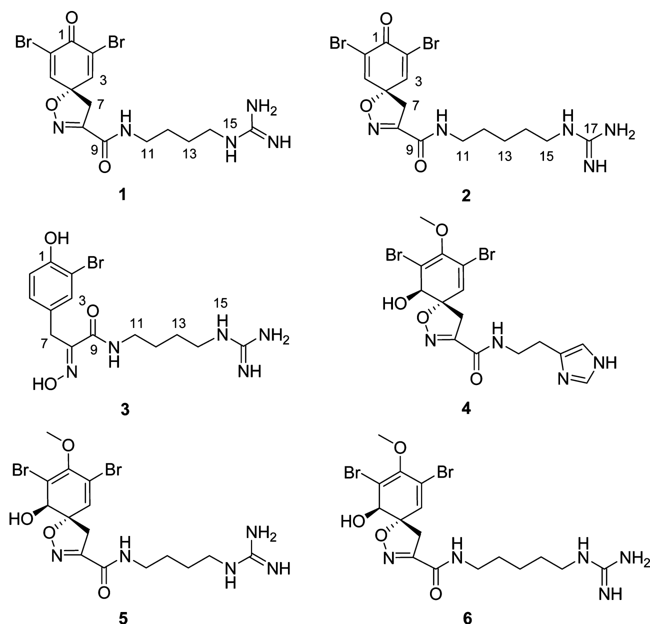
Three new marine alkaloids, clavatadines C−E (**1**−**3**), together with the three known compounds aerophobin 1 (**4**), purealdin L (**5**), and aplysinamisine II (**6**) were isolated from extracts of the sponge *Suberea clavata* by bioassay-guided fractionation using a serine protease factor XIa assay. Their structures were determined by 1D and 2D NMR spectroscopy. Compounds **1**−**6** exhibited weak inhibition of factor XIa.

Continuing our search to find new lead compounds for development into antithrombotic agents, two extracts of the marine sponge *Suberea clavata* Pulitzer-Finali, 1982 (Aplysinellidae) were further investigated since they showed inhibition of factor XIa (FXIa) with approximate IC_{50}'s of 0.4 and 0.5 $\mu g/\mu L$.[1−3] Purification of the active extracts led to the isolation of three new marine alkaloids, clavatadines C−E (**1**−**3**), together with the known compounds aerophobin 1 (**4**),[4] purealdin L (**5**),[5] and aplysinamisine II (**6**).[6] Recently we have reported the FXIa inhibitors clavatadines A and B from *S. clavata*.[3] An X-ray crystal structure revealed selective binding and irreversible inhibition of FXIa by clavatadine A. The isolation and structure elucidation of **1**−**6** are reported here, together with their inhibitory activity toward the serine proteases, factors IXa (FIXa) and FXIa.



The marine sponge *S. clavata*, collected at Swain Reefs (QMG317762), was freeze-dried, then pulverized to give 29.6 g of biota. This was extracted with CH_2Cl_2 followed by MeOH, and the combined extracts were chromatographed repeatedly by reversed-phase C_{18} HPLC to yield the five compounds, clavatadines C (**1**) and D (**2**), aerophobin 1 (**4**), purealdin L (**5**), and aplysinamisine II (**6**). The known compounds **4**−**6** were identified by comparison of their physical and spectroscopic data with previously published data.[4−6]

Clavatadine C (**1**) was obtained as an optically inactive amorphous solid. The (+)-LRESIMS of **1** displayed three pseudomolecular ion peaks at *m/z* 462, 464, and 466 $[M + H]^+$, which indicated the molecule contained two bromines. The molecular formula of **1** was determined to be $C_{14}H_{17}Br_2N_5O_3$ by (+)-HRESIMS of an $[M + H]^+$ ion at *m/z* 461.9757 (Δ 3.0 ppm). The ^1H NMR (Table 1) and gCOSY NMR data for clavatadine C (**1**) indicated that the molecule contained two α,β-unsaturated ketone β-protons (δ_H 7.80), an isolated methylene (δ_H 3.55), and a $NH(CH_2)_4NH$ spin system. Analysis of the ^{13}C and DEPT NMR spectra indicated that the molecule contained seven downfield quaternary carbons, two downfield methine carbons, and five upfield methylene carbons, giving a total of 14 carbon atoms. The correlations from a gHMBC spectrum helped to determine the structure. Analysis of gHMBC and ^{13}C NMR data (Table 1) allowed the ^1H NMR evidence for two α,β-unsaturated ketone β-protons (δ_H 7.80) to be expanded to a spiro 2,6-dibromocyclohexadienone ring. Some of the discorhabdin marine natural products contain the spiro 2,6-dibromocyclohexadienone moiety, and comparison of the NMR data of **1** with these known discorhabdins confirmed its presence in **1**.[7−9] The UV (λ_{max} 258 nm) and IR (ν_{max} 1683, 1651 cm^{-1}) spectra supported the presence of a dienone chromophore. An isoxazoline ring {δ_H 3.55 (s, 2H); δ_C 43.1, 85.2, 155.0} was clearly present forming a spirocyclohexadienoneisoxazoline ring system, compared to the spirocyclohexadienylisoxazoline system commonly found in Verongida sponge metabolites and present in compounds **4**−**6**.[10] Lastly, 1D and 2D NMR analyses confirmed the presence of an agmatine [1-(4-aminobutyl)guanidine] chain attached to the isoxazoline ring through an amide carbonyl, similarly to purealdin L (**5**).[5] Thus, the structure of clavatadine C was determined to be **1**.

The molecular weight of clavatadine D (**2**) (*m/z* 475.9930, representing $C_{15}H_{19}Br_2N_5O_3$) was greater than that of **1** by 14 Da. Analysis of the 1D (Table 1) and 2D NMR data of **2** clearly revealed that **2** had an extra methylene group {δ_H 1.29 (2H); δ_C 23.2). Thus, the 1-(4-aminobutyl)guanidine (agmatine) in **1** was replaced by a 1-(5-aminopentyl)guanidine in **2**. The latter chain was also present in aplysinamisine II (**6**).[6] The structure of clavatadine D was therefore established to be **2**.

A separate collection of *S. clavata* at Swain Reefs [(16.4 g), QMG317611] was similarly extracted and chromatographed repeatedly by reversed-phase C_{18} HPLC to yield clavatadine E (**3**). The (+)-LRESIMS of clavatadine E (**3**) displayed two equally intense pseudomolecular ion peaks at *m/z* 386 and 388 $[M + H]^+$, which indicated the molecule contained one bromine atom. A pseudomolecular ion in the (+)-HRESIMS at *m/z* 386.0825 (Δ +0.8 ppm) allowed the molecular formula $C_{14}H_{20}BrN_5O_3$ to be assigned to **3**. Clavatadine E (**3**) was clearly another bromophenol alkaloid like clavatadines A and B,[3] but with significant differences in the aromatic substituents. The ^1H NMR data (Table 2) for **3** indicated the presence of a 1,3,4-trisubstitued aromatic ring [δ_H 7.28 (d, 1.8), 7.01 (dd, 8.4, 1.8), 6.83

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Table 1. NMR Data[a] for Clavatadines C (**1**) and D (**2**) in DMSO-d_6

	1			**2**	
position	δ_C	δ_H (mult, J Hz)	$^{2,3}J_{CH}$ HMBC (C no.)	δ_C[c]	δ_H (mult, J Hz)
1	146.6, CH	7.80, s	2, 3, 5, 7	146.4, CH	7.79, s
2	121.6, qC			121.5, qC	
3	171.6, qC			171.6, qC	
4	121.6, qC			121.5, qC	
5	146.6, CH	7.80, s	1, 3, 4, 7	146.4, CH	7.79, s
6	85.2, qC			85.0, qC	
7	43.1, CH_2	3.55, s	1, 5, 6, 8	42.9, CH_2	3.55, s
8	155.0, qC			154.8, qC	
9	158.2, qC			158.1, qC	
10 (N)		8.63, t	9, 11		8.59, t
11	38.3, CH_2	3.18, dt (6.0, 6.0)	9	38.5, CH_2	3.16, dt (6.0, 6.0)
12	25.9, CH_2[b]	1.49, m		28.2, CH_2	1.49, tt (6.0, 6.0)[d]
13	26.0, CH_2[b]	1.48, m		23.2, CH_2	1.29, tt (6.0, 6.0)
14	40.4, CH_2	3.11, dt (6.0, 6.0)	16	27.9, CH_2	1.48, tt (6.0, 6.0)[d]
15 (N)		7.45, m	14, 16	40.4, CH_2	3.08, dt (6.0, 6.0)
16	156.6, qC				7.41, brt (6.0)
17				156.5, qC	

[a] ^{1}H, 600 MHz; ^{13}C, 125 MHz. [b] Chemical shifts are interchageable. [d] Chemical shifts are interchangeable. [c] Chemical shifts determined from 2D NMR experiments (^{13}C, 150 MHz).

Table 2. NMR Data[a] for Clavatadine E (**3**) in DMSO-d_6

position	δ_C[b]	δ_H (mult, J Hz)	COSY (H no.)	$^{2,3}J_{CH}$ HMBC (C no.)
1	152.2, qC			
2	108.7, qC			
3	132.4, CH	7.28, d (1.8)	5	1, 2, 7
4	128.8, qC			
5	128.8, CH	7.01, dd (8.4, 1.8)	3, 6	1, 3
6	115.9, CH	6.83, d (8.4)	5	1, 2
7	27.5, CH_2	3.69, s		3, 8, 9
8	152.0, qC			
9	163.0, qC			
1-OH		10.02, s		1, 2, 6
NOH		11.75, s		8
10 (N)		7.99, t (6.0)	11	9
11	37.9, CH_2	3.15, dt (6.0, 6.0)	10, 12	9
12	25.8, CH_2	1.43, m	11	
13	25.8, CH_2	1.42, m	14	
14	39.9, CH_2	3.08, dt (6.0, 6.0)	13, 15	16
15 (N)		7.39, t (6.0)	14	
16	156.5, qC			

[a] ^{1}H, 600 MHz; ^{13}C, 150 MHz. [b] Chemical shifts determined from 2D NMR experiments (^{13}C, 150 MHz).

(d, 8.4)]. The aromatic substituents were a bromine, a hydroxyl (δ_H 10.02), and a side chain described as follows. From the 1D and 2D NMR data (Table 2) of **3** a chain of four methylenes, ending in a guanidine (δ_C 156.5), was evident. The ^{13}C NMR signals at δ_C 152.0 and 163.0 were assigned to an amide-oxime conjugated system at the other end of the four-methylene chain. This was supported by the proton signals at δ_H 7.99 and 11.75. The amide-oxime moiety was attached to the aromatic ring through a methylene group (δ_H 3.69/δ_C 27.5). This linkage was established from gHMBC correlations between δ_H 3.69 (s, 2H) and δ_C 132.4, 152.0, and 163.0. The upfield ^{13}C NMR chemical shift at δ_C 27.5 suggested an *E* configuration for the oxime, as the corresponding value for a (*Z*)-oxime would be >35 ppm.[9] The carbon and proton chemical shifts for this side chain are consistent with those reported for other compounds containing this fragment.[10] Correlations in the gHMBC spectrum (Table 2) revealed the substitution pattern of the aromatic ring, in particular the correlations between H-7 (δ_H 3.69) and C-3 (δ_C 132.4), and the observed correlations of 1-OH (δ_H 10.02) and C-6 (δ_C 115.9), and C-1 (δ_C 152.2) and C-2 (δ_C 108.7). The above information indicated clavatadine E had structure **3**.

Compounds **1**−**6** showed weak inhibition against FXIa and no significant inhibition of FIXa (Table 3). The FIXa assay was run to determine selectivity of the isolated compounds. We predict that the inhibition of FXIa was due to weak interactions between the guanidine or imidazole of the compounds with Asp 189 in FXIa.[3]

Table 3. Factors XIa and IXa Inhibitory Activity for Compounds **1**−**6**[a]

compound	factor XIa	factor IXa
clavatadine C (**1**)	17% @ 222 μM	<5% @ 222 μM[b]
clavatadine D (**2**)	30% @ 222 μM	<5% @ 222 μM[b]
clavatadine E (**3**)	37% @ 222 μM	<5% @ 222 μM[b]
aerophobin 1 (**4**)	59% @ 222 μM	<5% @ 222 μM[b]
purealdin L (**5**)	12% @ 222 μM	<5% @ 222 μM[b]
aplysinamisine II (**6**)	30% @ 222 μM	<5% @ 222 μM[b]

[a] Assays performed in triplicate on two independent days. [b] No significant activity.

Experimental Section

General Experimental Procedures. UV and FTIR spectra were recorded on an Camspec M501 UV/vis spectrophotometer and a Bruker Tensor 27 FTIR spectrophotometer, respectively. NMR spectra were recorded at 30 °C on Varian Inova 500 and 600 MHz NMR spectrometers. Samples were dissolved in DMSO-d_6 (residual ^{1}H δ 2.50 and ^{13}C δ 39.5 ppm). Multiplicity was determined by DEPT (s = C, d = CH, t = CH_2, q = CH_3). Standard parameters were used for the 2D experiments, which included gradient COSY, HSQC ($^1J_{CH}$ = 140 Hz), and HMBC ($^nJ_{CH}$ = 8.3 Hz). HRESIMS spectra were measured on a Bruker Daltonics Apex III 4.7e Fourier transform mass spectrometer, fitted with an Apollo API source. A Betasil C_{18} 5 μm (50 mm × 150 mm i.d.) column was used for preparative HPLC, while Betasil C_{18} 5 μm (21.2 mm × 150 mm i.d.) and Gemini C_{18} 5 μm (21.1 mm × 250 mm i.d.) columns were used for semipreparative HPLC. A Waters 600 pump fitted with a 996 photodiode array detector and 717 Plus autosampler was used for the semipreparative separations. RP-18 was Sepra C_{18} (04K-4348) end-capped silica. Water was Millipore Milli-Q PF filtered, while all other solvents used were Laboratory-Scan HPLC grade. Trifluoroacetic acid (TFA) was Fluka spectroscopic grade. Ammonia solution (about 25% NH_3, sp. gr. 0.91) was from Merck. Assay materials: TrisHCl, NaCl, $CaCl_2$, bovine serum albumin (BSA), and ethylene glycol were purchased from Sigma. Factor IXa and factor XIa were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Perfachrome FIXa was purchased from Pentapharm (Switzerland), and substrate S2366 from Chromogenix (Italy). Clear 384-well polystyrene microtiter plates were purchased from Falcon. A Multiskan Ascent (Thermo) and a Powerwave (Biotek) were used as spectrophotmetric readers.

Animal Material. Two samples of the sponge *Suberea clavata* (phylum Porifera, class Demospongiae, order Verongida, family Aplysinellidae) were collected by scuba diving at Swain Reefs, Great Barrier Reef, Queensland, Australia. One sample was collected at a depth of 30 m on February 11, 2001 (voucher sample QMG317762). The other sample was collected at a depth of 17 m on February 8, 2001 (voucher sample QMG317611). Both were lodged at the Queensland Museum, South Brisbane, Queensland, Australia.

Extraction and Isolation. The freeze-dried sponge (QMG317762) was ground (29.6 g) and extracted with CH_2Cl_2 followed by MeOH. The combined CH_2Cl_2/MeOH extracts (8.25 g) were preadsorbed on C_{18} and

loaded into a column (80 mm × 40 mm) in line with a preparative C_{18} HPLC column. The following solvent conditions were used: a linear gradient from H_2O/1% TFA to MeOH/1% TFA in 50 min, then isocratic conditions for 20 min (flow rate 40 mL/min), 135 fractions were collected from time = 0 min. Bioactive fractions 43−69 were combined (1.02 g) and preadsorbed on C_{18} and loaded into a refillable preparative guard column (30 mm × 10 mm), in line with a semipreparative Gemini C_{18} HPLC column. The following solvent conditions were used: a linear gradient from H_2O/0.25% NH_3 to H_2O/0.25% NH_3 (50%):CH_3CN/0.25% NH_3 (50%) in 90 min, followed by a linear gradient to CH_3CN/0.25% NH_3 in 30 min (flow rate 5 mL/min); 60 fractions were collected from time = 0 min. Fractions 42−50 (68.9 mg) and fractions 53−60 (37.8 mg) were purified further by Betasil C_{18} HPLC. Fractions 42−50 (68.9 mg): a linear gradient from H_2O/1% TFA to H_2O/1% TFA (75%):MeOH/1% TFA (25%) in 2 min, followed by isocratic conditions for 18 min, then a linear gradient to H_2O/1% TFA (60%):MeOH/1% TFA (40%) in 25 min, and finally a linear gradient to MeOH/1% TFA in 15 min (flow rate 10 mL/min); 60 fractions were collected from time = 0 min. Fractions 45−48 (12 mg) was purified further by Betasil C_{18} HPLC: a linear gradient from H_2O/1% TFA to H_2O/1% TFA (70%):MeOH/1% TFA (30%) in 2 min, followed by isocratic conditions for 48 min, then a linear gradient to MeOH/1% TFA in 10 min (flow rate 10 mL/min); 60 fractions were collected from time = 0 min. Aerophobin 1 (**4**) (0.86 mg, 0.003% dry wt) eluted with a retention time of 51 min. Fractions 53−60 (37.8 mg): a linear gradient from H_2O/1% TFA to H_2O/1% TFA (75%):MeOH/1% TFA (25%) in 2 min, followed by isocratic conditions for 18 min, then a linear gradient to H_2O/1% TFA (60%):MeOH/1% TFA (40%) in 25 min, and finally a linear gradient to MeOH/1% TFA in 15 min (flow rate 10 mL/min); 60 fractions were collected from time = 0 min. Fractions 50−51 (6.6 mg) and fractions 52−53 (5.2 mg) were purified further by Betasil C_{18} HPLC. Fractions 50−51 (6.6 mg): a linear gradient from H_2O/1% TFA to H_2O/1% TFA (65%):MeOH/1% TFA (35%) in 3 min, followed by isocratic conditions for 42 min, then a linear gradient to MeOH/1% TFA in 15 min (flow rate 10 mL/min); 60 fractions were collected from time = 0 min. Clavatadine C (**1**) (0.64 mg, 0.002% dry wt) and purealdin L (**5**) (1.24 mg, 0.004% dry wt) eluted with retention times of 29 and 32 min, respectively. Fractions 52−53 (5.2 mg): a linear gradient from H_2O/1% TFA to H_2O/1% TFA (65%):MeOH/1% TFA (35%) in 3 min, followed by isocratic conditions for 42 min, then a linear gradient to MeOH/1% TFA in 15 min (flow rate 10 mL/min); 60 fractions were collected from time = 0 min. Clavatadine D (**2**) (0.58 mg, 0.002% dry wt) and aplysinamisine II (**6**) (2.84 mg, 0.01% dry wt) eluted with retention times of 38 and 50 min, respectively.

The sponge (QMG317611) was freeze-dried and ground (16.4 g), then extracted with CH_2Cl_2 followed by MeOH. The combined CH_2Cl_2/MeOH extracts (2.88 g) were further purified. A portion of this extract (1.44 g) was preadsorbed on C_{18} and loaded into a refillable preparative guard column (30 mm × 10 mm), in line with a semipreparative C_{18} HPLC column. The following solvent conditions were used: a linear gradient from H_2O/1% TFA to MeOH/1% TFA in 35 min, then isocratic conditions for 25 min (flow rate 9 mL/min); 60 fractions were collected from time = 0 min. Bioactive fractions 15−24 were combined (223 mg) and further purified. This fraction was again preadsorbed onto C_{18} and loaded into a refillable preparative guard column (30 mm × 10 mm), in line with a semipreparative C_{18} HPLC column. The following solvent conditions were used: a linear gradient from H_2O/1% TFA to H_2O/1% TFA (80%):MeOH/1% TFA (20%) in 5 min, then to H_2O/1% TFA (35%):MeOH/1% TFA (65%) in 45 min, then finally to MeOH/1% TFA in 10 min (flow rate 10 mL/min); 60 fractions were collected from time = 0 min. Bioactive fractions 27−30 (12.0 mg) were purified further by C_{18} HPLC: a linear gradient from H_2O/1% TFA to H_2O/1% TFA (70%):MeOH/1% TFA (30%) in 5 min, followed by isocratic conditions for 45 min, then a linear gradient to MeOH/1% TFA in 10 min; 60 fractions were collected from time = 0 min. Fractions 41−42 and 45 were combined (4.3 mg) and purified further by Betasil C_{18} HPLC: a linear gradient from H_2O/1% TFA to H_2O/1% TFA (70%):MeOH/1% TFA (30%) in 5 min, followed by isocratic conditions for 45 min, then a linear gradient to MeOH/1% TFA in 10 min; 60 fractions were collected from time = 0 min. Clavatadine E (**3**) (0.5 mg, 0.006% dry wt) eluted with a retention times of 45 min.

Compounds **1**−**6** were isolated as their trifluoroacetate salts.

Clavatadine C (1), *N*-(4-{[Amino(imino)methyl]amino}butyl)-7,9-dibromo-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide: amorphous solid; UV (MeOH) λ_{max} (log ε) 258 (3.90) nm; IR ν_{max} (film) 3304, 1683, 1651, 1206, 1134 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; (+)-HRESIMS *m/z* 461.9757 (calcd for $C_{14}H_{18}{}^{79}Br_2N_5O_3$, 461.9771).

Clavatadine D (2), *N*-(5-{[Amino(imino)methyl]amino}pentyl)-7,9-dibromo-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide: amorphous solid; UV (MeOH) λ_{max} (log ε) 258 (3.28) nm; IR ν_{max} (film) 3306, 1684, 1208, 1137 cm^{-1}; ^1H and ^{13}C NMR see Table 1; (+)-HRESIMS *m/z* 475.9930 (calcd for $C_{15}H_{20}{}^{79}Br_2N_5O_3$, 475.9927).

Clavatadine E (3), (2*E*)-3-(3-Bromo-4-hydroxyphenyl)-*N*-(4-carbamimidamidobutyl)-2-(hydroxyimino)propanamide: amorphous solid; UV (MeOH) λ_{max} (log ε) 196 (3.92), 287 (2.78) nm; IR ν_{max} (film) 3430, 1684, 1206 cm^{-1}; ^1H and ^{13}C NMR see Table 2; (+)-HRESIMS *m/z* 386.0825 (calcd for $C_{14}H_{21}{}^{79}BrN_5O_3$, 386.0822).

Factor IXa Assay. Stock solutions of factor IXa enzyme was diluted to a working concentration of 45 nM in assay buffer consisting of 50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 3 mg/mL BSA, and 33% ethylene glycol ("FIXa assay buffer"). The assay was performed in 384-well microtiter plates and consisted of the following: 1 μL of compound (stock 10 mM in DMSO), 4 μL of water, and 20 μL of factor IXa, followed by incubation at rt for 10 min. Then 20 μL of Pentachrome FIXa (1.125 mM in FIXa assay buffer) was added followed by another incubation of 90 min at rt. Absorbance was then read at 405 nm. In-plate controls consisted of 100% inhibition (1 μL of control compound, AZ12425961, 50 μM) and 0% inhibition (1 μL of DMSO).

Factor XIa Assay. Stock solutions of factor XIa enzyme was diluted to a working concentration of 0.225 nM in assay buffer consisting of 50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, and 0.1 mg/mL BSA ("FXIa assay buffer"). The assay was performed in 384-well microtiter plates and consisted of the following: 1 μL of compound (stock 10 mM in DMSO), 4 μL of water, and 20 μL of factor XIa, followed by incubation at rt for 15 min. Then 20 μL of substrate S2366 (0.54 mM in FXIa assay buffer) was added followed by another incubation of 2 h at rt. Absorbance was then read at 405 nm. In-plate controls consisted of 100% inhibition (1 μL of control compound, AZ10404570, 13.5 mM) and 0% inhibition (1 μL of DMSO).

Data Analysis. For both assays, percent inhibition for each compound was calculated as follows:

$$\% \text{ inhibition} = 100 - \{[(\text{Abs}_{\text{compound}} - 0\% \text{ control})/(100\% \text{ control} - 0\% \text{ control})] \times 100\}.$$

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