A New Meroditerpenoid Dimer from an Undescribed Philippine Marine Sponge of the Genus *Strongylophora*

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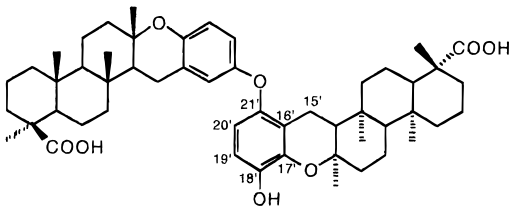
An undescribed Philippine marine sponge of the genus *Strongylophora* yielded a new meroditerpenoid−strongylophorine dimer (**1**) and the known meroditerpenoids, strongylophorine-2 (**2**), strongylophorine-3 (**3**), and strongylophorine-4 (**4**). The structures of the compounds were established on the basis of NMR spectroscopic and mass spectrometric data. The position of the inter-unit linkage in the new compound was elucidated after methylation and 1D ^{1}H NOE difference experiments. This is the first report wherein the ^{1}H and ^{13}C NMR data of the strongylophorine congeners are fully and unambiguously assigned on the basis of 2D NMR spectroscopy. Compounds **2** and **3** exhibited slight activity against *Micrococcus luteus* and *Salmonella typhii*, respectively. Compound **3** was active against the phytopathogenic fungus *Cladosporium cucumerinum* and also against the neonate larvae of the polyphagous pest insect *Spodoptera littoralis* (EC_{50} of 69 [±0.48 (S.E.)] ppm) when incorporated into artificial diet. Compound **1** was found to be the most active in the brine shrimp lethality test with a LC_{50} of 10.5 [±0.43 (S.E.)] μg/mL.

The ichthyotoxic meroditerpenoids were first isolated from the brown algae *Taonia atomaria*[1−3] and *Stypopodium zonale*.[4,5] Recently, related compounds have been isolated from the terrestrial cyanobacteria *Tolypothrix nodosa* [6] and *Nostoc commune*.[7] The same type of compound, called strongylophorines, was also isolated from the marine sponge *Strongylophora durissima* collected from Papua New Guinea.[8] The same sponge collected from Maricaban Island, Philippines, showed the presence of five other strongylophorine congeners.[9] In this paper, we describe the isolation and structure elucidation of a new strongylophorine dimer (**1**) and three other known derivatives (**2**−**4**) obtained from an undescribed Philippine species of *Strongylophora* and report on their insecticidal, antibacterial, fungicidal, and cytotoxic properties.

A species of the sponge of the genus *Strongylophora* (family Petrosiidae, order Haplosclerida) was collected from Ilocos Sur, Philippines, by scuba diving. The undescribed species appears to be closely related to *Strongylophora durissima* (Dendy), but that species is different in having a massive irregular shape, a grooved surface, and lacking the larger oxeas that are present in our material. From this information, we conclude that this material belongs to a new species, which will be described elsewhere.



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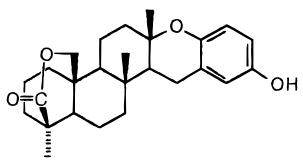

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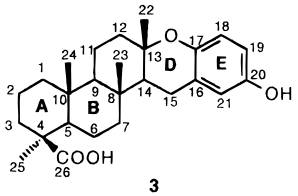

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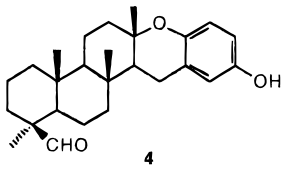

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The sponge samples were freeze-dried prior to extraction. The EtOAc-soluble material was subjected to Si gel column chromatography and yielded 18 fractions. The nonpolar fraction 6 afforded compound **4**. The semipolar fractions 11 and 12 contained the known compound **2** and the new strongylophorine dimer **1**, respectively. The major compound **3** was obtained from the polar fraction 13. The identities of the known strongylophorine derivatives (**2**−**4**) were established by comparison of their spectral data with those published.[8,9]

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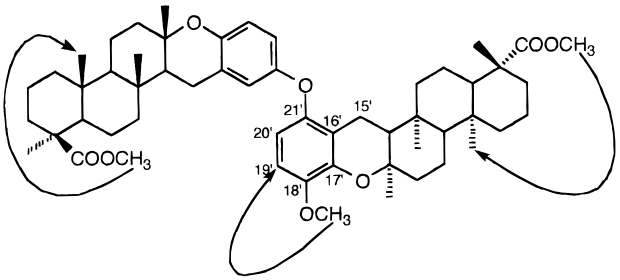
Chart 1. NOE experiment on the methylated distrongylophorine.



Compound **1**, which we have named distrongylophorine, was obtained as a cream-colored powder. The molecular formula of **1** was determined by HREIMS as $C_{52}H_{70}O_8$. It showed a molecular ion peak [M^+] at *m/z* 822 in ElMS suggesting it is a meroditerpenoid dimer, while an ion peak at *m/z* 412 indicates fragmentation to the constituent monomer, $[C_{26}H_{36}O_4]^+$. Comparison of the ^{13}C data with that of the three known strongylophorine congeners indicates that one-half of the molecule is related to **3**. The diterpene portion of the dimer was observed to be almost symmetrical, which led to a doubling of signals. The disruption of the symmetry was in the vicinity of the aromatic ring systems as 12 well-separated ^{13}C signals were observed. The ^{13}C NMR spectrum of **1** contains five aromatic carbons bearing oxygen (140.1, 142.5, 147.4, 148.5, and 150.6 ppm) and indicates the presence of an additional oxygen function in one of the subunits compared to a symmetrical dimer of **3**. From the correlations in the HMBC spectrum at highfield, one of the oxygen substituents participating in either the dimer linkage or new hydroxyl function must be attached to C-21′, as a long-range correlation is observed between H-15′A/15′B and the carbon signal at 147.4 ppm, in addition to their correlations with C-17′ at 140.1 ppm. This same aromatic system carries an isolated ortho-pair of protons in the ^1H NMR spectrum at 6.79 and 6.55 ppm, with a coupling constant of 8.9 Hz. The magnitudes of the long-range correlations of these two protons to the carbons in the system can only be satisfactorily explained when these are bound to C-19′ and C-20′, respectively. Consequently, C-18′ must also carry an oxygen substituent. The decisive experiment for ascertaining the position of the ether linkage was performed by methylation of the free hydroxyl function and subsequent investigation of this using 1D NOE difference data. Irradiation of the aromatic methoxyl group produced an NOE effect on the lowfield doublet that had been assigned to H-19′ in **1** and, consequently, indicated that the ether linkage was at C-21′ (Chart 1).The relative and absolute configurations of the various asymmetric centers in the molecules are assumed to be the same as those established by X-ray analysis for the known compounds. The optical rotation values obtained for the known compounds **2**−**4** were also similar to those previously reported.[8,9] However, we are the first to report the unambiguous assignment of the ^1H and ^{13}C NMR spectral data (Tables 1 and 2). As the NMR data for compound **3** have only been partially assigned in the literature, a full assignment of all the ^1H and ^{13}C signals was achieved at highfield (^1H 600 MHz) through the use of COSY and HMBC experiments. An HMBC experiment allowed unambiguous assignment of all of the ^{13}C signals, apart from C-2, C-6, and C-11, and a number of the ^1H signals, in particular the methyl groups. Assignment of the remaining signals was achieved through inspection of the COSY and HMQC spectra. The chemical shifts of all ^1H signals were readily determined from the latter experiment, and their multiplicities and coupling constants were assessed from well-resolved signals in the 1D ^1H spectrum. The data for **2** and **4** were initially compared to those of **3**, and finally the assignments were confirmed using HMQC and HMBC experiments at lower field strength (^1H 300 and 400 MHz).

Table 1. ^{13}C NMR Data of Compounds 1−4 in $CDCl_3$

C no.	1		2	3	4
1	40.2 t	1′	40.2 t	40.3 t	40.2 t	39.3 t
2	19.1 t	2′	19.1 t	21.0 t	19.1 t	18.4 t
3	38.0 t	3′	37.9 t	40.3 t	38.0 t	34.5 t
4	43.9 s	4′	43.8 s	43.2 s	43.7 s	48.4 s
5	56.9 d	5′	56.8 d	50.5 d	57.0 d	56.8 d
6	19.6 t	6′	43.2 s	19.6 t	20.4 t	19.6 t
7	40.8 t	7′	50.5 d	40.2 t	38.2 t	40.9 t
8	37.0 s	8′	36.8 s	36.6 s	37.0 s	37.0 s
9	60.1 d	9′	60.0 d	55.4 d	60.2 d	59.6 d
10	37.9 s	10′	37.9 s	36.7 s	38.0 s	37.8 s
11	18.8 t	11′	18.8 t	18.7 t	18.9 t	18.9 t
12	41.1 t	12′	40.9 t	41.4 t	41.2 t	41.1 t
13	76.3 s	13′	76.3 s	76.0 s	76.5 s	76.4 s
14	52.2 d	14′	51.6 d	52.5 d	52.4 d	52.4 d
15	22.5 t	15′	17.6 t	22.4 t	22.5 t	22.5 t
16	123.6 s	16′	116.7 s	122.6 s	123.2 s	123.1 s
17	148.5 s	17′	140.1 s	147.0 s	147.2 s	147.2 s
18	117.7 d	18′	142.5 s	117.7 d	117.6 d	117.6 d
19	113.1 d	19′	113.9 d	114.5 d	114.3 d	114.3 d
20	150.6 s	20′	113.9 d	148.8 5	148.6 s	148.7 s
21	116.9 d	21′	147.4 s	115.8 d	115.8 d	115.8 d
22	20.5 q	22′	20.3 q	20.8 q	20.6 q	20.6 q
23	15.7 q	23′	15.5 q	15.7 q	15.6 q	15.9 q
24	14.4 q	24′	14.4 q	73.5 t	14.2 q	15.2 q
25	28.8 q	25′	28.8 q	23.2 q	28.8 q	24.1 q
COOH	184.3 s		184.3 s	176.6 s	182.5 s	
CHO						205.9 s

All compounds were analyzed for their insecticidal, antibacterial, and fungicidal activities, as well as in the brine shrimp lethality assay. Insecticidal activity was studied by incorporating each compound into an artificial diet at an arbitrarily chosen concentration (530 ppm) and offering the spiked diet to neonate larvae of the vigorous pest insect *S. littoralis* in a chronic feeding experiment. After 6 days of exposure, larval survival and larval weight were monitored and compared to controls. Only compound **3** was active, causing 90% inhibition of larval growth at a concentration of 530 ppm. From the dose-response curve obtained, the EC_{50} for growth inhibition was calculated by probit analysis as 69 [±0.48 (S.E.)] ppm.

All isolated compounds were also tested for their antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, and *Salmonella typhii*. Compound **2** exhibited a slight activity against *M. luteus*, and compound **3** was antibacterial to *S. typhii*, causing inhibition zones of 7 and 9 mm in diameter, respectively, at concentrations of 100 μg per disk. No inhibition, however, was observed for *B. subtilis*, *S. aureus*, or *E. coli*.

The isolated compounds were also tested against the phytopathogenic fungus *Cladosporium cucumerinum*. Only compound **3** was found to be active at concentra-

Table 2. ^{1}H NMR Data of Compounds **1**−**4** in $CDCl_3$

H no.	1			2	3	4
1α	1.78 m	1A	1.78 m	1.88 m	1.78 m	
1β	0.90 m	1B	0.88 m	1.49 m	0.90 m	1.78 m
2α	1.85 m	2′A	1.77 m	1.68 m	1.92 qt 13.9, 3.5	0.90 m
2β	1.44 m	2′B	1.44 m		1.48 dm 14.2	1.64 m
3α	2.15 br t 13.3	3′A	2.15 br t 13.3	2.19 m	2.16 br d 13.3	1.48 dm 14.5
3β	1.01 m	3′B	1.01 m	1.14 m	1.03 td 13.4, 4.2	2.12 br d 14.6
5	1.10 d 13.7	5′	1.00 d 13.7	1.31 m	1.09 dm 13.6	1.08 m
6α	1.80 m	6′A	1.92 m	1.79 m	1.98 m	1.19 dd 12.1, 2.8
6β	1.62 m	6′B	1.65 m	1.28 m	1.81 m	1.88 m
7α	1.73 br q 13.5	7′A	1.47 m	1.82 m	1.79 m	
7β	1.02 m	7′B	0.71 br t ca. 12	1.08 m	0.97 td 12.4, 3.7	1.78 m
9	0.97 d 12.8	9′	0.87 d 12.4	1.20 m	0.95 d 13.2	1.08 m
11α	1.73 m	11′A	1.69 m	1.75 m	1.75 dm 13.5	1.00 m
11β	1.33 m	11′B	1.29 m	1.36 m	1.35 dd 13.7, 3.1	1.72 m
12α	2.01 br t 13.1	12′A	1.98 m	2.09 br d 2.9	2.02 m	1.35 dt 12.5, 3.3
12β	1.63 m	12′B	1.58 m	1.63 m	1.63 m	2.03 dt 12.3, 3.2
14	1.64 m	14′	1.41 dd 13.1, 5.0	1.65 m	1.61 t 9.0	1.64 m
15	2.62 bm	15′A	2.30 dd 17.0, 5.0	2.59 m	2.56 d 9.1	1.64 t 9.0
		15′B	2.18 dd 17.5, 13.3			2.57 d 9.0
18	6.59 d 9.0			6.62 d 9.0	6.61 d 9.1	6.62 d 9.6
19	6.41 dd 9.0, 2.6	19′	6.79 d 8.9	6.57 dd 9.0, 2.6	6.55 dd 9.1, 3.0	6.59 dd 9.6, 2.3
		20′	6.55 d 8.9			
21	6.81 d 2.6			6.58 br s	6.55 br s	6.56 d 2.3
22	1.14 s	22′	1.11 s	1.17 s	1.15 s	1.15 s
23	0.93 s	23′	0.81 s	1.01 s	0.90 s	0.90 s
24	0.79 s	24′	0.75 s		0.79 s	0.73 s
24A				4.78 d 12.3		
24B				4.02 d 12.4		
25	1.25 s	25′	1.19 s	1.21 s	1.24 s	1.02 s
CHO						9.80 s

tions of 0.80 and 0.10 μmol, producing inhibition zones of 15 and 10 mm in diameter, respectively, whereas the other compounds had no effect on fungal growth.

In the brine shrimp lethality assay, the strongylophorine dimer **1** was found to be the most active, with an LC_{50} of 10.5 [±0.43 S.E.] μg/mL, followed by its monomer **3** with an LC_{50} of 59.2 [±0.32 S.E.] μg/mL, then the aldehyde derivative **4** with an LC_{50} of 143.3 [±1.25 S.E.] μg/mL, while the acetate ester derivative **2** showed very weak activity, having an LC_{50} of 533.5 [±0.86 S.E.] μg/mL. The dimer **1** was found to be lethal to the brine shrimp, while the acid congener **3** was found to be both fungicidal and insecticidal. The fungicidal and insecticidal activity of **3** is definitely not due to a general toxicity but probably due to different modes of action or target requirements, which are influenced by the chemical structure of the compounds. The ring A structure of the diterpene portion appears to be responsible for the specificity of the mode of action. As observed, the aldehyde congener **4** was found to be inactive in most of the bioassays performed.

Experimental Section

General Experimental Procedures. ^{1}H NMR and ^{13}C NMR spectra (chemical shifts in ppm) were recorded at 300 K on Bruker DPX 300, ARX 400, or AVANCE DMX 600 NMR spectrometers. Mass spectra (EIMS) were measured on a Finnigan MAT 8430 mass spectrometer. Optical rotations were determined on a Perkin−Elmer 241 MC polarimeter. CD spectra were recorded on a CD6 ESA Jobin-YVIN/d Instrument S. A. using MeOH as solvent. UV spectra were recorded in MeOH using a Perkin−Elmer Lambda 2 UV/vis spectrophotometer. Percent purity of isolated compounds was determined by HPLC. For HPLC analysis, samples were injected into an HPLC system coupled to a photodiode-array detector (Gynkotek, Munchen, Germany). Routine detection was at 254 nm in aqueous MeOH. The separation column (125 × 4 mm, i.d.) was prefilled with Eurospher C-18 (Knauer, Berlin, Germany).

Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. TLC was performed on TLC plates precoated with Si gel 60 F_{254} (Merck, Darmstadt, Germany). The compounds were detected from their UV absorbance at 254 nm and by spraying the TLC plates with anisaldehyde reagent.

Animal Material. Specimens of the undescribed species of *Strongylophora* (1 kg) were collected by scuba diving at a depth of about 24.4 m in Candon, Ilocos Sur, Philippines, on 25 March 1996. The sponge has an olive color. It consists of rounded branches of 2−3 cm in diameter and has a smooth surface. The oscules are regularly spaced, 3 mm in diameter, with slightly raised rims. The ectosomal skeleton is a crust-like irregular reticulation of single megascleres or bundles of two or three, upon which large numbers of microstrongyles and microoxeas are found. The spicules include three size categories of strongyles (microstrongyles, kidney-shaped or angularly curved, 10−20 × 2−4 μm; middle-sized, curved or straight, 40−60 × 10−12 μm; large, straight or curved, 200−300 × 12−2 μm), and three size categories of oxeas (microoxeas angularly curved, toxa-like, 20−30 × 1−2 μm; middle-sized, straight, 55−65 × 2−3 μm; large, straight, 120−180 × 4−8 μm). The ectosome is carried by a subdermal reticulation of very thick tracts, 150−200 μm in diameter, forming meshes of 300−500 μm. Deeper in the choanosome, the reticulation becomes more irregular with many randomly distributed loose spicules. The samples were frozen immediately and then freeze-dried prior to transport to

University of Wurzburg, Germany. A voucher fragment is kept in 70% EtOH under the registration no. ZMA.POR. 11353 in the Zoölogisch Museum, Amsterdam.

Extraction and Isolation. The freeze-dried sample (ca. 50 g) of *Strongylophora* sp. were comminuted in a mill and extracted with Me_2CO and MeOH (300 mL × 2 for each) successively by maceration and with constant stirring. The total extract was evaporated under reduced pressure and partitioned between EtOAc (50 mL × 5) and H_2O (50 mL). The organic fraction was taken to dryness (ca. 5 g) and chromatographed over a Si gel column (mobile phase CH_2Cl_2−MeOH, 98:2), from which 18 fractions were obtained. The nonpolar fraction 6 afforded the pure compound **4** [R_f 0.85 (CH_2Cl_2−MeOH, 98:2); 158 mg, 0.316%]. The semipolar fractions 11 and 12 yielded the pure compound **2** [R_f 0.65 (CH_2Cl_2−MeOH, 98:2); 37.9 mg, 0.076%] and the strongylophorine dimer **1** [R_f 0.45 (CH_2Cl_2−MeOH, 98:2); 47.5 mg, 0.095%], respectively. The pure compound **1** was obtained by further purification of fraction 12 on a Si gel column with CH_2Cl_2−MeOH (85:15) as mobile phase. The major compound **3** [R_f 0.38 (CH_2Cl_2−MeOH, 98:2); 264.3 mg, 0.528%] was obtained from the polar fraction 13 and was purified by recrystallization from MeOH. The identity of the fractions was monitored by HPLC and UV spectra recorded online.

Methylation of Distrongylophorine. Methylation was accomplished by reacting the dimer (2 mg) with CH_2N_2, which was initially prepared by stirring ca. 50 mg of nitromethylurea in a mixture of 0.3 mL 40% KOH and 1.0 mL ether for 10 min in ice until the yellow CH_2N_2 was observed to be formed in the ether phase. Then 20 μL of CH_2N_2 in ether was pipetted out and added to a 1.0-mg sample of distrongylophorine dissolved in MeOH. The reaction mixture was continuously stirred for 24 h and later taken to dryness with N_2.

The methylated dimer was obtained as a white powder residue. It showed a molecular ion peak $[M + H]^+$ at *m/z* 851 in CIMS, which is compatible with the molecular composition of $C_{55}H_{76}O_8$, while a basis peak $[M + H]^+$ at *m/z* 441 indicates fragmentation to the constituent monomer, $[C_{28}H_{40}0_4]^+$: R_f 0.78 (CH_2Cl_2−MeOH, 98:2); percent purity 90% as determined by HPLC; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.89 (1H, br s, H-21), δ 6.78 (1H, d, J = 9.0 Hz, H-19′), δ 6.60 (1H, d, J = 9.0 Hz, H-20′), δ 6.59 (1H, dd, J = 9.0 Hz, H-18), δ 6.52 (1H, dd, J = 9.0 Hz, 2.5 Hz, H-19), δ 3.70 (3H, s, $COOCH_3$), δ 3.65 (3H, s, OCH_3), δ 3.60 (3H, s, $COOCH_3$), δ 1.25 (3H, s, Me-25), δ 1.25 (3H, s, Me-25), δ 1.18 (3H, s, Me-25′), δ 1.15 (3H, s, Me-22), δ 1.12 (3H, s, Me-22′), δ 0.88 (3H, s, Me-23), δ 0.81 (3H, s, Me-23′), δ 0.68 (3H, s, Me-24), δ 0.65 (3H, s, Me-24′); CIMS *m/z* $[M + H]^+$ 865 (2.5), 441 (100), 282 (10), 223 (9), 123 (4).

Distrongylophorine (**1**): cream-colored powder residue; percent purity 97% as determined by HPLC; ($C_{52}H_{70}O_8$); EIMS (70 eV) *m/z* $[M]^+$ 822 (100), 412 (75), 289 (78), 243 (42), 175 (24), 161 (26), 123 (42); UV λ_{max} (MeOH) 206 (ε 5000), 230 (ε 1500), 295 (ε 1200); $[\alpha]_D$ −12.90° (*c* 0.46, $CHCl_3$); CD (MeOH): Δε +500 (206 nm), Δε +460 (212 nm), Δε −145 (218 nm), Δε −150 (226 nm), Δε −50 (236 nm), Δε +25 (296 nm); HREIMS *m/z* $[M]^+$ 822.5069 (calcd for $C_{52}H_{70}O_8$, 822.5071).

Experiments with Insects. Larvae of *S. littoralis* were from a laboratory colony reared on an artificial diet under controlled conditions as described previously.[10] Feeding studies were conducted with neonate larvae (n = 20) that were kept on an artificial diet into which were incorporated various concentrations of the compounds under study. After 6 days, survival of the larvae and weight of the surviving larvae were determined and compared to controls. EC_{50} values were calculated from the dose−response curves by probit analysis.

Agar Plate Diffusion Assays. Susceptibility disks (5 mm in diameter) were impregnated with 100 μg of the isolated compound and placed on agar plates inoculated with the test bacteria: *B. subtilis* 168, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and clinical isolates of *M. luteus* and *S. typhii*. The plates were observed for zones of inhibition, after 24 h of incubation at 37 °C.

Bioautographic Detection of Fungicidal Activity. Spores of *C. cucurmerinum* were cultivated on carrot-nutrient agar and were inoculated into a liquid yeast culture medium (YCM) as previously described.[11,12] Si gel TLC plates were spotted with the isolated compounds at concentrations of 0.80 and 0.10 μmol, and then the plates were sprayed with the suspension of spores of *C. cucurmerinum* in liquid YCM. The fungitoxic compound **3** was observed to produce a clear white spot of inhibition in a dark layer of the mycelia covering the TLC plate after the inoculated plates were incubated for 2 days at 25 °C.

Brine Shrimp Lethality Test.[13,14] Eggs of *Artemia salina* (Dohse, Aquaristik GmbH, Bonn, Germany) were hatched in a small tank filled with artificial seawater that was prepared with a commercial salt mixture (Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany) and distilled H_2O. After 48 h, 20 phototropic nauplii were transferred into each sample vial using a pipet and artificial seawater was added to make 5 mL. The percent mortality at each dosage level, including the control, was determined. LC_{50} values were calculated from the dose−response curve by probit analysis.

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