Activities of 2,4-Dihydroxy-6-*n*-pentylbenzoic Acid Derivatives

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Esters of 2-hydroxy-4-methoxy-6-*n*-pentylbenzoic acid (**2**–**8**) (methyl, ethyl, butyl, pentyl, isopropyl, *sec*-butyl and benzyl), olivetol (**9**), methyl, ethyl, butyl perlatolates (**10**–**12**), 2,4-dihydroxy-6-*n*-pentylbenzoic acid (**15**), and methyl and ethyl esters of (**15**) were prepared through structural modifications of perlatolic acid (**1**) with the aim to detect new antifungal and antibacterial substances and also to evaluate the toxicity by the brine shrimp lethality assay against *Artemia salina*. The antifungal assays were carried out against the fungus *Cladosporium sphaerospermum* through the bioautography method, and methyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**13**) showed the highest antifungal activity (2.5 μg). Olivetol (**9**) and 2,4-dihydroxy-6-*n*-pentylbenzoic acid (**15**) are also potent inhibitors of the growth of the fungus (5.0 μg). Except for methyl (**10**), the ethyl (**11**) and butyl (**12**) perlatolates were less active than perlatolic acid (**1**). The activities presented by methyl (**2**) and ethyl (**3**) 2-hydroxy-4-methoxy-6-*n*-pentylbenzoates and methyl (**13**) and ethyl (**14**) 2,4-dihydroxy-6-*n*-pentylbenzoates suggest that compounds with a free hydroxy group in the aromatic ring (C-4) have a more pronounced effect against *C. sphaerospermum*. Antibacterial activities were tested by the disc diffusion method using pathogenic strains of *S. aureus* and *E. coli*. The compounds were weakly active with inhibition zones between 9–15 mm. The 2-hydroxy-4-methoxy-6-*n*-pentylbenzoic esters **2**–**8** and alkyl perlatolates **10**–**12** were selective against *E. coli*. Perlatolic acid (**1**) and methyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**2**) were the most active with LD_{50} values of 24.1 μM and 27.2 μM, respectively. The other compounds were not toxic to *Artemia salina* larvae.

Key words: Lichen, Perlatolic Acid, 2,4-Dihydroxy-6-*n*-pentylbenzoic Acid

Introduction

Among the compounds synthesized in nature, phenols are a group with a large structural diversity and several biological and/or pharmacological activities. They are simple or complex substances and possess a common characteristic: the presence of one or more aromatic rings with one or more hydroxy groups (Harbone, 1973, 1989). Besides, other substitutents, such as, alkyl chain, carboxy or carbonyl groups, are all present in a large number of natural compounds, such as those produced by lichens (Huneck and Yoshimura, 1996).

Lichens, a symbiotic association of a fungus and one or more algae, produce several classes of phenolic compounds, such as: depsides, depsidones, usnic acids, dibenzofuranes, xanthones, anthraquinones, naphthoquinones, besides the pulvinic acid derivatives and aliphatic acids. Many of these compounds are exclusive of lichens and some may be found also in fungi not lichenized and in superior plants (Hale, 1983).

Although many phenolic compounds present hazardous effects to health, they have been of great interest to researchers due to their large spectrum of activities, among these, the antifungal and antibacterial activities (Stich, 1991; Gomes *et al*., 2002, 2003; Ingólfsdóttir *et al*., 1985; Hickey *et al*., 1990). The search for antimicrobial agents has received attention, mainly as a result of increasing problems with multi-drug resistant microorganisms. Therefore, new agents are needed for the treatment of these diseases (Chaudhary *et al*., 2007).

Therefore, many relatively simple and sensitive bioassays may be performed as new strategies in

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order to obtain information about the activities of the substances. One of these assays is the bioautography for screening antifungal agents inhibiting the growth of a phytopathogenic fungus of the *Cladosporium* genus (Rahalison *et al.*, 1994; Hostettmann *et al.*, 2005). Another relatively simple and very important assay which acts as a screening for antibacterial activity is the disc diffusion method. The toxicity of the substances may be assayed by the use of *Artemia salina* larvae. This assay is rapid and has a high capacity to deliver rapid answer at relatively low costs (Meyer *et al.*, 1982).

Continuing our investigation on bioactive phenolic substances we relate here the results of the activity tests of 2,4-dihydroxy-6-*n*-pentylbenzoic acid and its derivatives against the fungus *Cladosporium sphaerospermum* through the bioautography method and the pathogenic strains of *Staphylococcus aureus* and *Escherichia coli* bacteria through the disc diffusion method. Since most phenolic compounds are toxic, mainly at elevated doses, their toxicity has been assayed by brine shrimp lethality.

Experimental

General experimental procedures

The ^{1}H NMR spectra were recorded in $CDCl_3$ at 300 MHz and the ^{13}C NMR spectra were recorded at 75 MHz in $CDCl_3$ on a Bruker DPX300 spectrometer. Solvent resonances were used as internal references. Column chromatography (CC) was carried out on a flash silica gel column (230–400 mesh). Purity of the samples was checked by TLC on pre-coated silica gel GF_{254} plates (0.25 mm thick, Merck), detected under UV light (254 nm), while methanol/sulfuric acid (10%) and *p*-anisaldehyde/sulfuric acid were used as spraying reagents.

Plant material, extraction and isolation of the compounds

The lichen *Cladina confusa* (Sant.) Folmm. & Ahti was obtained from a shop of decoration products. The identification was conducted by Prof. Dr. Mariana Fleig from UFRGS and Prof. Dr. Marcelo P. Marcelli from the Instituto de Botânica de São Paulo. A voucher specimen is kept in our laboratory for future reference.

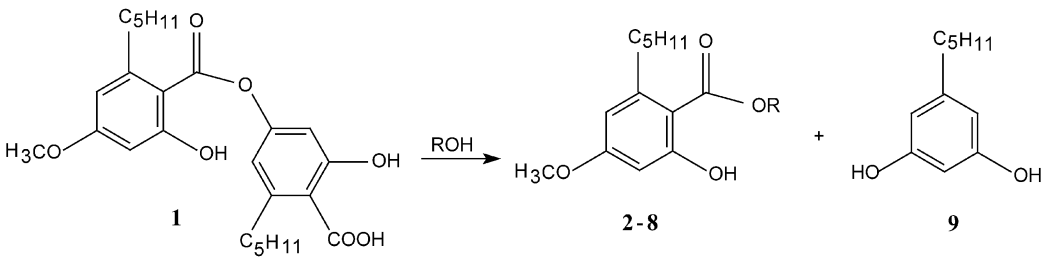
The dried lichen *C. confusa* (240.0 g) was extracted with hexane at room temperature, for four times. The extracts were concentrated and the residue was fractionated by silica gel CC, eluted with hexane/CH_2Cl_2 mixtures in gradient, resulting in perlatolic acid (**1**).

Obtaining derivatives

Perlatolic acid (**1**) (125 mg, 0.28 mmol) was treated with 50 mL of alcohol at 40 °C in a steam bath. After completation of the reaction, the mixture was concentrated and the compounds were separated by chromatography on a silica column with a hexane/CH_2Cl_2 gradient. In all reactions the corresponding esters 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate **2**–**8** and 1,3-dihydroxy-5-*n*-pentylbenzene (**9**) were obtained (Scheme I).

Methyl, ethyl and butyl perlatolates

Perlatolic acid (**1**) (0.54 mmol) was dissolved in acetone and 0.29 mmol of potassium carbonate was added. The mixture was cooled and stirred. After 10 min 5.7 mmol of the alkyl iodide were added. After 1 h the temperature was raised to room temperature and the mixture was stirred until the completion of the reaction (TLC control). The mixture was filtered, the solvent evaporated and the residue was purified by column chromatography. The elution was conducted with a hex-



Scheme I. Alcoholysis of perlatolic acid (**1**) producing 2-hydroxy-4-methoxy-6-*n*-pentylbenzoates **2**–**8** (methyl, ethyl, *n*-butyl, *n*-pentyl, isopropyl, *sec*-butyl and benzyl) and 1,3-dihydroxy-5-*n*-pentylbenzene (**9**).

ane/$CHCl_3$ gradient, except for *n*-butyl perlatolate (**12**), which was eluted with a hexane/acetone gradient. Methyl (**10**), ethyl (**11**) and *n*-butyl (**12**) perlatolates were obtained.

Methyl and ethyl 2,4-dihydroxy-6-n-pentylbenzoates

Methyl and ethyl perlatolates (0.32 mmol and 0.12 mmol, respectively) were dissolved in methanol and ethanol in excess, respectively. Each reaction was conducted in a steam bath at 60 °C (TLC control). After completion of each reaction the solvent was evaporated. The residue was fractionated by column chromatography with a hexane/acetone gradient and the pure fractions were joined. Methyl (**2**) and ethyl (**3**) 2-hydroxy-4-methoxy-6-*n*-pentylbenzoates and methyl (**13**) and ethyl (**14**) 2,4-dihydroxy-6-*n*-pentylbenzoates were obtained (Scheme II).

Alkaline alcoholysis of perlatolic acid

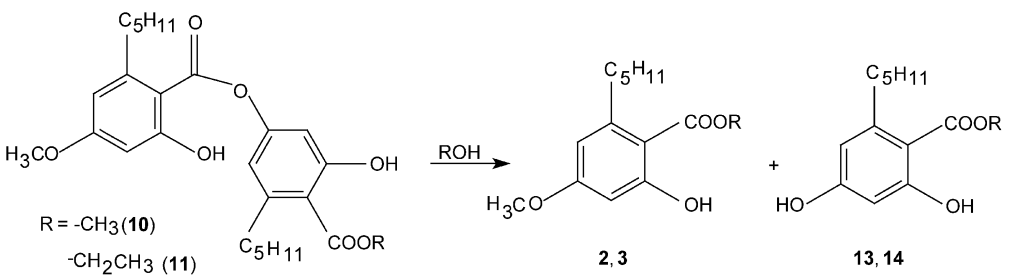
Methanol (15 mL) was added to 2.6 mmol of potassium hydroxide and 0.073 mmol of perlatolic acid. The mixture was stirred at room temperature, and after 2 h, HCl (0.1 M) was added until pH ~3.0. The solution was partitionated between water and chloroform. The chloroform layer was treated with water and NaCl, three times. The organic layer was dried with $MgSO_4$ and the solvent evaporated. The residue was fractionated on a silica gel column and the elution was conducted with a hexane/acetone gradient. Two compounds were isolated: methyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**2**) and the 2,4-dihydroxy-6-*n*-pentylbenzoic acid (**15**).

Antifungal assay

The evaluation of antifungal activity was conduced by bioautography methods. The fungus *Cladosporium sphaerospermum* was used in this study. This fungus was cultivated in potato dextrose agar (PDA), in the dark, at 28 °C until the formation of the appropriate spores. Solutions of pure compounds were freshly prepared and 25 μL of them were applied on Al-backed silica gel GF_{254} TLC sheets (Merck) to obtain the amounts of: 600.0, 500.0, 400.0, 300.0, 200.0, 150.0, 100.0, 50.0, 25.0, 10.0, 5.0, 2.5 and 1.0 μg. Amphotericin B (Fungizon®) was used as a positive control. After drying for complete solvent removal, a spore suspension in nutritious middle containing glucose was distributed over the plates and incubated for 48 h at 30 °C in polystyrene boxes in a moist atmosphere. The detection of fungitoxic activity of the compounds was performed by the observation of the inhibition zones of fungal growth, as described elsewhere (Homans and Fuchs, 1970).

Antibacterial assay

The *in vitro* antibacterial activity was tested by the disc diffusion method using pathogenic strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). The microorganisms were grown during 5 h at 37 °C in Mueller Hinton broth. The cultures were diluted in 0.45% of saline solution in Petri dishes containing Mueller Hinton agar. Solutions of compounds in DMSO at concentrations of 10 mg/mL each were prepared and impregnated on the discs (0.38 mm × 6 mm) at final doses of 200 μg per disc. Discs of amicacin were used as positive controls. The plates were incubated overnight at 37 °C and the diameter of any resulting inhibition zone (mm) was measured. LogP values were determined with the software



Scheme II. Alcoholysis of perlatolates **10** and **11** producing methyl (**2**) and ethyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoates (**3**) and methyl (**13**) and ethyl 2,4-dihydroxy-6-*n*-pentylbenzoates (**14**).

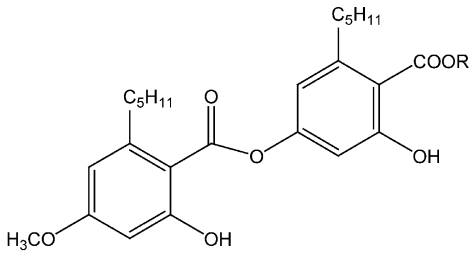
ACD logP (ACD/Labs 10.0, Advanced Chemistry Development INC., 2007).

Bioassays against Artemia salina

The assays against *A. salina* followed the methodology described by Meyer *et al.* (1982). The dried brine shrimp (*A. salina*) eggs were bred in saline solution (38 g L^{-1} in distilled water) and the recipient was illuminated with a 40 W lamp. After 48 h the larvae of *A. salina* were collected and added (ten per vial) to solutions of compounds **1**–**7**, which were solubilized in saline solutions with 1% DMSO. The bioassay were conducted in triplicate, and after 24 h contact the survivors were counted and LD_{50} values calculated using the software PROBITOS (Finney, 1971).

Results and Discussion

Fourteen compounds prepared by alcoholysis of perlatolic acid (**1**) or by alcoholysis of alkyl perlatolates, besides olivetol (**9**) and the 2,4-dihydroxy-6-*n*-pentylbenzoic acid (**15**), displayed activity against *C. sphaerospermum* using the bioautography method (Table I). Several of these compounds were potentially active such as methyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**13**) which showed the highest activity inhibiting the growth of the fungus at 2.5 μg. However, the ethyl ester **14** was less active (25.0 μg). 2,4-Dihydroxy-6-*n*-pentylbenzoic acid (**15**) and 1,3-dihydroxy-5-*n*-pentylbenzene (**9**) were very active inhibiting the fungus growth at 5.0 μg. The 2-hydroxy-4-methoxy-6-*n*-pentylbenzoates **2**–**8** were less active (100.0 to 300.0 μg). This difference may be related to the effect of the hydroxy group at C-4 in **13** and **14**, an important group for activity.



R = -CH_3 (**10**); -CH_2CH_3 (**11**); -$CH_2CH_2CH_2CH_3$ (**12**)

Fig. 1. Structures of methyl (**10**), ethyl (**11**) and *n*-butyl (**12**) perlatolates.

Methyl perlatolate (**10**) (Fig. 1) showed the same activity (10.0 μg) as perlatolic acid (**1**). Ethyl (**11**) and *n*-butyl perlatolates (**12**) were less active (50.0 μg) than perlatolic acid (**1**).

In vitro antibacterial activity was tested by the disc diffusion method using pathogenic strains of *S. aureus* and *E. coli*. Only compound **15** was not evaluated in this assay. In general, the experimental result of this test indicated a variable degree of efficacy of the compounds against the Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria (Table II). All evaluated compounds were active against *E. coli*, however, only perlatolic acid (**1**), 1,3-dihydroxy-5-*n*-pentylbenzene (**9**), methyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**13**) and ethyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**14**) were active against both bacteria. It is known that most Gram-positive bacteria are surrounded by a thick peptidoglycan cell wall. In contrast, Gram-negative bac-

Table I. Results of the bioautographic assay with *Cladosporium sphaerospermum* of compounds **1**–**15**.

Compound	Antifungal activity[a]
Perlatolic acid (**1**)	10.0
Methyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**2**)	100.0
Ethyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**3**)	300.0
Butyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**4**)	150.0
Pentyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**5**)	300.0
Isopropyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**6**)	150.0
sec-Butyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**7**)	150.0
Benzyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**8**)	200.0
1,3-Dihydroxy-5-*n*-pentylbenzene (olivetol) (**9**)	5.0
Methyl perlatolate (**10**)	10.0
Ethyl perlatolate (**11**)	50.0
n-Butyl perlatolate (**12**)	50.0
Methyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**13**)	2.5
Ethyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**14**)	25.0
2,4-Dihydroxy-6-*n*-pentylbenzoic acid (**15**)	5.0
Amphotericin B (Fungizon®)	5.0

[a] Minimal amount (μg) required for the inhibition of fungal growth on TLC plates.

Table II. Results of the antibacterial activity test of compounds **1**–**14** showing zone of inhibition (mm) against *S. aureus* and *E. coli.*

Compound	*S. aureus*	*E. coli*	LogP[a]
Perlatolic acid (**1**)	10	10	9.28 ± 0.42
Methyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**2**)	0	10	5.01 ± 0.26
Ethyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**3**)	0	10	5.54 ± 0.26
Butyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**4**)	0	10	6.61 ± 0.26
Pentyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**5**)	0	10	7.14 ± 0.26
Isopropyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**6**)	0	10	5.89 ± 0.26
sec-Butyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**7**)	0	9	6.42 ± 0.26
Benzyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**8**)	0	10	6.78 ± 0.27
1,3-Dihydroxy-5-*n*-pentylbenzene (**9**)	12	12	3.35 ± 0.21
Methyl perlatolate (**10**)	0	11	9.45 ± 0.42
Ethyl perlatolate (**11**)	0	10	9.98 ± 0.42
n-Butyl perlatolate (**12**)	0	10	11.05 ± 0.42
Methyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**13**)	14	12	4.50 ± 0.26
Ethyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**14**)	15	10	5.04 ± 0.26
Amicacin	27	28	–

[a] Values with a 95% confidence interval, obtained with the software ACD logP (ACD/Labs 10.00, Advanced Chemistry Development INC., 2007).

teria surround themselves with a second membrane, the outer membrane, rich in lipids and hydrophobic proteins (Nikaido, 1994). Probably the different susceptibilities of *S. aureus* and *E. coli* to the compounds assayed may be caused by the different permeability of the cell wall of the bacteria. The antibacterial activity of hydrophobic compounds may depend on their partition coefficients expressed as logP values (Togashi *et al.*, 2007). The logP values of compounds used in this study are listed in Table II. If activity to one or both bacteria is dependent on the partition coefficient, the activity would be expected to vary with logP values. However, our results do not support such a conclusion.

Perlatolic acid (**1**) exhibited the same inhibition zone for both bacteria (10 mm). However, its esters **10**–**12** were active only against *E.coli* (inibition zone of 10 mm), indicating that the free carboxy group of perlatolic acid seems to play an essential role on the antibacterial activity against *S. aureus.* The 2-hydroxy-4-methoxy-6-*n*-pentylbenzoic esters **2**–**8** exhibited inhibition zones between 9 and 10 mm showing that the elongation of the carbon chain does not change the antibacterial activity against *S. aureus* and *E. coli.* The 2,4-dihydroxy-6-*n*-pentylbenzoic esters **13** and **14** were active against both bacteria and they were the most active compounds against *S. aureus* (14–15 mm). Comparing the antibacterial activity results of esters **2**, **3** and **13**, **14** it should be noted that the presence of the free hydroxy group in the aromatic ring (C-4) of the compounds **13** and **14** is important for the antibacterial activity against *S. aureus.* Olivetol (**9**) showed inhibition zones of 12 mm against *S. aureus* and *E. coli*.

The LD_{50} values of toxicity assays obtained against *Artemia salina* of perlatolic acid (**1**) and 2-hydroxy-4-methoxy-6-*n*-pentylbenzoic esters **2**–**7** are listed in Table III. Perlatolic acid (**1**) and methyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**2**) were most active with LD_{50} values of 24.1 μM and 27.2 μM, respectively. The compounds **3**–**6** were not toxic against larvae (LD_{50} between

Compound	LD_{50} (μM)[a]
Perlatolic acid (**1**)	24.1 ± 8.78
Methyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**2**)	27.2 ± 12.7
Ethyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**3**)	1580.0 ± 123.0
Butyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**4**)	1090.0 ± 86.0
Pentyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**5**)	1750.0 ± 97.0
Isopropyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**6**)	> 1350.0
sec-Butyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**7**)	> 625.0

Table III. Toxicity against brine shrimp *A. salina* of compounds **1**–**7**.

[a] Values with a 95% confidence interval, obtained with the software PROBITOS.

1090.0 μM and 1750.0 μM). The esters **6** and **7** were not active in concentrations up to 1350.0 μM and 625.0 μM, respectively (Table III).

Conclusion

The results demonstrated that the fifteen compounds showed antifungal activity against *C. sphaerospermum*. Nevertheless, methyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**13**) was the most active compound, followed by 2,4-dihydroxy-6-*n*-pentylbenzoic acid (**15**), 1,3-dihydroxy-5-*n*-pentylbenzene (**9**), perlatolic acid (**1**), methyl perlatolate (**10**), and ethyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**14**)**.** Thus, these compounds are potent antifungal agents. Except for perlatolic acid (**1**) and methyl 2-hydroxy-4-methoxy-6-*n*-pentylbenzoate (**2**), the compounds **3**–**7** did not show toxicity against *A. salina*. Perlatolic acid (**1**), 1,3-dihydroxy-5-*n*-pentylbenzene (**9**), methyl (**13**) and ethyl 2,4-dihydroxy-6-*n*-pentylbenzoate (**14**) were active against *S. aureus* and *E. coli*. The other compounds were only active against *E. coli*, although the activities were not high.

Thus, perlatolic acid (**1**), methyl perlatolate (**10**), 2,4-dihydroxy-6-*n*-pentylbenzoic acid (**15**), the methyl ester **13** and olivetol (**9**) are potent antifungal agents.

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