STRUCTURE-ACTIVITY RELATIONSHIP OF BERBERINE AND DERIVATIVES ACTING AS ANTIFUNGAL COMPOUNDS

Enriz, R. D.

Facultad de Química, Bioquímica y Farmacia. Universidad Nacional de San Luis, Chacabuco 917. (5700) San Luis, Argentina.

Freile, M. L.

Facultad de Ciencias Naturales. Universidad Nacional de la Patagonia San Juan Bosco Km 4, Comodoro Rivadavia. (9000) Chubut, Argentina

FAX: +54-2652-431301, E-mail: mfreile@unpata.edu.ar

Received April 24th, 2006. In final form May 10th, 2006
Dedicated to Prof. Imre G. Csizmadia on the occasion of his 75th birthday

Abstract

A structure-activity relationship study of berberine and structurally related compounds is reported. Berberine, was chemically modified in order to obtain canadine and oxyberberine. The in vitro evaluation of antifungal activity of berberine, canadine, oxyberberine, liriodenine, o-methyl-mostachatoline and other four derivatives is also reported here. Our results allow us to determine the minimal structural requirements to produce the antifungal response and can provide a guide for the design of new compounds with these properties.

Resumen

Se presenta un estudio de correlación estructura-actividad de berberina y compuestos estructuralmente relacionados. Se modifico químicamente la berberina con el objeto de obtener canadina y oxiberberina. Se informa también la actividad antifúngica in vitro de bereberina, canadina, oxyberberina, liriodenina, o-metil-mostachatolina y otros cuatro derivados. Nuestros resultados nos han permitido determinar los mínimos requerimientos estructurales para producir la respuesta antifúngica y pueden dar una guía para el diseño de nuevos compuestos con estas propiedades.

Introduction

In the southern regions of Argentina (Patagonia), there are about sixteen different species of Berberis (*Berberidaceae*) commonly know as "calafate" or "michay". These plants are representative of this geographical zone and can vary in size from small shrubs to small trees.

Although some reports [1-4] indicate that other species of *Berberis* have been used as a remedy for different diseases, it appears that, at least in the south of Argentina, these plants had

not really been used with therapeutical purposes. First-hand interviews with local Indian communities in the state of Chubut (Argentina) indicate that the Indian people do not use "Calafate" as remedy, they use the roots only as a dye [5].

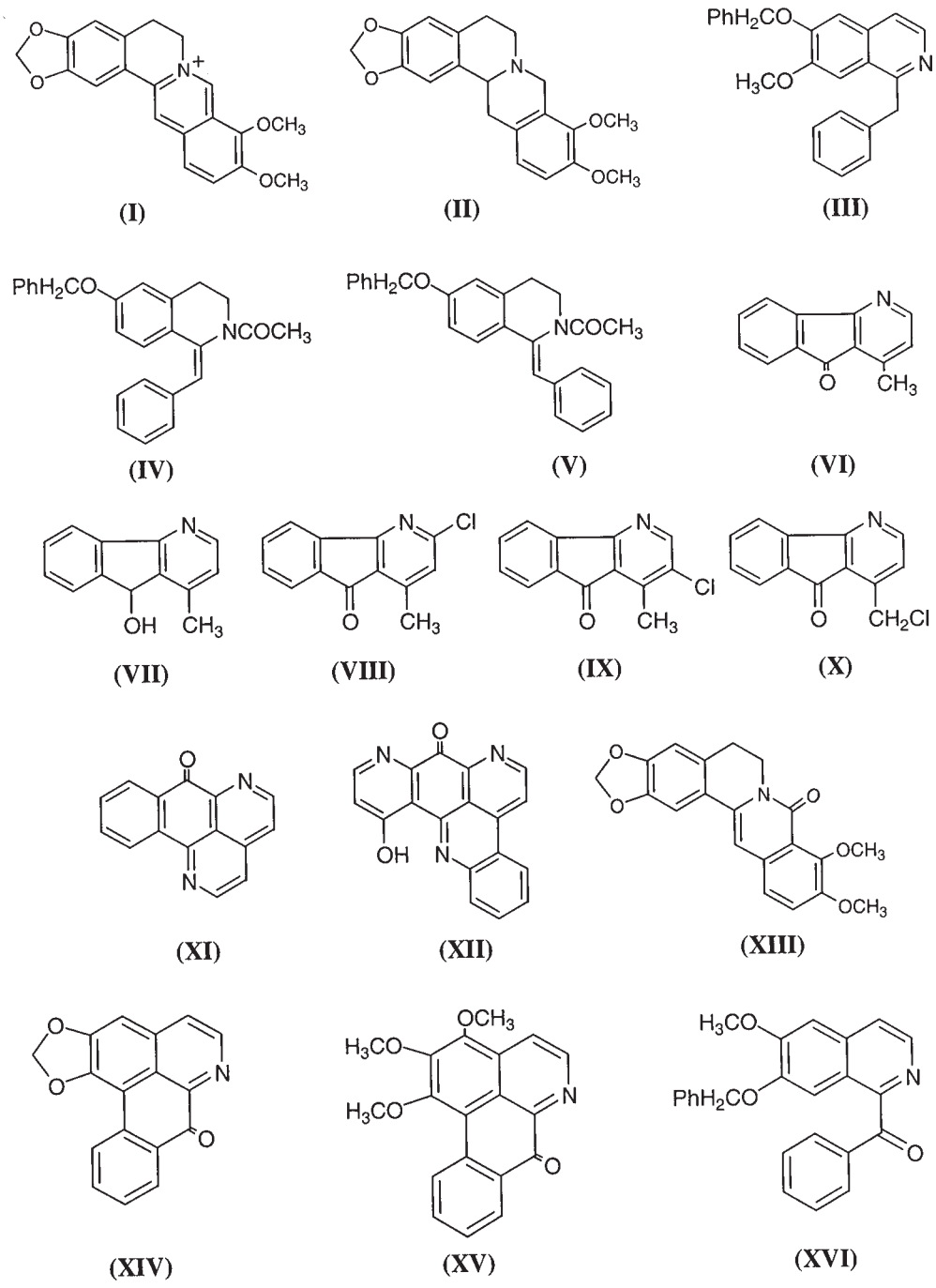

Figure 1 *–structures of alkaloids reported here and of structurally related compounds.*

Recently, we have reported that the aqueous extracts of *B.heterophylla* do not possess significant antimicrobial activity [6]. In addition the agar dilution method showed that none of the aqueous extracts tested displayed significant antifungal activity against dermatophytes fungi. These results account for, at least in part, the reason the Indian people of Patagonia do not use infusions of *Berberis heterophylla* for therapeutically. In contrast to those results, berberine (compound **I** in Figure 1) isolated from *B. heterophylla* displayed a moderate but significant antibacterial and antifungal activity against *Staphylococcus aureus* and different *Candida spp*. Also we evaluated berberine against a panel of *Candida spp* obtained from the clinical isolated. It is interesting to note that berberine displayed antifungal activity not only against standardized strains, but against clinical isolated of *Candidas.* We tested it against 7 clinical strains from different body humors from different immunocompromised patients. MIC (minimum inhibitory concentration) values ranged from >128 to 16 μg/ml were obtained for berberine indicating a moderate but significant activity against *C. glabrata*, *C. albicans*, *C. lusitaniae*, *C. krusei* and *C. Parapsilosis* [7].

A large number of studies have been performed in order to shed some light on the structural aspects and bioactivities of berberine and its congeners. However, compared with these aspects, the action mechanism of these alkaloids, at least at molecular level, has received relatively little attention. In this regard, previously we performed a computer-assisted study reporting the importance of aromatization within the putative bio-medical action mechanism of berberine and related cationic alkaloids with double iso-quinolinoid skeleton [8]. In this supposed mechanism of action of berberine, to prevent DNA replication, the first step is aromatization. In contrast to the covalent dehydrogenation, which is endothermic, the aromatization under ionic conditions was found to be exothermic [9]. Our results indicated that in the aromatization process the ease of hydride ion removal parallels the stabilization energy of the aromatic compounds to be formed. Comparing the nucleophylic additions to the pi-systems, LUMO (Lowest-Unoccupied-Molecular-Orbital) energy values suggested a greater accessibility of the N(+) heterocycles in comparison to the polycicle aromatic hydrocarbons. Thus, it appears that the quaternary nitrogen and the aromatic polycyclic and planar structure of berberine is the pharmacophoric pattern to produce the antifungal effect in this alkaloid.

With the aim to finding an experimental support for the above hypothesis, we report here the obtention of some analogues of berberine, their antifungal effects as well as the structure-activity relationship study performed on these alkaloids acting as antifugal compounds.

Experimental section

Chemistry

Canadine (compound II in Table 1)

Berberine chloride (105 mg) was dissolved in aqueous acetic acid 66.6 % (9 ml), Zn powder was added (10.54 g) with an aqueous HCl solution 35% (23 mL). The solution was refluxed for 2 h at 100 ºC, when TLC indicated a complete reaction. The reaction mixture was neutralized with an aqueous $HONH_4$ 30% (20 mL). It was extracted with CH_2Cl_2 (3 x 15 mL). The combined CH_2Cl_2 extracts were dried, filtered, and evaporated to yield Canadine (65.4 mg). Canadine was purified by column chromatography and then identified by ^{1}H and ^{13}C NMR and compared with spectral data from literature [10].

Oxyberberine (XIII)

Table 1. *MICs (¼g/ml) obtained for the different compounds. Active values are denoted in bold.*

Compounds	A	B	C	D	E	F	G
I*	**64**	**32**	**32**	**16**	**128**	**128**	**64**
II	>128	>128	>128	>128	>128	>128	>128
III	>128	>128	>128	>128	>128	>128	>128
IV	>128	**128**	**64**	**128**	**128**	>128	**128**
V	>128	**128**	**64**	**128**	**128**	>128	**128**
XIII	>128	>128	>128	>128	>128	>128	>128
XIV	>128	>128	>128	>128	>128	>128	>128
XV	>128	>128	>128	>128	>128	>128	>128
XVI	>128	**128**	**128**	**128**	**128**	>128	**128**

A) *C. albicans* (00-604); **B**) *C. glabrata*; **C**) *C. lusitaniae*; **D**) *C. krusei*; **E**) *C. parapsilosis* (951706); **F**) *C. albicans* (00-622); **G**) *C. parapsilosis* (00-629).
* Taken from reference 7

Berberine chloride (50 mg, 0.134 mmol) was dissolved in 20 % aqueous KOH (20 mL). The solution was refluxed for 6 h at 80 ºC, when TLC indicated complete reaction. The reaction mixture was extracted with CH_2Cl_2 (3 x 15 ml). The combined CH_2Cl_2 extracts were dried, filtered, and evaporated to yield oxyberberine (46.5 mg, 98.5%). The oxyberberine was purified by column chromatography and then identified by ^{1}H and ^{13}C NMR and compared with the spectral data from literature[11].

Compounds III (see reference [12]) **IV** and **V** [13]; and the oxoaporphines **XIV**, **XV** and **XVI** (reference [14]) have been previously reported and were kindly provided by Professor Dr. D. Cortes-Martinez (Universidad de Valencia, España).

Biological assays

Microorganism and media

The microorganisms used for the fungistatic evaluation were obtained from the Department of Mycology INEI ANLIS "Dr. Carlos G. Malbrán" Bs.As. Argentina: *Candida albicans* 00-604 (soft bone), *Candida glabrata* 00-547 (hisopado), *Candida haemulonii* 982822,1 (hemocultive), *Candida lusitaniae* 00-623 (BAL) and *Candida parapsilosis* 00-629 (cirugy injury) were used. All of them from clinical isolated.

Antifungal assays

The antifungal activity was evaluated using the microdilution technique in media RPMI 1640. These bioassays were performed following references [15 –17].

Results and Discussion

As was previously mentioned, in the putative mechanism of action for berberine to prevent DNA replication, the first step is aromatization. With the aim to finding an experimental support for the above hypothesis, as a first step of our study we obtain canadine (compound **[II]** in Figure 1) from berberine. Canadine was inactive against all the fungi tested here (Table 1). It should be noted that there are few structural differences between berberine and canadine. The nitrogen atom is not quaternary in canadine, besides one of the rings is not aromatic in this alkaloid (compare structures **(I)** and **(II),** Figure 1). At this stage of our study we assume that the electronic effects appear to be more important than conformational factors in the production of the antifungal response. Next we evaluate three compounds structurally related to berberine and canadine. They are compounds **III**, **IV** and **V**. These molecules were kindly provided by Professor Diego Cortes-Martinez (University of Valencia, Spain). It should be noted that these compounds were chosen considering their different molecular flexibility. Compounds **IV** and **V** are conformationally restricted due to the presence of a double bond in the connecting chain. In addition these molecules possess a $NCOCH_3$ moiety. None of these compounds displayed significant antifungal activity (Table 1). These results provide additional support for the hypothesis suggesting that the quaternary nitrogen, the aromatic polycyclic and the planar structure of berberine is the pharmacophoric pattern to produce the antifungal effect of these alkaloids.

Alkaloids possessing a carbonyl group

Evaluation of the antifungal activity of onychine **[VI]**, an azafluorene alkaloid, showed it to have anticandidal (MIC of 3.12 μg/ml) and anticryptococcal (MIC of 3.12 μg/ml) activities [18-19]. Subsequent structure-activity relationship studies revealed that the carbonyl moiety plays a key role in the activity of these compounds. Thus, partial reduction of the carbonyl moiety led to inactive dihydroonycine **(VII)**. Evaluation of the chlorinated isomeric agents **(VIII -X)**, revealed that modification of the 4-methyl group to a chloromethyl moiety enhanced as well as broadened the antifungal profile of azafluorenones [20-22]. In the last two decades some structurally related and biologically active polycyclic aromatic alkaloids were isolated or re-synthesized [23,24]. Several of these were evaluated for antifungal activity. Among these, sampagine **[XI]** has an EC_{50} (median effective concentration) of 1.6 μg/ml against *C. albicans* [23] and zones of inhibition of 25-31 nm against *C. albicans, C. glabatra* and *C tropicalis.* which are comparable to ketoconazole. Sampagine also showed potent activity against *C. Neoformans* and *A. Fumigatus*. Other polyaromatic structures were evaluated for their antifungal activity. Meridine **(XII)** has been reported to inhibit the growth of *C. Albicans*, *C neoformans* by interacting with nucleic acid biosynthesis [25].

Berberine was modified in order to obtain a polyaromatic derivative possessing a carbonyl group, oxyberberine **(XIII)**. Unfortunately oxyberberine was inactive against all the fungi tested (see Table 1).

In parallel we evaluate the oxoaporphines liriodenine **(XIV)**, o-methyl-mostachatoline **(XV)** and compound **XVI,** [14,26], all alkaloids possessing a carbonyl group in their structures and structurally related to oxiberberine. These compounds showed marginal or null antifungal activity. Thus, compound **XVI** only displayed a moderate antifungal effect (MIC=128 μg/ml); whereas compounds **XIV** and **XV** were inactive (Table 1).

Berberine isolated from *B. Heterophylla* displayed an interesting *in vitro/in vivo* antifungal activity against a panel of pathogenic fungi [6,7] In addition berberine displayed a lower toxic effect in comparison with ketoconazole. Unfortunately all the compounds tested here were inactive or at least displayed lower antifungal activity than those reported for berberine. On the basis of our results it appears that berberine is the best starting structure to look for new cationic alkaloids with double isoquinolinoid skeleton acting as antifungal agents.

Conclusions

Previously we have reported the importance of aromatization within the putative bio-medical action mechanism of berberine and related cationic alkaloids with double iso-quinolinoid skeleton. It appears that the quaternary nitrogen and the aromatic polyciclic and planar structure of berberine could be the pharmacophoric pattern for these alkaloids acting as antifungal agents. All the compounds tested and reported here give an additional support for this hypothesis.

Acknowledgements

This work was supported by grants from National University of San Luis, Argentina, ANPCyT (PICTR -00260). The authors thank Professor Dr. Diego Cortes for samples of some alkaloids. R.D Enriz is a member of CONICET, Argentina.

References

[1] Bottini, C.M. ; Bran, D., *Arbustos de la Patagonia. Calafates y Michay* In:*Presencia*, Casamiquela , C. (Ed.), INTA (30), Argentina **1993**, pp. 5-9.

[2] Donoso Zegers, C.; Ramírez Garcia, C. *Arbustos Nativos de Chile* In: Cúneo V.M. (Ed.), *Colección Naturaleza de Chile,* Valdivia-Chile, **1994**, pp. 27-32.

[3] Ivanoska, N.; Philipov, S. *Int. J. Immunopharmacol.* **1996,** *18 (10)*, 553.

[4] Pamplona, R.J. *Berberis vulgaris* In Safeliz, S.L. (Ed.), *Enciclopedia de las Plantas Medicinales*, Madrid (España) **1996**, pp. 22-54.

[5] Freile, M.L. Personal interview on June 30th, 2001 at the National University of Patagonia SJB with Nahuel Pan Chief's wife (Tehuelche community in the Nahuel Pan zone).

[6] Freile, M.L.; Giannini, F.; Sortino, M.; Zamora, M.; Juarez, A.; Zacchino, S.; Enriz, R. *Acta Farm Bonaerense.* **2006**, *25(1)*, 83.

[7] Freile, M.L.; Giannini, F.; Pucci, G.; Sturniolo, A.; Rodero, L.; Pucci, O.; Balzareti, V.; Enriz, R. *Fitoterapia*. **2003***, 74*, 702.

[8] Freile, M.L.; Masman, M.; Suvire, F.; Zacchino, S.; Balzaretti, V.; Enriz, R. *J. Mol. Struct.(TEOCHEM)*. **2001**, *546*: 243.

[9] Freile, M.L. *Busqueda de nuevos Compuestos Antifungicos y citotoxicos. Estudio de Correlacion estructura-actividad y Mecanismo de Acción.* Tesis Doctoral UNPSJB **2002**, pp. 128.

[10] Pinho, P.M.; Pinto, M.M.M.; Kijjoa, A.; Pharadi, K.; Diaz, J.G.; Herz, W. *Phytochemistry.* **2002**, *31*, 1403.

[11] Gonzalez, M.C.; Zafra-Polo, M.C.; Blázquez, M.A.; Serrano, A.; Cortes, D. *J. Nat Prod*. **1997**, *60 (2)* 108.

[12] Bermejo, A.; Andreu, I.; Suvire, F.; Leonce, S.; Caignard, D.H.; Renard, P.; Pierre, A.; Enriz, R.; Cortes, D. *J. Med. Chem*. **2002**, *45*, 5058.
[13] Andreu, I.; Cortes, D.; Protais, P.; Casesels, B.K.; Chagraoui, A.; Cabedo, N. *Bioorg. Med. Chem*. **2000,** *8*, 889.
[14] Leboeuf, M.; Cortes, D.; Hocquemiller, R.; Cavé, A. *Planta Medica.* **1983**, *48*, 185.
[15] Fromtling, R.F.; Galgiani, J.N.; Faller, M.A.P. *Antimicrob Agents Chemoter.* **1993**; *37*, 39.
[16] Faller, M.A.P.**;** Rinaldi, M.G.; Galgiani, J.N. *Antimicrob Agents Chemoter.* **1990**, *34*, 1648.
[17] Faller, M.A.P.; Grant, C.; Morthland, V.; Rhine Chalberg, J.J. *J. Clin. Microb*. **1994**; *32(2)*, 506.
[18] Koyama, J.; Sugita, T.; Suzuta, Y. *Heterocycles* **1979**, *12*, 1017.
[19] Hufford, C.D.; Liu, S.; Clark, A.M.; Oguntimein, B.O. *J. Nat Prod.* **1987,** *50*, 961.
[20] Bracher, F. *Prarmazie* **1993**, *48*, 521.
[21] Bracher, F. *Arch Pharm /Wheinheim*, **1994**, 327-371
[22] Koyama, J.; Okatani, T.; Tagahara, K. *Heterocycles* **1989**, *29*, 1649.
[23] Kitahara, Y.; Onikura, H.; Shibano, Y.; Watanabe, S.; Mikami, Y.; Kubo, Y. *Tetrahedrom*, **1997**, *53*, 6001.
[24] Feliu, L.; Ajana, W.; Alvarez, M.; Joule, J.A. *Tetrahedron,* **. 1997,** *53*, 4511.
[25] McCarthy, P.; Peterson, T.; Gunawardana, G. *31 st ICAAC*, **1991**, Abstract Nº 212.
[26] Cortes, D.; Hocquemiller, H.; Leboeuf, M.; Cavé, A. *J. Nat. Prod*. **1986** , *49(5*), 878.