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# Microbial Transformations of Natural Antitumor Agents. 3. Conversion of Thalicarpine to (+)-Hernandalinol by *Streptomyces punipalus*

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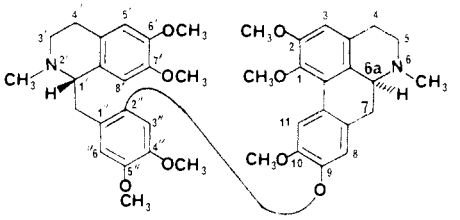
Microbial transformation studies were conducted with the antitumor alkaloid thalicarpine. *Streptomyces punipalus* (NRRL 3529) converted thalicarpine to (+)-hernandalinol, the structure of which was determined spectroscopically and by synthesis from the known alkaloid hernandaline. This unusual biotransformation reaction most likely occurs by oxidative cleavage of the isoquinoline ring from thalicarpine through the intermediate hernandaline, which then undergoes further reduction to hernandalinol.

Microbial transformation systems have been successfully utilized in the study of natural antitumor drug biotransformations.[1-4] We have been developing microbial transformations as a general means for (a) providing quantities of potentially active metabolites of complex antitumor compounds from nature; (b) preparing difficult to synthesize metabolites of such compounds which may be used as analytical standards to facilitate mammalian drug metabolism studies; and (c) determining potentially important pathways of bioactivation, bioinactivation, and cytotoxicity which may also occur in mammalian species. Previous reports from our laboratory concerned the microbial hydroxylation of acronycine,[1] an antitumor acridone alkaloid, and microbial N-dealkylation of *d*-tetrandrine,[2] a bis(benzyltetrahydroisoquinoline) alkaloid. This report describes the results of microbial transformation studies with the alkaloid thalicarpine (1a).



1a

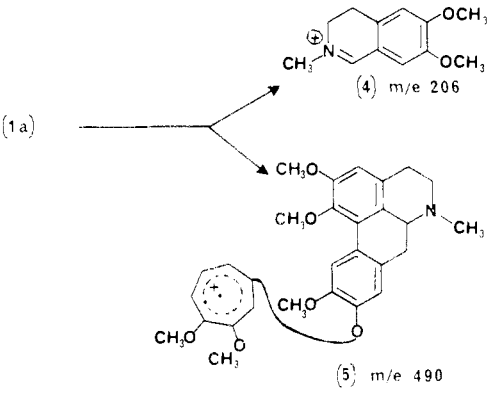
1b 6a,7-dehydro-

Thalicarpine, the first reported example of a benzyltetrahydroisoquinoline–aporphine dimeric alkaloid,[5] was originally isolated from *Thalictrum*[5,6] and *Hernandia*[7] species, and the correct structure for the alkaloid was shown to be **1a** by total synthesis.[8] Thalicarpine has demonstrated significant antitumor activity against the Walker 256 carcinosarcoma[9] and cytotoxicity against monolayer cultures of KB cells.[10]

## Results and Discussion

Of 22 cultures used in initial screening work those consistently yielding metabolites with thalicarpine were

Scheme I. Mass Spectral Fragments of Thalicarpine (1a)



(4) m/e 206

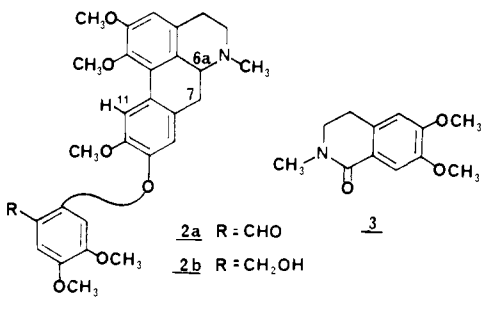
(5) m/e 490

*Fusarium solani* (ATCC 12823), *Streptomyces punipalus* (NRRL 3529), *Streptomyces griseus* (UI 1158), *Cunninghamella blakesleeana* (ATCC 8688a), and *Mucor mucedo* (UI 4605).

Since yields of metabolites were low, medium variation studies were performed in order to optimize the production of metabolites by these organisms. Each culture was grown in each of the media described under the Experimental section. Both the yields and numbers of metabolites were found to be highly dependent on the medium employed. *S. punipalus* gave relatively good yields of three metabolites on medium B containing glucose, maltose, or sucrose as the primary carbon and energy source. Since results with this organism were most reproducible, it was selected for a preparative scale fermentation.

A fermentation of *S. punipalus* containing 3.0 g of thalicarpine yielded 0.27 g of the major metabolite as well as two minor compounds after chromatography. Most of the thalicarpine added to the medium was recovered unchanged. The structure of the major metabolite was determined principally on the basis of NMR and mass spectral measurements and was confirmed by chemical synthesis.

The mass spectrum of thalicarpine is characterized by the presence of ions at $m/e$ 696, 490, and 206 (Scheme I). These are consistent with the molecular ion, the molecular ion minus an isoquinolinium ion (**5**), and the isoquinolinium ion (**4**). The NMR spectrum of thalicarpine possesses overlapping singlets for the 7-methoxyl groups and two well-separated *N*-methyl signals.[11] The fragment ions of greatest $m/e$ for the metabolite occurred at 507 and 506, with no signal evident at $m/e$ 206. In the NMR spectrum of the metabolite, only one *N*-methyl signal is evident and signals for only 5-methoxyl groups could be seen. In addition, a two-proton signal at $\delta$ 4.60 and a one-proton exchangeable signal at $\delta$ 3.39 indicated the presence of a benzyl alcohol moiety on the metabolite. The IR spectrum also indicated the presence of the alcohol through the absorption band at 3431 $cm^{-1}$. The UV spectrum of the metabolite gave $\lambda_{max}$ values at 283 and 303 nm, consistent with the UV spectra obtained with aporphines and thalicarpine itself.[11,12] The elemental composition of the metabolite was $C_{29}H_{33}NO_7$ by high-resolution mass spectrometry and by analysis. Chemical-ionization mass spectral data indicated an abundant $MH^+$ minus $H_2O$ peak at $m/e$ 490 indicative of an aliphatic OH. On the basis of these data, we postulated a hernandaline-like structure (**2b**) for the metabolite. Since it could easily be derived from hernandaline (**2a**) we labeled the new metabolite hernandalinol.




To confirm the structure of the metabolite as **2b**, it was synthesized by reduction of the known alkaloid hernandaline (**2a**), which was obtained by $KMnO_4$ oxidation of thalicarpine.[13] The isolated hernandaline was optically pure based on values reported in the literature. Hernandalinol (**2b**) was obtained both by $NaBH_4$ reduction of hernandaline and by using a modification of Kupchan's method for reducing dehydrothalicarpine.[14] The synthetic compound produced by $NaBH_4$ reduction of **2a** was identical in all respects with the metabolite **2b**.

The synthetic metabolite produced by reduction of **2a** with $PtO_2$[14] was identical with the metabolite with the exception of its optical rotation value, +19.4°, which was nearly 16° lower than that obtained with the metabolite and with **2b** obtained by $NaBH_4$ reduction of **2a**. It has been reported that racemization occurs when alkaloids are reduced under the conditions used in the $PtO_2$ reduction of hernandaline to synthetic hernandalinol (**2b**),[15] and this most likely accounts for the lowered optical rotation of synthetic **2b**. Racemic **2b** was previously obtained by catalytic reduction of 6a,7-dehydrohernandaline,[16] but it was not fully characterized.

Initial attempts to accomplish metal hydride reduction of hernandaline to hernandalinol met with limited success. Cava et al.[17] obtained an amorphous product by treating **2a** with $NaBH_4$. Although spectral data indicated the loss of the carbonyl function, the reduction product was not fully characterized. With $NaBH_4$ in methanol, we also obtained an amorphous product which was different than **2b**. The amorphous material was chromatographically identical with the product formed when hernandalinol was refluxed with 10% Pd/C in acetonitrile. Since Pd/C has been successfully used to dehydrogenate aporphines under similar conditions,[12,18] it is reasonable to suggest that the amorphous hydride reduction product is dehydrohernandalinol. Later efforts demonstrated that when great excesses of $NaBH_4$ were used to reduce **2a** to **2b** in methanol, the reaction went to completion rapidly (30 min) and yielded only the desired product.

To determine the optical purity of the metabolite, the optically active shift reagent Eu(facam)$_3$ was used according to a modification of the procedure described by Shaath and Soine.[19] To establish working conditions with the shift reagent, (*S*)-(+)-glaucine and racemic glaucine[12] were used with substrate–reagent molar ratios of 7.5:1. Under these conditions, the C-11 proton signal of (*S*)-(+)-glaucine experiences a shift of 0.255 ppm further downfield than the C-11 proton signal of the (*R*)-(−)-glaucine isomer. With the metabolite **2b**, substrate–reagent ratios of 7.5:1 and 3.75:1 failed to cause observable splitting of the C-11 proton signal. Thus, within experimental error, it appears that hernandalinol (**2b**) produced by microbial degradation of thalicarpine is optically pure. Conclusive experiments to demonstrate the optical purity of the metabolite will require more abundant quantities of **2b**.

The mechanism by which *S. punipalus* converts thalicarpine to hernandalinol is unknown. However, we have demonstrated that the organism can efficiently reduce hernandaline to **2b**. On this basis, it is likely that the metabolite is formed in two steps: first, by oxidative cleavage of the isoquinoline ring from thalicarpine to yield (**2a**), and second, by reduction of **2a** to hernandalinol (**2b**). Whether the initial oxidation reaction is a mono- or dioxygenase mediated enzyme transformation is uncertain at this time. It is interesting that hernandaline (**2a**) is found in *Hernandia ovigera*[17] along with the isoquinolone **3**, presumably the two products of oxidation of thalicarpine in this plant, and that similar compounds may be obtained by $KMnO_4$ or photochemical oxidation[20] of alkaloids.

It has been proposed that the active antitumor portion of thalicarpine resides in the aporphine moiety connected to a part of the benzylisoquinoline moiety.[10] This conclusion was derived partly because hernandaline (**2a**) demonstrates cytotoxicity in the KB cell test system.[10] Further studies relating to the antitumor properties of hernandalinol and its derivatives and to the characterization of other thalicarpine metabolites are in progress.

## Experimental Section

Melting points were determined in open-ended capillaries in a Thomas-Hoover capillary melting point apparatus and are corrected. IR spectra were obtained using a Beckman IR-5A spectrophotometer through KBr disk and were standardized against polystyrene. Low-resolution mass spectra were determined with a Finnigan Model 3200 spectrometer. High-resolution mass spectral data were obtained through the services of Battelle Columbus Laboratories, Columbus, Ohio. UV spectra were taken on a Pye-Unicam SP1800 instrument, and optical rotations were determined with a Perkin-Elmer 141 instrument. Measurements of pH were done with an Ionalyzer Model 801 digital pH meter (Orion Research). The elemental analysis was performed by Midwest Microlab, Ltd.

Thin-layer chromatography (TLC) was performed on 0.25-mm thick layers of silica gel $GF_{254}$ (Merck) prepared on glass plates with a Quickfit Industries spreader. Preparative TLC plates (1 mm thick) were prepared and used in the final purification of hernandalinol (**2b**). Preparative alumina plates (0.5 mm) were purchased from Analtech, Inc. Compounds were detected on developed chromatograms by fluorescence quenching under 254- or 365-nm UV irradiation and were later visualized by spraying

with one of the following reagents: Dragendorff; ferric chloride; diazotized sulfanilic acid; dinitrophenylhydrazine (DNPH).[21] Solvent systems used in developing TLC plates were A, $C_6H_6$–MeOH–58% $NH_4OH$ (80:30:0.1); B, $C_6H_6$–MeOH–58% $NH_4OH$ (80:30:0.3); C, $Me_2CO$–100% EtOH (50:1); D, cyclohexane–$CHCl_3$–diethylamine (12:4:1). Column chromatography was performed with silica gel (Baker 3405) which was activated in an oven at 120 °C for 30 min prior to use. The silica gel was then slurried in developing solvent and packed into a glass column wet. Fractions were collected using a Fractomette 200 instrument.

**NMR Spectral Methods.** NMR spectra were obtained with a Varian Associates Model T-60 spectrometer with an $Me_4Si$ lock, using $Me_4Si$ as an internal standard and $CDCl_3$ as solvent. Experiments with Eu(facam)$_3$ (Eu-Opt, Ventron) were conducted by modification of the procedure described by Shaath and Soine.[19] Samples of 40 mg of (*S*)-(+)-glaucine, racemic glaucine, and the microbial metabolite **2b** were dissolved in 0.5 mL of $CDCl_3$, and NMR spectra were determined. The shift reagent was then used in substrate–reagent molar ratios of 7.5:1 and 3.75:1 and was added to NMR tubes as a solid. Under these conditions, resolution of pertinent peaks in the NMR spectra of the alkaloids remained satisfactory.

**Fermentation Methods.** All cultures described are maintained in the culture collection of the University of Iowa, College of Pharmacy. Those bearing the designation UI are unique to our culture collection; NRRL cultures were obtained from the Northern Regional Research Laboratories of the Agriculture Research Service, Peoria, Ill.; ATCC cultures were obtained from the American Type Culture Collection, Rockville, Md.

The fermentation procedure employed was identical with the two-stage incubation procedure previously described.[1] Several media were employed in the course of this study.

**Medium A**: soybean meal–glucose medium consisting of glucose (20 g), soybean meal (5 g), yeast extract (5 g), NaCl (5 g), and $K_2HPO_4$ (5 g), and 1000 mL of distilled water, adjusted to pH 7.0 with 6 N HCl.

**Medium B**: glucose (10 g), corn steep liquor (6 g), $KH_2PO_4$ (3 g), $CaCO_3$ (3.5 g), soybean oil (2.2 mL), yeast extract (2.5 g), distilled water to 1000 mL, pH ranging between 7.0 and 7.3. This medium was used for both stage I and stage II cultures. Variations in this medium were achieved by replacing glucose with 10 g of maltose, lactose, or sucrose as the main carbon source in stage II cultures.[22]

**Medium C**: glucose (30 g), lactose (10 g), corn steep liquor (30 g), $(NH_4)_2SO_4$ (2 g), $KH_2PO_4$ (0.5 g), $CaO_3$ (5 g), and distilled water to 1000 mL.

**Medium D**: lactose (75 g), Pharmamedia (40 g), $CaCO_3$ (10 g), $CaSO_4$ (5 g), $Na_2SO_4$ (4 g), distilled water to make 1000 mL. On occasion, yeast extract was substituted for Pharmamedia.

All media were sterilized in an autoclave at 15 lb/in.$^2$ for 15 min at 121 °C.

**Fermentation Sampling Procedure.** A standard sampling procedure was employed in all fermentations. Samples (4 mL) of culture medium were withdrawn from substrate containing cultures and controls at various time intervals. These were adjusted to pH 8.5 with saturated $NaHCO_3$ solution, extracted with 1 mL of ethyl acetate, and spotted (30–40 μL) onto TLC plates. TLC plates were developed with one or more of the previously mentioned solvent systems and sprayed with one or more visualizing reagents.

**Thalicarpine.** Thalicarpine (NSC No. 68075) was obtained from the National Cancer Institute and it gave the following physical properties: mp 160–162 °C (reported[5] mp 160–161 °C); NMR[7,11] ($CDCl_3$) δ 2.40 and 2.45 (s, 3 H each, $NCH_3$ groups), 2.53–3.33 (m, complex signals representing benzylic and other nonaromatic protons, 14 H), 3.55 (s, 3 H, 7′-$OCH_3$), 3.67 (s, 3 H, 1-$OCH_3$), 3.70–4.00 (several overlapping singlets representing the remaining methoxyl group signals, 15 H), 6.15 (s, 1 H, 8′-ArH), 6.38–6.67 (m, 5 H, ArH), 8.12 (s, 1 H, 11 H); mass spectrum *m/e* (% rel abundance) 696 (2.5), 490 (28), 472 (23), 457 (35), 444 (33), 428 (29), 308 (25), 206 (100); $[\alpha]^{26}_D$ MeOH (*c* 0.80) +126.1° [reported[5] +133° (*c* 0.83, MeOH)].

**Screening of Microorganisms.** A total of 22 cultures were selected for preliminary screening to determine their abilities to metabolize thalicarpine. Their selection was based on previous studies in our laboratories which identified cultures capable of metabolizing aporphine and benzylisoquinoline alkaloids. Experiments were conducted in 25 mL of medium held in cotton-plugged 125-mL Erlenmeyer flasks. Thalicarpine (10 mg in 0.1 mL of DMF) was added to each flask 24 h after stage II cultures were initiated. Samples were taken at 24, 48, 72, 144, 240, and 312 h and were examined with solvent system A on silica gel $GF_{254}$ TLC plates. Metabolites of thalicarpine were evident in culture extracts taken between 72 and 144 h. This experiment was repeated using suitable controls, and the influence of each of the media described on the yield and number of metabolites was determined. Of all cultures examined, *F. solani* (ATCC 12823), *S. punipalus* (NRRL 3529), *S. griseus* (UI 1158), *C. blakesleeana* (ATCC 8688a), and *M. mucedo* (UI 4605) consistently yielded significant levels of metabolites. *S. punipalus* gave the highest levels of three metabolites on medium B containing glucose, maltose, or sucrose in stage II media. This organism was selected for larger scale production of the metabolite.

**Microbial Transformation of Thalicarpine (1a) into (+)-Hernandalinol with *S. punipalus* (NRRL 3529).** Stage II cultures of *S. punipalus* were grown in 6.0 L of medium B (glucose) held in 30 cotton-plugged 1000-mL Erlenmeyer flasks. A total of 3.0 g of thalicarpine (**1a**) was dissolved in 30 mL of DMF with brief warming in a hot water bath and was distributed evenly among the cultures flasks. Metabolite formation was followed by TLC (solvent system B). Developed TLC plates were viewed under UV, sprayed with Dragendorff's reagent, and warmed with a heat gun. The major metabolite was evident in the incubation after 48 h. After 10 days, the fermentation was harvested, pooled (pH 7.2), and exhaustively extracted with diethyl ether (6 × 0.8 vol) using large separatory funnels. The ether extracts were dried over anhydrous $Na_2SO_4$ and evaporated to yield 4.7 g of oil. The oil was adsorbed onto 20 g of activated silica gel (Baker) (giving an orange pink mass), applied to the top of a silica gel column (500 g, 68 × 5.0 cm), and eluted with solvent system C at a flow rate of 4 mL/min, while 16-mL fractions were collected. Fractions 210–310 yielded 270 mg of the metabolite **2b** as a glass which could be induced to crystallize in methanol: mp 95–100 °C; NMR δ 2.39, 2.52–3.17 (complex signals representing benzylic and other nonaromatic protons, 8 H), 2.45 (s, 3 H, $NCH_3$), 3.39 (s, 1 H, OH), 3.65 (s, 3 H, $OCH_3$), 3.73 (s, 3 H, $OCH_3$), 3.85 (overlapping singlets, 9 H, $-OCH_3$), 4.60 (s, 2 H, $ArCH_2O-$), 6.47 (s, 1 H, ArH), 6.53 (m, 2 H, ArH), 6.88 (s, 1 H, ArH), and 8.04 (s, 1 H, ArH C-11); high-resolution mass spectrum *m/e* (% rel abundance) 508 (30), 507 (100), 506 (63), 492 (24), 476 (12); elemental composition, by high-resolution mass spectrometry, calculated for $C_{29}H_{33}NO_7$ 507.2257, found 507.2211; by analysis, calcd C 68.62, H 6.55, N 2.76, found C 68.25, H 6.48, N 2.59; IR 3431 cm$^{-1}$ (OH); UV max (EtOH) 283 nm (log ε 4.241), 303 (4.162); optical rotation $[\alpha]^{26}_D$ +35.0° (*c* 1.03, EtOH).

**Synthesis of (+)-Hernandaline by $KMnO_4$ Oxidation of Thalicarpine (1a).**[13] A 3.0-g sample of thalicarpine was suspended in 80 mL of acetone to which was added 0.75 g of $KMnO_4$ in 80 mL of acetone. The reaction mixture was stirred for 10 min and filtered, and the solids were washed with MeOH (2 × 10 mL). The combined dark amber filtrates were evaporated to a light tan oil, adsorbed onto 17.5 g of silica gel, applied to the top of a silica gel column (350 g, 46 × 5 cm), and eluted with solvent system C at a flow rate of 7 mL/min, while 15-mL fractions were collected. TLC analysis of the eluent fractions indicated that fractions 81–135 contained hernandaline (**2a**) by comparison with an authetic sample of **2a**. Evaporation of the solvent and addition of cold 100% EtOH yielded a crystalline solid after standing in the cold for 18 h. The crystals were collected by filtration, washed with cold 1:1 EtOH–$H_2O$, and dried under vacuum to give 350 mg of (+)-hernandaline: mp 170–170.5 °C (reported[17] mp 170–171.5 °C). The product exhibited spectral properties identical with those reported in the literature:[17] optical rotation $[\alpha]^{26}_D$ +33.3° (*c* 0.82, $CHCl_3$) (reported[17] +33.8 and +35.6°); the product was cochromatographically identical with authentic (±)-hernandaline on silica gel $GF_{254}$ TLC using solvent systems B and C.

**Synthesis of (+)-Hernandalinol (2b) by Reduction of Hernandaline (2a). Method 1.** Hernandaline (**2a**) (60 mg, 0.12 mM) was dissolved in 50 mL of MeOH, and 200 mg of $NaBH_4$ (5.3 mM) was added gradually over the course of 5 min while the reaction mixture was cooled in an ice bath. After stirring for 30

min, the reaction was diluted with 200 mL of water and extracted with five 15-mL portions of $CHCl_3$. After drying over $Na_2SO_4$, the chloroform was removed under vacuum to leave a residue of 55 mg of pure synthetic **2b** (92%) which was crystallized from MeOH: mp 95–98 °C; mmp (with the microbial metabolite **2b**), 94–98 °C. The synthetic product was chromatographically identical with the microbial metabolite on three separate TLC systems: silica gel $GF_{254}$, solvent system B ($R_f$ 0.5), solvent system C ($R_f$ 0.35); alumina $GF_{254}$ solvent system D ($R_f$ 0.2). The synthetic hernandalinol was spectrally identical with the microbial metabolite in all cases; optical rotation $[\alpha]^{26}_D$ +36.2° (*c* 1.01, EtOH).

**Method 2.** Using a modification of Kupchan's procedure,[14] a 100-mg quantity of $PtO_2$ was suspended in 30 mL of glacial acetic acid and prereduced for 1 h under 25 psig of hydrogen. A solution of 120 mg of (+)-hernandaline (**2a**) in 5 mL of glacial acetic acid was added and reduced under 30 psig of hydrogen for 24 h. The catalyst was removed by filtration and washed twice with MeOH, and the combined filtrates were evaporated to an oily residue. The residue was suspended in 40 mL of 2 N HCl, allowed to stand for 20 min, alkalinized with solid $NaHCO_3$, and extracted with $CHCl_3$ (6 × 15 mL). The $CHCl_3$ extract was dried over anhydrous $Na_2SO_4$ and concentrated to an oil. The oil was then uniformly adsorbed onto 200 mg of silica gel, applied to the top of a silica gel column (6 g, 29 × 1 cm), and eluted with solvent system C at a flow rate of 4 mL/min, while 15-mL fractions were collected. Fractions 8–11 gave 20 mg (17%) of synthetic hernandalinol (**2b**). The noncrystalline product was chromatographically and spectrally identical with the microbial metabolite and with the product obtained by $NaBH_4$ reduction of hernandaline by method 1; optical rotation $[\alpha]^{26}_D$ +19.4° (*c* 1.138, MeOH).

**Microbiological Reduction of Hernandaline (2a) to Hernandalinol (2b).** To determine whether *S. punipalus* was capable of reducing **2a** to **2b** the following small-scale fermentation experiment was performed. Stage I and stage II *S. punipalus* cultures were grown in medium B containing glucose. The stage II fermentation consisted of duplicate flasks to which 10 mg of hernandaline (**2a**) was added and control flasks containing only medium plus **2a**. Substrate-containing cultures were incubated with shaking, and samples were withdrawn as usual. Within 48 h, all of the hernandaline substrate had been consumed and converted to a single metabolite, hernandalinol (**2b**). The fermentations and controls were evaluated by TLC using silica gel $GF_{254}$ and solvent system C [$R_f$ values, hernandaline (**2a**), 0.4; hernandalinol (**2b**) 0.35], and alumina $GF_{254}$, solvent system D [$R_f$ values (**2a**), 0.7; and (**4b**), 0.2]. Differential visualization was accomplished by use of DNPH and $FeCl_3$ spray reagents.

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