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# Inhibitory Effects of *Serenoa repens* on the Kinetic of Pig Prostatic Microsomal 5α-Reductase Activity

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**The pathogenesis of benign prostatic hyperplasia is linked to the accumulation of dihydrotestosterone (DHT), the active form of testosterone (T), in prostatic tissue. We have defined characteristics of 5α-reductase enzyme which catalyzes the conversion of T into DHT in prostatic microsomes of growing pigs. Peaks for the 5α-reductase activity were found at pH 5.5 and 8.0, which indicates the presence of both type 1 and type 2 isozymes. Kinetic parameters of porcine 5α-reductase in the presence of *Serenoa repens* extracts revealed uncompetitive, noncompetitive, and mixed types of inhibitions. Our results show the inhibitory action of *S. repens* on prostate porcine microsomal 5α-reductase activity.**

**Key Words:** Steroid 5α-reductase enzyme; *Serenoa repens*; benign prostatic hyperplasia (BPH); porcine; pig; swine; testosterone (T); dihydrotestosterone (DHT); enzyme kinetics; inhibitors; prostate, NADPH-dependent reductase.

## Introduction

The NADPH-dependent steroid 5α-reductase enzyme (EC 1.3.99.5) reduces testosterone (T) into dihydrotestosterone (DHT), a more potent androgen that regulates prostatic cell growth and proliferation *(1)*. The 5α-reductase activity is related to the etiology of two important diseases: benign prostatic hyperplasia (BPH) and prostatic carcinoma *(2–4)*. Two different steroid 5α-reductase isoforms, type 1 and type 2, have been identified in human. Type 1 isozyme is localized mainly in liver and skin, whereas type 2 isozyme seems to predominate in the prostatic tissues. These isozymes display maximum activity at different pHs, which are neutral-basic for type 1 (pH between 7.0 and 8.0) and acidic for type 2 (pH between 5.0 and 6.0) *(5–9)*.

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Overproduction and accumulation of DHT seems to be a crucial element responsible for different pathologies, such as benign prostatic hyperplasia, acne vulgaris, androgenic alopecia, male pattern baldness, and hirsutism *(10–12)*. Treatments have been developed to control prostatic growth rate, which are directed toward the development of steroid 5α-reductase inhibitors *(13)*. These inhibitors include plant and lipid extracts as well as steroidal and nonsteroidal compounds. Recently, a lipido-sterol extract of the fruit of the dwarf American palm *Serenoa repens* demonstrated an inhibitory effect on the rat 5α-reductase enzyme activity *(14,15)*. The beneficial effects of *S. repens* extract on the treatment of BPH seem to be related to its anti-inflammatory potential *(16,17)*, as well as its effects on the androgen, estrogen *(15)*, and prolactin receptors *(18)*.

In order to examine the effect of the *S. repens* lipido-sterol extract on the 5α-reductase activity, pig prostatic microsomal preparations were used. Kinetic parameters of the porcine prostatic 5α-reductase enzyme type 2 and the ability of the lipido-sterol extract to inhibit the enzyme activity are reported. In this study, we report that *S. repens* extract has inhibitory effects on porcine prostatic steroid 5α-reductase activity. In fact, the lipido- sterol preparations show three types of inhibition: mixed, uncompetitive, and noncompetitive, related to its mixed fatty acids content.

## Results

### *pH Dependency of Kinetic Parameters*

Pig prostatic microsomal 5a-reductase assays at different pHs revealed two different activity peaks: one at a pH optimum of 5.5 and the other much lower at pH optimum of 8.0 (Fig. 1). The activity of pig prostatic steroid 5α-reductase was assessed using increasing concentration of T at pH 5.5 and also at pH 7.0, which are the reported pH optima for the type 1 and type 2 isozymes, respectively (Fig. 2). At pH 7.0, the $K_m$ value of 5α-reductase calculated from these data was 213.7 n*M* and the $V_{max}$ was 11.5 pmol DHT/mg protein/min. At pH 5.5, the $K_m$ value of 5α-reductase calculated from the kinetic data was 331.4 n*M* and the $V_{max}$ was 319 pmol DHT/mg protein/min. Therefore, the pH dependency results indicated that the microsomal

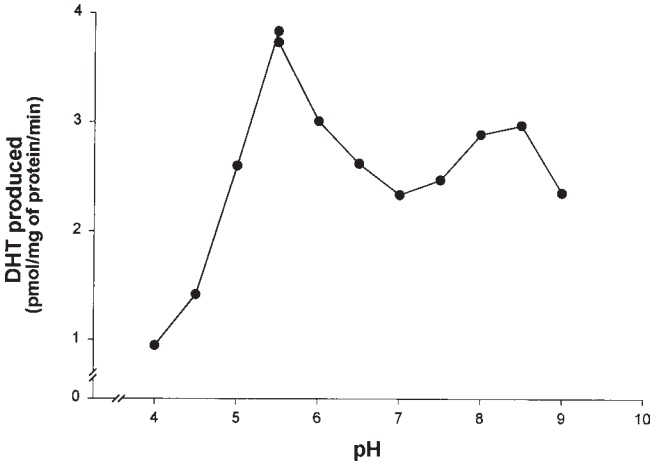


**Fig. 1.** pH dependency of porcine microsomal steroid 5α-reductase. Assays were carried out at the indicated pH in the presence of 250 μg of porcine prostatic microsomes, 0.4 m*M* NADPH, and 50 n*M* [4-$^{14}$C]-testosterone for 30 min at 37°C.

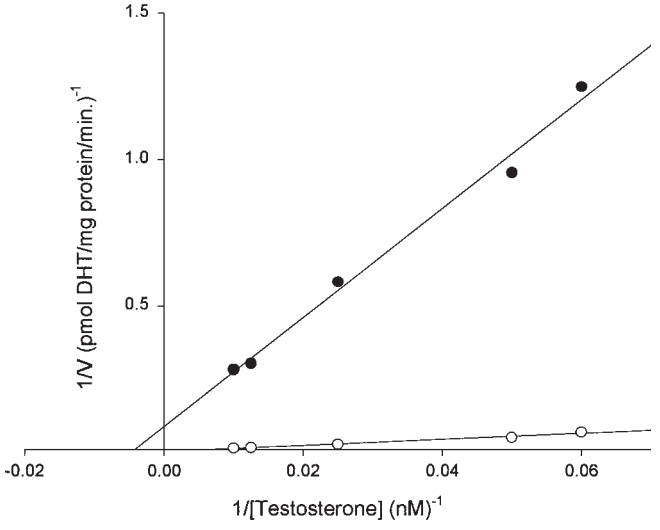


**Fig. 2.** Kinetics parameters evaluation at pH 5.5 and 7.0. Double-reciprocal plot showing linear Michaelis-Menten kinetics of steroid 5α-reductase in porcine prostatic microsomal preparations using [4-$^{14}$C]-testosterone concentration of 15, 20, 40, 80, and 100 n*M*. Enzyme activity was evaluated at pH 5.5 (closed circles) and at pH 7.0 (opened circles).

porcine steroid 5α-reductase has similar affinity for T at both pHs, but a higher $V_{max}$ (27-fold) at pH 5.5 than at pH 7.0 was noticed. These results also suggest that the acidic 5α-reductase isoform type 2 is active in this pig microsomal preparation. All subsequent kinetics were made at pH 5.5.

### *Steroid 5α-Reductase Activity Assay*

On incubation of the pig prostatic microsomal preparation with [4-$^{14}$C]-testosterone at pH 5.5, the formation of [4-$^{14}$C]-dihydrotestosterone was linear for a period of 90 min (data not shown). A 30-min incubation time was therefore selected in all subsequent experiments. At pH 5.5,

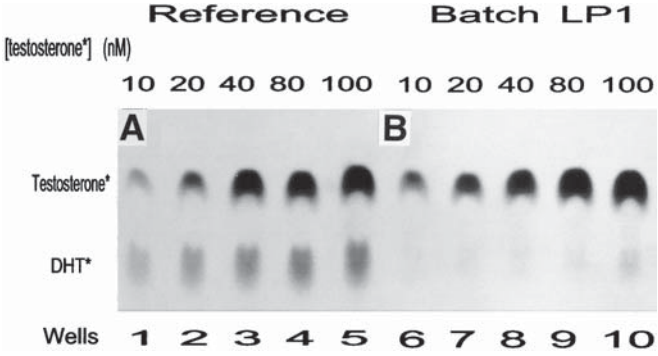


**Fig. 3.** TLC representation of 5α-reductase activity. Enzymatic conversion of [4-$^{14}$C]-testosterone to [4-$^{14}$C]-dihydrotestosterone using a porcine prostatic microsomal preparation was measured without inhibitor (Fig. 4A) and with *S. repens* extract #LPI (Fig. 4B). Cold T, DHT, and 5α-androstane-3α-diol were used as internal standards.

$V_{max}$ and $K_m$ values of 5α-reductase for T were reproductible for the different microsomal preparations used in this study. Reaction products were separated by thin-layer chromatography (TLC) and revealed on films (Fig. 3). As expected, autoradiograms revealed the production of [4-$^{14}$C]-dihydrotestosterone, which is associated with 5α-reductase activity. No significant amounts of 5α-androstane-3α-17β-diols were detected under our experimental conditions. The 5α-androstane-3α-17β-diols were separated by more than 3 cm from T. We have only used 3α-diol as internal standard, because only minor amounts of 3β-diol, but mainly 3α-diol, are formed from the substrate DHT in the prostate *(19)*. Figure 3 also shows a decreased transformation of [4-$^{14}$C]-testosterone to its 5α metabolite DHT in the presence of *S. repens* extract batch #Lp1.

### *Inhibition Assays*

The inhibitory potential of different *S. repens* extracts was tested. Pig prostatic microsomes were incubated in the presence of *S. repens* extract to examine its effect on the ability of steroid 5α-reductase to convert [4-$^{14}$C]-testosterone into [4-$^{14}$C] DHT. Inhibition is associated with a decrease in 5α-reductase enzyme activity, which results in a lower production of [4-$^{14}$C]-DHT. Five different *S. repens* extraction mixtures were assayed and were compared with a control without inhibitor (Figs. 4 and 5). Three different types of inhibition were obtained. Lineweaver-Burk plots of extracts #95K04M and #95L01M showed an uncompetitive type of inhibition with a lower $K_m$ of 40.8 and 21.0 n*M*, and a lower $V_{max}$ of 3.69 and 1.83 pmol DHT/mg protein/min, respectively (Fig. 4). *S. repens* extract #96C01A showed a noncompetitive type of inhibition with lower $V_{max}$ of 3.68 pmol DHT/mg protein/min and a similar affinity for T ($K_m$ of 50.3 n*M*) (Fig. 5). Finally, extracts #Lp1 and #95K01M presented a mixed type of inhibition, which is characterized by a lower $V_{max}$ of 4.31 and 6.52 pmol DHT/mg protein/min, respectively, and higher affinity constant ($K_m$) of 58.0 and 78.7 n*M*, respectively (Fig. 5).

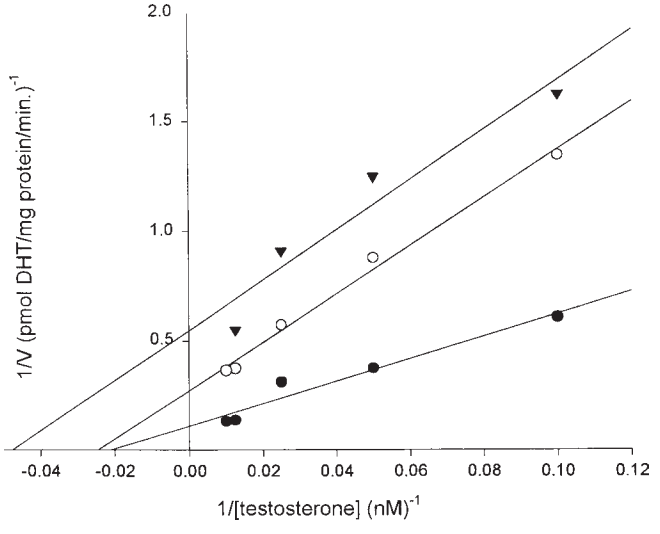


**Fig. 4.** Inhibitory effect of *S. repens* on porcine steroid 5α-reductase enzyme. Double-reciprocal plot showing the effect of two preparations, #95K04M (open circles) and #95L01M (closed triangles) vs control (closed circles) on 5α-reductase activity in porcine prostate microsomal preparations.

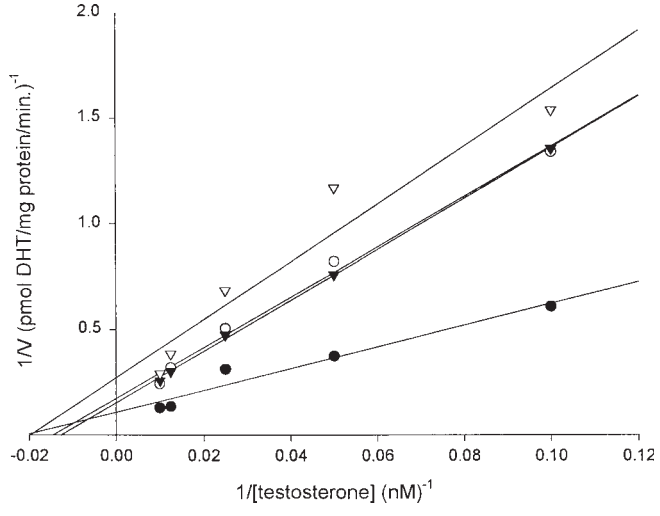


**Fig. 5.** Inhibitory effect of *S. repens* on porcine steroid 5α-reductase enzyme. Double-reciprocal plot showing the effect of three *S. repens* preparations, #96C01A (opened triangles), #Lp1 (open circles), and #95K01M (closed triangles) vs control (closed circles), on 5α-reductase activity in porcine prostate microsomal preparations.

## Discussion

In this study, we have characterized the porcine prostatic steroid 5α-reductase enzyme. We have also demonstrated the inhibitory action of *S. repens* on the porcine 5α-reductase activity. Our results revealed that optimal 5α-reductase activity occurs at pH 5.5, indicating that type 2 isozyme is expressed in porcine prostatic microsomal preparations. These results are in agreement with those of Martin et al. *(20)*, which indicated that the 5α-reductase isozyme type 2 is also expressed in human BPH tissues. Moreover, in porcine prostatic microsomal preparations, another 5α-reductase activity peak was found at pH 8.0, suggesting that the type 1 isozyme is also expressed in that tissue. It was earlier reported that the relative contributions of the two isozymes to the 5α-reductase activities ($V_{max}$) can be estimated by calculating the ratio of pH 7.0/5.0 activity *(4)*; tissues harboring a ratio of < 1, express predominantly the 5α-reductase type 2. Our kinetic results of 5α-reductase activity revealed a pH 7.0/5.0 ratio of 0.036, which is in agreement with a higher expression of type 2 over type 1 isozyme in the microsomal pig prostatic tissues. Although the alkaline and acidic pH optima are used as a diagnostic of the presence of type 1 and type 2 5α-reductase respectively *(21)*, exceptions to this rule have been reported. For example, equivalent activity at acidic and neutral pH was observed in CHO cells transfected with 5α-reductase type 2 isozyme; in this case CHO cells were treated with digitonin to permealize the plasma membrane *(22)*. The reported discrepancies in the 5α-reductase activity profile for type 2 isozyme in relation to pH variation could result from the physical lysis method used to disrupt cells, the isozyme being active at neutral pH inside the cell and at pH 5.5 on cell disruption. This pH shift may result from a conformational change of the isozyme *(21)*. The activity of porcine prostatic 5α-reductase was examined at pH 5.5 and 7.0. Lineweaver-Burk plots of these data showed that the higher activity observed at pH 5.5 is owing to a higher activity ($V_{max}$) of 5α-reductase for testosterone at pH 5.5 (319 pmol DHT/mg protein/min) than at pH 7.0 (11.5 pmol DHT/mg protein/min). Our decision to perform kinetic assay at pH 5.5 was thus based on the presence of a low 5α-reductase activity for testosterone at pH 7.0 and also on the literature reported for 5α-reductase type 2 activities at acidic pH *(23)*.

Overproduction of DHT in the prostatic tissues often results in benign prostate hypertrophy, which affects a majority of elderly men *(24)*. Moreover, if not treated, the accumulation of DHT can increase the risk of prostate cancer *(25,26)*. Because of its premier role in the conversion of T into DHT, the enzyme 5α-reductase has become the main target for BPH inhibitor development. Over the last few years, inhibitors of 5α-reductase isozymes have been developed. Among these inhibitors, finasteride has been approved for the clinical treatment of benign prostatic hyperplasia *(27)*. However, adverse effects occurred from a total blockade of the enzyme by finasteride *(28)*. For example, a significant increase in testosterone was observed *(29)*. Furthermore, serious side effects were reported, including impotence, decreased libido, ejaculatory disorders, such as low ejaculate volume, and mild headaches *(27,30,31)*. Finally, administration of finasteride is usually followed by a decrease in serum prostate-specific antigen (PSA) levels.

This treatment increases the risk of prostate cancer by masking its development. Clinical studies on humans clearly demonstrated the absence of such risk when using

*S. repens (32)*. The inhibitor extracts used in this study were made from berries of *S. repens*, which is a small palm tree native of the Atlantic coast of the North America. Magistral® is a concentrated liquid herbal extract obtained from *S. repens* berries. Results presented herein also demonstrated that the lipidosterolic extract of *S. repens* can decrease the 5α-reductase activity in porcine prostatic microsomes. More specifically, we report three different modes of inhibition for different *S. repens* extractions. Uncompetitive, noncompetitive, and mixed-type inhibitions (Figs. 4 and 5) were identified. These suggest that different active inhibitors are present in the *S. repens* lipidosterol fractions. The inhibitory action of *S. repens* can be tentatively associated with the modulatory action of its lipid component on the 5α-reductase environment. Indeed, steroid 5α-reductase is a membrane-bound enzyme whose native conformation is influenced by its lipidic vicinity whose solubilization requires lipids *(33)*. It has been reported previously that specific phospholipids (phosphatidyl-serine, phosphatidyl-choline, phosphatidyl-ethanolamine, and phosphatidyl-inositol) and unsaturated fatty acids, such as linoleic, palmitoleic, arachidonic, and oleic acids, can exert a range of inhibitory activities on 5α-reductase *(34,35)*. The *S. repens* extract is composed of 85% fatty acids, mainly, lauric, myristic, palmitic, oleic, and stearic acids. It was previously reported that any modification of the 5α-reductase lipid environment can lead to conformational changes of the enzyme, which may result in a reduced access to T and to NADPH *(36)*. The different inhibition patterns observed in this study could be explained by the diversity of fatty acids in the *S. repens* extracts, these fatty acids having different modulatory effects on the membrane lipid bilayer, which in turn could also lead to a reduced access of the enzyme to its substrate or cofactor.

In addition to its direct inhibitory effect on 5α-reductase, *S. repens* also has known antiandrogenic and antiestrogenic activities *(14,15)*. Moreover, it was demonstrated that separate fractions of the *Saw palmetto* extract are responsible for these effects *(37)*. Taken together, these results suggest that the overall antiprostatic activity of the lipidosterol extract may result from a more complex multiloci mechanism, which may be owing to the fatty acid diversity of *S. repens*.

## Materials and Methods

### *Chemicals*

[4-$^{14}$C]testosterone (57.3 mCi/mmol) was purchased from New England Nuclear (Mississauga, Ontario, Canada). Cold T and DHT were obtained from Steraloids (Wilson, NH). Chemicals used for tissue extraction, activity buffers, and TLC running solvent were obtained from Sigma-Aldrich (Mississauga, Ontario, Canada). TLC plates (K6F Silica gel 60 Å) were purchased from Whatman (Clifton, NJ). Protein concentration was measured by the Bradford method commercialized as the Bio-Rad protein assay kit (Bio-Rad, Mississauga, Ontario, Canada) using bovine serum albumin as standard. *S. repens* liquid extracts (Magistral) were supplied by "Les produits naturels Magistral inc." (Blainville, Quebec, Canada).

### *Animals*

Three noncastrated male Duroc pigs were purchased from a local pig breeder. Pigs had free access to commercial food and water. These pigs were slaughtered at 150 kg of body wt by stunning and exsanguination, according to the recommend code of practice for the care and handling of farm animals *(38)*. Pig dorsal and ventral prostates were removed by dissection and rapidly frozen in liquid nitrogen. These tissues were used as a source of steroid 5α-reductase enzyme. The 5α-reductase activity was stable for at least 1 yr at –80°C.

### *Porcine Microsomal Preparation*

Microsomes containing porcine steroid 5α-reductase enzymes were prepared according to Sargent and Habib *(33)* with minor modifications. Two grams of frozen prostatic tissue were homogenized with a Kinematica homogenizer (Rexdale, Ontario, Canada), at 6, for 30 s. The pellets obtained after ultracentrifugation were pooled, resuspended in 1 mL of extraction buffer (100 m*M* Tris, 100 m*M* sodium citrate, 100 m*M* potassium chloride, 1 m*M* EDTA, 15 m*M* β-mercaptoethanol, 20% glycerol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL Nα-*p*-tosyl-L-arginine methyl ester [TAME], 5 m*M* NADPH, pH 7.4) and homogenized for total protein concentration determination. The resulting microsomal preparation was then kept at –20°C until used. Frozen microsomal preparations were stable for at least 1 mo.

### *5α-Reductase Activity Assay*

Determination of kinetic parameters, $K_m$ and $V_{max}$, for 5α-reductase at pH 5.5 and 37°C was based on protocol published by Faller et al. *(39)*. Enzyme activity was measured using [4-$^{14}$C]-testosterone (15–100 n*M*) into the following activity buffer: 100 m*M* Tris-citrate, 100 m*M* potassium chloride, 1 m*M* EDTA, 5 m*M* dithiothreitol (DDT), and 20% glycerol. The final concentration of ethanol in the reaction medium was 1% (v/v), 0.4 m*M* NADPH was added to the reaction mixture, which was preincubated at 37°C for 5 min. Microsomes (83 μg/mL, 3 mL total volume) were added to initiate the reaction. Five hundred-microliter aliquots of the reaction products were transferred to fresh tubes after 10, 20, 30, 40, 50, and 60 min of incubation. Reactions were stopped by the addition of 2 mL ethyl ether, and steroids were extracted by high vortexing agitations. The organic phase was evaporated to dryness in a water bath at 40°C. Stock solutions of T, DHT, and 5α-androstane-3α-diol were prepared in 95% ethanol. Steroids were resuspended in 50 μL of 95% ethanol containing cold T, DHT, and 5α-androstane-3α-diol (2 mg/mL), used as internal standards. Reaction products and internal standards were separated by TLC (silica gel) using the follow-

ing running solvent: methylene chloride/ethyl ether (9:1). Cold steroids were visualized by exposing the TLC plates to UV light (366 nm) for 10 min. The conversion of [4-$^{14}$C]testosterone to [4-$^{14}$C]- dihydrotestosterone was measured by densitometry (Densitometer GS-670 model from Bio-Rad). Kinetic constants, $K_m$ and $V_{max}$, were estimated using Molecular analyst software (Bio-Rad) and SigmaPlot for windows (Jandel Corporation, Mississauga, Ontario, Canada).

### *pH Dependency of Kinetic Parameters*

pH optimum of microsomal 5α-reductase was evaluated as follows. Activity assays was performed at 37°C for 30 min with 250 μg of porcine prostatic microsomal preparation, 0.4 m*M* NADPH, and 50 n*M* [4-$^{14}$C]-testosterone in activity buffer of a range of pH strengths from 4.0 to 9.0. Microsomal 5α-reductase activity was also estimated at pH 5.5 and 7.0. All kinetics were done at 37°C in activity buffer containing 0.4 m*M* NADPH and an increasing amount of [4-$^{14}$C]-testosterone. Enzymatic reactions were initiated by the addition of microsomes (250 μg) in a 3-mL total mixture volume. Five hundred-microliters aliquots were taken after 10, 20, 30, 40, 50, and 60 min, and the amount of [4-$^{14}$C]-dihydrotestosterone was quantified as described previously.

### *Inhibition Assays*

Inhibition assays were performed according to procedures described in the 5α-Reductase Activity Assay section. Inhibition assays were done at pH 5.5. Inhibitors were added (16 μg/mL) prior to the addition of microsomal preparations, and reactions were stopped after a 30-min reaction by adding 2 mL of ethyl ether.

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