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BIOTRANSFORMATION OF LAPACHOL: AN ALTERNATIVE TO CYCLIZATION OF NAPHTHOQUINONES BIOATIVES

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Abstract: This work describes the cyclization of Lapachol induced by *Penicillium sp*. Intended for transformation to be performed correctly, the substrate with the fungus were placed in a rotating incubator for a period of seven days and for the purification of the product used to chromatographic techniques. From the biotransformation reaction was possible to obtain two products biotransformed, called V1 and V2. Spectrometric analysis suggests that V2 was obtained from a Lapachol cyclization reaction between the hydroxyl group and the prenyl group forming a third ring which may be a linear, saturated or not, pyran or furan. The spectrometric analysis of compound V1 suggests alteration of the naphthoquinone ring, because when compared to the spectra IR and ^{1}H NMR of Lapachol note that the signals corresponding to the quinone skeleton are absent. Despite the difficulties encountered in the processes of isolation, purification and elucidation of the products obtained, as well as the low yield, the technique of biotransformation is well diffused in Brazil and several researchers use it searching for more pharmacologically active compounds from natural substrates.

Keywords: Lapachol. Biotransformation. Cyclization.

BIOTRANSFORMAÇÃO DO LAPACHOL: uma alternative para ciclização de naftoquinonas bioativas

Resumo: Este trabalho descreve a ciclização de Lapachol induzida por *Penicillium sp*. Para que a transformação seja realizada corretamente, o substrato com o fungo foi colocado em incubadora rotativa por um período de sete dias e para a purificação do produto foram utilizadas técnicas cromatográficas. A partir da reação de biotransformação foi possível obter dois produtos, denominados V1 e V2. A análise espectrométrica sugere que V2 foi obtido de uma reação de ciclização do Lapachol entre o grupo hidroxil e o grupo prenila formando um terceiro anel que pode ser um pirano ou furano, linear, saturado ou não. A análise espectrométrica do composto V1 sugere alteração do anel naftoquinona, pois quando comparado aos espectros de IV e RMN^{1}H do Lapachol percebe-se que os sinais correspondentes ao esqueleto quinônico estão ausentes. Apesar das dificuldades encontradas nos processos de isolamento, purificação e elucidação dos produtos obtidos, bem como, o baixo rendimento, a técnica de biotransformação é bem difundida no Brasil e vários pesquisadores a utilizam procurando por compostos farmacologicamente mais ativos a partir de substratos naturais.

Palavras-chave: Lapachol. Biotransformação. Ciclização.

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Introduction

Quinones are cyclic conjugated dienones, and are classified according to the type of aromatic system in benzoquinones, naphthoquinones and anthraquinones. Intensified in recent years the interest in these substances, not only due to its vital importance in biochemical processes but also to highlight increasing presenting in various pharmacological studies (MURRAY et al., 2003).

Among the natural naphthoquinones stands out Lapachol which can be considered one of the main representatives of the group of the genus Tabebuia quinines (Bignoniaceae). The Lapachol in Brazil is known as Pau D'Arc, Ipê-purple, Ipê-Black, and Yellow-Ipê. Lapachol and several natural related 1,4- and 1,2-naphthoquinones are associated with numerous biological activities like antibacterial, fungicidal, antimalarial, trypanocidal, and antitumoral (DA SILVA et al., 2003; FONSECA et al., 2003; LIRA et al., 2004).

On the basis ofthe biological and structural properties, 1,2- and 1,4-naphthoquinonesare considered privileged structures in medicinal chemistry. This term describes selected structural types like polycyclic heteroatomic systems, capable of orienting varied substituent patterns in a well-defined three-dimensional spaceand binding to multiple, unrelated classes of protein receptors as high-affinity ligands (FRANCISCO et al., 2009; FERREIRA et al., 2010).

Due to the biological activities of cyclic derivatives of Lapachol, many research groups have been studying their preparations. Among the different methods for obtaining the cyclic derivatives, we can mention those that involve direct cyclization of lapachol. Among these processes that seek synthesis of new molecules have the biotransformation (SILVA JR et al., 2009).

Biotransformation by microorganisms is an important method to convert cheap and plentiful organic compounds into more useful ones, according to the ability of microorganisms specifically to produce secondary metabolites. The reactions involved in the biotransformation of organic compounds by microorganisms include oxidation, reduction, hydroxylation, esterification, methylation, isomerization, hydrolysis, and glycosylation (SHIMODA et al., 2006; ISHIHARA et al., 2003)

Natural products have been widely used as substrates for biotransformation reactions in order to obtain products that are potentially more active. Since the side effects can be minimized when a molecular modification is successful, come up the commitment by obtaining new chemical entities with pharmacological interest that meet the new demand of the pharmaceutical industry (Kebano et al., 2015).

Due to our interest in this type of reaction, we decided to study the application of biotransformation reaction to promote the cyclization of Lapachol, aimed at preparing natural cyclic derivatives potentially active.

Materials and Methods

Lapachol extraction

In a percolator was placed 20g of powdered ipe bark and extracted with 300mL of aqueous 5% Na_2CO_3 and let stand for 3 min. To the obtained solution was added slowly concentrated HCl while stirring continuously until pH=1.This acidic solution was allowed to stand for about 5 minute and then vacuum filtered. The precipitate obtained was transferred to an Erlenmeyer flask and hot ethanol was added gradually. Once all the solid was solubilized in the smallest amount of alcohol heat, the flask was closed and left undisturbed for obtaining Lapachol in crystal form. Filter the precipitate obtained, collecting in the flask weighed and labeled. Lapachol obtained was compared with a standard by TLC using as eluent CH_2Cl_2/hexane (8:2).

Microorganism

The filamentous fungus used in the procedure of biotransformation was isolated from Laboratory of Chemistry, Federal University of Mato Grosso and identified as a *Penicillium* sp according to Ribeiro (2000). The culture was cultivated on Malt Extract Agar (MEA) for 10 days at 28 °C ±2 °C.

Media

MEA medium containg malt extract, 20 g; peptone, 1 g; dextrose, 20 g; agar, 20 g; distilled water, 1 l was used for grown culture. SDB medium, composed of 10g peptone and 40 g glucose, at pH 5.6 in 1 l distilled water, was used for transformation experiment. The components of SDB as well as the maintenance media were purchased from Biobrás (Belo Horizonte, State of Minas Gerais, Brazil) and Isofar (Duque de Caxias, State of Rio de Janeiro, Brazil).

Transformation by *Penicillium sp*

The ability this fungus to transform Lapachol was examined by fermentation procedure. Incubations were carried out in cotton-plugged 500 mL Erlenmeyer flasks containing about one-fifth of their volumes of medium. The biotransformation of Lapachol by *Penicillium* sp. was carried out as follows: one loop from a slant of *Penicillium* sp was inoculated into 30 mL of SDB-broth and incubated at 30ºC for 48 hours on a rotary shaker at 150 rpm; 3 mL of culture was used as inoculum for 500 mL Erlenmeyer flasks containing 100 mL of the same medium which was incubated as described above. After 24 hours of incubation, 0.18 g of the substrate dissolved in 2 mL of dimethylsulfoxide (DMSO) was added to the culture medium. Two sets of control were included, one flask contained cells of this fungus and sterile SDB broth and another contained sterile SDB broth and Lapachol (controls of metabolic products and Lapachol stability, respectively). The incubation was continued for 7 days. This experiment was repeated four times. Samples (1 mL) of culture broth were withdrawn at various time intervals and submitted to TLC analysis to detect metabolites of reaction. The TLC plates were visualized under UV light and by iodine vapor.

Extraction and analysis of biotransformation products

After 7 days of incubation, the mycelium was removed by filtration and the culture filtrate was extracted with 3 equal volumes of ethyl acetate. The extracts were combined and the solvent being removed under reduced pressure in a rotary evaporate.

Silica gel column chromatography and TLC preparative were employed for analytical separations. Two metabolic products denominated V1 and V2 were isolated.

Infrared (IR) spectra were recorded on a Bomem spectrophotometer in KBr discs. Proton nuclear magnetic resonance spectra (^{1}H and ^{13}C NMR) were determined with a Mercury 300 spectrometer, using tetramethylsilane (TMS) as internal standard.

Results and Discussion

TLC-anallyses, eluting with hexane-ethyl acetate (1:1), indicated that the most frequent products of biotransformation of Lapachol (V1 and V2) showed a red color spot and a lower Rf (V1) and same Rf (V2) values than the starting compound. The highest yield of these compounds was produced by 7 days of incubation. It was noticed that the strain of *Penicillium* isolated in the Laboratory of Chemistry/UFMT, had an ability to survive in harsh environments and therefore can be considered a microorganism that present a potential to resist the reaction medium giving it the ability to transforming chemical compounds for use as substrates (TAKAHASHI et al., 2008). This microorganism was selected for the preparative scale transformation aiming to obtain sufficient amount of the crude product for isolation, purification and structural elucidation of the components. The methodology used for this experiment was continuous culture in SDB medium using DMSO as substrate diluent.

In such conditions the reaction, in a preparative scale, was first monitored by sampling at regular intervals and analysis by TLC. It was observed that the transformation products were produced continuously during the reaction and were partially metabolized by the cells. Lapachol was consumed progressively but not disappeared completely and it was stable under the conditions and reaction time tested.

Some of the difficulties encountered in these reactions are due to the fact that part of the substrate was adhered to mycelium and the fungus selected metabolized the Lapachol producing a variety of metabolites of low yield and difficult separation.

Therefore, in this work, the reaction was repeated four times and the crude product was submitted to sílica gel colunm chromatography and, then, purified by TLC preparative.

As can be seen, in the four biotransformation reactions above, the metabolite V1 was produced in greater quantity than V2. Probably the enzymatic pathway responsible for the metabolism of Lapachol priority is to form the metabolite V1 as the major product, or there is more than one enzyme system acting in this biotransformation. This variation occurs because the main disadvantage of systems for microbial transformation which is the unpredictability of the process, except sometimes in the case of purified enzymes (ARAKAWA et al., 2006).

Transformation products V1 and V2 were identified by IR and ^{1}H and ^{13}C NMR spectral data.

Compound V1 was obtained as a red gum or amorphous solid. In this IR spectrum a hydroxyl absorption band was found at 3450 cm^{-1}, in the region between 2952 - 2853cm^{-1} was attributed to aliphatic CH while double-bond were observed at 1659 and 1629 cm^{-1}. Comparison of this IR data for compound V1 with those of lapachol revealed the absence of the absorption bands attributed to C=O quinone groups.

That ^{1}H NMR spectrum showed the presence of two tertiary methyl at □1.25 and □1.53. Differences were found in this spectrum comparised at the lapachol, The presence of quinine moietie wasn't evident and $–CH_2$-CH- system of the lateral chain not observed.The presence of the signals in the aromatic region centraded in □7.25 and a singlet at □3.48 attributed at hydrogen bonded to carbon oxygenated were confirmed by spectrum analysis.

Spectrum of ^{13}C NMR was not enlightening but these signals were consistent with ^{1}H NMR spectrum for –H-C-O- at □77.00 and $–CH(CH_3)_2$ system at □29.89. Based on the above data and the negative test for quinones of V1 indicated that the quinone ring has been broken.

Another component isolated V_2 showed, in the IR spectrum, in the region between 2919-2849 cm^{-1} absorption band corresponding to aliphatic axial deformation, at 1692-1659 cm^{-1} was attributed to axial deformation of C=C double-bond in aromatic system and 1710 to 1737 cm^{-1} attributed to axial deformation of the carbonyl group. The absorption band at 3000 cm^{-1} for the alcoholic hydroxyl group observed in the IR spectrum of the Lapachol was absent in the spectrum of V_2. By ^{1}H and ^{13}C NMR spectra are observed signals corresponding to aromatic hydrogen centered at δ 8.00, δ 7.25, δ 6.70; at δ 3.49 and 77.40 were assigned to H-C-O and signal centered at δ 1.25 and δ 29.91 relatived to H of methyl groups.

The complexity of signals in these spectra indicated a mixture of substances. However, the analysis of these spectra and colorimetric test positive for quinones (MATOS, 1998) of V_2 allowed conclude that there were structural changes in substrate inoculated, although it was not possible to reach the structure of this metabolite.

The above data, compared with that found in the literature (FILHO e YUNES, 1998) suggest that there was a cyclization reaction between the side chain and hydroxyl group of Lapachol forming a thrid ring may be furan or pyran, linear, saturated or unsaturated (Figure 1).

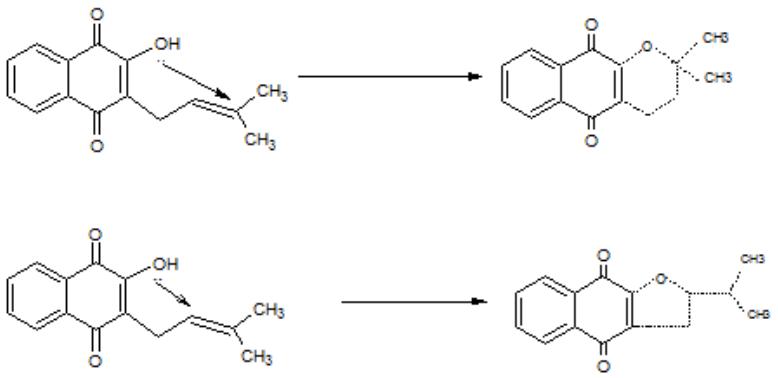

Figure 1: Possible Lapachol biotransformation pathway

Conclusion

This paper describes the application of fungi in cyclization reactions of alkenols, in this case the cyclization of lapachol by fermentation procedure. It is worth noting that the reaction time influences the amount and yield of metabolites formed. It was observed that much of the substrate added to the reaction medium was adhered to the fungal mycelium, hampering thereby their metabolism. Nevertheless, this fungus was able to perform interesting structural changes in the Lapachol, selectively, resulting in a cyclic compound, called V2 Approximation with 10-13% yield. This value is considerable compared to that obtained by natural plant metabolism. Finally, these results demonstrate the possibility of using this methodology for reactions with derivatives of more complex systems or other natural naphthoquinones. Further studies should be conducted to elucidate the structural and

pharmacological tests can be performed, since naphthoquinone compounds have various pharmacological activities of medicinal interest.

References

ARAKAWA, N. S.; AMBROSIO, S. R.; NOMIZO,A; DA COSTA, F. B.; Avaliação da atividade citotóxica e de apoptose da budleína A isolada de *Viguiera robusta Gardn*(Asteraceae). In: **XIX Simpósio de Plantas Medicinais do Brasil**, Salvador- BA, 2006.

DA SILVA, M. N.; FERREIRA, V. F.; SOUZA, M. Um panorama atual da química e da farmacologia de naftoquinonas, com ênfase na □-lapachona e derivados. **Quimica Nova**, v. 26 (3), p. 407-416, 2003.

FERREIRA, S. B.; GONZAGA, D. T. G.; SANTOS, W. C.; ARAÚJO, K. G. L.; FERREIRA, V. F. β-Lapachona: Sua importância em química medicinal e modificações estruturais. **Revista Virtual da Química**, v. 2 (2), p. 140-160, 2010.

FILHO, V. C.; YUNES, R. A.; Estratégias para a Obtenção de Compostos Farmacologicamente Ativos a partir de Plantas Medicinais. Conceitos sobre Modificação Estrutural para Otimização da Atividade. **Quimica Nova***,* v. 21 (1)*, p. 99-105, 1998.*

FONSECA, C. G. S.; BRAGA, C. M. R.; SANTANA, P. D. Lapachol . química, farmacologia e métodos de dosagem**. Revista Brasileira de Farmacologia.** v. 84 (1), p. 9 - 16, 2003.

FRANCISCO, I. A.; CASELLATO, A.; VARGAS, D. M. New Synthetic Routes for the Preparation of 2-amino-3-hydroxy-1,4-naphthoquinone. **Organic preparations and procedures international.** v. 41 (4), p. 323-326, 2009.

ISHIHARA K.; HAMADA H.; HIRATA T.; NAKAJIMA N. Biotransformation using plant cultured cells. **J mol catalysit b: enz.** v. 23, p. 145-170, 2003.

KEBAMO, S.; TESEMA, S.; GELETA, B. The Role of Biotransformation in Drug Discovery and Development. **J Drug Metab Toxicol** v. 6, p.196-209, 2015.

LIRA M. A. A.; SESTER A. E.; ABREU P. R. L.; SILVA L. B. L.; WANDERLEY G. A.; SANTANA P. D. Desenvolvimento preliminar de gel de lapachol: estudo de permeação in vitro. **Brazilian Journal of Pharmaceutical Sciences** v. 40 (1), p. 35-41, 2004.

MATOS, F. J. A; **Farmácias Vivas:** sistema de utilização de plantas medicinais projetado para pequenas comunidades /F. J. Abreu Matos. 3ª ed. rev. e atual. Fortaleza: EUFC, 1998.

MURRAY, K. R.; GRANNER, D.K., MAYES, P.A.; RODWELL, V. W. **Harper's Illustrated Biochemistry**. 26ª ed. Mcgraw-Hill, 2003.

RIBEIRO, M. C. **Microbiologia pratica:** roteiro e manual: bacterias e fungos. São Paulo: Atheneu, 2000.

SHIMODA, K.; KONDO Y.; NISHIDA T.; HAMADA H.; NAKAJIMA N. Biotransformation of thymol, carvacrol and eugenol by cultured cells of eucalyptus perriniana**. Phytochemistry**, v. 67, p. 2256 - 2261, 2006.

DE SILVA JUNIOR, E. N.; DE MOURA, M. A. B. F.; PINTO, A. V.; PINTO, M. C. F. R.; DE SOUZA, M. C. B. V.; ARAÚJO, A. J.; CLAUDIA PESSOA, C.; COSTA-LOTUFO, L. V.; MONTENEGROR. C.; DE MORAE, M. O.; FERREIRA, V. F.; GOULART, M. O. F. Cytotoxic, trypanocidal activities and physicochemical parameters of nor-^{2}-lapachone-based 1,2,3-triazoles. ***J. Braz. Chem. Soc.*** **[online]**, v.20 (4), p. 635 - 640, 2009.

TAKAHASHI, J. A.; LUCAS, E. M. F. Ocorrência e diversidade estrutural de metabólitos fúngicos com atividade antibiótica. **Química Nova**, v. 31 (7), p. 1807-1813, 2008.

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