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4′-*N*-Methyl-5′-hydroxystaurosporine and 5′-Hydroxystaurosporine, New Indolocarbazole Alkaloids from a Marine *Micromonospora* sp. Strain

LIBRADA M. CAÑEDO HERNÁNDEZ, JESÚS ANGEL DE LA FUENTE BLANCO, JULIA PÉREZ BAZ*, JOSÉ L. FERNÁNDEZ PUENTES, FRANCISCO ROMERO MILLÁN*, FERNANDO ESPLIEGO VÁZQUEZ and ROSA ISABEL FERNÁNDEZ-CHIMENO

Instituto Biomar S.A., Pol. Ind., Edificio CEI.
Mód. 2.02 24231 Onzonilla, León, Spain

DOLORES GARCÍA GRÁVALOS

Pharma Mar S.A.,
La Calera 3, 28760 Tres Cantos, Madrid, Spain

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Two new indolocarbazole alkaloids, 4′-*N*-methyl-5′-hydroxystaurosporine (**2**) and 5′-hydroxystaurosporine (**3**), were isolated together with the known staurosporine (**1**) from the culture broth of a marine *Micromonospora* sp. (strain L-31-CLCO-002). The fermentation, structural data and cytotoxic activities of these compounds against various tumor cell lines are given.

Marine microorganisms are potentially a prolific source of highly bioactive secondary metabolites[1] that might represent useful leads in the development of new pharmaceutical agents.

In the course of our screening program for novel antitumor metabolites produced by microorganisms inhabiting the marine environment, we have isolated the known staurosporine (**1**) and two new minor staurosporine analogues, 4′-*N*-methyl-5′-hydroxystaurosporine (**2**) and 5′-hydroxystaurosporine (**3**), from a marine *Micromonospora* sp. strain L-31-CLCO-002.

Staurosporine was first isolated in 1977 from *Saccharothrix* sp. AM-2282[2]. Its structure and relative stereochemistry were determined by X-ray crystallographic analysis[3,4] and by ^{1}H and ^{13}C NMR studies[5]. The absolute stereochemistry could only recently be established[6]. Since TAMAOKI *et al.* reported staurosporine to have potent inhibitory activity for protein kinase C (PKC)[7], staurosporine and analogues have become very important compounds, which are used frequently for various biochemical experiments, and some of them are potential medicines[8].

The active compounds were obtained by aerobic fermentation under controlled conditions and the structures of (**2**) and (**3**) have been elucidated by NMR and mass spectra, mainly by comparison of their data with those of staurosporine (**1**). The new compounds (**2**) and (**3**) possess the indolo[2,3-a]carbazole chromophore of staurosporine, but differ in the substitution pattern of the sugar moiety (Figure 1). We describe herein the taxonomical characterization of the producing strain, fermentation, isolation, structural elucidation and cytotoxic activity of these metabolites.

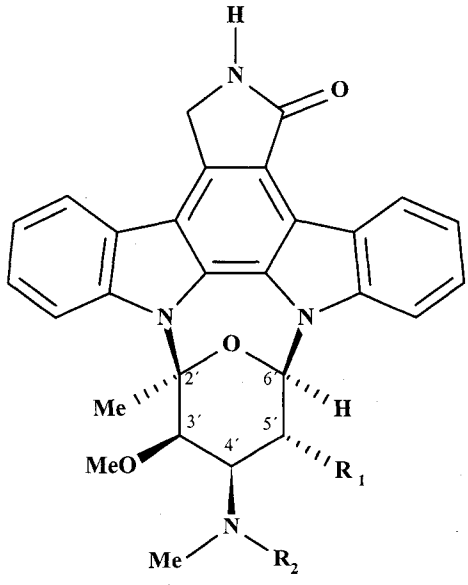
Experimental

Taxonomy of the Producing Strain

Strain L-31-CLCO-002 was isolated from a homogenate of the sponge *Clathrina coriacea* collected on the coast of Fuerteventura Island in the Canary Islands archipielago. The strain was deposited with an accession number of CECT-3347 at the Colección Española de Cultivos Tipo (Valencia, Spain). All other strains used in this study were from different culture collections.

Morphological studies were carried out with a light microscope and a scanning electron microscope. The cultures were grown at 28°C for 21 days on chitin agar

Fig. 1. Structure of staurosporine **1** and the compounds **2**, and **3**.



	R_1	R_2
Staurosporine	**H**	**H**
Compound 2	**OH**	**Me**
Compound 3	**OH**	**H**

medium and ATCC's 172 agar medium. The isomer of diaminopimelic acid from the cell wall was determined by the method of HASEGAWA *et al.*[9]. Carbon source requirements were studied by the method of SHIRLING and GOTTLIEB[10]. Fatty acids profile was obtained by the method of VAN DER AUWERA *et al.*[11]

Fermentation

Staurosporine (**1**) and staurosporine analogues (**2**) and (**3**) were produced in 2 liter Erlenmeyer flasks containing 250 ml of fermentation medium. Inoculum was grown in a medium consisting of dextrose 0.5%, soluble starch 2%, beef extract 0.3%, yeast extract 0.5%, peptone 0.5%, calcium carbonate 0.4%, sodium chloride 0.4%, sodium sulfate 0.1%, potassium chloride 0.05%, magnesium chloride 0.2%, and monobasic potassium phosphate 0.05%, distilled water to one liter, and the pH was adjusted to 7 prior to sterilisation at 121°C for 20 minutes. Seed preparation was a sequence of two steps, the first in 50 ml Erlenmeyer flasks containing 10 ml of inoculum medium and the second in 250 ml Erlenmeyer flasks containing 40 ml of the same medium. Both flasks were incubated at 28°C on a rotary shaker at 250 rpm, for 48 hours. The medium consisted of dextrose 0.5%, dextrin 2%, soybean meal 0.3%, yeast extract 0.5%, peptone 0.1%, calcium carbonate 0.4%, sodium chloride 0.2%, sodium sulfate 0.25%, potassium chloride 0.05%, magnesium chloride 0.05%, ammonium sulphate 0.05% and monobasic potassium phosphate 0.05%, distilled water to one liter, and the pH was adjusted to 7 prior to sterilisation. The fermentor was inoculated with 12.5 ml of culture from the 250 ml flasks. The culture was grown in shake flasks with rotary agitation at 250 rpm and the flasks were incubated for 96 hours at 28°C.

General Procedures

Analytical TLC was done on pre-coated silica gel 60 F254 plates (0.2 mm thick, 20×20 cm, Merck) and the spots were detected under UV light (254 nm) or visualized with vanillin in conc. H_2SO_4. Silica gel 60 (40~63 μm, Merck) and LiChroprep RP-18 (40~63 μm, Merck) were used for column chromatography.

HPLC analysis was performed using an analytical radial pack cartridge Resolve C18 (10 μm, Waters Chromatography), using as a mobile phase CH_3CN - 0.025 M Na_2HPO_4 pH=3 (75 : 25), at a flow rate of 2.0 ml/minute and with detection at 290 nm. The HPLC equipment was a Waters 991 with a photodiode-array detector and a Rheodyne injector.

Optical rotations were measured with an Optical Activity AA-10 polarimeter. IR spectra were recorded on a Perkin Elmer 881 spectrophotometer. NMR spectra were acquired on a Varian Unity 300 NMR spectrometer (300 MHz for ^1H, 75 MHz for ^{13}C). Chemical shifts are reported in ppm

Table 1. Cultural characteristics of strain L-31-CLCO-002.

Medium	Growth	Substrate mycelium	Pigments
ISP-1	Poor	Orange	None
ISP-2	Fair	Orange	None
ISP-3	Fair	Orange	None
ISP-4	Fair	Orange	None
ISP-5	Fair	Orange	None
ISP-6	Good	Orange	None
ISP-7	Good	Orange	None
Czapek's agar	Fair	Orange	None
Bennet's agar	Good	Orange	None
ATCC 172	Very good	Orange	None

All of the mediums were supplemented with sea water 25%.

Fig. 2. Scanning electron micrograph of *Micromonospora* sp. L-31-CLCO-002 grown in ATTC172 medium for 21 days at 28°C.

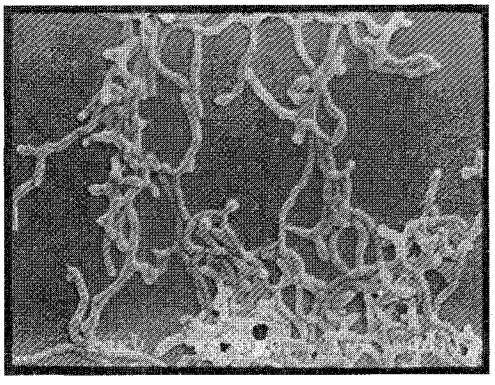


Table 2. Physiological characteristics of strain L-31-CLCO-002.

Growth at 20-28°C	+
Melanoid pigment production:	-
NaCl resistance	1%-4%
Carbon source:	
D-Glucose:	+
D-Mannose	+
D-Fructose:	±
Galactose:	±
Xylose	±
Rammnose:	±
Saccarose:	±
Raffinose:	±
Mellibiose:	±
myo-Inositol:	±
Nitrogen source:	
Glutamic acid	+
Amomiun	-
L-Asparragin	+
Valine	+
Histidine	±
L-Arginine	±
L-Tyrosine	+
D-Glucosamine	±
L-Methionine	+

++= good; += fair; ±= doubtful; -= absent;

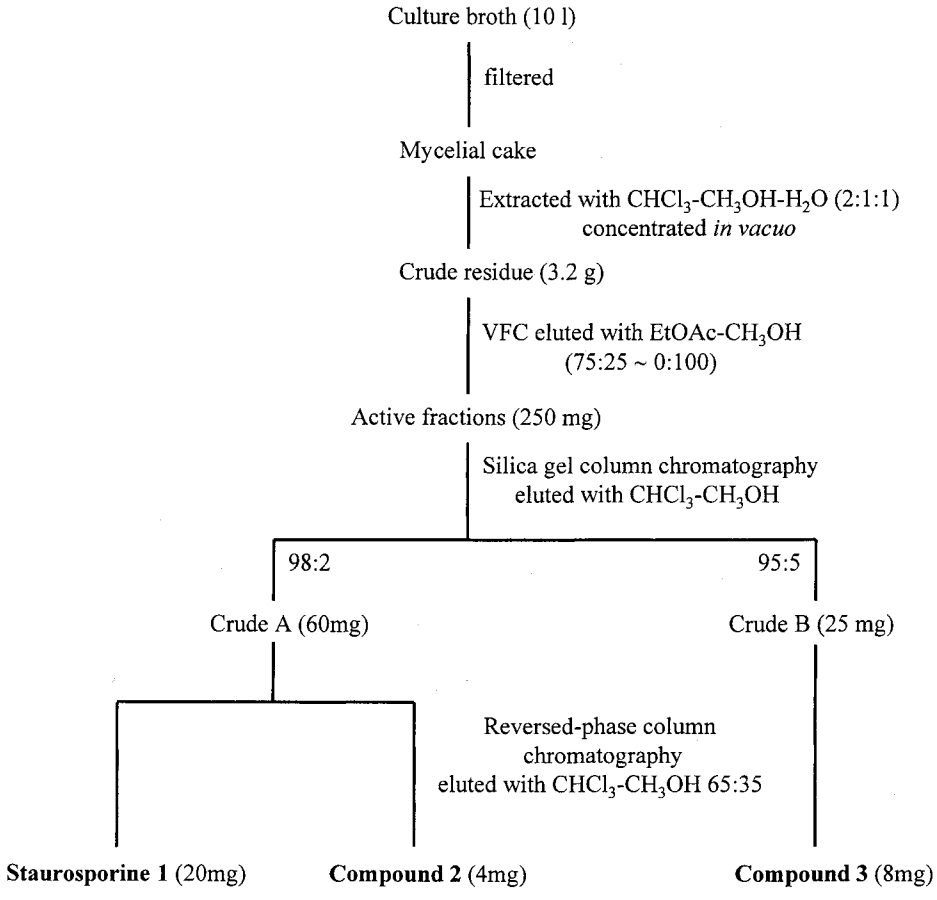
referenced to the $CHCl_3$ peak at 7.26 ppm for ^1H and 77.0 ppm for ^{13}C. FAB-MS spectra were carried out with a VG ZAB-SE spectrometer.

Results and Discussion

Taxonomy and Fermentation

Strain L-31-CLCO-002 forms branched mycelia with the absence of aerial mycelium. The spores are single and they are formed directly from the hyphae (Figure 2). The vegetative mycelium showed an orange colour in most media (Table 1) and there was an absence of secretion of pigments in any of the media tested. The physiological properties of strain L-31-CLCO-002 studied are shown in Table 2. One relevant result is the dependence on salt for growth. The optimal growth occurred in the presence of 1~4% of sodium chloride. The isomer for the diaminopimelic acid found after whole-cell hydrolysis was the *meso* form. The predominant fatty acids are *iso*-C15:0, *iso*-C16:0

Fig. 3. Isolation procedure of staurosporine **1** and the new compounds **2** and **3**.



and C17:1. The microscopical, cultural and chemical characteristics are that of a *Micromonospora* species.

The production of active metabolites reached a maximum around 144 hours of fermentation. At this point, the packed cell volume was 6% of total broth volume and the pH had reached 7.6 from a starting value of 7.

Isolation and Purification

A flow diagram of the isolation procedure is shown in Figure 3. The pooled 10 liters of culture broth was filtered with diatomaceous earth. The mycelial cake was extracted three times with 3 liters of chloroform - methanol - water (2 : 1 : 1). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to dryness to give a crude residue (3.2 g). The residue was applied to a silica gel "vacuum flash" column for chromatography VFC (L=9 cm, ϕ_i=9 cm). After washing with 300 ml of *n*-hexane - ethyl acetate (1 : 1), the column was developed with an ethyl acetate - methanol gradient (75 : 25→0 : 100, v/v). The progress of the elution was checked for cytotoxicity against A 549 cells and monitored by TLC ($CHCl_3$ - CH_3OH 9 : 1) and analytical reversed phase HPLC photodiode-array.

The active fractions were combined (250 mg) and chromatographed on a silica gel column (2×30 cm) with a chloroform - methanol gradient (100 : 0→90 : 10) as the eluent. An active fraction was eluted with chloroform - methanol 98 : 2 and a second active fraction was eluted with chloroform - methanol 95 : 5. Each of these fractions were chromatographed on a column of C18 reversed phase (1.8× 20 cm) and eluted with methanol - water 65 : 35 to give staurosporine (**1**) (20 mg), the compound (**2**) (4 mg) and the compound (**3**) (8 mg) respectively.

Analytical HPLC was carried out and the compounds of interest showed retention times of 3.29, 3.92, and 4.05 minutes to (**3**), (**2**) and staurosporine (**1**) respectively.

Table 3. Physico-chemical properties of compounds **2** and **3**.

	Compound **2**	Compound **3**
Appearance	Pale yellow powder	Pale yellow powder
Molecular formula	$C_{29}H_{28}O_4N_4$	$C_{28}H_{26}O_4N_4$
FAB-MS $(M+H)^+$	497.2	483.2
MP(°C)	>220°C (dec.)	>220°C (dec.)
$[\alpha]_D^{25}$	+30.0° (c = 0.11, $CHCl_3$)	+53.0° (c = 0.10, CH_3OH)
UV $\lambda_{máx}$ nm	206, 242, 291, 320, 334, 354, 370 (in CH_3OH)	
IR $\nu_{máx}$ (KBr) cm^{-1}	3400, 2940, 1670, 1590, 1460, 1400, 1360, 1320, 1280, 1230, 1120, 1070, 750	
TLC[a] Rf value[b]	0.35	0.25
Rf value[c]	0.55	0.40
HPLC (Rt, minutes)[d]	3.92	3.29

[a] Silica gel 60 F_{254}, Merck
[b] Solvent I: CH_2Cl_2:2-PrOH (10:1)
[c] Solvent II: $CHCl_3$:MeOH (9:1)
[d] C18 radial pack cartridge (10μm); mobile phase: CH_3CN-Na_2HPO_4 0.025M pH=3 (75:25); flow rate: 2 ml/minute; detection: 290 nm

Structural Elucidation

The structures of the new compounds (**2**) and (**3**) have been elucidated by NMR and mass spectra, mainly by comparison of their data with those of the known staurosporine. The physico-chemical properties of the molecules are summarized in Table 3. The molecular formula was established as $C_{29}H_{28}O_4N_4$ [*m/z* 497.2 $(M+H)^+$] for (**2**) and $C_{28}H_{26}O_4N_4$ [*m/z* 483.2 $(M+H)^+$] for (**3**) by FAB-MS spectra and ^{13}C NMR spectra demonstrating the presence of additional CH_2O and O atoms respectively compared to staurosporine $C_{28}H_{26}O_3N_4$. The UV spectra of both compounds suggested the presence of the indolo[2,3-a]carbazole chromophore of staurosporine (206, 242, 291, 320, 334, 354, and 370 nm in MeOH). The IR spectra (KBr) contained bands at 3400, 2940, 1670, 1590, 1460, 1400, 1360, 1320, 1280, 1230, 1120, 1070 and 750 cm^{-1}.

The ^1H and ^{13}C NMR spectral data of (**2**) and (**3**) are summarized in Tables 4 and 5. Comparison of NMR data defined the same aglycone structure (for atoms 1~13a) for staurosporine and the new compounds (**2**) and (**3**). Some differences were observed in the chemical shift for sugar moiety signals.

The relative configuration (Figure 1) and the solution conformation (Figure 4) of the tetrahydropyran ring of these two new staurosporine analogues were determined by NMR analysis, in the free base forms, on the base of the coupling constants for protons, chemical shifts, and the cross-peaks from NOESY experiments. The vicinal couplings in (**3**) for protons 3′4′, 4′5′, and 5′6′ (see Table 4) are those expected for a slightly distorted chair conformation[12,13]; proton 6′ has coupling constant of 1.2 Hz to 5′ showing a 6′α5′β relative configuration; the NOESY cross-peak (see Fig. 4) to the proton 5′ and the N-Me shows a 5′β4′α relative configuration; the NOESY cross-peaks to the protons 4′ and 3′ and to this proton 3′ and the methyl 2′ show a 4′α3′α2′Meα relative

Table 4. ^{1}H NMR chemical shifts (δ_H, ppm), multiplicities[a] and coupling constants (J, Hz) of compounds **2**, **3** and staurosporine **1** in $CDCl_3$.

Position	Compound **2**	Compound **3**	Staurosporine **1**
1	7.64 (d, 7.7)	7.53 (d, 8.1)	7.26 (t, 7.6)
2	7.53 (t, 7.7)	7.44 (t, 7.4)	7.46 (t, 7.6)
3	7.38 (t, 7.7)	7.31 (t, 7.4)	7.35 (t, 7.6)
4	9.43 (d, 7.7)	9.40 (d, 7.4)	9.42 (t, 7.6)
6	6.50 (s)	6.43 (s)	6.81 (br s)
7	4.99 (s)	4.98 (s)	4.99 AB
8	7.90 (d, 7.7)	7.89 (d, 7.4)	7.87 (d, 7.8)
9	7.34 (t, 7.7)	7.31 (t, 7.4)	7.30 (d, 7.8)
10	7.45 (t, 7.7)	7.44 (t, 7.4)	7.41 (t, 7.8)
11	7.76 (d, 7.7)	7.85 (d, 7.4)	7.91 (d, 7.8)
3'	3.95 (s)	4.14 (d, 2.8)	3.86 (d, 3.6)
4'	3.02 (d, 9.9)	3.09 (dd, 6.8, 2.8)	3.33 (t, 3.6)
5'	4.43 (d, 9.9)	4.26 (dd, 6.8, 1.2)	2.71 (dd, 14.7, 3.6)
6'	6.52 (s)	6.49 (d, 1.2)	6.52 (d, 5.2)
2'-CH_3	2.48 (s)	2.45 (s)	2.33 (s)
3'-OCH_3	2.03 (s)	2.71 (s)	3.37 (s)
4'-NCH_3		2.17 (s)	1.54 (s)
4'-$N(CH_3)_2$	2.37 (s)		

[a] Multiplicities, s: singlet, d: doublet, t: triplet, br: broad

configuration.

This product, 5′-hydroxystaurosporine (**3**), was transformed into 4′-*N*-methyl-5′-hydroxystaurosporine (**2**) (60%) by treatment of a solution of 4 mg of (**3**) in acetone (1.5 ml) with K_2CO_3 (1 mg) and methyl iodide (1 eq.), showing that the relative configuration for both compounds are the same.

Substantial changes in the NMR spectra were observed for (**2**); the chemical shift of the *N*-Me_2 is 2.37 ppm [2.17 ppm for *N*-Me in (**3**)] and the chemical shift of the *O*-Me is 2.03 ppm [2.71 ppm for *O*-Me in (**3**)], the coupling constant for 4′5′ is 9.9 Hz (6.8 Hz in **3**), there are NOESY cross-peaks (see Fig. 4) to 2′Me and 4′H, and also to 3′H and *N*-Me_2, all these values and cross-peaks can only be explained by a boat conformation[12,13] for this (**2**).

In contrast to other staurosporine analogues carrying a free hydroxy group in the sugar moiety, compounds (**2**) and (**3**) contain a hydroxy group in position 5′ while retaining the pharmacologically important aminomethyl group in position 4′.

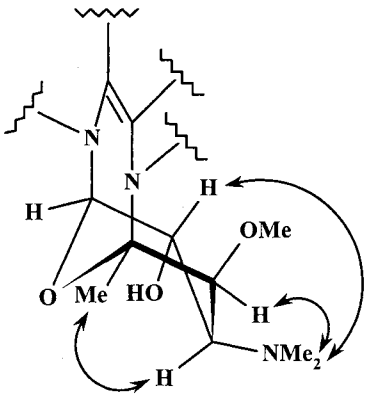
In Vitro Biological Activities

The cytotoxic activities (Table 6) of the new compounds (**2**) and (**3**), and the known staurosporine (**1**) were determined *in vitro* in cell cultures of $P388D_1$ (ATCC CCL-46), A549 (ATCC CCL-185), HT-29 (ATCC HTB-38), and SK-MEL-28 (ATCC HTB-72) following the procedure of BERGERON *et al.*[14]. Staurosporine showed the strongest activity. Since the chromophore is the same in the three compounds, the results suggest that the sugar moiety of indolocarbazole group antibiotics is important for their

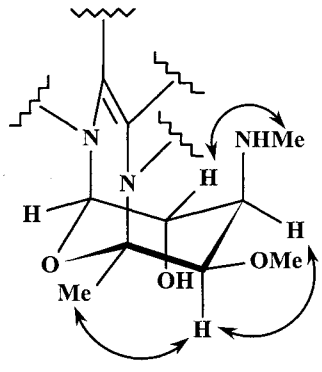
Table 5. ^{13}C NMR chemical shifts (δ_C, ppm) of compounds **2**, **3** and staurosporine **1** in $CDCl_3$.

Position	Compound **2**	Compound **3**	Staurosporine **1**
1	108.97	108.10	106.90
2	125.46	125.33	124.96
3	119.98	119.92	119.66
4	126.13	126.13	127.06
7	45.79	45.88	45.95
8	121.49	121.01	120.57
9	120.43	120.32	119.94
10	124.94	124.67	124.12
11	111.46	113.50	115.13
3'	79.30	80.14	84.05
4'	66.75	60.11	50.35
5'	69.50	70.03	30.14
6'	91.54	88.38	80.10
2'-CH_3	28.00	28.96	30.00
3'-OCH_3	58.36	59.02	57.27
4'-NCH_3		33.68	33.27
4'-$N(CH_3)_2$	41.67		
4a	123.22	123.24	123.38
4b	115.86	115.64	115.34
4c	118.89	118.56	132.17
5	173.65	173.81	173.62
7a	131.94	132.17	118.43
7b	114.14	114.19	114.00
7c	124.54	124.52	124.57
11a	137.86	138.86	139.67
12a	130.64	130.50	130.69
12b	126.79	126.89	128.26
13a	137.12	137.05	138.58
2'	94.92	92.37	91.09

Fig. 4. Solution conformation of the tetrahydropyran ring for compounds **2** and **3**.



Compound **2**



Compound **3**

Table 6. Cytotoxic activities of the new compound **2** and **3**, and staurosporine **1**.

Cell line	IC_{50} (μM)		
	Compound **2**	Compound **3**	Staurosporine **1**
$P388D_1$	0.04	0.02	0.01
A 549	0.002	0.002	0.0005
HT-29	0.004	0.004	0.02
SK-MEL-28	0.004	0.002	0.001

biological activity.

Acknowledgments

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