

Cytotoxic Activity of Orsellinates

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The series of 2,4-dihydroxy-6-methylbenzoates **2–10** (methyl to hexyl orsellinates) prepared by alcoholysis of lecanoric acid (**1**) – a natural product from the lichen *Parmotrema tinctorum* (Nyl.) Hale – was submitted to the brine shrimp lethality test (BST), which was also performed for 2,4-dihydroxy-6-methylbenzoic acid (**11**) (orsellinic acid) and the derivative ethyl-2-hydroxy-4-methoxy-6-methylbenzoate (**12**) (4-methoxy-ethyl orsellinate), in order to detect new substances with probable antineoplastic activity. Results showed that chain elongation – increase in lipophilicity (log *P*) – causes a rise in the cytotoxic activity of orsellinates. Hexyl orsellinate (**7**) showed the highest cytotoxic activity (LC₅₀ = 31 μM). A correlation between lipophilicity (log *P*) and cytotoxic activity (log 1/LC₅₀) is presented. Compounds with ramified chains – *iso*-propyl, *sec*-butyl and *tert*-butyl orsellinates (**8–10**) – were less active than those with the correspondent linear chain. The activities presented by 4-methoxy-ethyl orsellinate (**12**) and ethyl orsellinate (**3**) suggest that the hydroxy group at the C-4 position causes effect in the cytotoxic activity of orsellinates against *Artemia salina*.

Key words: Orsellinates, Lichen, *Artemia salina*, *Parmotrema tinctorum*

Introduction

Because many natural compounds, upon being isolated and characterized, are not submitted to biological assays, their biological properties may remain unknown for years. This lack may be explained by difficulties in performing biological assays in laboratories of natural products, since the search for specific pharmacological activities, such as antineoplastic activity, relies on expensive and time-consuming procedures. Generally bioassays are therefore employed if the detection of large spectra of pharmacological activities is seen as desirable. For this purpose, the brine shrimp lethality test (BST) has proved to be an excellent procedure for performing an early scanning for bioactive compounds that can subsequently be submitted to more refined tests for biological activities (Meyer *et al.*, 1982).

Solis *et al.* (1993), evaluating the cytotoxic activities of 21 compounds against the microcrustacean *Artemia salina* (brine shrimp), found that nearly all of the active substances tested also presented toxicity against KB cells (human nasopharyngeal carcinoma), the exceptions being two compounds that required metabolic activation in humans.

The cytotoxic activity against *A. salina* can thus be regarded as a useful method for carrying out a preliminary search for candidate substances to be later tested for their ability to act as antineoplastic agents.

Many phenolic substances isolated from lichens, as well as their derivatives obtained by structural modification, are known for their ample variety of pharmacological activities, including antineoplastic ones (Fournet *et al.*, 1997; Neamati *et al.*, 1997; Ingólfssdóttir *et al.*, 1998; Perry *et al.*, 1999; Kumar and Müller, 2000; Hirayama *et al.*, 1980; Takai *et al.* 1979).

In order to obtain potentially bioactive compounds, and mainly those with the desirable antineoplastic activity, lecanoric acid (**1**) – the main constituent of the lichen *Parmotrema tinctorum* (Nyl.) Hale – was chemically modified to produce as derivatives the 2,4-dihydroxy-6-methylbenzoates **2–10** (methyl to hexyl orsellinates). The derivatives 2,4-dihydroxy-6-methylbenzoic acid (**11**) (orsellinic acid) and ethyl-2-hydroxy-4-methoxy-6-methylbenzoate (**12**) (4-methoxy-ethyl orsellinate) were also obtained, so that the influence of selected functional groups on the cytotoxic activity

against *A. salina* could be investigated. Studies concerning quantitative relationships between cytotoxic activity and chemical structures (QSARs) were also performed for the derivatives obtained.

Material and Methods

Preparation of derivatives

Lecanoric acid (**1**) was isolated and purified from *Parmotrema tinctorum* according to Ahmann and Mathey (1967). This lichen was collected in Mato Grosso do Sul state, Brazil, in March 1999, and a voucher specimen is kept in our laboratory for future reference.

The 2,4-dihydroxy-6-methylbenzoates **2–10** (orsellinates) were obtained through alcoholysis of **1** (Scheme 1) according to the methodology described by Bachelor *et al.* (1979). These compounds were then separated in a flash silica gel chromatographic column with CHCl_3 . Orsellinic acid (**11**) was separated with CHCl_3 /acetone (93:7 v/v).

The derivative 4-methoxy-ethyl orsellinate (**12**) was obtained through methylation of ethyl orsellinate (**3**), following the methodology described by Elix *et al.* (1990). It was separated in a flash silica gel chromatographic column with hexane/ethyl acetate (70:30 v/v).

The ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained with the use of the solvents DMSO-d_6 for **1**, acetone-d_6 for **2–11** and CDCl_3 for **12**. FT-IR and EI mass spectra were also obtained. The spectroscopic data of compounds **1–12** were in accordance with those of Huneck and Yoshimura (1996).

Bioassays against *Artemia salina* (BST)

BST followed the methodology described by Meyer *et al.* (1982). The dried brine shrimp (*A.*

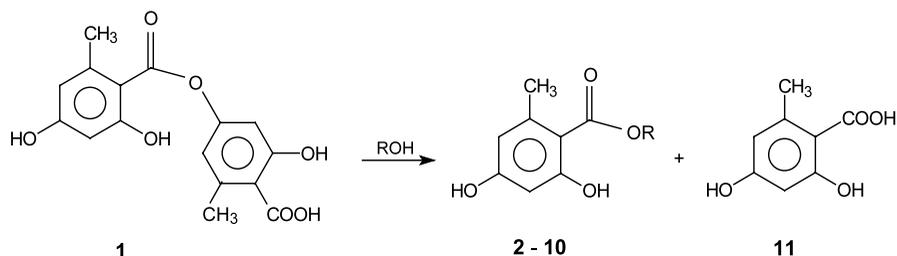
salina) eggs were bred in saline solution (38 g L^{-1} in distilled water) and the recipient was illuminated with a 40 W lamp. After 48 h the larvae of *A. salina* were collected and added (ten per vial) to solutions of the compounds **1–12**, which were solubilized in saline solution with 1% DMSO. Atropine sulfate was used as control. The bioassays were conducted in triplicate, and after 24 h contact the survivors were counted and LC_{50} values calculated using the software PROBITOS (Finney, 1971). QSAR studies were conducted by relating the cytotoxic activities against *A. salina* and the lipophilicities of the derivatives expressed by their $\log P$ values. Such $\log P$ values were determined with the software ACD LogP (ACD/LogP 1.0, Advanced Chemistry Development INC.; ACD/LOG 1.0, Toronto, 1994). The pK_a values were calculated with SPARC (2004).

Results and Discussion

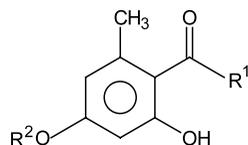
All the derivatives obtained were active against *A. salina*. Some of them, as was the case for *n*-pentyl orsellinate (**6**) and *n*-hexyl orsellinate (**7**), displayed pronounced activities as compared with known cytotoxic agents such as podophyllotoxin and emetine. The $\log P$ values and the results for the cytotoxic activity of the compounds against *A. salina* are listed in Table I.

Table I reveals that an increase in lipophilicity, mainly because of chain elongation and evidenced by an increase in the $\log P$ values, causes an increment in the cytotoxic activity. This can be seen in the homologous series methyl orsellinate (**2**) – hexyl orsellinate (**7**).

Lipophilicity was also affected by interchanges in the functional groups, which significantly altered the biological activity within the series. The validity of the $\log P$ values as a predictor of biolo-



Scheme 1. Alcoholysis of lecanoric acid (**1**) producing 2,4-dihydroxy-6-methylbenzoates **2–10** (methyl to hexyl orsellinates) and 2,4-dihydroxy-6-methylbenzoic acid (**11**) (orsellinic acid).



Compound	R ¹	R ²	log <i>P</i> ^a	LC ₅₀ [μM] ^b
2	OCH ₃	H	2.38 ± 0.33	1100 ± 108
3	OCH ₂ CH ₃	H	2.91 ± 0.33	495 ± 36
4	O(CH ₂) ₂ CH ₃	H	3.44 ± 0.33	137 ± 13
5	O(CH ₂) ₃ CH ₃	H	3.97 ± 0.33	85 ± 25
6	O(CH ₂) ₄ CH ₃	H	4.50 ± 0.33	39 ± 8
7	O(CH ₂) ₅ CH ₃	H	5.03 ± 0.33	31 ± 6
8	OCH(CH ₃) ₂	H	3.26 ± 0.33	229 ± 24
9	OCH(CH ₃)(CH ₂ CH ₃)	H	3.79 ± 0.33	123 ± 11
10	OC(CH ₃) ₃	H	3.61 ± 0.33	210 ± 26
11	OH	H	2.06 ± 0.33	> 800
12	OCH ₂ CH ₃	CH ₃	3.42 ± 0.34	93 ± 45
Atropine sulfate	–	–	–	>1000
Emetine HCl	–	–	–	29 ± 6,8 ^c
Podophyllotoxin	–	–	–	15 ± 36 ^c

Table I. Cytotoxic activity against brine shrimp (*Artemia salina*) and log *P* values for the compounds tested.

^a Values with a 95% confidence interval, obtained with the software ACD-LogP.

^b Values with a 95% confidence interval, obtained with the software PROBITOS.

^c Values obtained by Solis *et al.* (1993).

gical activity can be perceived when the activities of ethyl orsellinate (**3**) and 4-methoxy-ethyl orsellinate (**12**) are compared: indeed, the latter, being more active because of its greater log *P* value. Another interesting aspect is the similarity in the activities of substances with similar log *P* values, as, for example, propyl orsellinate (**4**) and 4-methoxy-ethyl orsellinate (**12**). Also noteworthy is the decreasing activity shown in BST of the compounds with chain ramification. Indeed, propyl orsellinate (**4**) is more potent than *iso*-propyl orsellinate (**8**), and butyl orsellinate (**5**) is more active than *sec*-butyl orsellinate (**9**) and *tert*-butyl orsellinate (**10**).

Most of the phenols exert their toxicity via hydrophobicity by perturbing the protective lipid membrane bilayer surrounding cells. However, p*K*_a is also another parameter that contributes to the toxicity of phenols. The effects of phenoxy radicals would depend on the type of chemical entities with which they interact, their location in the cells and their importance in the various phases of the cell cycle. The problem is complex and has been the aim of several studies (Hansch *et al.*, 2000; Selassie *et al.*, 1998). The compounds **2–10** have p*K*_a values (C4–OH) around 8.0 and they are nearly 10% ionized at pH 7.0. Although these compounds produce phenoxy radicals, the difference of toxicity of them depends mainly on the hydrophobicity and in this case on the chain elongation of the ester. There are many studies about

toxicity of phenols where good correlations have been obtained based on hydrophobicity alone (Selassie *et al.*, 1998).

A quantitative correlation between lipophilicity and cytotoxic activity against *A. salina* was performed for substances **2–11** according to the model proposed by Hansch *et al.* (2000), namely log (1/LC₅₀) × log *P*:

$$\log (1/LC_{50}) = -5.9 (\pm 2.56) + 1.48 (\pm 1.39) \log P - 0.12 (\pm 0.19) (\log P)^2, \quad (1)$$

$$n = 10, \quad r^2 = 0.9284, \quad s = 0.1428, \quad F = 45.42, \\ p < 0.0001, \quad q^2 = 0.9356, \quad s_{\text{press}} = 0.0513.$$

The correlation presented a suitable extinction coefficient ($r^2 = 0.9284$), and extrapolation to maximum activity led to a log *P* value close to 7, which would correspond to decyl orsellinate. Cross validation (Gaudio and Zandonade, 2001) confirmed the applicability of the model ($q^2 = 0.9356$, $s_{\text{press}} = 0.0513$). In Table II the predictions for cytotoxic activity obtained by using the equation (1) of the above-mentioned model are compared with the experimental values.

Overall, the results shown in Table II reveal that the activities of the orsellinates were predicted with good precision. Another important aspect is the possibility of inferring the activities of substances within the same series. These mathematical models can also be used for predicting the activities of analogous compounds, for which it is suffi-

Table II. Comparison between experimental and calculated values of LC_{50} for the cytotoxic activity of orsellinates against *A. salina*.

Compound	LC_{50} [μM] experimental	LC_{50} [μM] calculated by regression using $\log P \times \log (1/LC_{50})$
2	1100 \pm 108	1140
3	495 \pm 36	407
4	137 \pm 13	169
5	85 \pm 25	82
6	39 \pm 8	47
7	31 \pm 6	31
8	229 \pm 24	224
9	123 \pm 11	103
10	210 \pm 26	132
12	93 \pm 45	172

cient to substitute the $\log P$ value of the substance into the equation of the model proposed.

According to Gaudio and Zandonade (2001), the validation of this model, and hence of the predictions shown in Table II, can be confirmed by obtaining the linear correlation between the values of experimental $\log (1/LC_{50})$ and calculated $\log (1/LC_{50})$. Fig. 1 shows the results of this analysis.

For the model (Fig. 1), only two out of 10 compounds studied lie outside the area defined by the solid line, which corresponds to the graph region where there is a 95% probability for the experimental-versus-calculated LC_{50} line to be located. These two compounds [*tert*-butyl orsellinate (**10**)

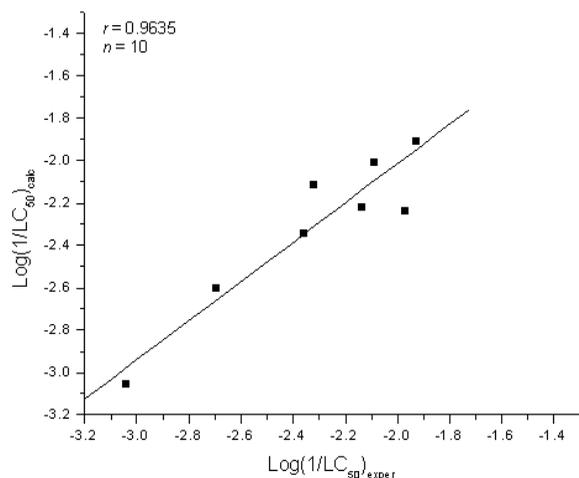


Fig. 1. Linear correlation between experimental cytotoxic activity against *A. salina* and calculated activity for orsellinates.

and 4-methoxy-ethyl orsellinate (**12**)], however, are not far enough from this region to be regarded as outlying. The mathematical model thus satisfies the correlation between cytotoxic activity against *A. salina* and lipophilicity of orsellinates.

It is important to point out that, because the mathematical model presented only takes lipophilicity into account, it may fail to predict whether other variables can have an influence on the activity. Such variables might include, for example, spatial and electronic order, or might be related to a specific metabolism of action which is different from that one presented by the substances of this work. It is also important to point out that, until the moment, the elucidative mechanism for the cytotoxic action of this class of compounds against *A. salina* is unknown.

Conclusion

Through alcoholysis of lecanoric acid (**1**) the homologous series methyl orsellinate (**2**) – hexyl orsellinate (**7**) was obtained, along with orsellinic acid (**11**) and compounds with ramified chains – *iso*-propyl (**8**), *sec*-butyl (**9**) and *tert*-butyl orsellinates (**10**). 4-Methoxy-ethyl orsellinate (**12**) was also prepared.

SAR and QSAR studies were employed to investigate the derivatives obtained regarding their cytotoxic activities against the microcrustacean *Artemia salina*. Results showed that chain elongation – increase in lipophilicity evidenced by $\log P$ values – causes a rise in the cytotoxic activity of orsellinates. Hexyl orsellinate (**7**) was the compound that displayed the highest cytotoxic activity ($LC_{50} = 31 \mu M$). In contrast, ramification in the carbon lateral chain led to a decreased activity. The compounds with chain ramification – *iso*-propyl orsellinate (**8**), *sec*-butyl orsellinate (**9**) and *tert*-butyl orsellinate (**10**) – were less active than the correspondent compounds of linear chain – propyl orsellinate (**4**) and butyl orsellinate (**5**). The activity of 4-methoxy-ethyl orsellinate (**12**) indicates that the hydroxy group in position 4 is an accessory site to the cytotoxic activity.

The quantitative correlation between lipophilicity and cytotoxic activity against *A. salina* presented a suitable extinction coefficient and the mathematical model reveal that the activities of the orsellinates were predicted with good precision. The validation of the model was confirmed by obtaining the linear correlation between the

values of experimental log (1/LC₅₀) and calculated log (1/LC₅₀).

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