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New Antifouling Sesquiterpenes from Four Nudibranchs of the Family Phyllidiidae

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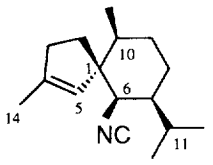
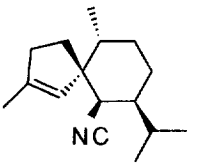
Abstract: Three new antifouling sesquiterpene isocyanides were isolated from nudibranchs of the family Phyllidiidae along with a new sesquiterpene peroxide and six known sesquiterpenes. Their structures were determined mainly on the basis of 2D NMR data. These compounds showed potent antifouling activity against larvae of the barnacle *Balanus amphitrite*.

INTRODUCTION

Sessile marine organisms, such as algae, hydroids, mussels, and barnacles, often cause serious problems by settling on ships' hulls, cooling systems for power plants, and aquaculture cages.[2] Organotin compounds were widely used for control until recently, when they were found toxic to marine organisms.[3] Therefore, development of environmentally safe antifouling substances is urgently needed.[4-6] Sessile marine invertebrates are believed to maintain epibiont-free surfaces by chemical defense; hence their secondary metabolites might be potentially nontoxic antifouling agents. A variety of antifouling substances against barnacle settlement have been isolated, among others, from the sponge *Agelas conifera*,[7] the gorgonian *Leptogorgia virgulata*,[8] the sea pansy *Renilla reniformis*,[9] and the bryozoan *Zoobotryon pellucidum*.[10] We also reported antifouling substances from the marine sponges *Acanthella cavernosa*,[11,12] *Agelas mauritiana*,[13] and *Pseudoceratina purpurea*.[14] Similarly, nudibranchs are well known to sequester secondary metabolites from their sponge diets to protect their soft bodies from predators.[15] Their chemical defense could be used for development of antifouling strategy. In fact, during our search for antifouling substances from Japanese marine invertebrates, we found that extracts of four nudibranchs of the family Phyllidiidae inhibited larval settlement and metamorphosis of the barnacle *Balanus amphitrite*. Bioassay-guided fractionation of the ethanol extract of the nudibranchs yielded three



1



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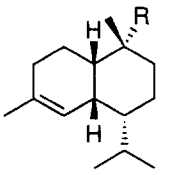
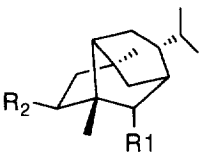
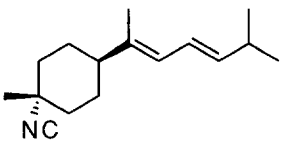


3 R=NCS
8 R=NC



	R_1	R_2
4	SCN	H
5	H	SCN



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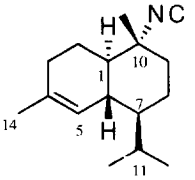

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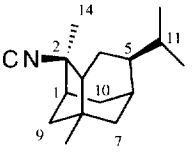

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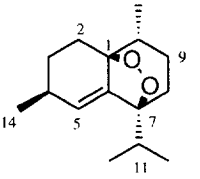

10

new sesquiterpene isocyanides, 10-*epi*-axisonitrile-3 (**1**), 10-isocyano-4-cadinene (**7**), and 2-isocyanotrachyopsane (**9**) along with the known sesquiterpenes, axisonitrile-3 (**2**),[16] (–)-10-isothiocyanato-4-amorphene (**3**),[17] 2-thiocyanato*neo*pupukeanane (**4**),[18] 4-thiocyanato*neo*pupukeanane (**5**),[18] 3-isocyanotheonellin (**6**),[19] and 10-isocyano-4-amorphene (**8**)[17] and a new sesquiterpene peroxide, 1,7-epidioxy-5-cadinene (**10**). In this paper we describe the isolation and structure elucidation of these antifouling substances.

RESULTS AND DISCUSSION

Three nudibranchs, *Phyllidia ocelata*, *P. varicosa*, and *Phillidiopsis krempfi*, were collected off the Koshiki-jima Islands, west of Kyushu by Scuba. Specimens of *Phyllidia pustulosa* were collected from Yakushima, Kuchinoerabu-jima, and Tanegashima Islands, south of Kyushu in addition to the Koshiki-jima Islands. Animals were steeped in EtOH immediately after collection. The EtOH extract was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 layer which inhibited larval settlement in the barnacle *B. amphitrite* was fractionated by silica gel column chromatography (hexane-ether). The active fractions were purified by HPLC on a silica gel column with hexane-ether, and if necessary on chiral, NH_2, and ODS columns. Interestingly, *P. pustulosa* from Yakushima, Kuchinoerabu-jima, and Tanegashima Islands showed similar profiles of antifouling compounds, while the Kamikoshiki-jima collection contained a different compound **7** (see Table 1). Moreover, nudibranchs from the Koshiki-jima Islands showed similar profiles of active substances. Known compounds were identified by comparison of spectral data reported for axisonitrile-3 (**2**),[16] (–)-10-isothiocyanato-4-amorphene (**3**),[17] 2-thiocyanato*neo*pupukeanane

Table 1. Yield and antifouling activity of sesquiterpenes from nudibranchs of the family Phyllidiidae

(mg/animal)

Species	Collection site	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
P. pustulosa	Yakushima	1.2	0.4	0.7							
P. pustulosa	Kuchinoerabu	0.1	0.4		0.2						
P. pustulosa	Tanegashima		0.3		0.4	0.8	1.6				
P. pustulosa	Kamikoshiki							13.0			
P. ocelata	Kamikoshiki								1.0		
P. varicosa	Shimokoshiki							2.1		2.5	
P. krempfi	Shimokoshiki							1.8	0.8		1.3
Antifouling activity IC_{50} (μg/mL)		10	3.2	7.2	4.6	2.3	0.13	0.14	0.70	0.33	>50

(**4**),[18] 4-thiocyanato-*neo*pupukeanane (**5**),[18] 3-isocyanotheonellin (**6**),[19] and 10-isocyano-4-amorphene (**8**).[17]

The first new compound **1** was obtained pure from a mixture of **1** and **2** by using recycled HPLC on a chiral column. The molecular formula of **1** was established to be $C_{16}H_{25}N$ by HREIMS (*m/z* 231.1980, Δ -0.7 mmu). The presence of an isocyanide functionality was supported both by IR (2126 cm^{-1}) and ^{13}C NMR data (δ 157.3 and δ 59.4, both triplets, which are typical chemical shifts of an isocyano carbon and a carbon attached to an isocyano nitrogen). The planar structure of **1** was readily determined by comparison of the COSY, HMQC, and HMBC spectra with those of **2**. NOESY correlations; H-5/H-6 and H-5/H-7 implied α-orientation for C-5, H-6, and H-7 in the cyclohexane. Thus, H-6 was equatorial, while H-7 was axial, which was the same as in axisonitrile-3 (**2**); stereochemistry of C-10 remained to be determined. However, the ^1H NMR spectrum of **1** measured in $CDCl_3$ was too congested around 1.6 ppm, which hampered determining stereochemistry of C-10 by NOESY experiments; ^1H NMR spectra measured in mixtures of $CDCl_3$ and C_6D_6 were unimproved. Nevertheless, NMR data of **1** and **2** were apparently different, which suggested the stereochemistry of C-10 should be different. Thus, **1** had to be 10-*epi*-axisonitrile-3.

Compound **7** had a molecular formula of $C_{16}H_{25}N$, which was established by HREIMS (*m/z* 231.1976, Δ -1.1 mmu). The presence of an isocyano group in **7** was also suggested by IR (2122 cm^{-1}) and ^{13}C NMR data (δ 152.1 and δ 60.7). The planar structure was straightforward from COSY correlations as well as from HMBC cross peaks (H-1/C-15, H-1/C-10, H-8/C-10, H-9/C-1, H-9/C-10, H-15/C-1, H-15/C-9, and H-15/C-10). The isocyano group was placed on C-10, which was a triplet with a coupling constant of *J*=5 Hz. Stereochemistry was deduced by the NOESY spectrum: H-7 was correlated to both H-1 and H-8α (δ 1.57), while Me-15 to H-6 and H-8β (δ 1.09). Thus, **7** was (1*R**, 6*R**, 7*S**, 10*R**)-10-isocyano-4-cadinene.

Table 2. ^{13}C NMR Data for New Sesquiterpenes ($CDCl_3$)

Carbon	1	7	9	10
1	54.9 (s)	48.0 (d)	47.3 (d)	89.4 (s)
2	34.7 (t)	23.7 (t)	70.8 (s)[a]	36.6 (t)
3	34.7 (t)	30.7 (t)	53.6 (d)	32.4 (t)
4	141.8 (s)	135.3 (s)	24.7 (t)	38.3 (d)
5	129.8 (d)	121.2 (d)	46.8 (d)	117.9 (d)
6	59.4 (d)[a]	37.9 (d)	31.2 (d)	153.0 (s)
7	43.8 (d)	46.2 (d)	37.2 (t)	83.2 (s)
8	19.5 (t)	20.2 (t)	39.3 (s)	31.0 (t)
9	29.4 (t)	40.6 (t)	45.3 (t)	28.2 (t)
10	36.8 (d)	60.7 (s)[a]	34.2 (t)	38.7 (d)
11	29.8 (d)	25.9 (d)	31.8 (d)	35.7 (d)
12	20.8 (q)	15.0 (q)	20.8 (q)	16.9 (q)[b]
13	20.0 (q)	21.3 (q)	21.5 (q)	17.1 (q)[b]
14	16.8 (q)	23.6 (q)	22.5 (q)	16.5 (q)
15	16.0 (q)	20.0 (q)	27.3 (q)	13.8 (q)
16	157.3 (s)[a]	152.1 (s)[a]	152.3 (s)[a]	

[a] Signal appears as a triplet.

[b] Signals may be interchanged.

Compound **9** had the same molecular formula, $C_{16}H_{25}N$, as **7**; the presence of an isocyano group was again suggested by IR and ^{13}C NMR data (δ 152.3 and δ 70.8). No sp^2 signal was observed in 1D NMR spectra, thus suggesting that **9** is tricyclic. The COSY spectrum revealed two partial structures, (CH-3)-(CH_2-4)-(CH-5)-(CH-11)-(Me-12,13) and (CH_2-9)-(CH-1)-(CH_2-10)-(CH-6)-(CH_2-7). HMBC correlations from H-15 to C-3, 7, 8, and 9 indicated that the C-8 quaternary carbon was linked to C-3, 7, 9, and 15. W-coupling observed between H-7 (δ 1.22) and H-9 (δ 2.25) supported the presence of a cyclohexane ring (C-1, 10, 6, 7, 8, 9). Similarly, HMBC cross peaks; H-14 to C-1, 2, and 3 led to connectivities from triplet carbon C-2 to C-1, 3, and 14. Finally, the connection between C-5 and C-6 was inferred from HMBC correlations (H-5/C-6, H-5/C-7, H-5/C-10, and H-6/C-5), thereby constructing the trachyopsane skeleton; 2-isothiocyanatotrachyopsane was reported from the marine sponge *Trachyopsis aplysinoides*.[20] Furthermore, NOESY cross peaks (H-4α/Me-14, H-5/H-10eq, H-5/Me-14, and H-10eq/Me-14) indicated that these six protons were positioned on the same face of the seven-membered ring. Thus, **9** was 2-isocyanotrachyopsane.

The last new compound **10** had a molecular formula of $C_{15}H_{24}O_2$ as established by HREIMS. ^1H and ^{13}C NMR spectra (Table 2) revealed the presence of four methyls, four methylenes, three methines, and two quaternary sp^3 carbons, in addition to two sp^2 carbons, which accounted for $C_{15}H_{24}$; two

oxygens were missing from the molecular formula. Interpretation of the COSY spectrum led to three partial structures, (Me-15)-(CH-10)-(CH_2-9)-(CH_2-8), (Me-12)-(CH-11)-(Me-13), and (CH_2-2)-(CH_2-3)-(CH-4)-(Me-14)(CH-5); C-5 was connected to the remaining olefinic carbon C-6. HMBC correlations from H-2, H-5, and H-15 to C-1 indicated that the quaternary carbon C-1 (δ 89.4) was connected to C-2, 6, and 10. Furthermore, HMBC cross peaks from H-5, H-12, and H-13 to C-7 implied connectivities from another quaternary carbon C-7 (δ 83.2) to C-6 and C-11. Chemical shifts of these two quaternary carbons and the presence of two oxygens suggested the presence of an epidioxy group between C-1 and C-7. Finally, connection between C-7 and C-8 completed the planar structure of **10**. In order to determine the relative stereochemistry, all coupling patterns in the ^{1}H NMR spectrum were simulated by using the computer program named "Let's Spin".[21] Coupling constants of H-10 (J=5.8, 3.8 Hz) with H_2-9 indicated its equatorial configuration, while those of H-4 (J=7.1, 10.5 Hz) with H_2-3 its axial orientation. NOESY correlations between 10α-Me and H-2α (ax), and between H-2α (ax) and H-4α (ax) supported the 4β (eq)-Me orientaion.

Sesquiterpenes containing isocyano, isothiocyanato, and thiocyanato functionalities (**1**-**9**) inhibited settlement and metamorphosis of cyprid larvae of *B. amphitrite* (Table 1). Especially, **6** and **7** showed potent antifouling activity with IC_{50}'s of 0.13 and 0.14 μg/mL, respectively, while no toxicity was found at these concentrations.[22] Their activity was comparable to that of $CuSO_4$ (IC_{50} 0.15 μg/mL). It should be noted that **10** showed no activity at 50 μg/mL, though it contained a peroxide moiety.

EXPERIMENTAL

General methods: NMR spectra were recorded on a BRUKER ARX 500 spectrometer in $CDCl_3$ and C_6D_6 at 500.14 MHz for ^{1}H and 125.77 MHz for ^{13}C at 300 K. Chemical shifts were referenced to solvent peaks: δ_H 7.24 and δ_C 77.0 for $CDCl_3$. Optical rotations were determined with a JASCO DIP-1000 digital polarimeter. EI mass spectra were measured with a JEOL JMS-SX102A mass spectrometer. IR spectra were recorded on a JASCO IR-700 infrared spectrometer.

Antifouling assay: Adult barnacles, *Balanus amphitrite*, attached to bamboo poles were procured from oyster farms in Lake Hamana, Shizuoka, and maintained in an aquarium at 25 °C by feeding on *Artemia salina* nauplii. Broods released I-II stage nauplii upon immersion in seawater after being dried for 2 days. Nauplii thus obtained were cultured in 80 % filtered seawater at 25 °C by feeding with the diatom *Chaetoceros gracilis*. The seawater and diet were renewed everyday. Larvae reached the cyprid stage in 5 days. The cyprids were stored at 5 °C until used.

Test samples were dissolved in ethanol; aliquots of the solution were supplied to wells of 24-well polystyrene tissue culture plates and air-dried. To each well were added 2 mL of 80 % filtered seawater and six one-day-old cyprids. Four wells were used for each experiment. The plates were kept in the dark

for 48 h at 25 °C, and the numbers of larvae which attached, metamorphosed, died, or did not settle were counted under a microscope.

Extraction and isolation: Samples of the nudibranch *Phyllidia pustulosa* were collected by hand using Scuba at depths of 3-15 m either off Yakushima Island (3 specimens), Kuchinoerabu-jima Island (3 specimens), Tanegashima Island (3 specimens), or Kamikoshiki-jima Island (1 specimen). Specimens of *P. ocelata* (1 specimen), *P. varicosa* (2 specimens) and *Phillidiopsis krempfi* (1 specimen) were collected at a depth of 10 m off Koshiki-jima Islands. All nudibranch samples were steeped in ethanol immediately after collection and kept at room temperature. The ethanol extracts were obtained by decantation of the ethanol solution which contained the nudibranchs, concentrated, and partitioned between H_2O and CH_2Cl_2. The CH_2Cl_2-soluble fractions were fractionated by silica gel column chromatography with increasing amounts of ether in hexane. Potent antifouling activity was observed in the fractions eluted with hexane/ether (98:2) from all specimens.

The potent active fraction of three specimens of *P. pustulosa* from Yakushima Island was separated by HPLC on Develosil 60-5 (4.6 × 250 mm, mobile phase hexane/ether 99:1, flow rate 1.0 mL/min, UV 210 nm) to yield 3:1 mixture of **1** and **2** (4.8 mg), which furnished pure **1** (0.5 mg) by using recycled HPLC on Chiralpak AD (4.6 × 250 mm, mobile phase hexane 100 %, flow rate 1.2 mL/min, UV 220 nm). Moderate activity was also shown in the hexane/ether (99:1) eluate from the silica gel column, which yielded compound **3** (2.2 mg) by HPLC on Develosil 60-5 (4.6 × 250 mm, mobile phase hexane 100 %, flow rate 1.0 mL/min, UV 210 nm).

The active fraction of three specimens of *P. pustulosa* from Kuchinoerabu-jima Island was separated by HPLC on Shodex SIL-5B (4.6 × 250 mm, mobile phase hexane/ether 99:1, flow rate 1.0 mL/min, UV 220 nm) and then on Develosil NH_2 (4.6 × 250 mm, mobile phase hexane 100 %, flow rate 1.0 mL/min, UV 220 nm) to yield compound **4** (0.6 mg) and a 1:4 mixture of **1** and **2** (1.7 mg).

The active fraction of three specimens of *P. pustulosa* from Tanegashima Island was separated by HPLC on Senshupak silica 1251-N (4.6 × 250 mm, mobile phase hexane/ether 99:1, flow rate 1.0 mL/min, UV 220 nm) to yield compound **6** (4.9 mg). Further purification of another active peak by HPLC under the same condition yielded compound **5** (2.5 mg) and a single peak containing **2** and **4**. These compounds (**2**; 0.8 mg and **4**; 1.1 mg) were isolated by HPLC on Develosil NH_2 (4.6 × 250 mm, mobile phase hexane 100 %, flow rate 1.0 mL/min, UV 220 nm).

The active fractions of one each specimen of *P. pustulosa* and *P. ocelata* from Kamikoshiki-jima Island were separated by HPLC on Develosil 60-5 (4.6 × 250 mm, mobile phase hexane/ether 98:2, flow rate 1.0 mL/min, UV 220 nm) to yield **7** (13.0 mg) and **8** (1.0 mg), respectively.

The active fraction of two specimens of *P. varicosa* from Shimokoshiki-jima Island was separated by HPLC on Shodex SIL-5B (10 × 250 mm, mobile phase hexane/ether 98:2, flow rate 3.0 mL/min, UV 220 nm) and then on Develosil ODS HG-5 (4.6 × 250 mm, mobile phase 80 % MeOH, flow rate 0.8 mL/min, RI) to yield **7** (4.2 mg) and **9** (4.9 mg).

The active fraction of a specimen of *Phillidiopsis krempfi* from Shimokoshiki-jima Island was separated by HPLC on Develosil 60-5 (4.6 × 250 mm, mobile phase hexane/ether 97:3, flow rate 1.0 mL/min, UV 220 nm) to obtain two active fractions. Each fraction was separated by HPLC on Develosil 60-5 (4.6 × 250 mm, mobile phase hexane/ether 99:1, flow rate 1.0 mL/min, UV 220 nm) to yield **7** (1.8 mg), **8** (0.8 mg) and **10** (1.3 mg), respectively.

10-*epi*-Axisonitrile-3 (1): $[\alpha]_D^{23}$ -5.6° (*c* 0.025, $CHCl_3$); IR (neat) 2920, 2126 cm^{-1}; HREIMS *m/z* 231.1980 (calcd for $C_{16}H_{25}N$, 231.1987); ^1H NMR ($CDCl_3$) δ 5.39 (1H, s, H-5), 3.48 (1H, brs, H-6), 2.32 (1H, m, H-3), 2.20 (2H, m, H-2 and H-3), 1.88 (1H, m, H-2), 1.69 (3H, s, H-14), 1.63 (2H, m, H-8 and H-10), 1.62 (2H, m, H-9 and H-11), 1.48 (2H, m, H-8 and H-9), 1.14 (3H, d, *J*=7.5 Hz, H-15), 1.09 (1H, m, H-7), 0.96 (3H, d, 6.7, H-12), 0.87 (3H, d, 6.6, H-13); ^{13}C NMR See Table 2.

10-Isocyano-4-cadinene (7): $[\alpha]_D^{23}$ +63.6° (*c* 0.60, $CHCl_3$); IR (neat) 2934, 2122 cm^{-1}; HREIMS *m/z* 231.1976 (calcd for $C_{16}H_{25}N$, 231.1987); ^1H NMR ($CDCl_3$) δ 5.44 (1H, s, H-5), 2.14 (1H, m, H-11), 2.05 (1H, m, H-9), 2.04 (2H, m, H-2 and H-3), 1.98 (1H, m, H-3), 1.80 (1H, ddd, *J*=13.4, 13.3, 3.7 Hz, H-9), 1.71 (1H, dd, 10.8, 9.4, H-6), 1.65 (3H, s, H-14), 1.57 (1H, m, H-8), 1.48 (1H, brdd, 12.0, 10.8, H-1), 1.33 (1H, m, H-2), 1.27 (3H, s, H-15), 1.09 (1H, m, H-8), 1.05 (1H, m, H-7), 0.89 (3H, d, 7.0, H-13), 0.73 (3H, d, 7.0, H-12); ^{13}C NMR See Table 2.

2-Isocyanotrachyopsane (9): $[\alpha]_D^{23}$ +74.4° (*c* 0.23, $CHCl_3$); IR (neat) 2914, 2118 cm^{-1}; HREIMS *m/z* 231.1983 (calcd for $C_{16}H_{25}N$, 231.1987); ^1H NMR ($CDCl_3$) δ 2.25 (1H, brd, *J*=11.6 Hz, H-9), 2.13 (1H, br, H-1), 1.97 (1H, br, H-3), 1.90 (1H, br, H-6), 1.76 (1H, ddd, 12.5, 6.4, 3.6, H-10), 1.69 (1H, ddd, 13.9, 7.0, 3.3, H-4), 1.54 (3H, s, H-14), 1.37 (1H, brd, 12.5, H-10), 1.33 (1H, m, H-11), 1.28 (1H, m, H-9), 1.22 (2H, m, H-7), 1.08 (1H, m, H-4), 1.04 (3H, s, H-15), 1.02 (1H, m, H-5), 0.85 (3H, d, 6.8, H-12), 0.83 (3H, d, 6.5, H-13); ^{13}C NMR See Table 2.

1,7-Epidioxy-5-cadinene (10): $[\alpha]_D^{23}$ +36.6° (*c* 0.080, $CHCl_3$); IR (neat) 2950 cm^{-1}; HREIMS *m/z* 236.1791 (calcd for $C_{15}H_{24}O_2$, 236.1776); ^1H NMR ($CDCl_3$) δ 5.70 (1H, d, *J*=2.5 Hz, H-5), 2.59 (1H, ddqd, 10.5, 7.1, 6.5, 2.5, H-4α), 1.91 (1H, ddd, 14.2, 8.0, 2.5, H-2β), 1.84 (1H, ddd, 14.0, 11.1, 5.0, H-8α), 1.83 (1H, dddd, 12.0, 7.1, 7.0, 2.5, H-3α), 1.83 (1H, qq, 6.9, 6.9, H-11), 1.77 (1H, qdd, 7.0, 5.8, 3.8, H-10β), 1.69 (1H, ddd, 14.2, 10.5, 7.0, H-2α), 1.62 (1H, ddd, 14.0, 5.2, 4.7, H-8β), 1.47 (1H, dddd, 13.9, 11.1, 5.8, 5.2, H-9β), 1.40 (1H, dddd, 13.9, 5.0, 4.7, 3.8, H-9α), 1.31 (1H, dddd, 12.0, 10.5, 10.5, 8.0, H-3β), 1.16 (3H, d, 6.5, H-14), 1.03 (3H, d, 7.0, H-15), 0.96 (3H, d, 6.9, H-13), 0.95 (3H, d, 6.9, H-12) The values of chemical shifts and coupling constants were assigned by comparison of spectral data with those obtained by simulation using the program "Let's Spin";[21] for ^{13}C NMR data see Table 2.

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