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Antimicrobial activity of 1,4-naphthoquinones by metal complexation

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*The effect of metal complexation on the antimicrobial activity of 1,4-naphthoquinones was investigated. Nickel-, chromium-, iron-, copper-, and cobalt-containing metal chelates of 5-amino-8-hydroxy-1,4-naphtoquinone (**2**) and its acyl-derivatives (**3-8**) were synthesized and characterized, and their antimicrobial activity was evaluated. Data from infrared spectroscopy indicate that naphthoquinones coordinate through oxygen and nitrogen atoms for **2**, and through oxygen atoms when ligands were acyl derivatives **3-8**. Susceptibility tests for antimicrobial activity showed that **2** and its acyl derivatives were effective on inhibiting the growth of pathogenic bacteria such as* Staphylococcus aureus, Streptococcus uberis *and* Bacillus cereus*, but not Gram-negative bacteria. The metal complexation often caused decrease of biological activity. Nickel complex of **2** was the most effective against Gram-positive bacteria, showing MIC values ranging from 375 to 1400 mg/ml. Metal chelates may be useful tools for the understanding of the antimicrobial mechanism of 1,4-naphthoquinones on these bacteria.*

Uniterms
- Antimicrobial
- 5-Amino-8-hydroxy-1,4-naphtoquinone
- Metal chelate
- Hydroxyquinone
- Staphylococci

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INTRODUCTION

Naphthoquinones are largely found in plants, microorganisms, and some animals. These compounds have been widely used in diverse cultures such as colorants for cosmetics, fabrics, foods, and for medicinal purposes, including antitumor, anti-inflammatory, and antimicrobial agents (Thomson, 1971; Masuda *et al.*, 1987; Papageorgiou *et al*., 1999). Naphthoquinones are also described as precursors of more complex structures (Couladouros, Plyta, Papageorgiou, 1996). The 5,8-dihydroxy-1,4-naphthoquinone and the 5-amino-8-hydroxy-1,4-naphthoquinone are known as precursors of the anthracyclinone system and aglycones of the anthracyclines (Fariña *et al.*, 1985).

The investigation on new antimicrobial agents is important due to the resistance acquired by several pathogenic microorganisms. The prevalence of strains of *Staphylococcus aureus* resistant to conventional antibiotics has increased to high levels in some hospitals (Emori, Gaynes, 1993). Increased resistance among strains of streptococci and enterococci are also described (Lipsitch, 2001).

The biological activity of several well-known and widely used anthracycline antibiotics such as daunomycin and doxorubicin is thought to be associated to the hydroxyquinone structure (Young, Ozols, Myers, 1981). Moreover, equivalent active sites are also present in the

tetracycline antibiotics as well as in myxopyronin (Smilack, Wilson, Cockerill, 1990; Chopra, Hawkey, Hinton, 1991). The antibacterial effect is also related to naphthoquinones from vegetal origin (Didry, Pinkas, Dubreil, 1986; Machado *et al*., 2003), synthetic naphthoquinones (Oliveira *et al*., 2001; Riffel *et al*., 2002) and isoxazolyl-naphthoquinones (Bogdanov *et al*., 1995). In addition, the fungitoxic effect of 1,4-naphtoquinones (Gershon, Shanks, 1975; Sasaki, Abe, Yoshizaki, 2002) and the antiviral activity of some hydroxyquinones (Meruelo, Javie, Lavie, 1988; Brinkworth, Fairlie, 1995) have been described.

The effect of metal complexation on some antimicrobial agents has been mentioned in the literature. Tetracyclines often lose their antimicrobial activity when coordinated with metals (Chopra, Hawkey, Hinton, 1991). Some cations such as magnesium and aluminium are complexed by fluoroquinolones, resulting in decrease in oral bioavailability, and may cause therapeutic failure (Lecomte *et al*., 1994; Wallis *et al*., 1996). However, increased activity has been described for some hydroxyquinone metal-chelates (Christianopoulou, Ecateriniadou, Sarris, 1986) and a copper(II) complex of cephalexin (Iqbal *et al.*, 1999).

In this paper we report on the screening of *in vitro* activity of naphthoquinones derived from 5-amino-8-hydroxy-1,4-naphthoquinone and their metal chelates as part of our interest on the synthesis and characterization of the naphtoquinones derivatives and their transition metal complexes.

MATERIALS AND METHODS

Naphthoquinones and metal complexes

All organic compounds used in this work were synthesized and characterized in this laboratory and will be assigned as: 5-amino-8-hydroxy-1,4-naphthoquinone (**2**), and a series of derivatives 5-acetylamino-8-hydroxy-1,4-naphthoquinone **(3)**, 5-octanoylamino-8-hydroxy-1,4-naphthoquinone **(4)**, 5-decanoylamino-8-hydroxy-1,4-naphthoquinone **(5)**, 5-dodecanoylamino-8-hydroxy-1,4-naphthoquinone **(6)**, 5-tetradecanoylamino-8-hydroxy-1,4-naphthoquinone **(7)** and 5-hexadecanoylamino-8-hydroxy-1,4-naphthoquinone **(8)**. Their complexes will be referred as M**(2)** when the chelate is formed by metal (M) and compound 5-amino-8-hydroxy-1,4-naphthoquinone (**2**); M**(3)** for chelates 5-acetylamino-8-hydroxy-1,4-naphthoquinone **(3)** and there forth. Their structures are shown in Figure 1.

The synthesis of 5-amino-8-hydroxy-1,4-naphtoquinone (**2**) have been described in the literature

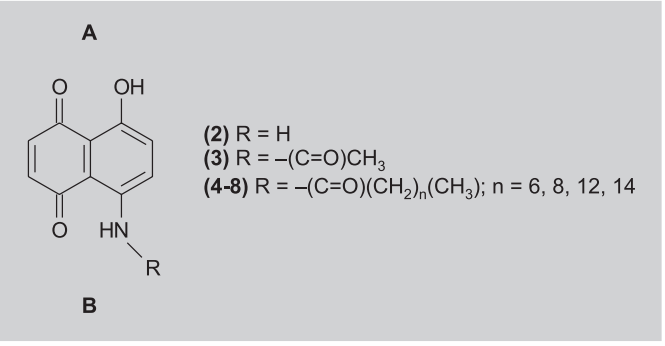

FIGURE 1 - Chemical structures of the naphthoquinones tested. The metal ion coordinates through site B for ligand (**2**) and site A for the acyl-derivatives (**3** to **8**).

(Fariña *et al.*, 1995), while the synthesis of new derivatives (**3-8**) and their metal chelates have been subject of recent studies of this group (Martinelli *et al*., 1999, 2000). The structures of the synthesized compounds were confirmed by ^{1}H-NMR, ^{13}C-NMR, UV-Visible, and elemental analysis. Infrared spectra (4000-200 cm^{-1}) were recorded on a Mattson GL3020 instrument using KBr discs.

Microorganisms

The strains tested for antibacterial activity were clinical isolates obtained at the Faculdade de Medicina Veterinária, UFRGS. Screening tests were carried out on the following bacteria: *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus intermedius, Enterococcus faecalis, Streptococcus uberis, Escherichia coli, Pseudomonas fluorescens, Bacillus cereus, Salmonella* Typhimurium. Strains from collection were *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Stock cultures were kept frozen at –21 °C in nutrient broth (Difco) containing 20% (v/v) glycerol. The organisms were propagated and maintained on nutrient agar dishes at 4 °C during the susceptibility experiments. Strains of *S. uberis* were submitted to susceptibility tests after isolation on 5% blood sheep agar.

Susceptibility testing

The susceptibility tests were performed following the NCCLS recommendations (NCCLS, 2001). Screening tests regarding the inhibition zone were carried out by the filter paper disk method. Bacterial suspension was prepared from colonies grown overnight on an agar plate, and inoculated into Mueller-Hinton broth (Merck) to give a 0.5 McFarland turbidity standard solution. A sterile swab was immersed in

TABLE I - Most relevant infrared frequencies of some characteristic bands of 5-amino-8-hydroxy-1,4-naphthoquinone (2),5-acetylamino-8-hydroxy-1,4-naphthoquinone (3) and their Ni-chelates Ni(2) and Ni(3), respectively

Band	$\nu(N\text{-}H)_2$	ν(N-H)	ν(O-H)	ν(NHC=O)	$\nu(C{=}O)_1$	$\nu(C{=}O)_4$	ν(C-OH)	ν(C-OM)	ν_{ass}(M-O)	ν_{sim}(M-O)
(2)	3346w 3259m		3154w		1606vs		1242s			
Ni(2)		3275m			1640m	1580vs		1240br,vs	459w	268w
(3)		3208w	3081w	1697s	1623vs		1269vs 1220vs		456w	256w
Ni(3)		Broad		1669m	1566vs	1590vs		111280s 1250sh,s		

the bacterial suspension and used to inoculate Mueller-Hinton agar plates. The disks were applied to the surface of inoculated plates using a sterile forceps. The compounds were diluted in phosphate buffered saline solution from a 10 mg/ml stock dimethylsulfoxide (DMSO) solution and then applied on disks (50 mg/disk). The inhibition zone was measured around each disk after 24 h at 37 °C. Strains of *E. faecalis* and *S. uberis* were tested on Mueller-Hinton agar plates supplemented with 5% sheep blood. Controls with DMSO were adequately done.

To access the Minimum Inhibitory Concentration (MIC) – defined as the drug concentration at which no growth was visible – 96-well sterile microplates (Corning) were filled with 0.1 ml of serial twofold dilutions (2000 to 4 µg/ml) of the different naphthoquinones. The compounds were diluted in Mueller-Hinton broth from a 10 mg/ml stock DMSO solution. A standardized number of bacteria (0.1 ml of a 10^6 CFU/ml suspension in Mueller-Hinton broth) were inoculated into each well. A growth well (broth plus inoculum) and a sterility control well (broth only) were included in each panel. Microplates were incubated at 37 °C for 24 h, and then MIC was determined as the last dilution where no increase in visual turbidity was observed.

RESULTS

The naphthoquinone compounds and their chelates were synthesized as described elsewhere (Martinelli *et al*., 1999, 2000). All the chelates were made of bivalent metal, except Cr(III). They are neutral complexes and were isolated as fine powders. They are quite insoluble in common organic solvents despite the lipophylic chain on compounds **4** to **8** and are thermally more stable than the free organic ligands, as should be expected. The most important changes in the IR spectrum of ligands **2** and **3** and their nickel chelates, Ni**(2)** and Ni**(3)**, can be seen in Table I.

Compounds **2** to **8** and their metal chelates were initially tested against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. Antimicrobial activity of **2-8** was observed on *S. aureus* but not on *E. coli* (not shown). The acyl-derivatives presented lower inhibition zones than the starting compound **2** (Table 2). Metal chelates showed either decreased antibacterial activity or lack of inhibitory effect. The nickel complexes of **2** and **3** and the cobalt complex of **2** were the most active (Table II).

The biological effect of **2**, and their metal-chelates on bacterial growth is shown in Table III. The ligand **2** was capable of inhibiting the growth of *S. aureus*, *S. intermedius*, *S. epidermidis*, *B. cereus*, *S. uberis* and *E. faecalis*. Nickel, cobalt and iron complexes of **2** showed a decreased inhibitory effect when compared with the ligand alone. Lack of inhibitory effect was observed for copper and chromium chelates. The activity of these compounds was also tested on Gram-negative microorganisms, such as *Escherichia coli*, *Pseudomonas fluorescens*, and

TABLE II - Effect of 1,4-naphthoquinones and metal chelates on growth of *Staphylococcus aureus**

	Metal Complex					
Compound	**None**	**Ni**	**Co**	**Fe**	**Cu**	**Cr**
2	21	16	14	12	-	-
3	16	12	10	-	-	-
4	12	-	-	-	-	-
5	10	9	-	-	9(v)	-
6	12	-	9(v)	-	-	-
7	10	-	-	-	-	-
8	9	-	-	-	-	-

* Inhibition zone in mm. Results are the means of five independent determinations for each isolate. (-) no inhibition was observed. (v) inhibition was not observed against all isolates.

TABLE III - Susceptibility testing of 5-amino-8-hydroxy-1,4-naphthoquinone metal-chelates*

Microorganism	Compound					
(No. of isolates)	2	Ni(2)	Co(2)	Fe(2)	Cu(2)	Cr(2)
S. aureus ATCC 25923	23	18	14	14	-	-
S. aureus (10)	21	16	14	12	-	-
S. intermedius (5)	26	18	14	15	-	-
S. epidermidis (10)	21	16	15	-	-	-
B. cereus (4)	18	11	-	-	-	-
S. uberis (3)	19	17	14	-	-	-
E. faecalis (5)	20	15	12	-	-	-
E. coli ATCC 25922	-	-	-	-	-	-
E. coli (10)	-	-	-	-	-	-
P. fluorescens (5)	-	-	-	-	-	-
S. Thyphimurium (7)	-	-	-	-	-	-

* Inhibition zone in mm. Results are the means of five independent determinations for each isolate. (-) no inhibition was observed.

Salmonella Thyphimurium. None of the compounds were able to inhibit the growth of those Gram-negative bacteria (Table III).

The effect of nickel and cobalt complexation of **2** on MIC values was determined against Gram-positive bacteria (Table IV). Compound **2** presented MIC values in the 30 to 125 mg/ml range, whereas the Ni**(2)** complex resulted in MIC values ranging from 375 to 1400 mg/ml. Nickel complexation of **3** or **5** caused increase of MIC to values higher than 2000 mg/ml (not shown).

TABLE IV - Minimal inhibitory concentrations of compound 2 and its nickel and cobalt complexes against Gram-positive bacteria

Microorganism	MIC (µg/mL)		
(No. of isolates)	(2)	Ni(2)	Co(2)
S. aureus ATCC 25923	30	500	500
S. aureus (10)	32	475	1100
S. intermedius (5)	34	600	800
S. epidermidis (10)	50	455	1050
B. cereus (4)	125	1400	-
S. uberis (3)	52	375	>2000
E. faecalis (5)	81	700	>2000

Results are the means of five independent determinations for each isolate. (-) no inhibition was observed.

DISCUSSION

In the previous work we showed that naphthoquinone ligands have two potential sites to coordinate a metal ion (Martinelli *et al*., 1999, 2000). Spectroscopic data allowed us to assign that the metal ion coordinates through site B for ligand **2**, while site A is preferred by the metal in the acyl-derivatives **3** to **8** (Figure 1).

Metal chelates of compound **2** and its derivatives **3** to **8** have been synthesized and were tested against Gram-positive and Gram-negative bacteria. In Tables 2 and 3 it is possible to observe that all the ligands showed antibacterial activity. Compound **2** showed higher activity compared with its derivatives **3** to **8**. All compounds possess the free naphthazarin site (site A, Figure 1), which has been associated with antibacterial properties (Papageorgiou, *et al*.1999).

Studies on antimicrobial activity of 1,4-naphtoquinones demonstrated that those with electron-releasing or weak electron-withdrawing groups at position 2 or 3 may enhance their antimicrobial activity (Ambrogi *et al*., 1970; Gershon, Shanks, 1975). An explanation for this behavior is related to the electronic effect of the groups directly bonded at the naphthoquinone ring. This effect is attributed to the enhancement of the hydrogen bonding, allowing stronger binding at its site of action. Nevertheless, in our case, the acyl compounds **3** to **8** showed a decrease in activity, although they present the site A free. Such behavior could be explained considering that the group R is not directly bound to the naphthoquinone ring and does not cause a positive electronic effect. Furthermore, the decrease in activity observed for the acyl derivatives may be related to the presence of the lipophylic group, since excess of hydrophobicity causes loss of activity (Gershon, Shanks, 1975).

Antimicrobial activity of metal complexes of naphthoquinones has been described. Some metal chelates of juglone often maintain their antibacterial effect (Joshi, 1986). (II) and Ni(II) complexes of 5-hydroxy-1,4-naphthoquinone result in higher antibacterial effect against *Bacillus spp*. and *S. aureus* (Christianopoulou, Ecateriniadou, Sarris, 1986). In addition, the Ni(II) complex of vitamin K3-thiosemicarbazole has been recently reported to increase the activity against Gram-positive bacteria (Li *et al*., 2000). Therefore, the effect of metal complexation on antimicrobial activity was a matter of our interest since the chelation of **2** occurred in site B, leaving the naphthazarin site A free.

The data obtained for the metal chelates showed lower activity than their respective ligands, and total loss of activity was observed in some cases. Considering that the naphthazarin site A is the active one, its blocking by complexation may be related with the decrease or loss of activity. In fact, site A remains free after coordination in the case of ligand **2**, but it is involved in metal complexation in **3** to **8**. Assuming that the antibacterial mechanism is interfering in the electron transport chain, this free site should be required to participate in a redox reaction. This idea may be supported by electrochemical studies on **3** and Ni**(3),** in which the reduction potential becomes more negative with complexation (Piatnicki *et al*., 1996), i.e., more energy is required for the reduction process. In addition, the presence of an imino group instead of a keto group in position 1 or 4 results in loss of antimicrobial activity (Riffel *et al*., 2002). This may indicate that both free C=O groups are required for full activity.

The set of data of compound **2** and its metal chelates showed the highest activity compared to the other chelates. The Ni**(2)** complex was found to be slightly less active than the ligand itself, nevertheless it followed the behavior of **2** being the most active against all Gram-positive bacteria tested. This was an unexpected result since the naphthazarin site is kept uncoordinated in the chelates unless the electrochemical behavior of the ligand has been modified by the metal ion.

As the ligands and some complexes were active against *S. aureus*, which is a very relevant pathogen, metal chelates may be useful tools for the understanding of the antimicrobial mechanism of 1,4-naphthoquinones on this bacterium.

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RESUMO

Atividade antimicrobiana de 1,4-naftoquinonas por complexação com metais

*O efeito da complexação com metais sobre a atividade antimicrobiana de 1,4-naftoquinonas foi investigado. Complexos contendo níquel, cromo, ferro, cobre e cobalto da 5-amino-8-hidroxi-1,4-naftoquinona (**2**) e seus acil-derivados (**3-8**) foram sintetizados e caracterizados e sua atividade antimicrobiana foi avaliada. Dados de espectroscopia de infravermelho indicaram que as naftoquinonas coordenam os metais através dos átomos de oxigênio e nitrogênio para **2** e através de átomos de oxigênio, quando os ligantes são os acil-derivados **3-8**. Testes de sensibilidade antimicrobiana demonstraram que **2** e seus derivados foram efetivos na inibição do crescimento de bactérias patogênicas como* Staphylococcus aureus, Streptococcus uberis e Bacillus cereus*, mas não apresentaram efeito contra bactérias Gram-negativas. A complexação de metais geralmente causou diminuição da atividade biológica. O complexo de níquel de **2** foi o mais eficaz contra bactérias Gram-positivas, apresentando valores de MIC de, 375 a 1400 μg/mL. Os complexos metálicos podem ser ferramentas úteis para o estudo do mecanismo antimicrobiano de 1,4-naftoquinonas nestas bactérias.*

UNITERMOS: Antimicrobiano. 5-Amino-8-hidroxi-1,4-naftoquinona. Complexo metálico. Hidroxiquinona. Estafilococos

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