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Preparation of berberrubine. Chemistry and Biological activity

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Introduction

Natural Products are undoubtedly one of the main sources for obtaining "leader structures". This is essentially due to the plant "adaptation" power by production of secondary metabolites, which are able to respond to a wide variety of external stimuli, and especially because of the tremendous structural diversity of these metabolites that escapes the imagination of any synthetic chemist. It is worth to remember that after a relative interest decrease in this kind of products, in the last decade a systematic increase of the interest in natural products as suppliers of potential new "leader compounds" has been observed.

Recent studies reported by our research group (Freile *et al.*, 2001, 2003; Freile, 2002; Károlyházy *et al.*, 2003) have shown that the studied species of the *Berberis* genus (Berberidaceae) have benzylisoquinolinic, protoberberinic and bisbenzylisoquinolinic alkaloids, some of which have an interesting antibacterial activity, and especially a potent antifungal activity. Moreover, theoretical calculations have allowed us to propose a mechanism of action at molecular level (Freile *et al.*, 2001; Freile, 2002). A number of chemical modifications that have been made to these structures allowed to assess the minimum structural requirements, and a possible pharmacophoric pattern for this type of alkaloids. To complete this study in the present work we report a chemical modification on the main alkaloid extracted from *B. heterophylla* (berberine) and its potential biological activities (antifungal and antibacterial).

Methodology

Structural modification of berberine

Berberine (**Fig. 1)** (113.2 mg) was heated to 220 ºC in a vacuum stove (20-30 mm_{Hg}) for 10 minutes. The crude product obtained was purified by preparative TLC to give berberrubine (76.1 mg), a red alkaloid soluble in MeOH.

The modified compound was separated by column chromatography (CC) and was purified by preparative TLC. Silica Gel 60 (Aldrich, 0.063-0.2 mm) was used for CC, Silica Gel 60 F_{254} chromatoplates (Merck) were used for TLC analysis. Solvents were purified by distillation.

The compound was identified using spectroscopic data. Nuclear Magnetic Resonance (^{1}H-NMR and ^{13}C-NMR) studies were performed on a Bruker AM-500 spectrometer using deuterated methanol as solvent. deuteric.

Bioassays

Whole-cell *in vitro* tests

a) Antifungal Tests

The antifungal activity was assessed by the agar dilution method using Saboureaud Agar-Chloramphenicol. The compounds were diluted in DMSO in dilutions that added to culture medium resulted in a concentration range of 100-250 µg/ml for the compound to be evaluated. The final DMSO concentration in the test medium was not higher than 2%. A drug-free solution was used as blank, and the antifungal agent miconazole was included as a positive control. Culture plates were incubated at 28 ºC for 24, 48 or 72 h (according to the growth of fungi control). MIC (Minimum Inhibitory Concentration) was defined as the minimum compound concentration that showed neither development nor growth of the fungus after incubation time.

Microorganisms and media for measuring antifungal action

Candida albicans ATCC 10231, *Candida tropicalis*, *Candida guillermondi* and *Saccharomyces cerevisiae* ATCC 9763 were provided by CEIMA (UNPSJB). Strains were cultured in a Sabouraud Agar Chloramphenicol for 48 hours at 30 °C. A cell suspension was performed in sterile distilled water, which was adjusted to a final concentration of 10^6 viable yeasts/ml.

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Dermatophytes: *Microsporum gypsium* C 115 and *Trichophyton mentagrophytes* ATCC 9972 provided by CEIMA. The microorganisms were cultured in Sabouraud Agar Chloramphenicol, and were subcultured every 15 days to prevent pleomorphic transformations. Spore suspensions were obtained according to previously reported procedures (Wright *et al.*, 1983) and were adjusted to 10^6 colony-forming units/ml.

***b*) Antibacterial Tests**

Antibacterial activity was assessed by the agar dilution method using the Müller-Hinton agar. The compounds were diluted in DMSO leading dilutions that added to the culture medium resulted in a concentration range of 100-250 µg/ml for the compound to be evaluated. The final DMSO concentration in the test medium was not higher than 2%. A drug-free solution was used as blank. Chloramphenicol was used as positive control. Culture plates were incubated at 37 ºC for 24-48 h. MIC was defined as the minimum compound concentration that showed neither development nor growth of bacteria after incubation time.

Microorganisms and media for measuring antibacterial action

Enterococcus faecalis, *Pseudomonas aeruginosa, Acinetobacter* spp. and *Proteus mirabilis* were obtained from clinical isolates, and provided by the Central Laboratory of the Regional Hospital of Comodoro Rivadavia.

Staphylococcus aureus ATCC 25923 and *Staphylococcus aureus* ATCC 29203 were provided by CEIMA (UNPSJB).

Microorganisms were cultured in Müller-Hinton Agar at 37 ºC for 24-48 h. Inocula were prepared from 4-5 colonies of equal morphology in a suspension of 4 ml of triptein- soy broth incubated at 37 °C until reaching standard turbidity (0.5 of Mc Farland).

Results

Structural modification of berberine

In order to study whether a hydroxyl group at C-9 of the berberine structure could improve its antimicrobial activity, a structural modification of this alkaloid was carried out. Berberine after being subjected to extreme temperature conditions was transformed into a compound that when analysed by TLC showed an R_f value lower than that observed for berberine, a red colouration unlike berberine which is yellow, and then gave positive Dragendorff, thus showing that it was another alkaloid. This new alkaloid was obtained with 67.23% yield. This alkaloid was identified by ^{1}H-NMR y ^{13}C-NMR spectroscopic data, and subsequently compared with literature data (Iwasa and Kamigauchi, 1996). Therefore, the structure accounted for the alkaloid berberrubine (Fig. 2), which has a hydroxyl group at C-9.

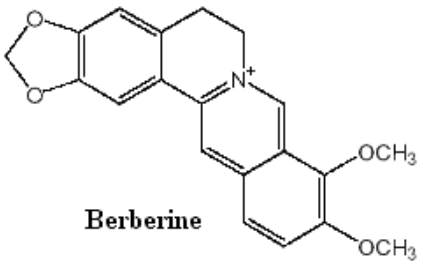

Figure 1. Structure of berberine

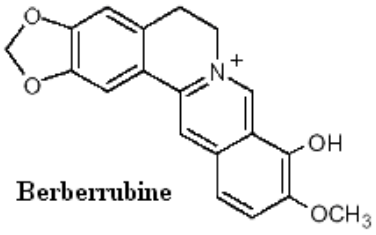

Figure 2. Structure of berberrubine

Antimicrobial activity of berberine and berberrubine

There are reports that indicate that the inclusion of hydroxyl groups on protoberberinic structures has increased antimalarial activity (Iwasa *et al.*, 1999).

On the basis of these data the antimicrobial activity of berberine and its structural modification (berberrubine) was determined.

Antifungal activity

The results of antifungal activities of both alkaloids are shown in Table 1, thus showing that berberrubine does not show a better antifungal activity than berberine against the tested fungi species, except against

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Saccharomyces cerevisiae ATCC 9763, which showed a MIC lower than 250 µg/ml.

Table 1: Antifungal effect (MIC, µg/ml) of berberine and berberrubine.

	A	B	C	D	E	F
Berberine (µg/ml)	**> 125**	**< 250**	> 250	> 250	> 250	> 250
Berberrubine (µg/ml)	> 250	> 250	> 250	**< 250**	> 250	> 250

A) Candida albicans ATCC 10231, *B) Candida tropicalis*, *C) Candida guillermondi*i, *D) Saccharomyces cerevisiae* ATCC 9763, *E) Microsporum gypsium* C 115, *F) Trichophyton mentagrophytes* ATCC 9972

Antibacterial activity

Results of antibacterial activities of berberine and berberrubine are shown in Table 2. According to the table, berberrubine did not show best antibacterial activity than berberine against tested bacterial strains.

Table 2: Antibacterial effect (CIM, µg/ml) of berberine and berberrubine.

	A	B	C	D	E	F
Berberine (µg/ml)	> 250	> 250	> 250	> 250	**> 125**	> 250
Berberrubine (µg/ml)	> 250	> 250	> 250	> 250	> 250	> 250

A) Enterococcus faecalis, *B) Proteus mirabilis*, *C) Acinetobacter* spp. *D) Pseudomonas aeruginosa*, *E) Staphylococcus aureus* ATCC 25923, *F) Staphylococcus aureus* ATCC 29203.

Conclusions

Our results indicate that the structural modification carried out on berberine has no significant antimicrobial activity. This would indicate that the presence of hydroxyl groups in these kind of protoberberinic structures does not improve antifungal and antibacterial activity.

Note: This study was presented at the "XXVI Congreso Argentino de Química" , San Luis, Argentina, 2006

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