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GLIOCLADINS A – C AND GLIOPERAZINE ; CYTOTOXIC DIOXO- OR TRIOXOPIPERAZINE METABOLITES FROM A *GLIOCLADIUM* SP. SEPARATED FROM A SEA HARE

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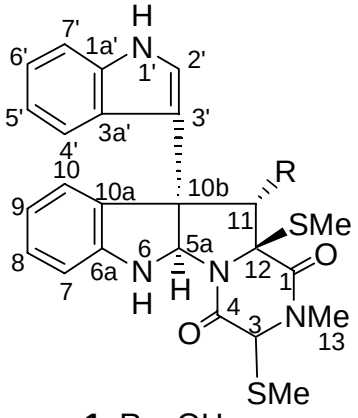
Abstract – New dioxo- or trioxopiperazine metabolites named gliocladins A–C (**1** – **3**) and glioperazine (**4**) have been isolated from a strain of *Gliocladium* sp., originally separated from the sea hare. Their structures have been elucidated by spectroscopic analyses using various NMR spectroscopic techniques. Structurally unique trioxopiperazine (**3**) exhibited significant cytotoxicity against cultured P388 cells.

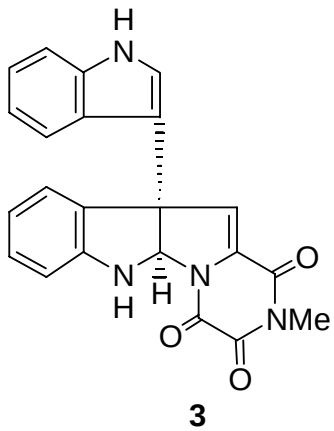
INTRODUCTION

Based on the fact that some of the bioactive materials isolated from marine animals have been produced by bacteria,[1] we have focused our attention on new antitumour metabolites from microorganisms inhabiting the marine environment. So far we have reported isolation and structure determination of a various antitumour and/or cytotoxic compounds from various fungi and an actinomycete originally separated from marine sources, and elucidated their structures.[2–7] As a part of this program, we have isolated a strain *Gliocladium roseum* OUPS-N132 as producing microorganisms of cytotoxic materials from the sea hare *Aplysia kurodai*. Investigation for metabolites of this fungal strain has led to isolation

Figure 1. Structures of gliocladins A – C (**1** – **3**) and glioperazine (**4**)



1: R = OH
2: R = H



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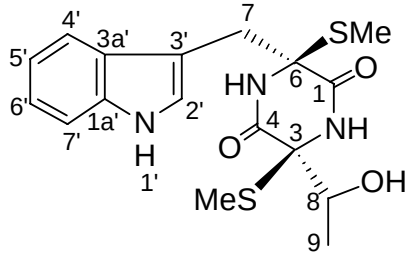

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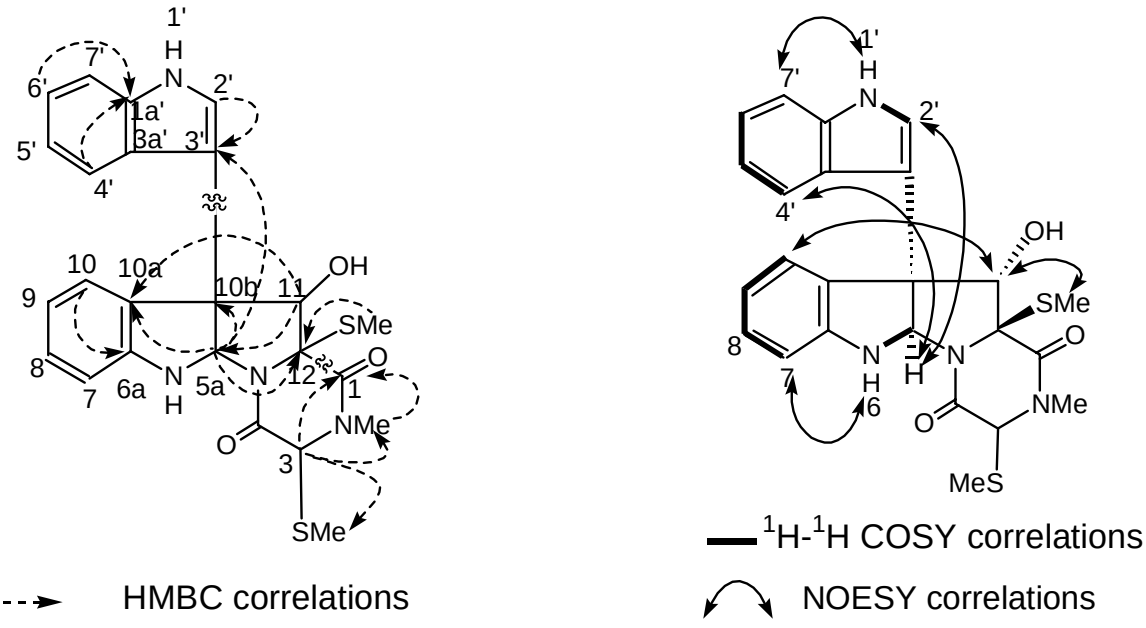
of four new tryptophan derived di- or trioxopiperazine compounds, designated gliocladins A – C (**1**– **3**) and glioperazine (**4**), respectively. They exhibited cytotoxicity against the murine P388 lymphocytic leukemia cells. We report herein the isolation, structure determination and biological activity of these compounds.

RESULTS AND DISCUSSION

The fungal strain was cultured at 27 ^{o}C for 4 weeks in a medium containing 2 % glucose, 1 % peptone and 0.5 % yeast extract in artificial seawater adjusted to pH 7.5. The MeOH extract of the mycelium was purified by bioassay-directed fractionation employing a combination of Sephadex LH-20 and silica gel column chromatography and high-performance liquid chromatography (HPLC) to afford gliocladins A–C (**1**–**3**) and glioperazine (**4**).

Gliocladin A (**1**) had the molecular formula $C_{24}H_{24}N_4O_3S_2$ established by high-resolution electron impact mass spectrometry (HREIMS). Its IR spectrum exhibited absorption at 3408, 1661, 1609 cm^{-1}, characteristic of hydroxyl, amino, amido functions and aromatic ring. A close inspection of the ^{1}H and ^{13}C NMR spectra of **1** (Table 1) by DEPT, HMQC experiments revealed the presence of following functionalities: two *S*-methyl groups, one *N*-methyl group (C-13), one hydroxymethine (C-11), one methine (C-5a) bearing two nitrogen atoms, one methine (C-3) and one quaternary sp^3 carbon (C-12) each bearing both nitrogen and sulfur atoms, two amide carbonyls (C-1, C-4), one sp^2 methine (C-2') bearing one nitrogen atom, one sp^2-carbon (C-3') bearing no hydrogen atom, two 1, 2-disubstituted benzenes, each bonding to an amino group as one substituent, one benzylic quaternary carbon (C-10b). The connection of these functional groups was demonstrated on the basis of ^{1}H-^{1}H- COSY, HMBC and NOESY correlations summarized in Figure 2, and the planar structure of **1** was elucidated.

Figure 2. Selected HMBC, ^{1}H-^{1}H COSY and NOESY correlations of **1**



The relative stereochemistry of **1** was deduced from analysis of its NOESY spectra except for C-3. NOEs observed between H-5a to H-2' and H-4' suggested *cis* configuration of H-5a and indolyl substituent. NOE between H-10 and H-11 indicated that H-11 and the C-10b–C-3' bond have *trans* configuration. And NOE between H-11 and 12-SMe was indicative of their *cis* configuration. Stereochemistry about C-3 remained undefined because informative NOESY cross peaks, such as H-3/12-SMe, 3-SMe/12-SMe, H-5/H-3 or H-5/3-SMe, were not observed.

Table 1. NMR spectral data of gliocladins A, B (**1, 2**) in $CDCl_3$ and gliocladin C (**3**) in acetone-d_6

	1				**2**				**3**			
Position	δH[a]		*J* /Hz	δC	δH[a]		*J* /Hz	δC	δH[a]		*J* /Hz	δC
1				164.6 (q)[b]				165.52 (q)[b]				158.62 (q)[b]
3	4.60	s		67.59 (t)	4.62	s		68.28 (t)				158.01 (q)
4				164.78 (q)				164.46 (q)				150.71 (q)
5a	6.35	s		81.55 (q)	6.07	s		82.37 (q)	6.24	d	3.2	84.72 (q)
6	5.12	br s			5.10	br s			6.61	br d	3.2	
6a				147.42 (q)				148.58 (q)				149.94 (q)
7	6.65	br d	7.8	109.89 (t)	6.70	d	7.8	109.33 (t)	6.86	br d	8.2	110.59 (t)
8	7.09	td	7.8, 1.1	128.74 (t)	7.14	t	7.8	128.64 (t)	7.13	br dd	8.2, 7.6	129.68 (t)
9	6.73	td	7.8, 0.9	119.10 (t)	6.75	t	7.8	119.14 (t)	6.72	dddd	7.6, 7.3, 1.6, 1.1	119.66 (t)
10	7.32	br d	7.8	123.21 (t)	7.17	d	7.8	123.08 (t)	7.18	br d	7.3	125.47 (t)
10a				131.65 (q)				132.41 (q)				131.12 (q)
10b				58.94 (q)				53.72 (q)				61.00 (q)
11a	5.32	br d	3.9	80.26 (t)	3.21	d	14.3	44.1 (s)	6.96	s		126.97 (t)
11b					3.33	d	14.3					
12				73.15 (q)				68.64 (q)				133.08 (q)
13-NMe	3.11	s		32.13 (p)	3.12	s		32.17 (p)	3.25	s		27.10 (q)
3-SMe	2.47	s		18.06 (p)	2.46	s		18.23 (p)				
12-SMe	2.08	s		15.42 (p)	1.99	s		15.23 (p)				
11-OH	3.28	d	3.9									
1'	8.04	br s			8.02	br s			10.3	br d	1.6	
1a'				136.91 (q)				137.19 (q)				138.49 (q)
2'	7.11	d	2.5	122.91 (t)	6.99	d	2.5	121.64 (t)	7.23	d	1.6	123.71 (t)
3'				114.89 (q)				118.58 (q)				122.75 (q)
3a'				125.78 (q)				125.10 (q)				123.55 (q)
4'	7.87	br d	7.8	121.08 (t)	7.43	d	8.0	119.65 (t)	7.33	br d	8.2	119.71 (t)
5'	7.15	td	7.8, 1.4	119.90 (t)	7.04	br t	8.0	122.47 (t)	6.90	ddd	8.0, 7.1, 0.9	119.71 (t)
6'	7.19	td	7.8, 1.4	122.34 (t)	7.04	ddd	8.2, 8.0, 0.9	119.98 (t)	7.10	ddd	8.2, 7.1, 1.1	120.12 (t)
7'	7.32	br d	7.8	111.53 (t)	7.34	dd	8.2	111.47 (t)	7.43	br d	8.2	112.64 (t)

a ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (*J* /Hz). b Letters, p, s, t and q, in parentheses indicate respectively primary, secondary, tertiary and quaternary carbons, assigned by DEPT.

Gliocladin B (**2**) had the molecular formula $C_{24}H_{24}N_4O_2S_2$ established by HREIMS. The general features of its spectral data (Table 1) closely resembled those of **1** expect for presence of isolated sp^3-methylene instead of lacking of a hydroxymethine (C-11). Structure determination of **2** was supported by analysis of ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY spectra. In the NOESY spectra, correlations H-11α/H-5a, H-11α/H-2', H-11α/H-4' were observed in addition to similar cross peaks observed in NOESY spectra of **1**.

Gliocladin C (**3**) had the molecular formula $C_{22}H_{16}N_4O_3$ established by HREIMS. The general features of its spectral data also closely resembled those of **1**. ^{1}H and ^{13}C NMR spectra of **3** implied the lack of two *S*-methyl groups but presence of another sp^2-methine (C-11), one sp^2-carbon (C-12) bearing a nitrogen atom, and the third corbonyl (C-3). Then structure of **3** was deduced as trioxopiperazine shown in Figure 1. Assignments of signals in ^{1}H and ^{13}C NMR spectrum were supported by careful analysis of ^{1}H-^{1}H-COSY, HMQC, HMBC and NOESY spectra. In HMBC spectrum cross peaks were observed between proton of *N*-CH_3 and two carbonyls (C-1: 158.62 ppm and C-3: 158.01 ppm). Correlations H-5a/C-11, H-11/C-12 also supported the deduced planar structure. Cross peaks, H-5a /H-2' and H-5a /H-4',

observed in NOESY spectra of **3** clarified the relative stereochemistry as shown in Figure 1.
In our knowledge there are few examples of natural occurring trioxopiperazines, dethiosecoemestrin[8, 9] and neoechinulin.[10, 11] This is the first case for isolation of trioxopiperazine metabolite from *Gliocladium* sp. though dioxopiperazines such as Sch 52900, Sch 52901[12] and verticllin D–F[13] have been known as metabolites of *Gliocladium* sp..

Glioperazine (**4**) had the molecular formula $C_{17}H_{21}N_3O_3S_2$ established by HREIMS. Its IR spectrum exhibited absorptions at 3322, 1724, 1662 cm^{-1}, characteristic of hydroxyl, amino, amido functions and

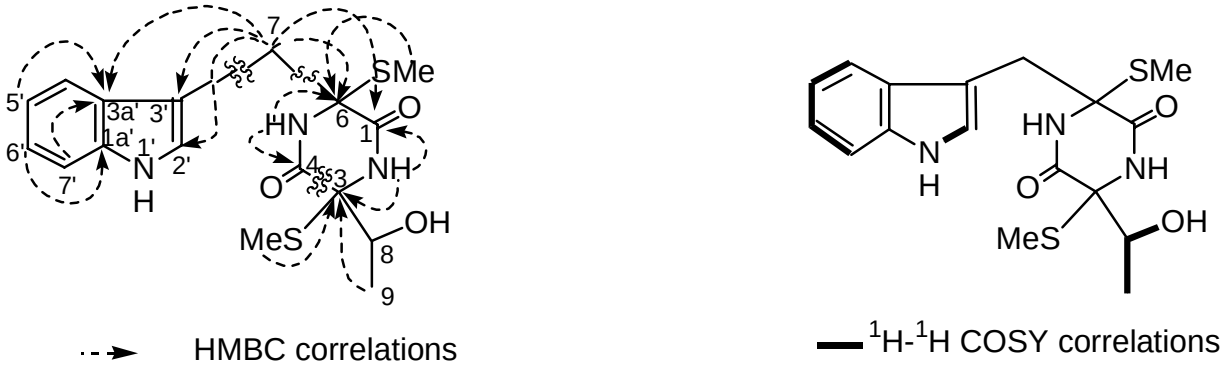
Table 2. NMR spectral data of glioperazine (**4**) in DMSO-d_6

Position	δH[a]	*J/Hz*	^{1}H-^{1}H COSY	δ C	HMBC	NOESY
1				165.52 (q)[b]		
2	8.94 s				1, 3	4', 6'
3				69.07 (q)		
4				164.98 (q)		
5	7.41 s				4, 6	2'
6				66.05 (q)		
7 A	3.64 d	14	7B	34.63 (s)	2', 3', 3a', 6	
B	3.05 d	14	7A		1, 2', 3', 3a', 6	
8	3.62 dd	7.6, 6.4	9, -OH	68.58 (t)		
9	-0.31 d	6.4	8	15.86 (p)	3, 8	-OH, 8
1'	10.89 br d	2.3	2'			2', 7'
1a'				135.76 (q)		
2'	7.07 d	2.3	1'	125.79 (t)	1a', 3', 3a'	
3'				107.65 (q)		
3a'				127.69 (q)	1a', 3', 3a'	
4'	7.7 br d	7.8	5'	119.31 (t)	1a', 3', 6'	
5'	6.91 ddd	7.8, 7.1, 1.1	4', 6'	118.39 (t)	3a', 7'	
6'	6.98 ddd	8.0, 7.1, 1.1	7', 5'	120.68 (t)	1a', 4'	
7'	7.25 br d	8	6'	110.92 (t)	3a', 6'	
3-SMe	2.13 s			13.38 (p)	3	
6-SMe	2.36 s			13.75 (p)	6	
8-OH	4.66 d	7.6	8			

[a, b.] As in Table 1.

aromatic ring. A close inspection of the ^{1}H and ^{13}C NMR spectra of **4** (Table 2) by DEPT, HMQC experiments revealed the presence of following functionalities: two quaternary sp^3 carbon (C-3, C-6) each bearing both nitrogen and sulfur atoms, two amide carbonyls (C-1, C-4), two *S*-methyls, one isolated sp^3-methylene (C-7), one sp^2 (C-2') bearing one nitrogen atom, one sp^2-carbon (C-3') bearing no hydrogen atom, one 1, 2-disubstituted benzenes bonding to an amino group as one substituent, one hydroxymethine (C-8), one methyl group (C-9). The connection of these functional groups was demonstrated on the basis of ^{1}H-^{1}H-COSY, HMBC correlations summarized in Figure 3, and the planar structure of **4** was elucidated. The fact of observation of C-9 methyl group in extraordinary high-field (–0.31 ppm) implied that C-8 – C-9 side chain and aromatic side chain on dioxopiperazine ring have *cis* configuration.[7] The stereochemistry of C-8 remained undefined.

Figure 3. HMBC and ^{1}H-^{1}H COSY correlations of **4**



Cytotoxic activities of **1–4** were examined in the P388 lymphocytic leukemia test system in cell culture, according to the method reported previously.[14] Compounds (**1–4**) exhibited cytotoxic activities (ED_{50} 6.5, 20, 2.4, 6.7 μg/mL respectively). Among them trioxopiperazine metabolite (**3**) showed more potent activity (ED_{50} 2.4 μg/mL) than others.

In conclusion, new di- or trioxopiperazine metabolites named gliocladins A–C (**1**– **3**) and glioperazine (**4**) have been isolated from a strain of *Gliocladium* sp., isolated from the sea hare. The structures of these compounds have been elucidated by spectroscopic analyses. Though all of them showed cytotoxicity against the murine P388 lymphocytic leukemia cells, structurally unique **3** exhibited more potent activity than others.

EXPERIMENTAL

General. UV spectra were recorded on a Shimazu spectrophotometer and IR spectra on a Perkin Elmer FT-IR spectrophotometer 1720X. Optical rotations were obtained on a JASCO ORD/UV-5 spectropolarimeter. NMR spectra were recorded at 27 ^{o}C on a Varian UNITY-500 spectrometer, operating at 500 and 125 MHz for ^{1}H and ^{13}C, respectively, in $CDCl_3$, acetone-d_6 or DMSO-d_6 with TMS as internal reference. EI-MS spectra were measured using a Hitachi M-4000H mass spectrometer. Liquid chromatography was performed over Sephadex LH-20 and silica gel 69 (mesh 230–400, Nacalai Tesque) in a ordinary and medium pressure, equipped with a differential refractometer (R40l) and Shim-pack PREP-ODS (25 cm x 20 mm i.d.). Analytical TLC was performed on precoated Merck aluminum sheets (DC-Alufolien Kieselgel 60 F254, 0.2 mm) and precoated plates RP-18 $F_{254}S$, and compounds were viewed under UV lamp and sprayed with 10 % H_2SO_4 followed by heating.

Fermentation. The sea hare *Aplysia kurodai*, collected in coast of Kata (Wakayama Prefecture of Japan) in May of 1994, was homogenized with sterile artificial sea water and applied to the surface of nutrient agar layered in a Petri dish. Serial transfers of one of the resulting colonies provided a pure strain of *Gliocladium roseum* OUPS-N132. The fungal strain was grown in a liquid medium (120 L) containing 2% glucose, 1% peptone, and 0.5% yeast extract in artificial sea water adjusted to pH 7.5 for 4 weeks at 27 ^{o}C.

Isolation of metabolites. The mycelium extracted three times with MeOH (3x3 L) at 22 ^{o}C for 6 h. The combined extracts were evaporated in *vacuo* to give a crude residue (110 g), which was resolved with AcOEt. The AcOEt solution was concentrated to give a mixture of crude metabolites (16.0 g). The mixture was passed through Sephadex LH-20 using CH_2Cl_2-MeOH (1:1) as the eluent to give an active fraction F1 (1.68 g), which was further separated with a silica-gel column chromatography using CH_2Cl_2-MeOH gradient (100:0–0:100) to give F2 (9.7 mg) , F3 (76.2 mg) and F4 (10.7 mg).

n-Hexane insoluble part (18.2 mg) of F3 was applied to RPHPLC (MeOH : H_2O = 80 : 20) to give **1** (6.2 mg). Similarly, *n*-hexane insoluble part (8.2 mg) of F2 was applied to RPHPLC (MeOH : H_2O = 80 : 20) to give **2** (1.4 mg) and **3** (3.0 mg). F4 was separated by RPHPLC (MeOH : H_2O = 80 : 20) to afford **4** (2.6 mg).
All new compounds were determined to be >95% pure by ^{1}H-NMR spectroscopy.

Gliocladin A (**1**) : colorless crystal (CH_2Cl_2-MeOH); mp 174~176 °C; $[\alpha]_D^{16}$ +263 ° (*c* 0.14, $CHCl_3$); HREIMS Found: 480.1283 (M^+), Calcd: 480.1292 (for $C_{24}H_{24}N_4O_3S_2$); UV λ_{max} (EtOH) nm (log ε) 242 (4.36), 283 (4.12), 291 (4.11); IR ν_{max} (KBr) 3408 (OH and NH), 1661 (C=O), 1609 (C=C) cm^{-1} ;CD λ_{max} (EtOH) nm ($\Delta\varepsilon$) 340 (0), 296 (+12.6), 277 (+8.28), 256 (+14.9), 239.7 (0), 219 (–9.52) (*c* 5.85 x 10^{-5} M in EtOH).

Gliocladin B (**2**) : colorless crystal (CH_2Cl_2-MeOH); mp 145~148 °C; $[\alpha]_D^{16}$ +200.0 ° (*c* 0.06, $CHCl_3$); HREIMS Found: 464.1332 (M^+), Calcd: 464.1329 (for $C_{24}H_{24}N_4O_2S_2$); UV λ_{max} (EtOH) nm (log ε) 283 (3.78), 292 (3.77); IR ν_{max} (KBr) 3355 (OH and NH), 1666 (C=O), 1610 (C=C) cm^{-1} ; CD λ_{max} (EtOH) nm ($\Delta\varepsilon$) 327 (0), 297 (+0.43), 280 (+4.09), 253 (+13.8), 237 (0), 219 (–30.7) (*c* 2.37 x 10^{-5} M in EtOH).

Gliocladin C (**3**) : pale yellow powder (CH_2Cl_2-MeOH); mp 180~183 °C; $[\alpha]_D^{16}$ +131.4 ° (*c* 0.07, $CHCl_3$); HREIMS Found: 384.1222 (M^+), Calcd: 384.1221 (for $C_{22}H_{16}N_4O_3$); UV λ_{max} (EtOH) nm (log ε) 242 (5.11), 283 (4.88), 292 (4.89); IR ν_{max} (KBr) 3320 (OH and NH), 1694 (C=O), 1675 (C=C) cm^{-1} ; CD λ_{max} (EtOH) nm ($\Delta\varepsilon$) 367 (–6.1), 323.5 (0), 296 (+13.93), 274 (+4.76), 263 (+4.42),252 (+0.68) 235.5 (+13.59), 226 (0), 219 (–10.87) (*c* 3.57 x 10^{-5} M in EtOH).

Glioperazine (**4**) : colorless crystal (CH_2Cl_2-MeOH); mp 170~173 °C; $[\alpha]_D^{16}$ +52.6 ° (*c* 0.11, $CHCl_3$); HREIMS Found: 379.1030 (M^+), Calcd: 379.1027 (for $C_{17}H_{21}N_3O_3S_2$); UV λ_{max} (EtOH) nm (log ε) 274 (3.61), 283 (3.62), 292 (3.56); IR ν_{max} (KBr) 3322 (OH and NH), 1724 (C=O) , 1662 (C=O or C=C) cm^{-1} ; CD λ_{max} (EtOH) nm ($\Delta\varepsilon$) 278 (0), 271 (–0.2), 263 (0), 254 (0) 241 (–1.21), 234 (0), 226 (+1.21), 224 (0), 217 (–3.23) (*c* 6.01 x 10^{-5} M in EtOH).

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