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Full Paper

Design, Synthesis and *in vitro* Antimicrobial Activity Evaluation of Novel Hybrids of Lichexantone-THC Derivatives

Ana Camila Micheletti[a*], Neli Kika Honda[a], Nadia Cristina Pereira Carvalho[b], Dênis Pires de Lima[a], and Adilson Beatriz[a]

[a]*Instituto de Química – INQUI, Universidade Federal do Mato Grosso do Sul, Av. Senador Felinto Müller, 1555, Cidade Universitária, 79074-460, Campo Grande, MS, Brazil.*
[b]*Núcleo do Hospital Universitário da Universidade Federal do Mato Grosso do Sul – NHU/UFMS, Av. Senador Felinto Müller, S/N, Cidade Universitária, 79.002-970, Campo Grande, MS, Brazil.*

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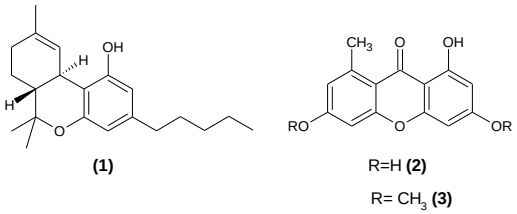
Abstract: Two novel hybrid lichexantone-THC (benzopirane group) derivatives as potential antimicrobial agents were designed and prepared from norlichexanthone and (*S*)-*cis*-verbenol in two steps. The newly synthesized compounds were characterized by ^{1}H-NMR, ^{13}C-NMR and MS. In addition, we evaluated the *in vitro* antimicrobial activity against Gram-positive (*Staphylococcus aureus, Enterococcus faecalis and Enterococcus faecium*) and Gram- negative (*Escherichia coli, Enterobacter cloacea, Klebisiella pneumoniae, Acinetobacter baumannii and Pseudomonas aeruginosa*) bacterial strains. The bioassay results indicate that the compounds namely **5** and **6** proved active against Gram-positive strains (standard and clinical) in comparison with reference drugs.

Keywords: xanthones; cannabinoids; structure modification; antibiotic activity

1. INTRODUCTION

Xanthones are a class of polyphenolics widespread in nature, commonly occurring in a few higher plant families (mainly Clusiaceae and Gentianaceae), lichen and fungi. Xanthone chemistry is considerable rich, with the conjugated donor–acceptor motif of the central ring ensuring that these compounds display a degree of feature greater than their apparently simple core structure might suggest. Many xanthones have been found to exhibit pronounced biological activities, such as antibacterial, antifungal, antioxidant, antiviral, anti-inflammatory, and anti-carcinogenic activities [1-7].

The active components of *Cannabis sativa* (marijuana) and their derivatives are classified as cannabinoids, tricyclic terpenoids having a benzopirane group, being Δ^9-tetrahydrocannabinol (Δ^9-THC **(1)** – Figure 1) one of the most famous members of this class. Another important structural feature of cannabinoids is the presence of a five-atom alkyl chain, which is a key pharmacophore that plays a crucial role in determining ligand affinity and selectivity towards cannabinoid receptors [8]. These compounds have important physiological effects, such as anti-emetic, analgesic, as well as inhibition of the enzyme acetylcholinesterase [9], anti-inflammatory [10], antimalarial [11], antimicrobial, antiparasitic [12, 13] and antitumor [14, 15], and can serve as prototypes for drug development. The behavioral effects of cannabinoids in humans and animals are complex, and the mechanism(s) that elicits these effects has been a subject of investigation for many years [16].



(1) R=H **(2)** R= CH_3 **(3)**

Figure 1. Chemical structures of Δ^9-THC (**1**), norlichexanthone (**2**) and lichexanthone (**3**).

The resistance to antimicrobials has become a global hazard with the irrational use of antibiotics leading to the infections caused by drug resistant

*Corresponding author. E-mail: ana.micheletti@ufms.br

bacteria to become a serious threat to human health. Currently there is an urgent need for the development of novel antimicrobial agents [17]. Recently, there has been a move toward multicomponent drugs whereby two or more agents are coformulated in a single tablet, and an alternative strategy is to develop a single chemical entity (hybrid) that is able to modulate multiple targets simultaneously. As anticipated, xanthones and cannabinoids are substances with a wide range of important biological activities, including antibiotic activity, and it could be interesting to prepare hybrid compounds having the basic structural features of each class, with the goal of discovering and developing potential candidates for prototypes of new drugs [18, 19].

Our approach was to develop merged hybrids, which have their frameworks merged by taking advantage of commonalities in the structures of the starting compounds, which give rise to smaller and simpler molecules [18, 19]. Herein, we describe the synthesis of two new hybrid molecules, starting from norlichexanthone **(2)**, a xanthone derivative prepared by semisynthesis from lichexanthone **(3)** (Figure 1), a natural lichen compound [20].

2. MATERIAL AND METHODS

General

Silica gel (Carlo Erba 70 - 270 mesh) was used for column chromatography. NMR spectra were taken in a Bruker DPX-300 spectrometer ($CDCl_3$). Mass spectra (EI, 70 eV) were run on a Shimadzu CGMS QP2010 Plus gas chromatography mass spectrometer, in direct injection mode, melting points were recorded in a Uniscience do Brasil apparatus, model 498. Optical rotation measurements were performed on a Perkin Elmer 341 polarimeter, with optical path of 1 dm.

Synthesis

Norlichexanthone **(2)** was prepared from lichexantone **(3)**, according to the procedure described by Micheletti et al. [20]. 60 mg (0.24 mmol) of **2**, 14 mg (0.08 mmol) of *p*-toluenesulfonic acid and 50 mg (0.3 mmol) of *S*-(-)-*cis*-verbenol **(4)** were dissolved in CH_2Cl_2 (50 mL) and the solution was refluxed for 6 hours. Subsequently, water was added to the reaction mixture and it was extracted with CH_2Cl_2. The organic phase was dried with Na_2SO_4 and the solvent was evaporated. The mixture was then dissolved in CH_2Cl_2 (30 mL) and subjected to reaction with $BF_3.EtO_2$ under reflux for 24 hours. The reaction was finished by the addition of a saturated aqueous $NaHCO_3$ solution and extracted with CH_2Cl_2. The resulting organic phase was dried over Na_2SO_4, evaporated under vacuum and purified by column chromatography, using hexane / ethyl acetate in gradient, leading to the isolation of two tetrahydroisochromenexanthones, **5** and **6**.

(3'R,4'R)-1,6-dihydroxy-1',7',7',8-tetramethyl-2', 3', 4', 7'-tetrahydro-4H,9H-isochromene[3,4-c]xanthen-9-one ***(5)****:* Yield 15%; $[\alpha]_D^{25}$: - 54 ($CHCl_3$); Mp 140 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.13 (3H, bs), 1.40 (3H, bs), 1.73 (3H, bs), 1.81 – 1.87 (3H, m), 2.15 – 2.20 (1H, m), 2.82 (3H, s), 2.86 (1H, m), 3.15 – 3.18 (1H, bd), 5.47 (1H, bs), 6.17 (1H, s), 6.59 (1H, bs), 6.67 (1H, bs), 13.29 (1H, s). ^{13}C NMR (75MHz, $CDCl_3$): δ 18.7; 23.4; 23.6; 27.4; 27.7; 30.9; 36.8; 44.4; 78.5; 99.8; 100.8; 103.8, 104.0, 113.0; 115.7; 119.5; 134.2; 144.2; 155.0; 160.0; 160.8; 161.3; 182.5; MS (EI) *m/z* (%): 392 ($[M]^+$, 43%), 349 (11), 309 (100), 271 (17).

(3'S,4'S)-1,6-dihydroxy-1',7',7',8-tetramethyl-2', 3', 4', 7'-tetrahydro-4H,9H-isochromene[2,3-c]xanthen-9-one ***(6):*** Yield 7.5%; $[\alpha]_D^{25}$: - 12 ($CHCl_3$); Mp 120 - 122°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.15 (3H, bs), 1.45 (3H, bs), 1.66 (3H, bs), 1.71 – 1.83 (3H, m), 2.17 (1H, m), 2.79 (3H, s), 2.84 – 2.88 (1H, m), 3.41 – 3.47 (1H, bd), 5.41 (1H, bs), 6.33 (1H, s), 6.58 (1H, bs), 6.68 (1H, bs). ^{13}C NMR (75MHz, $CDCl_3$): δ 19.2; 23.5; 23.8; 27.3; 27.6; 30.9; 34.9; 44.0; 80.5; 95.5; 101.2; 103.8, 104.0, 110.7; 117.6; 118.6; 134.9; 144.7; 154.5; 160.5; 163.5; 166.2; 182.1; MS (EI) *m/z* (%): 392 ($[M]^+$, 16%), 357 (17), 309 (46), 83 (88), 55 (100).

Antibiotic assay

Microorganisms and media

The test organisms used in this study were as follows: *S. aureus* ATCC 25923, *E. faecalis* ATCC 51299, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, clinical strains (oxacillin resistant *S. aureus*, vancomicin resistant *E. faecium*, *E. cloacea*, *K. pneumonia* and *A. baumannii*). All media were purchased from Oxoid.

Determination of minimum inhibitory concentrations

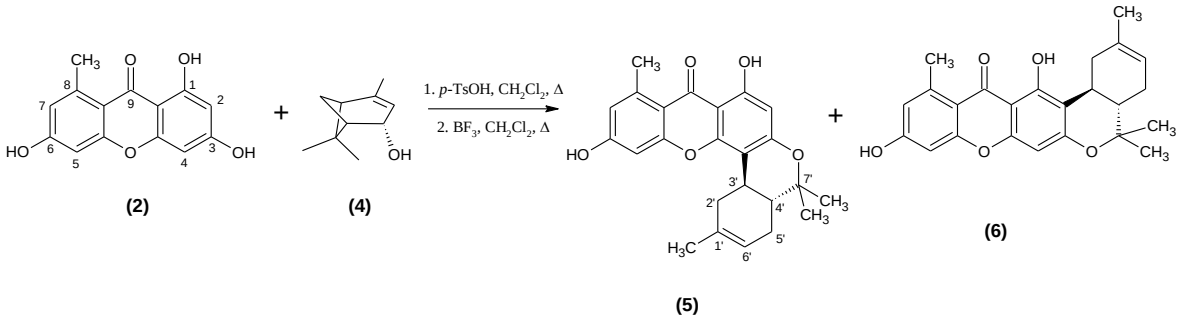
The 96-well plates were prepared by

dispensing 100 µL Mueller-Hinton broth into each well. A stock solution was prepared at a concentration of 2 mg/mL and serial dilutions were performed to reach a final concentration within a 1 µg/mL to 1000 µg/mL range, with a 100 µL final volume in each well. For gentamycine, final concentration ranged from 64 µg/mL to 0.5 µg/mL. The inoculum was an overnight culture of each bacterial species in Mueller-Hinton agar diluted in saline sterile solution (0.45%) to a concentration of approximately 10^8 CFU/mL. This solution was diluted 1/10 in saline solution (0.45%) and 5 µL (10^4 CFU/mL) were added to each well containing the test samples.

All experiments were performed in triplicate and the microdilution trays were incubated at 36ºC for 18 h. Then, 20 µL of an aqueous solution (0.5 %) of triphenyl tetrazolium chloride (TTC) were added to each well and the trays were again incubated at 36ºC for 2 h. Afterwards, in those wells where bacterial growth did occur, TTC changed from colorless to red. MIC was defined as the lowest concentration of each substance at which no color change occurred, and was expressed in µg/mL.

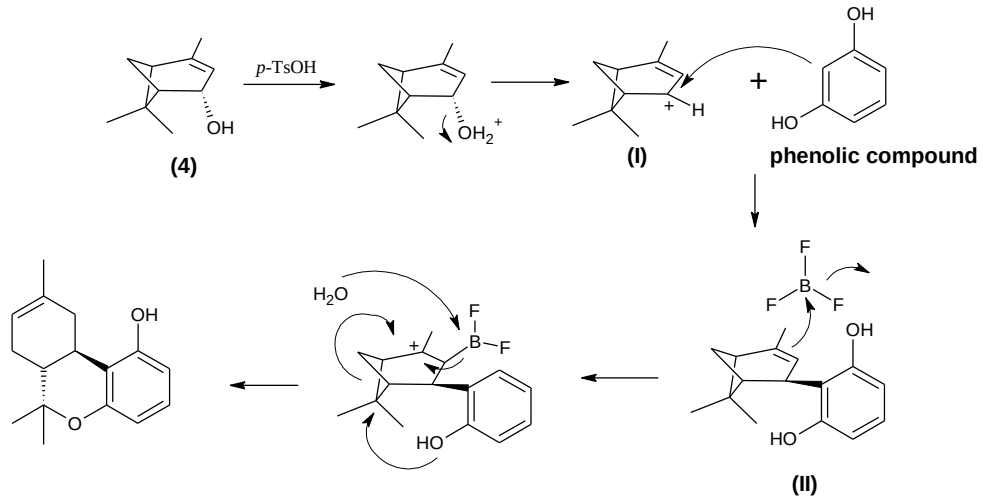
3. RESULTS AND DISCUSSION

Two tetrahydroisochromenexanthones (**5** and **6**) were obtained in the reaction between norlichexanthone **(2)** and *S*-(-)-*cis*-verbenol **(4)**, in two steps (Scheme 1).



Scheme 1. Synthesis of compounds **5** and **6**.

The first step to obtain the desired compounds was the coupling reaction between **2** and **4**, catalyzed by *p*-toluenesulfonic acid, according to the method described by Mechoulam et al. [21]. Based on experimental evidence, these authors proposed a mechanism (Scheme 2) and suggested that the stereochemistry of the final product is determined by steric factors, since any one of the configurations (*R* or *S*) of the carbinolic carbon in **4** give the same result.



Scheme 2. Schematic representation of the mechanism for the theoretical coupling reaction between a phenolic substrate (in one of steps one hydroxyl was removed from the structure just for better visualization) and *S*-(-)-*cis*-verbenol **(4)** [21].

The coupling catalyzed by *p*-toluenesulfonic acid gave a complex mixture, since norlichexanthone

(2) has four free positions for this reaction, and in each possible product, more than one position of the phenolic moiety can attack the electrophile. The reaction mixture was subjected to the reaction with BF_3 in CH_2Cl_2 for intramolecular closure of the pyran ring. After separation of the complex mixture by column chromatography, it was possible to isolate just two of the various components, whose structures were elucidated by NMR spectral analysis (data shown in Supplementary Material) and mass spectrometry.

The two compounds showed very similar NMR spectra and since they showed molecular ions $[M]^+$ 392 in their mass spectra, it can be concluded that they are isomers. The one with higher Rf isolated in the first column fractions, showed a quite complex ^1H NMR spectrum, however, it was observed signals to three hydrogens attached to aromatic carbons, between 6 and 7 ppm, four singlets to methyl groups, a broad signal at 5.5 ppm, referring to an olefinic hydrogen and many overlapping multiplets between 1 to 3.2 ppm. Correlations in *g*HSQC and *g*HMBC experiments were used to assign all hydrogen signals in the spectrum.

The observation of ^{13}C NMR spectrum confirmed that the coupling has occurred and that the terpene moiety was attached to the xanthone framework at position 4, once the signal related to this methyne group, which has a chemical shift of about 93 ppm, is not present. After the coupling reaction, the chemical shift of the signal concerning this carbon was shifted to 104 ppm.

Also, it can be observed signals for the carbons of the two rings inserted in the xanthone basic structure: signals for three methyl groups: 18.7 and 27.4 ppm for those connected to the pyran ring, and 23.6 ppm for the methyl group attached to the sp^2 carbon; two methylene groups, at 27.7 and 36.8 ppm; two methyne groups, at approximately 31.0 and 44.4 ppm, and a signal to a tetrasubstituted carbon at 78.5 ppm. The spectral data confirms the success of the second stage of reaction, the intramolecular formation of the pyran ring. The other signals are related to the basic xanthone skeleton, used as starting material. According to the proposed mechanism, the sp^3 carbon that supports two geminal methyls in intermediate (II) is facing one side of the cyclohexene ring, and the aromatic ring is facing other side (Scheme 2). When there is the nucleophilic attack of the aromatic hydroxyl to this carbon, the stereochemistry of the adjacent carbon is mantained, then forming a product where the hydrogens attached to carbons at the junction of rings have a *trans* configuration [21].

With all this information, it was possible to assign this derivative as the (3'*R*,4'*R*)-1,6-dihydroxy-1',7',7',8-tetramethyl-2',3',4',7'-tetrahydro-4*H*,9*H*-isochromene[3,4-*c*]xanthen-9-one **(5)**, a tetrahydroisochromenexanthone described for the first time, with 15% yield.

It is interesting to note that the two geminal methyl groups show a difference of nearly 10 ppm in chemical shift values in ^{13}C NMR spectrum. This is due to the different positions they occupy in space, one group out of the plane formed by the xanthone framework and other group, approximately in the plane (Figure 2), indicating that the two groups are in different chemical environments. For the correlations observed in the NOESY experiment it can be seen the spatial correlation between the protected methyl group, and the signal H-3', thus the two groups are facing the same side.

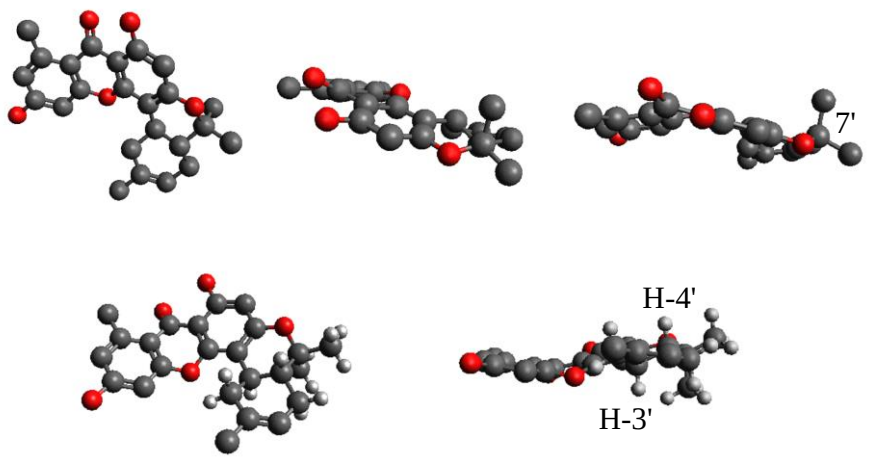


Figure 2. Three-dimensional structure of **5**, obtained with RM1 optimization with the Gaussian program (five different ways to facilitate visualization) [22].

The second compound isolated from the complex reaction mixture was an isomer of **5**. ^{1}H NMR spectrum was equally complex, but, again, the ^{13}C NMR spectrum provided the necessary data. Signal for carbon 2, at about 99 ppm was not observed, indicating that coupling with the terpene occurred at this position. The other signals observed in both spectra were very similar to those observed in the isomer spectra, though, in this case both the hydroxyl groups, at positon 1 or 3 carbon may serve as nucleophiles for intramolecular substitution reaction. In the ^{1}H NMR spectrum of the compound is not observed the characteristic signal of phenolic hydroxyl in approximately 13 ppm, however, the hydroxyl at position 1 does not behave as a good nucleophile due to hydrogen bonding to the carbonyl, and there are no reports on our previous work in which there has been any reaction in this position [20].

Furthermore, according to the literature, when there is no substituent at the position 2 of xanthonic skeleton of 1-methoxyxanthones, the chemical shift expected for the methoxyl carbon is approximately 55 ppm, with a deshielding effect of about 8 ppm when there is a substituent at position 2 [23]. Thus, similarly, a large deshielding of carbon 7 would be expect if the hydroxyl 1 had reacted, which was not observed, confirming that hydroxyl 3 was again the nucleophile in the intramolecular reaction that gave (3'*S*,4'*S*)-1,6-dihydroxy-1',7',7',8-tetramethyl-2', 3', 4', 7'-tetrahydro-4*H*,9*H*-isochromene[2,3-*c*]xanthen-9-one **(6)**, with 7.5% yield.

It is important to emphasize that, as observed in previous studies [20], the A ring of norlichexanthone **(2)** has a higher electron density than the B ring, and the atoms of the fist (carbon and oxygen) are stronger nucleophiles than the corresponding ones in B ring.

There are few reports in the literature on antimicrobial activity of cannabinoids. Novak et al. [24] and Nissen et al. [25] reviewed the antibiotic action of *Cannabis* essential oils, which do not contain significant amounts of these substances. Muhammad et al. [12] described the activity of hexahydrodibenzopiranes, structurally related to cannabinoids, on a number of microorganisms, including bacteria, fungi and protozoa, giving good results against *S. aureus* (including resistant strains), *P. falciparum* and *L. donovani*. Appendino et al. [13] evaluated the antimicrobial activity of *Cannabis sativa* cannabinoids with promising results. All majority cannabinoids, cannabidiol **(7)**, cannabichromene **(8)**, cannabigerol **(9)**, Δ^{9}-tetrahydrocannabinol **(1)** and cannabinol **(10)** (Figure 3), showed potent activity against a variety of methicillin-resistant *S. aureus* (MRSA) strains, with MIC values between 0.5 and 2 μg/ mL. The authors suggest some important structural features for the activity, such as the presence of the phenolic hydroxyl, and a good balance between the polar and nonpolar portion of the structure (alkyl residue and terpenic group).

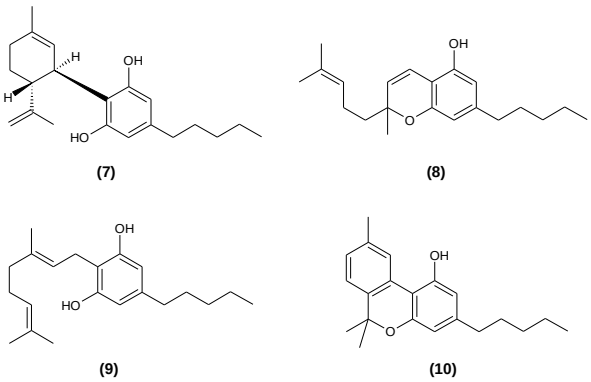


Figure 3. Structures of major of *Cannabis sativa* cannabinoids.

Compounds **5** and **6** were evaluated for antimicrobial activity against Gram-positive (*Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium*) and Gram-negative (*Escherichia coli*, *Enterobacter cloacea*, *Klebisiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*) bacterial strains, however, they proved to be active only against Gram-positive strains (Table 1). For this assay, we have selected standard sensible strains (*S. aureus* ATCC 25923, *E. faecalis* ATCC 51299, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) and clinical resistant strains (oxacillin resistant *S. aureus*, vancomycin resistant *E. faecium*, *E. cloacea*, *K. pneumonia* and *A. baumannii*).

The synthesized compounds showed significant results, including against resistant bacterial strains whose treatment has been a challenge for health systems. For the tested Gram-positive bacteria it is interesting to note the increased activity on resistant strains and, for both species, the isomer **5** was more active. There was a huge increment in antibiotic effectiveness with the terpenic moiety insertion, since norlichexanthone **(2)** has no antibiotic activity [20]. The two compounds have the same

structural characteristics, however, the spatial distribution of the groups seems to influence the biological activity. Despite not having the 5 atom alkyl chain, both isomers have comparable antibacterial activity to that of Δ^9-THC **(1)**, against resistant *S. aureus* [13], what may indicate that this group is not crucial when the activity evaluated does not involve cannabinoid receptors.

Table 1. MIC values (μg/ mL) for **5** and **6**, against four bacterial strains.

Compounds	**MIC (μg/ mL)**			
	S. aureus (ATCC 25923)	*S. aureus* (clinical)	*E. faecalis* (ATCC 51299)	*E. faecium* (clinical)
5	2	1	7.8	3.9
6	7.8	2	15.6	7.8
standard*	<0.5	64	<0.5	≤0.12

*tigecycline for *E. faecium;* gentamicin for the other strains.

4. CONCLUSION

In this work, we have synthesized two new tetrahydroisochromenexanthones that showed to be very effective against bacteria that are a real risk to human health. The results achieved indicate the importance of the strategy employed, by constructing hybrids comprising structural characteristics from biologically relevant classes, to design new biologically active compounds. Synthetic yields were mild, given that the structural characteristics of the phenolic substrate, with many free positions for the coupling reaction. On the other hand, regarding their biological importance, other conditions could be tested in order to optimize the reactions.

5. ACKNOWLEDMENTS

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