*Article*

# Antioxidant and Antimicrobial Properties of 2-(4,5-Dihydro-1*H*-pyrazol-1-yl)-pyrimidine and 1-Carboxamidino-1*H*-pyrazole Derivatives

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Cinco derivados de 4-trifluorometil-2-(5-aril-3-stiril-1*H*-pirazol-1il)-pirimidinas e seis 5-aril-3-estiril-1-carboxamidino-1*H*-pirazois previamente sintetizados foram avaliados de acordo com suas propriedades antioxidantes e antimicrobianas. Estas atividades foram avaliadas por ensaios de DPPH e HRP/luminol/$H_2O_2$ quimioluminescência e suas atividades antimicrobianas (CIM). Os resultados foram bons para alguns compostos da série em certas concentrações em comparação direta com padrões.

Five previously synthesized 4-trifluoromethyl-2-(5-aryl-3-styryl-1*H*-pyrazol-1yl)-pyrimidines and six 5-aryl-3-styryl-1-carboxamidino-1*H*-pyrazole derivatives were screened for their antioxidant proprieties. The antioxidant activities were evaluated by using the DPPH and the HRP/luminol/$H_2O_2$ chemiluminescence assay systems and for their antimicrobial activity (MIC). The results were good for those series in some concentration in comparison with the standards.

**Keywords:** heterocycles, 4,5-dihydropyrazoles, antioxidant activity, antimicrobial activity

## Introduction

Pyrazole derivatives represent a class of compounds of great importance in heterocyclic chemistry, primarily due to the fact that many 1*H*-pyrazoles are subunits of pharmaceutical and agrochemical agents.[1] Structurally novel 2-(1*H*-pyrazol-1-yl)-pyrimidine derivatives have been reported to have interesting biological activity. Epirazole is a nonsteroidal anti-inflammatory and analgesic agent therapeutically applied in Japan. In addition, many 2-(1*H*-pyrazol-1-yl)-pyrimidine derivatives show potent inhibition of induced ulcers in rats, as well as herbicidal, fungicidal and cardiotonic activities.[2] Moreover, the 1,3,5-triaryl-4,5-dihydropyrazole and 5-aryl-3-styryl-4,5-dihydropyrazole derivatives are well-known fluorescent compounds with high emission quantum yields and are widely used as optical brighteners, and as fluorescence
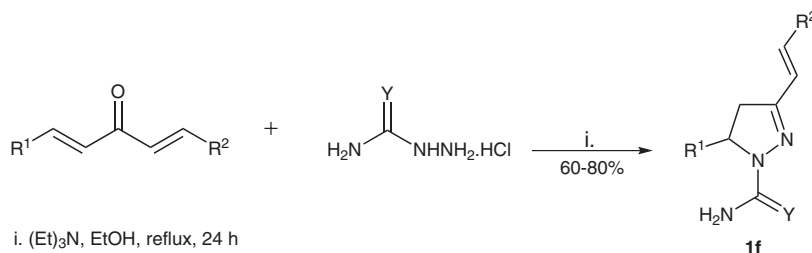
probes in chemosensors.[3] Also, polycyclic heterocycles have been reported to be antioxidant and antimicrobial agents.[4-6]

Reactive oxygen species (ROS) and free radicals *in vivo* are involved in a wide range of human diseases.[7] ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radical are by-products of a variety of pathways of aerobic metabolism. They are unstable and react readily with a wide range of biological substrates, such as lipids, DNA, and proteins, resulting in cell damage.[8-10] Therefore, antioxidants may prevent damage and death of cells.

The use of antimicrobial drugs to treat public health is growing and it is possible to see an emergence of bacterial resistance. To maintain the efficiency of the treatment of disease caused by bacteria, new active compounds are always being searched for.[11]

The aim of this work is to evaluate the antioxidant and antimicrobial activity of a series of five 2-(4,5-dihydro-1*H*-pyrazol-1-yl)pyrimidines and six 1-carboxamidino-

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i. (Et)$_3$N, EtOH, reflux, 24 h

| | 1a | 1b | 1c | 1d | 1e | 1f |
|---|---|---|---|---|---|---|
| R$^1$ | 2-CH$_3$C$_6$H$_4$ | 3,4,5-(CH$_3$O)$_3$C$_6$H$_2$ | 4-ClC$_6$H$_4$ | 4-ClC$_6$H$_4$ | 3,4-(CH$_3$O)$_2$C$_6$H$_3$ | 4-(CH$_3$O)C$_6$H$_4$ |
| R$^2$ | 2-CH$_3$C$_6$H$_4$ | 3,4,5-(CH$_3$O)$_3$C$_6$H$_2$ | 4-ClC$_6$H$_4$ | 4-thienyl | 3,4-(CH$_3$O)$_2$C$_6$H$_3$ | 4-(CH$_3$O)C$_6$H$_4$ |
| Y | NH | NH | NH | NH | SH | NH |



ii. BF$_3$·OEt$_2$ or Ti(Oi-Pr)$_4$, EtOH, r.t., 15 mim
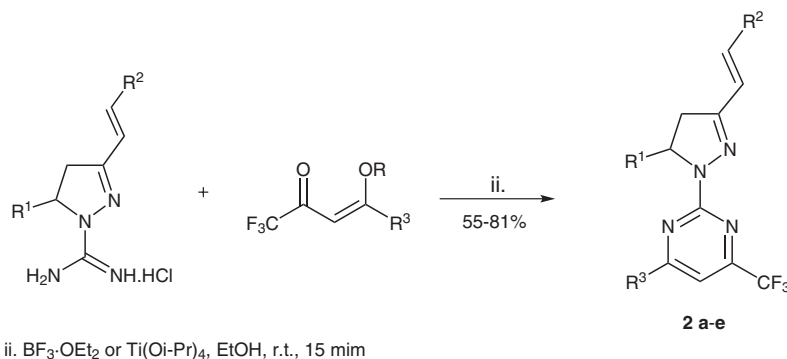
| | 2a | 2b | 2c | 2d | 2e |
|---|---|---|---|---|---|
| R$^1$ | 2-CH$_3$C$_6$H$_4$ | 2-CH$_3$C$_6$H$_4$ | C$_6$H$_5$ | C$_6$H$_5$ | 2-CH$_3$C$_6$H$_4$ |
| R$^2$ | 2-CH$_3$C$_6$H$_4$ | 2-CH$_3$C$_6$H$_4$ | C$_6$H$_5$ | C$_6$H$_5$ | 2-CH$_3$C$_6$H$_4$ |
| R$^3$ | 2-furyl | CH$_3$ | 2-thienyl | CH$_3$ | H |

**Figure 1.** Chemical structures of evaluated compounds.

1H-pyrazoles (Figure 1) that were previously synthesized.[3] These new heterocyclic systems were of interest principally for antioxidant properties due to the presence of a conjugated π-system. Thus, some of these molecules might be important in preventing for the treatment of diseases related to the imbalance between formation and detoxification.

Antioxidants may be classified according to their mode of action as free radical scavengers, chelators of metal ions involved in catalyzing lipid oxidation, or oxygen scavengers that react with oxygen in closed systems.[12] Several different methods are available and have been used to assess the total antioxidant capacity of numerous molecules. Two commonly used antioxidant evaluation methods, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and chemiluminescence determination in the presence of HRP, were applied to determine the antioxidant capacity. The target compounds were compared with vitamin E and N-acetyl-L-cysteine, respectively, as reference compounds.

# Experimental

## Synthesis

The 5-aryl-1-carboxamidino-3-styryl-4,5-dihydro-1H-pyrazoles **1a-e** were synthesized by the reactions of 1,5-diarylpenta-1,4-dien-3-ones with aminoguanidine hydrochloride in the presence of triethylamine in ethanol, under reflux for 24 h.

Reactions of 1-carboxamidino-4,5-dihydro-1H-pyrazole compounds with 4-alkoxy-1,1,1-trifluoro-alk-3-en-2-ones were carried out in refluxing ethanol (2-72 h) with a catalytic amount of BF$_3$·OEt$_2$ or Ti(O-iPr)$_4$, affording the 2-(5-aryl-3-styryl-4,5-dihydro-1H-pyrazol-1-yl)-4-(trifluoromethyl) pyrimidines **2a-e**.

## DPPH radical scavenging assay

The DPPH radical scavenging model is extensively used to evaluate antioxidant activities faster than with

other methods. Radical scavenging activity of solutions at several concentrations of the compounds (**1a**-**f** and **2a**-**e**) were evaluated against stable DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) spectrophotometrically according to Yen and Chan,[13] with some modifications. The absorbance decrease was measured at 517 nm (10 min) on a UV-Visible spectrophotometer (Spectrum Power Wave X 340, Bio-Tec Instruments, INC). The DPPH solution ($1 \times 10^{-3}$ mol $L^{-1}$ in methanol) was freshly prepared. 10 μL of this solution were mixed with 100 μL of solutions of final concentrations of 500, 250, 100, 50 and 5 μg $mL^{-1}$ in a 96-well plate. The samples were kept in the dark for 20 min at room temperature and then the decay of absorption was followed. Vitamin E was used as a positive control. The experiment was carried out in triplicate. Radical scavenging activity was calculated as follows:

$$\text{Inhibition \%} = \frac{(\text{Abs}_{\text{DPPH}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{DPPH}}} \times 100$$

where $\text{Abs}_{\text{DPPH}}$ – absorption of blank sample (MeOH + DPPH) and $\text{Abs}_{\text{sample}}$ – absorption of tested solution + DPPH (t = 30 min).

*Chemiluminescence determination in the presence of HRP*

This method is based on light emission and, for antioxidant determination, solutions of 1 mg $mL^{-1}$ of the test sample in phosphate buffer 5% DMSO were mixed with $H_2O_2$, yielding a final concentration of $5 \times 10^{-5}$ mol $L^{-1}$. Then luminol (5-amino-2,3-dihydrophthalazine-1,4-dione, in DMSO) was added to a final concentration of $1.13 \times 10^{-4}$ mol $L^{-1}$. The 96-well plate was incubated for 3 min at 30 °C and then HRP at a final concentration of 0.2 IU $mL^{-1}$ was added to initiate the chemiluminescence reaction.[14] Phosphate buffer / 5% DMSO was used as a blank for the maximum luminescence and *N*-acetyl-*L*-cysteine was used as the positive control. Chemiluminescence was measured for 15 min at 25 °C with a microplate reader (Tecan-infinite®M200). Final concentrations of 500, 250, 100, 50 and 5 μL $mL^{-1}$ of the solutions were analyzed and the experiment was carried out in triplicate. The antioxidant activity was calculated by the following formula:

$$\text{Inhibition \%} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

where $A_{\text{control}}$ is the area under the curve of the blank (phosphate buffer / 5% DMSO + $H_2O_2$ + luminol + HRP) during 15 min and $A_{\text{sample}}$ is the area under the curve of the sample (sample + $H_2O_2$ + luminol + HRP) during 15 min.

*Antimicrobial testing (MIC)*

*In vitro* antimicrobial studies were carried out against eight bacterial strains (*Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 49619, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhi* ATCC 19430, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853), and one yeast strain (*Candida albicans* ATCC 10231). The minimal inhibitory concentration (MIC) was determined in 96-well culture plates by a micro-dilution method using a microorganism suspension with a density of $10^5$ CFU $mL^{-1}$ in Mueller Hinton broth (MHB) incubated for 24 h at $35 \pm 1$ °C for bacteria, and Sabouraud broth (SB) incubated for 72 h at $35 \pm 1$ °C for yeast, as recommended by NCCLS for determination of the MIC (NCCLS M100-S15 and M38-A).[15,16] Proper blanks were assayed simultaneously with samples tested in triplicate. The concentrations tested were 500, 250, 125, 62.5, 31.25 and 16.125 μg $mL^{-1}$. The minimum lethal concentration (MLC) was carried out by inoculating the suspension of the concentrations tested in a Petri plate with Nitrient agar and incubated 24 h at $35 \pm 1$ °C for bacteria and Sabouraud agar dextrose for 72 h at $35 \pm 1$ °C for yeast. After the incubation period, the MLC was determined by the absence of microorganism growth.

## Results and Discussion

The DPPH antioxidant assay measures the hydrogen donating capacity of the molecules in the sample.[13,17] When the free radical DPPH is reduced by the sample, its colour changes from violet to yellow. This absorbance decline is measured and the antioxidant capacity can be determined. On the other hand, the chemiluminescence method is based on the light emission produced by a chemical reaction. In this case, the chemiluminescence oxidation of luminol involves the formation of a complex between the oxidant ($H_2O_2$) and the HRP enzyme, oxidized HRP leading to luminol radical production. The native enzyme (+3) reacts with $H_2O_2$ and, in this way, catalyzes the luminol oxidation ($LH^-$) through the enzymatic cycle. Luminol reacts with the HRP intermediates [complex I (+5) and II (+4)] producing the luminol radical ($L^{\bullet-}$), which then reacts with oxygen resulting in the formation of an endoperoxide ($LO^{2-}$). This endoperoxide decomposes to a excited electronically 3-aminophthalate dianion ($AP^{*2-}$), which returns to its ground state with emission of light. In the presence of antioxidant molecules (RH), the luminol radical reacts more quickly with these molecules than with oxygen, preventing the luminol radical from oxidation to 3-aminophthalate and

inhibiting light emission. This light emission reduction is considered to be a measure of the antioxidant activity.[18]

The samples **2a**, **2c** and **2e** were not totally soluble in the DMSO/phosphate buffer system and, because of the lack of stability of HRP to higher levels of DMSO, the concentrations tested were ten times lower (50, 25, 10 and 5 µg mL⁻¹) in the chemiluminescence antioxidant assay. The higher concentrations (100, 250 and 500 µg mL⁻¹) were estimated according to each curve profile in order to compare the results with those obtained in the DPPH assay.

The evaluation *via* more than one assay is interesting for comparison between possible mechanisms of action for antioxidant properties. Profiles of the antioxidant potentials of the tested compounds are shown in Figure 2 and in Table 1. Overall, DPPH assay was fairly consistent with the chemiluminescence assay. Thus, the compounds in series 1 (**1a**-**f**) shown in Figure 2A have higher potency of radical scavenging with good $IC_{50}$ (Table 1). However, the compounds of series 2 (**2a**-**e**) were inactive or showed low activity in both tests, not being potent enough to cause 50% inhibition (Figure 2B). All the samples presented a better

sensitivity in the chemiluminescence method. This method is known to be more sensitive because of the high light emission produced by the $H_2O_2$/luminol/HRP system.[19]

This result might also indicate that both series of compounds have higher antioxidant action in a mechanism different of electron scavenging. For the two methods tested, an increase of the activity was observed with increasing concentration. For compounds **1a** to **1f**, the antioxidant profile appears to follow the Michaelis-Menten curve.

Considering these results, an explanation for the potent antioxidant activity of compounds of series **1a**-**f** might be found in the possible stabilization of the radical that is formed after hydrogen abstraction. Figure 3 shows the antioxidant reaction and the hydrogens that can be abstracted in the molecules. For the series **2a**-**e**, resonance stabilization is not possible because the molecules do not have chain flexibility.

Some differences in antioxidant activity for the series are related to the substituents present. In this case, for example, when we look at **1c** and **1d**, the presence of thienyl exerts a significant influence on the radical
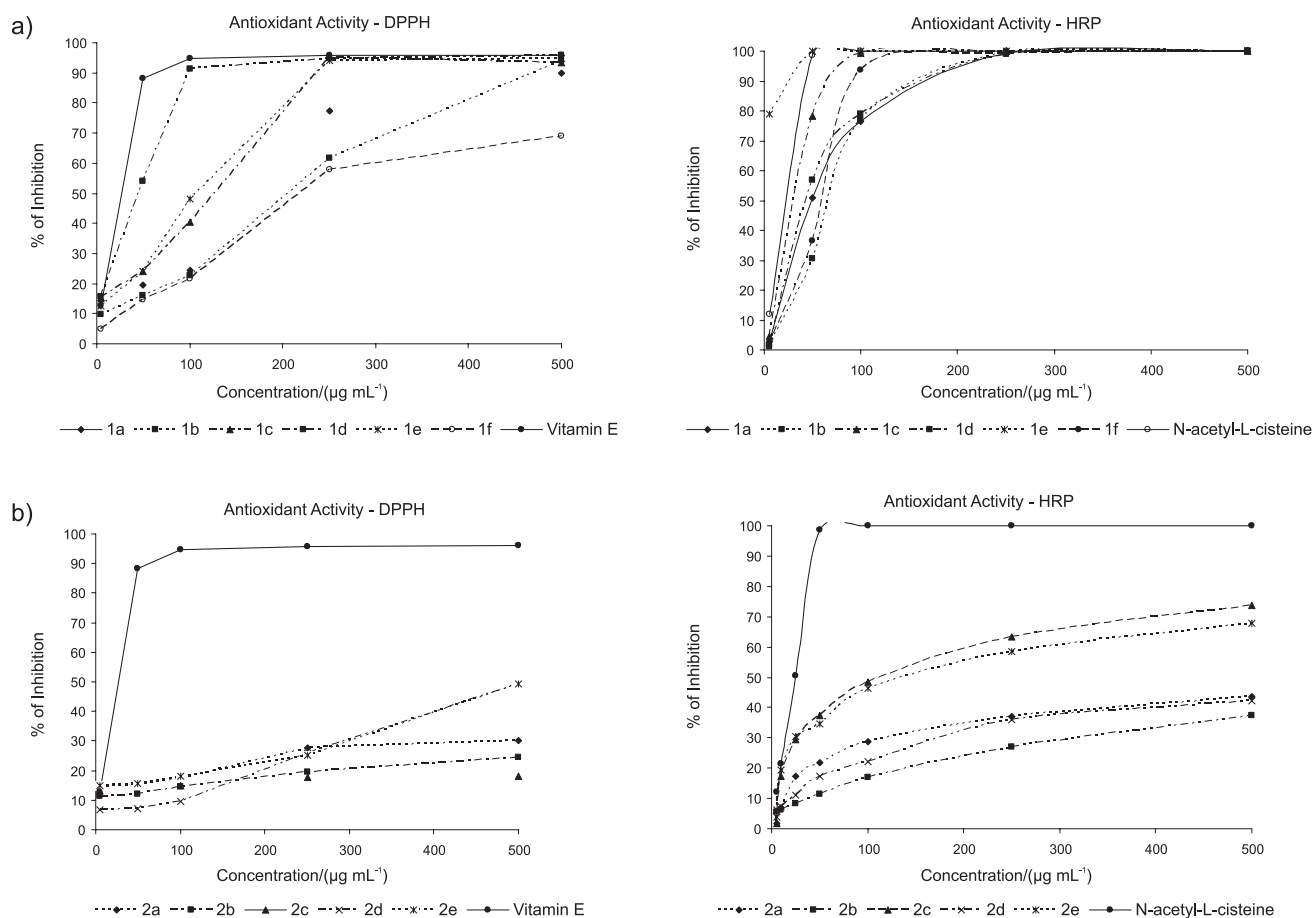


**Figure 2.** Comparative graphs of the antioxidant activity of the compounds in: a) series 1; b) series 2.

**Table 1.** Assay results for compounds **1a-f** and **2a-e**

| DPPH radical scavenging assay | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Compouds | 5 µg mL$^{-1}$ | 50 µg mL$^{-1}$ | 100 µg mL$^{-1}$ | 250 µg mL$^{-1}$ | 500 µg mL$^{-1}$ | IC$_{50}$/(µg mL$^{-1}$) | Absorbance | λ max/ nm |
| **1a** | 14.18 (± 1.9) | 19.45 (± 3.6) | 24.24 (± 1.1) | 77.21 (± 2.5) | 89.82 (± 1.1) | 172.96 | 0.83 | 319.10 |
| **1b** | 9.70 (± 1.3) | 16.12 (± 1.3) | 22.55 (± 1.8) | 61.82 (± 1.5) | 93.94 (± 0.6) | 204.88 | 0.75 | 341.20 |
| **1c** | 15.27 (± 1.0) | 24.12 (± 3.3) | 40.55 (± 3.4) | 95.15 (± 0.2) | 93.45 (± 0.4) | 125.99 | 0.47 | 317.70 |
| **1d** | 15.82 (± 0.9) | 54.11 (± 1.1) | 91.32 (± 1.4) | 94.60 (± 0.4) | 96.40 (± 2.1) | 45.17 | 0.89 | 353.00 |
| **1e** | 12.60 (± 1.2) | 23.91 (± 2.6) | 47.94 (± 3.7) | 93.96 (± 1.5) | 94.86 (± 0.2) | 106.69 | 0.81 | 343.60 |
| **1f** | 4.95 (± 2.5) | 14.65 (± 1.8) | 21.47 (± 1.8) | 57.97 (± 1.8) | 69.02 (± 2.4) | 217.22 | 0.84 | 344.00 |
| **2a** | 14.43 (± 0.7) | 15.31 (± 0.5) | 17.20 (± 0.9) | 27.55 (± 1.3) | 30.03 (± 0.1) | - | 0.95 | 351.40 |
| **2b** | 11.31 (± 1.9) | 12.01 (± 1.3) | 14.49 (± 0.6) | 19.55 (± 1.2) | 24.38 (± 4.3) | - | 0.87 | 357.60 |
| **2c** | 12.97 (± 0.8) | 15.16 (± 2.3) | 15.01 (± 1.4) | 17.78 (± 1.2) | 18.22 (± 0.1) | - | 0.84 | 351.20 |
| **2d** | 6.71 (± 0.1) | 7.24 (± 0.2) | 9.54 (± 0.0) | 25.91 (± 7.0) | 49.12 (± 3.1) | - | 0.93 | 358.60 |
| **2e** | 14.87(± 1.5) | 15.60(± 4.4) | 17.93(± 2.4) | 25.22(± 1.2) | 49.27(± 0.6) | - | 0.90 | 351.20 |
| Vit. E | 12.60 (± 2.6) | 88.24 (± 4.1) | 94.73 (± 0.2) | 95.76 (± 0.1) | 95.95 (± 0.3) | 27.25 | | |
| Chemiluminescence determination in the presence of HRP | | | | | | | | |
| **1a** | 2.82 (± 0.2) | 50.97 (± 2.9) | 76.63 (± 2.3) | 99.34 (± 0.4) | 99.95 (± 0.0) | 49.09 | 0.83 | 319.10 |
| **1b** | 1.28 (± 0.2) | 30.62 (± 1.6) | 77.03 (± 1.9) | 99.66 (± 0.2) | 99.96 (± 0.0) | 70.88 | 0.75 | 341.20 |
| **1c** | 1.19 (± 0.1) | 56.90 (± 1.6) | 78.82 (± 2.5) | 98.81 (± 0.9) | 99.98 (± 0.0) | 32.79 | 0.47 | 317.70 |
| **1d** | 4.54 (± 0.7) | 78.16 (± 2.0) | 99.42 (± 0.3) | 99.93 (± 0.0) | 99.96 (± 0.0) | 44.43 | 0.89 | 353.00 |
| **1e** | 78.84 (± 2.6) | 99.92 (± 0.0) | 99.96 (± 0.0) | 99.89 (± 0.0) | 99.99 (± 0.0) | 3.17 | 0.81 | 343.60 |
| **1f** | 1.96 (± 0.1) | 36.40 (± 1.4) | 93.76 (± 0.6) | 99.3 (± 0.2) | 99.83 (± 0.1) | 61.85 | 0.84 | 344.00 |
| **2a** | 1.59 (± 0.8) | 6.96 (± 0.8) | 17.41 (± 1.6) | 21.82 (± 1.4) | 28.81[b] | - | 0.95 | 351.40 |
| **2b** | 5.69 (± 0.9) | 6.34[b] | 8.29[b] | 11.53 (± 0.7) | 16.91 (± 1.4) | - | 0.87 | 357.60 |
| **2c** | 1.77 (± 1.7) | 17.40 (± 3.1) | 29.42 (± 0.8) | 37.31 (± 2.4) | 48.36[b] | 105.54 | 0.84 | 351.20 |
| **2d** | 6.12 (± 1.1) | 7.36[b] | 11.07[b] | 17.26 (± 1.8) | 22.15 (± 0.4) | - | 0.93 | 358.60 |
| **2e** | 3.70 (± 0.5) | 19.30 (± 1.2) | 30.31 (± 0.9) | 34.72 (± 2.3) | 46.49[b] | 130.28 | 0.90 | 351.20 |
| Control[a] | 11.98 (± 1.1) | 21.62 (± 0.0) | 50.55 (± 0.0) | 98.76 (± 1.1) | 99.89 (± 0.0) | 24.72 | | |

[a]*N*-acetyl-*L*-cisteine; [b]estimated value.

stability in both methods of analysis. On the other hand, the presence of chlorine substituents does not appear to affect significant the antioxidant potency in comparison with the other compounds of the series. Another correlation with substituents can be seen with **1b** and **1f**. These two molecules have the same W substituent, but **1b** is more potent in the DPPH method, probably because **1b** has six –OCH$_3$ substituents in the aromatic ring in contrast with only two in **1f**. But when we compare **1b**, **1e** and **1f**, although **1e** has a weak donating group in R$^1$ and R$^2$ in comparison with **1b**, **1e** shows stronger activity because of the presence of the –SH group at the W position.

The results of the chemiluminescence method for **1e**, with good antioxidant activity (78.85%) even at the lower concentration (5 µg mL$^{-1}$), show that the –SH group exerts a strong influence in this system. This activity could reflect the rapidity of the reaction that consumes the luminol radical, preventing formation of 3-aminophthalate.

The DPPH and chemiluminescence methods have been extensively used for antioxidant activity determination for pure molecules and extracts, but, when we compare our results by both methods, is not possible to see a clear correlation between them. It denotes that the molecules tested may act by different mechanisms, principally when we consider the complex system present in the chemiluminescence method in comparison with the simple redox reaction in the DPPH method.

The antibacterial and antifungal activities of the eleven pyrazole derivatives were evaluated. Only four molecules were active against some of the microorganisms (Table 2) The compounds showed only a weak effect toward *C. albicans*. The bacteria *S. typhi*, *S. aureus* and *S. pneumoniae* were the most susceptible strains inhibited between 1.95 to 15.625 µg mL$^{-1}$ by **1a**, **1c** and **1f**. This potent antibacterial activity is only observed for compounds **1a**, **1c** and **1f** and not for the other closely related molecules
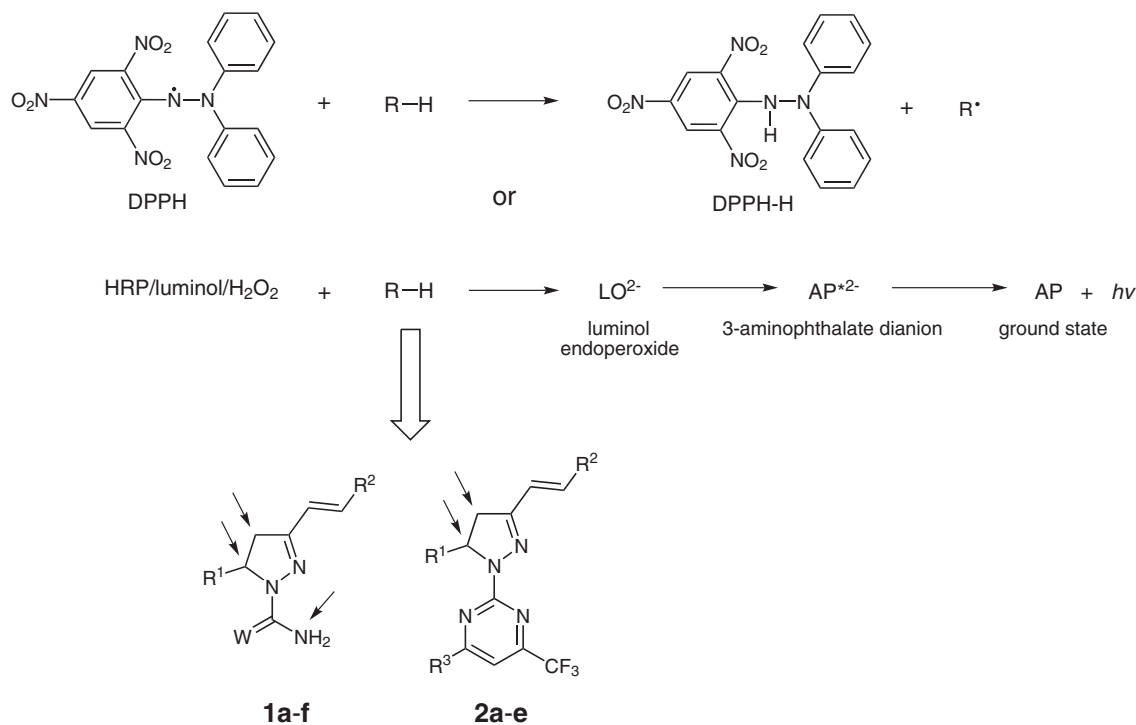
**Figure 3.** Antioxidant reaction and indication of the hydrogens that are probably abstracted in the reaction.

**Table 2.** Antimicrobial potency of the active molecules **1a**, **1c**, **1e** and **1f** (concentrations are given in $\mu g\ mL^{-1}$)

| Microorganisms | 1a | | 1c | | 1e | | 1f | |
|---|---|---|---|---|---|---|---|---|
| | MIC | MLC | MIC | MLC | MIC | MLC | MIC | MLC |
| *E. faecalis* | n.a. | ND | n.a. | ND | n.a. | ND | n.a. | ND |
| *K. pneumonae* | 500 | 500 | 125 | ND | n.a. | ND | 250 | ND |
| *S. typhi* | 15.625 | ND | 15.625 | 15.625 | n.a. | ND | 15.625 | 15.625 |
| *S. aureus* | 1.95 | 3.90 | 3.90 | 7.81 | n.a. | ND | 15.625 | ND |
| *S. pneumoniae* | 1.95 | 7.81 | 7.81 | ND | n.a. | ND | 15.625 | ND |
| *B. subtilis* | n.a. | ND | n.a. | ND | n.a. | ND | n.a. | ND |
| *E. coli* | 250 | ND | 250 | ND | n.a. | ND | 250 | ND |
| *P. aeruginosa* | 500 | ND | n.a. | ND | n.a. | ND | n.a. | ND |
| *C. albicans* | 62.5 | 125 | 500 | ND | 500 | ND | 125 | 250 |

n.a. not active. ND: not detected. Positive control: tetracycline (8 to 32 $\mu g\ mL^{-1}$) for bacteria and fuconazole (0.125 to 65 $\mu g\ mL^{-1}$) for yeast.

(**1b**, **1d** and **1e**), suggesting a mode of action that is structure specific. The presence of chlorine appears to affect the activity, but when the thienyl group is present, no cell inhibitory effect can be seen. The substituent –SH in the W position probably is responsible for the weak activity of **1e** against the fungus *C. albicans*. We also note that only small substituents can affect antimicrobial potency. Comparing **1b** and **1f**, when there are more –OCH$_3$ substituents in the aromatic ring, the antimicrobial activity is lost.

For the antimicrobial active compounds, we also tested the Minimum Lethal Concentration (MLC). As shown in Table 2, most of the compounds were able to kill the strains at concentrations not much higher than the MIC.

## Conclusions

In summary, we tested two different series of heterocycles at several concentrations to evaluate their antioxidant and antimicrobial activity. Our results have shown that 1-carboxyamidino-1*H*-pyrazole derivatives exhibit good antioxidant properties in both tests. These activities are related to the concentration and type of substituents present in each compound and resonance stabilization (Figure 3) may be the determining factor. In the antimicrobial test, we also observed a substituent dependence of the activity.

## Acknowledgments

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