MICROBIAL CONVERSION OF DAUNORUBICIN INTO *N*-ACETYL-13(*S*)-DIHYDRODAUNOMYCIN AND BISANHYDRO-13-DIHYDRODAUNOMYCINONE

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By using a strain of *Streptomyces willmorii*, daunorubicin (daunomycin) was stereoselectively converted into *N*-acetyl-13(*S*)-dihydrodaunomycin and bisanhydro-13-dihydrodaunomycinone. The absolute stereochemistry of the new chiral center in *N*-acetyl-13(*S*)-dihydrodaunomycin was established by means of nuclear Overhauser effect measured in the 9,13-*O*-isopropylidene derivative.

In connection with our search for new biosynthetic anthracyclines with potentially high biological activity, we have undertaken a systematic study of the microbial conversion of daunorubicin (**1**) by means of microorganisms selected from a broad taxonomic range. Previous works[1,2] indicated that conversion of **1** in microbial and mammalian cells consists predominantly in the reduction of the C-13-oxo function into the corresponding carbinyl group; reductive cleavage of the carbon-oxygen linkage at C-7[3], 4-*O*-demethylation[4] as well as *N*-acetylation of the amino sugar moiety[5] have also been reported as further steps of the bioconversion.

We have found that microbial conversion of **1** by a strain of *Streptomyces willmorii* leads to multiple modification of the daunorubicin structure giving *N*-acetyl-13(*S*)-dihydrodaunomycin (**2**) *via* enantioselective reduction of the oxo group and simultaneous *N*-acetylation and bisanhydro-13-dihydrodaunomycinone (dihydro-η-daunomycinone) (**3**) *via* reduction, deglycosylation and dehydration of **1** (Fig. 1).

This paper describes our results on the bioconversion of **1** by a strain of *S. willmorii*; the isolation and identification of the conversion products, and determination of the absolute configuration of the new chiral center in **2**.

Results and Discussion

Bioconversion experiments were performed by using whole cells of *S. willmorii* in the fermentation process. The strain was grown according to the usual two stage fermentation procedure. In stage I, the culture was incubated for 24 hours on a rotary shaker at 29°C and then used as inoculum (10%) for the stage II culture. Daunorubicin was added to the 24-hour old stage II culture and incubation was continued as above. The progress of microbial conversion was monitored by TLC on silica gel ($CHCl_3$ - MeOH, 10: 1). After 48 hours, **1** disappeared and the conversion products were isolated from the whole culture broth by extraction with $CHCl_3$ - MeOH (1: 1). Column chromato-

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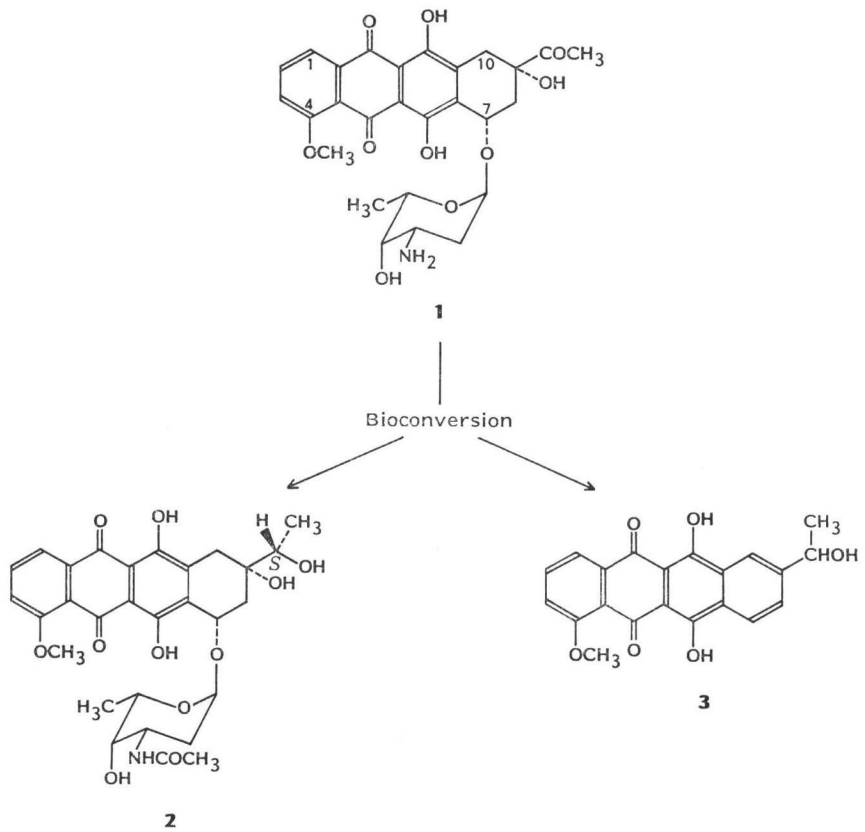
Fig. 1. Bioconversion of daunorubicin (**1**) into **2** and **3**.

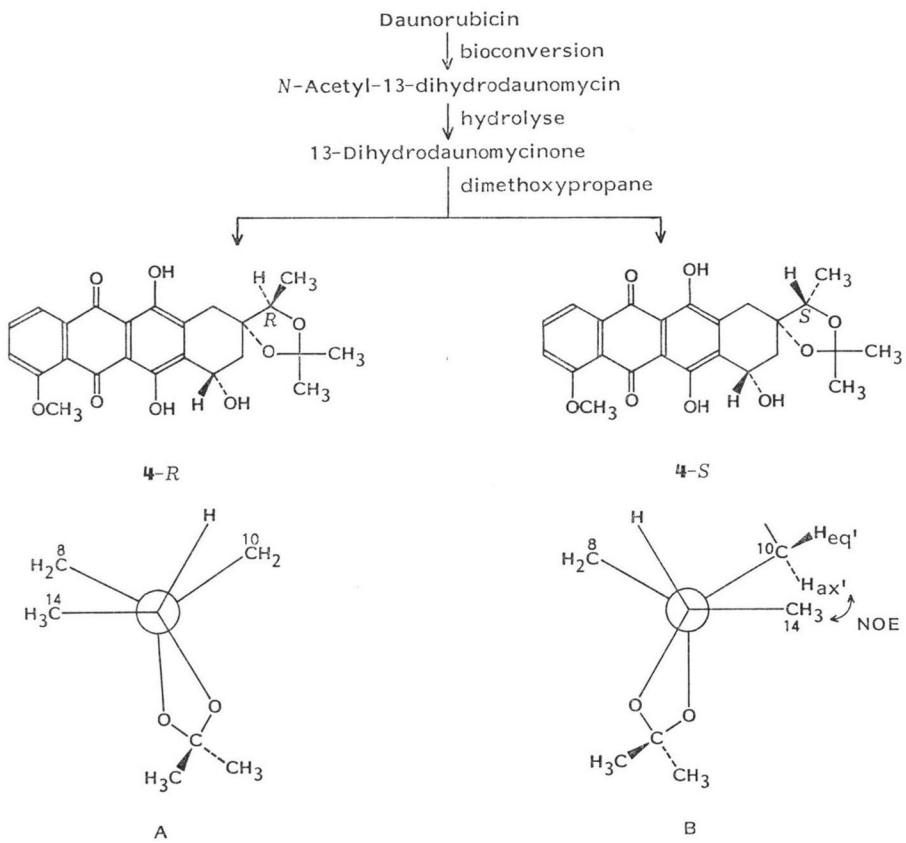


graphy on silica gel with $CHCl_3$ - MeOH (10: 1) as eluent gave two products in ratio 1: 1 which, by means of conventional spectral methods (UV/VIS, IR, MS, ^1H and ^{13}C NMR), were shown to be *N*-acetyl-13(*S*)-dihydrodaunomycin and bisanhydro-13-dihydrodaunomycinone (see Experimental). Besides these two conversion products daunomycinone was recovered.

To the best of our knowledge, reduction of **1** combined with deglycosidation and dehydration yielding **3** has not yet been observed either in microbial or mammalian cells. Since the conditions of fermentation used in our work were identical or very similar to those reported by other laboratories on microbial conversion of **1**, deglycosidation and dehydration of **1** must proceed *via* enzymic reaction triggered by *S. willmorii*, rather than through chemical processes. Supporting this contention is the complete lack of chemical conversion of **2** into **3** when the former is left to stand overnight in a phosphate buffer of pH 8, the final pH value of the culture broth of *S. willmorii*. It may be added that the formation of 11-deoxy **3** and other fully aromatic anthracyclinones in a biosynthetic reaction (by a mutant strain of *Streptomyces coeruleorubidus*) has recently been reported by JIZBA *et al.*[6].

Despite numerous studies on microbial reduction of the C-13-oxo function of **1**, very little is known on the chirality of the new asymmetry center of the product molecules. In rubeomycin B[7] and 13-dihydro-4-demethoxydaunomycin[8], the two cases where the absolute stereochemistry of the reduced side chain has been established, the reduced carbon atom (C-13) proved to be of *R* configuration.

Scheme 1. Conversion of daunorubicin for NOE measurements.
Determination of the absolute configuration at C-13 position.

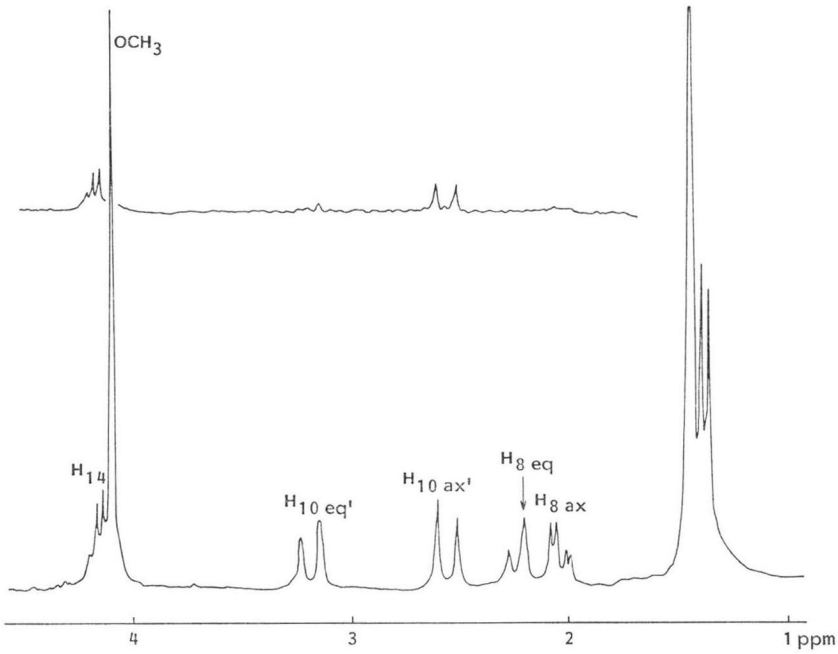


In the present work, the absolute stereochemistry of C-13 carbon atom in **2** was inferred from nuclear Overhauser effect (NOE) experiments. To this end, first the rotational uncertainty of the side chain groups was removed by transforming the vicinal diol function of **2** into the corresponding 9,13-*O*-isopropylidene derivative, **4**, as shown in the Scheme 1. Formation of the alternative 7,9-*O*-isopropylidene structure in the last step could safely be excluded on the basis of the ^{1}H NMR spectrum of the product molecule, **4**, which showed the 7-H proton resonance (the X part of an ABX system) to have an additional splitting due to vicinal coupling (J=9.3) to an H/D exchangeable proton appearing at δ_H 4.22 ppm (C-7-OH).

The NOE experiments were performed by using the difference spectrum technique[6] and the results are shown in the partial 200 MHz ^{1}H NMR spectrum of **4** displayed in Fig. 2. It can be seen that irradiation of the CH_3-14 doublet at δ_H 1.36 ppm induces a positive NOE effect (approx 8%) at the 10-H_{ax}, proton resonance. This finding is readily accounted for by considering the partial structure B which represents the *S* absolute configuration of the new chiral center at C-13. (It may be noted that spatial proximity to the CH_3-14 group induces a positive NOE effect of nearly the same size at the H-14 proton resonance in this experiment.)

This stereochemical conclusion is fully supported by recent ^{1}H NMR data published on synthetic diastereomers of 9,13-*O*-isopropylidene-4-demethoxy-13-dihydrodaunomycinone[8] which indicate

Fig. 2. Partial 200 MHz ^1H NMR spectrum of **4**, 0.05 M in $CDCl_3$ solution.
Below is the control spectrum; above is the NOE difference spectrum (×4) arising from irradiation of the CH_3-14 doublet at δ_H 1.36 ppm.



significant variations in the differential shieldings for C-8 and C-10 methylene protons depending on the configuration of the C-13 carbon atom. The chemical shift values measured for **4** (see Experimental) are in excellent agreement with the data reported for the stereoisomer with an *S*-configured C-13 carbon atom.

Although **2** is antibiotically inactive, the value of this finding is that this product may serve as a nontoxic competitive inhibitor for the active efflux mechanism of **1** in resistant tumor cells, like much in the same way as reported for *N*-acetyldaunomycin[10,11]. Moreover, **2** can also compete with **1** or with doxorubicin for binding sites on structural proteins and thereby protect myocardial cells against cardiotoxic effect of the parent drugs[12].

Experimental

Melting points were determined on a Kofler hot stage and are uncorrected. UV/VIS spectra were measured with a Zeiss spectrophotometer Specord UV/VIS. IR spectra were taken in KBr pellets using a Perkin Elmer 325 spectrometer. EI mass spectra were determined on a Jeol JMS D-100 mass spectrometer. NMR spectra were recorded on a Bruker WP-200/SY spectrometer using tetramethylsilane as an internal standard.

Analytical TLC was performed on silica gel precoated aluminum sheets (Silufol, Kavalier, CSSR). Column chromatography was carried with Silica gel 60, 0.04~0.06 mm (Merck, F.R.G.) and Sephadex LH-20 (Pharmacia, Sweden).

Daunorubicin (Leukaemomycin) was obtained as detailed in the previous paper[13].

S. willmorii IMET 40343 was obtained from the strain collection of Central Institute of Microbiology and Experimental Therapy, Jena.

Cultivation and Bioconversion

The following medium M 79 was used for all cultures: glucose 1%, peptone 1%, Casamino Acids 0.1%, yeast extract 0.2%, NaCl 1%, distilled water, pH 7.2.

The strain *S. willmorii* was grown at 29°C for 7 days on a M 79 agar slant. The inoculum for shake cultures was prepared by washing the surface of a slant with 5 ml M 79 nutrient broth and gently scrapping the surface with a sterile pipet. The resulting spore suspension was added to 50 ml of medium M 79 in a 500-ml flask and incubated at 29°C on a rotary shaker (180 rpm) for 3 days (stage I culture). 5 ml of this inoculum were added to a 500-ml flask containing 50 ml of medium M 79 and cultivated as above for 24 hours (stage II culture).

For a large-scale conversion test, a total of 40 mg of **1**, dissolved in 20 ml filter-sterilized EtOH was distributed evenly among 500-ml flasks containing 50 ml of a 24-hour stage II culture. 0.5 ml EtOH containing 1 mg of **1** was added to each 500-ml flask (final concentration of **1**: 20 μg/ml). After 48 additional hours of incubation, more than 95% of **1** was utilized.

Isolation of *N*-Acetyl-13(*S*)-dihydrodaunomycin (**2**) and Bisanhydro-13-dihydrodaunomycinone (**3**)

The conversion products were extracted from the whole broth by vigorous mixing with $CHCl_3$ - MeOH, 1: 1 at pH 8.2. The organic extract was concd and the residue (340 mg), dissolved in small amounts $CHCl_3$ - MeOH, 10: 1, was chromatographed on a silica gel column (150 g) eluted with $CHCl_3$ - MeOH, 10: 1. The eluate was monitored by TLC (Silufol sheets, $CHCl_3$ - MeOH, 10: 1), conversion product **3** (Rf 0.48) eluted first from the column followed by **2** (Rf 0.20). Fractions containing the separated products were evaporated to a small volume and thereafter rechromatographed on silica gel as mentioned above to give 18 mg of **3** and 14.5 mg of **2**. Each compound was finally purified by Sephadex LH-20 chromatography (20 g) with MeOH as eluent for analytical purposes.

Characterization of these compounds is as follows:

Compound **2**: MP 129~130°C; $[\alpha]_D^{20}$ −83° (*c* 0.05, $CHCl_3$). The MS did not display the molecular ion, however, in all other respects it was identical to that of 13-dihydrodaunomycinone published by KARNETOVÁ *et al.*[14]. The presence of *N*-acetyldaunosamine followed from the cleavage of a fragment with *m/z* 172.0974 (calcd for $C_8H_{14}NO_3$: 172.0974). IR ν_{max} (KBr) cm^{-1} 3800 (OH), 1645 (amide), 1612 (quinone C=O), 1575 (aromatic C=C), the band at 1700 cm^{-1} of the C-13-oxo group was absent; UV/VIS λ_{max} nm ($E_{1cm}^{1\%}$) $CHCl_3$ 291 (250), 480 (sh, 290), 508 (300), 546 (sh, 180); DMF 480 (sh, 235), 508 (237), 544 (133); piperidine 568 (109), 604 (100); H_2SO_4 303 (616), 546 (375), 592 (558); ^1H NMR (200 MHz, $CDCl_3$ - DMSO-d_6, 20: 1) δ_H 14.00 (1H, s, C-10-OH), 13.35 (1H, s, C-6-OH), 8.03 (1H, dd, $J_{1,2}$=7.7 Hz, $J_{1,3}$=1.0 Hz, H-1), 7.76 (1H, dd, $J_{2,3}$=7.7 Hz, H-2), 7.38 (1H, dd, H-3), 6.50 (1H, d, C-3′-NH), 5.49 (1H, dd, $J_{1',2'ax}$=3.5 Hz, $J_{1',2'eq}$=1.5 Hz, H-1′$_{eq}$), 5.24 (1H, dd, $J_{7,8eq}$=1.8 Hz, $J_{7,8ax}$= 4.5 Hz, H-7), 4.22 (1H, qd, $J_{5'ax,6'}$=7.0 Hz, H-5′$_{ax}$), 4.12 (1H, dddd, $J_{3'ax,4'eq}$=2.5 Hz, $J_{3'ax,NH}$=8.4 Hz, H-3′$_{ax}$), 4.08 (3H, s, C4-OCH$_3$), 3.66 (1H, $J_{13,14}$=6.3 Hz, H-13), 3.58 (1H, dd, $J_{4'eq,5'ax}$=0.9 Hz, H-4′$_{eq}$), 3.15 (1H, dd, $J_{10eq',10ax'}$=19 Hz, H-10$_{eq}$′), 2.62 (1H, d, H-10$_{ax}$′), 2.53 (1H, ddd, $J_{8eq,8ax}$= 14 Hz, $J_{8eq,10eq'}$=1.3 Hz, H-8$_{eq}$), 1.92 (3H, s, NCOCH$_3$), 1.83 (1H, ddd, $J_{2'ax,2'eq}$=14 Hz, $J_{2'ax,3'ax}$= 11 Hz, H-2′$_{ax}$), 1.82 (1H, dd, H-8$_{ax}$), 1.76 (1H, ddd, $J_{2'eq,3'ax}$=5.5 Hz, H-2′$_{eq}$), 1.31 (3H, d, H-14), 1.29 (3H, d, CH$_3$-6′); ^{13}C NMR (50 MHz, $CDCl_3$ - DMSO-d_6, 20: 1), δ_C 186.65[a] (C-5), 186.44[a] (C-12), 169.48 (CH$_3$*C*ON), 160.92 (C-4), 156.70 (C-11), 156.12 (C-6), 136.15 (C-2), 135.54[b] (C-10a), 135.38[b] (C-6a), 119.53[c] (C-4a), 119.45[c] (C-3), 118.48 (C-1), 110.97[d] (C-11a), 110.85[d] (C-5a), 100.57 (C-1′), 72.93 (C-9), 71.71 (C-13), 70.16 (C-7), 68.99 (C-5′), 67.38 (C-4′), 56.60 (OCH$_3$), 45.60 (C-3′), 34.00 (C-8), 32.77 (C-10), 29.57 (C-2′), 23.17 (*C*H$_3$CON), 16.88 (CH$_3$-5′ and C-14). (Assignments marked with a, b, c and d may be interchanged.)

Compound **3**: MP 140~143°C; MS *m/z* 364.0958 (M, calcd for $C_{21}H_{16}O_6$: 364.0947), 346 (M−H_2O), 331 (M−H_2O−CH_3), 321 (M−C_2H_3O), 306 (M−C_2H_3O−CH_3), 303 (M−H_2O−C_2H_3O); IR ν_{max} (KBr) cm^{-1} 3500 (OH), 1590 (overlapping bands of quinone C=O and aromatic C=C), the band at 1700 cm^{-1} of the C-13- oxo group was absent. UV/VIS λ_{max} nm ($E_{1cm}^{1\%}$) $CHCl_3$ 481 (230), 508 (376), 546 (353); DMF 475 (sh, 225), 506 (350), 539 (325); piperidine 558 (138), 599 (133); H_2SO_4 300 (1,093), 546 (366), 592 (660); ^1H NMR (200 MHz, $CDCl_3$ - DMSO-d_6, 2: 1) δ_H 16.07 (1H, s, C-10-OH), 15.21 (1H, s, C-6-OH), 8.44 (1H, s, H-10), 8.43 (1H, d, $J_{7,8}$=7.6 Hz, H-7), 8.11 (1H, dd, $J_{1,2}$=7.6 Hz,

$J_{1,3}$=1.5 Hz, H-1), 7.78 (1H, dd, $J_{8,9}$=1.5 Hz, H-8), 7.75 (1H, dd, $J_{2,3}$=7.6, H-2), 7.35 (1H, dd, H-3), 5.06 (1H, q, $J_{13,14}$=6.5 Hz, H-13), 4.06 (3H, s, C-4-OCH_3), 1.52 (3H, d, H-14). (Mutual couplings are given only once).

Mild Acid Hydrolysis of *N*-Acetyl-13(*S*)-dihydrodaunomycin (**2**)

10 mg of **2** were dissolved in 1 ml of THF and 0.5 ml of 3.0 N HCl, and the solution was stirred at 45°C for 2 hours. After stirring, the solution was neutralized with saturated $NaHCO_3$ solution, then extracted with $CHCl_3$ (2×5 ml). The extract was washed with saturated brine, dried and concd to dryness to give 13-dihydrodaunomycinone (6.6 mg) in 94% yield.

MS *m/z* 400.1160 (M, calcd for $C_{21}H_{20}O_8$: 400.1158).

13(*S*)-Dihydro-9,13-*O*-isopropylidenedaunomycinone (**4**)

6.6 mg of 13-dihydrodaunomycinone were dissolved in 3 ml of 2,2-dimethoxypropane. After addition of catalytic amounts of *p*-toluenesulfonic acid (0.3 mg), the mixture was stirred at ambient temperature for 3 hours, then poured in 20 ml of H_2O and extracted with $CHCl_3$ (2×10 ml). The extract was washed with H_2O, dried and concd to afford a crude product which was purified by column chromatography on silica gel (12.5 g) using $CHCl_3$ - MeOH, 10: 1 as eluent to give 5.1 mg of the isopropylidene derivative. Rechromatography on Sephadex LH-20 (20 g, $CHCl_3$ - MeOH, 10: 1) afforded 4.5 mg of **4** in high purity for NMR measurements.

^{1}H NMR (200 MHz, $CDCl_3$) δ_H 14.00 (1H, s, C-10-OH), 13.37 (1H, s, C-6-OH), 8.02 (1H, dd, $J_{1,2}$=7.6 Hz, $J_{1,3}$=1.0 Hz, H-1), 7.76 (1H, dd, $J_{2,3}$=8.0 Hz, H-2), 7.40 (1H, dd, H-3), 5.17 (1H, m, $J_{7,OH}$=9.3 Hz, H-7), 4.22 (1H, d, C-7-OH), 4.14 (1H, q, $J_{13,14}$=6.4 Hz, H-13), 4.08 (3H, s, C-4-OCH_3), 3.18 (1H, dd, $J_{10eq',10ax'}$=19 Hz, $J_{10eq',8eq}$=1.9 Hz, H-$10_{eq'}$), 2.54 (1H, d, H-$10_{ax'}$), 2.19 (1H, ddd, $J_{8eq,8ax}$=14 Hz, $J_{8eq,7}$=2.4 Hz, H-8_{eq}), 2.02 (1H, dd, $J_{8ax,7}$=5.1 Hz, H-8_{ax}), 1.43 and 1.42 (dimethyl ketal), 1.36 (3H, d, CH_3-14). (Mutual couplings are given only once).

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