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Investigación

Inhibition of Phosphofructokinase by Molluscicidal Sesquiterpene Lactones

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Dedicated to Professor Fernando Walls

Abstract. The effect of ten molluscicidal sesquiterpene lactones was tested on the enzyme phosphofructokinase (PKF) from rabbit muscle. PFK was inhibited irreversibly by all the sesquiterpene lactones tested. 7α-Hydroxy-3-desoxyzaluzanin C (**2**) exhibited the highest efficacy of inhibition with a K_i value of 90 nM. This is also the lowest K_i reported for inactivation of this enzyme by sesquiterpene lactones. The molluscicidal action of the sesquiterpene lactones tested in this study correlates closely with their potency as PFK inhibitors. Preincubation of **2** with dithiothreitol resulted in loss of inhibitory power of the lactone. The substrates adenosine-5'-triphospate and fructose-6-phosphate protected phosphofructokinase against inhibition by **2**. The PFK inhibitory action and molluscicidal activity are most significant when a hydroxyl group is in proximity of the nucleophile receptor site, the α-methylene-γ-lactone moiety.
Keywords: Sesquiterpene lactones, phosphofructokinase inhibitors, molluscicidal activity.

Resumen. Se evaluó el efecto de diez lactonas sesquiterpénicas molusquicidas sobre la enzima fosfofructocinasa (FFC) obtenida a partir de músculo de conejo. La FFC se inhibió irreversiblemente por todas las lactonas probadas. 7α-hidroxi-3-desoxizaluzanina C (**2**) mostró la mayor eficiencia de inhibición con un valor de K_i de 90 nM. Este es también el valor de K_i mas bajo informado para la inactivación de esta enzima para lactonas sesquiterpénicas. La acción molusquicida de las lactonas sesquiterpénicas evaluadas en este estudio correlaciona con su potencia como inhibidores de FFC. La preincubación de **2** con ditiotreitol resultó en la pérdida del poder inhibitorio de la lactona. Los substratos 5'-trifosfato de adenosina y 6-fosfato de fructosa protegieron a la fosfofructocinasa contra la inhibición por **2**. La actividad inhibitoria de la FFC y la actividad molusquicida son mas significativas cuando un grupo hidroxilo se encuentra en la proximidad del sitio receptor, el fragmento de la α-metilen-γ-lactona-
Palabras clave: Lactonas sesquiterpénicas, inhibidores de las fosfofructocinasa, actividad molusquicida.

Introduction

Sesquiterpene lactones have been shown to have anti-tumor, anti-microbal, cytotoxic and phytotoxic activity [1-3]. They have been known to cause allergic contact dermatitis in humans, livestock poisoning and to act as mollucidides against the snails of the genus *Biomphalaria* [4].

All experimental evidence about the biological activities of sesquiterpene lactones seems to indicate that the α-methylene-γ-lactone portion of the molecule is essential for biological activity. The main reason for activity of this moiety is its reactivity towards nucleophiles to form a Michael-adduct. A possible mechanism of action is the reaction with biological nucleophiles found in the amino acid side chains of proteins (*e.g.* cysteine, lysine or histidine).

Previous investigations have revealed that sesquiterpene lactones act as inhibitors of certain enzymes of the energy producing pathways [5,6]. In a previous study we have reported the role of thiol groups in the activity and allosteric characteristic of mammalian phophofructokinase (PKF) [7]. This prompted us to test a series of sesquiterpene lactones which we had previously isolated and determined their structure by spectroscopic and X-ray diffraction techniques [8-10].

In this report evidence is presented that the molluscicidal sesquiterpene lactone **2** irreversibly inhibits the *in vitro* activity of phophofructokinase and that the inhibitory efficacy correlates closely with the molluscicidal action [8, 11].

Experimental

Materials. Dehydrocostuslactone (**1**), 7α-hydroxy-3-desoxyzaluzanin C (**2**), 11,13-dihydro-7,11-dehydro-13-hydroxy-3-dosoxyzaluzanin C (**3**) and 11,13-dihydro-7α-hydroxy-3-desoxyzaluzanin C (**4**) were isolated from *Ambrosia confertiflora* [11] and parthenin (**7**) was isolated from *Parthenium hysterophorous* [10]. Allodesacetylconfertiflorin (**8**), 8α-acetoxyambrosin (**9**) and 2,3-dehydro-8α-acetoxypsilostachin C (**10**) were prepared from confertiflorin [11].

a, b: New addresses.

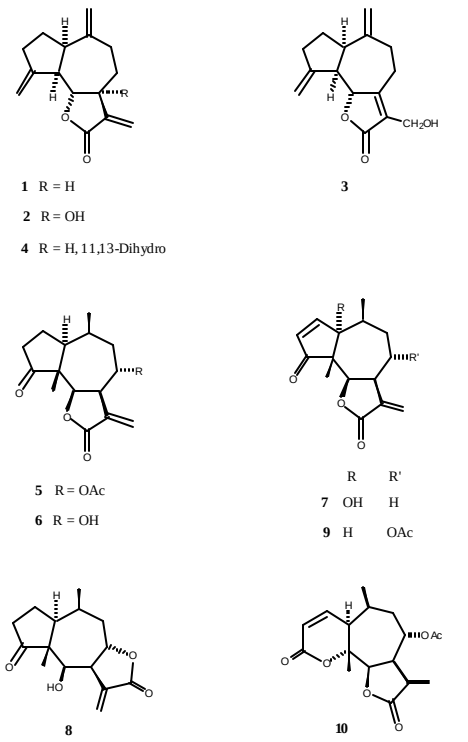

Fig. 1. Structure of the sesquiterpene lactones tested for their inhibition of phosphofructokinase from rabbit muscle.

Table 1. Apparent K_i and LC_{100} values for the guaianolide and pseudoguaianolide series. Assays were carried out under optimal conditions.

Compound	Apparent K_i (mM)*	LC_{100} (24 h)**
Guaianolides		
(1) Dehydrocostulactone	1.88	NA
(2) 7α-hydroxy-3-desoxyzaluzanin	0.076	1.0 (0.75 in 40 h)
(3) 11,13-dihydro-7,11-dehydro-13-hydroxy-3-desoxyzaluzanin	2.36	NA
(4) 11,13-dihydro-7α-hydroxy-3-desoxyzaluzanin C	3.23	NA
Pseudoguaianolides		
(5) Confertiflorin	14.20	50
(6) Desacetylconfertiflorin	7.39	NA
(7) Parthenin	21.49	60
(8) Allodesacetylconfertiflorin	3.24	25
(9) 8α-acetoxyambrosin	4.82	NA
(10) 2,3-dehydro-8α-acetoxy psilostachin C	5.25	50

*Apparent K_i is defined as the concentration required for 50 % inhibition of enzyme activity.

**LC_{100} (lethal concentration for killing 100 % of the snails within 24 h); values (in ppm) are those reported in references 8 and 11; NA = no activity.

Rabbit muscle phosphofructokinase (ATP: D-Fructose-6-phosphate-1-phosphotransferase [EC 2.7.1.11], was extracted and purified by the method of Ling *et al.* [12]. The enzyme was stored in potassium phosphate buffer pH 8.0, 0.2 mM EDTA, 60 % ammonium sulfate. Before use, it was dialyzed against the same buffer but without the ammonium sulfate.

The auxiliary enzymes, rabbit muscle triose phosphate isomerase [EC 5.3.1.1 (TPI) (α-GDH) α-Glycerophosphate dehydrogenase [EC 1.2.1.12] and rabbit muscle aldolase [EC 4.1.2.13] were purchased from Sigma.

Methods. PFK was assayed spectrophotometrically by measuring the decrease in absorbance of NADH at 340 nm using the aldolase/triosephosphate isomerse/glycerophosphate dehydrogenase coupled assay method [7]. The sesquiterpene lactone inhibitors were dissolved in methanol, and the control assay contained the same volume of pure methanol as that of inhibitor solution being added.

Optimal PFK activity assays were carried out following the conditions described in the literature [7, 12] at pH 8.0. The final reaction mixture pH 8.0, 0.01 mM EDTA, 40 mM KCl, 4 mM $MgCl_2$, 4 mM NH_4Cl, 2 mM ATP, 0.48 units of aldolase, 4.6 units triose phosphate isomerase, 0.5 units of α-glycerophosphate dehydrogenase, 0.2 mM fructose-6-phosphate and approximately 0.2 μg of PFK. The reaction was usually started by the addition of fructose-6-phosphate. When the effect of dithiothretiol (DTT) on the inhibitor was tested, the reaction was started with the addition of the auxiliary enzymes system followed by PFK.

Assays of PFK under allosteric conditions were run at pH 7.2 [14]. The reaction mixture contained 0.16 mM NADH, 1.0 mM DTT, 50 mM imidazole-Cl pH 7.2, 0.2 mM fructose-6-phosphate, varied concentrations of Mg^{++}. ATP, 4.6 units TPI, 0.5 units α-GDH, PFK and 0.1 mM of the inhibitor. The reaction was started by addition of the Mg^{++}/ATP solution to the reaction mixture.

Results

Inhibition of PFK by lactones 1-10. The structures of the molluscicidal sesquiterpene lactones tested for their inhibition of PFK are shown in figure 1. The results of the inhibition studies are presented in table 1. From the apparent K_i values it is clear that the most active inhibitor is 7α-hydroxy-3-desoxyzaluzanin C (**2**), with an apparent K_i value of 0.076 mM. The other three guaianolides **1**, **3** and **4** also inhibited PFK to a lesser extent with apparent K_i values of 1.88, 2.36 and 3.23 mM, respectively. Lactone **2** inhibited 47 % of enzyme activity at concentrations as low as 0.029 mM. Above this value, the extent of inhibition was linear with respect to the concentration of lactone **2**.

The pseudoguaianolides (**5-10**) also inhibited PFK but to a lesser extent than any of the guaianolides. The strongest inhibitor of this series was allodesacetylconfertiflorin (**8**) with an apparent K_i value of 3.24 mM. The least active of all the lactone inhibitors was parthenin (**7**) with an apparent K_i value of 21.49 mM.

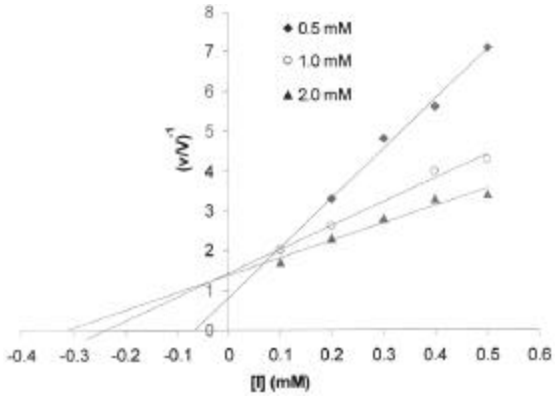

Fig. 2. Inhibition of phosphofructokinase by varying concentrations of 7α-hydroxy-3-desoxyzaluzanin C (**2**). Dixon plots at three concentrations of fructose-6-phosphate and 2.0 mM concentration of AT under optimal assay conditions (pH 8.0).

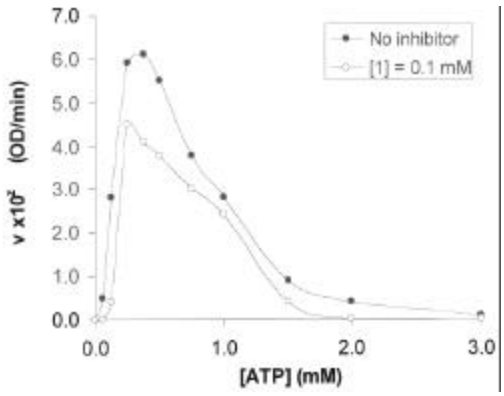

Fig. 3. Effect of ATP concentration on inhibition of phosphofructokinase by 7α-hydroxy-3-desoxyzaluzanin C (**2**) under allosteric conditions (pH 7.2).

Addition of DTT to the assay mixtures affected the extent of inhibition of PFK by the lactones. When DTT and PFK were added together in the reaction mixture it had very little effect in reducing the inhibitory power of the lactones. Incubation of DTT and the lactone inhibitors for five minutes prior to the addition of PFK resulted in lowering the extent of inhibition. Complete neutralization of the inhibitory power of the lactones was achieved when equimolar amounts of DTT and the inhibitor were incubated together.

Further studies were carried out with the most active inhibitor (**2**). The effect of **2** on the values of K_m and V_{max} under optimal conditions for enzyme activity at pH 8.0 are shown in Table 2. It is clear from these data that compound **2** lowers V_{max} and increases K_m of PFK with respect to its substrate, fructose-6-phosphate.

Protection of PFK by the substrate fructose-6-phosphate under optimal conditions (pH 8.0) can be seen in the Dixon plot [15] depicted in figure 2. An increase in the concentration of fructose-6-phosphate results in an increase of the initial velocity of the enzymatic reaction.

Assays were performed under allosteric conditions at pH 7.2, varying the concentration of ATP in presence and in absence of the lactone inhibitor (**2**). The results, shown in figure 3, indicate that compound **2** diminished the activity of PFK but did not change its sensitivity to inhibition by excess ATP. Thus the action of compound **2** seems to influence the active site of the enzyme without affecting its ATP allosteric site.

Discussion

The fact that the least active of the guaianolides **3** and **4** were those that do not contain an exocyclic α-methylene-γ-lactone moiety seems to indicate that there is an enhancement of the inhibitory power when this group is present. This is exemplified by 7α-hydroxy-3-desoxyzaluzanin C (**2**), which is approximately 42 times more inhibitory than its dihydro derivative (**4**).

However, the most outstanding result comes from the fact that the presence of the C-7α-OH group in 7α-hydroxy-3-hydroxy-3-desoxyzaluzanin C (**2**), when compared with dehydrocostus lactone (**1**), decreases the apparent K_i value approximately 25-fold (from 1.88 mM for **1** to 0.076 for **2**). It is noteworthy that **2** is the most effective sesquiterpene lactone inhibitor of phosphofructokinase reported so far in the literature [5, 6].

The most active compound of the pseudoguaianolide series was allodesacetylconfertiflorin (**8**), with an apparent K_i value of 3.24 mM. The isomeric desacetylconfertiflorin (**6**) on the other hand, was about 2.2 times less inhibory than **8**. When confertiflorin (**5**) with an apparent K_i of 14.2 mM is compared to 8α-acetoxyambrosin (**9**) and 8α-acetoxy-2,3-dehydropsilostachin C (**10**) the introduction of an additional α,β-unsaturated carbonyl function resulted in a significant increase in inhibitory power with apparent K_i values of 4.82 mM for **9** and 5.25 mM for **10**. The presence of additional reactive alkylating sites in lactones **9** and **10** could explain the increase in activity of these two compounds. Surprisingly, parthenin (**7**), which also contains a cyclopentenone moiety, exhibits a significantly lower inhibition of PFK with an apparent K_i value of 21.5 mM. This could be due to steric hindrance caused by the angular allylic OH group plus the methyl

Table 2. Effect of 7α-hydroxy-3-desoxyzaluzanin C (**2**) on the K_m and V_{max} of phosphofructokinase with respect to fructose-6-phosphate at pH 8.0, 2.0 mM ATP and 0.2 μg PFK per 1.0 mL of reaction mixure.

Inhibitor*	K_m (mM)	V_{max} (24 h)**
0.00	0.2.70 + 0.040	0.099 + 0.003
0.37	0.412 + 0.098	0.031 + 0.001
0.56	0.985 + 0.144	0.026 + 0.001

*Inhibitor concentration in mM; ** Velocity in Δ OD/min.

group at C-10, which may block access to the α,β-unsaturated carbonyl group of the cyclopentenone portion of the molecule. It is also possible, however, that the overall conformation of parthenin is affected by the presence of the allylic hydroxyl group, thus influencing its reactivity. These results are in agreement with previous observations that, although an electrophilic site in the sesquiterpene lactone is essential for its inhibitory action on PFK, the efficacy of this action is influenced by other structural and steric characteristics of the molecule [6]. The general acceptance that the substates protect the enzyme against irreversible inhibition by stabilizing the active form of the enzyme was confirmed by the experiments of Hanson *et al.* [5]. Our results are in agreement with their observations that the substrates ATP and fructose-6-phosphate protect PFK against inhibition by the lactones. However, these results differ slightly from the reported by Gaspar *et al.* [6] who observed this protection to occur only with ATP.

The manner in which lactone **2** exerts its action can very well be due to the uncommon OH group attached to C-7. We believe that this group could possibly assist the nucleophilic attack at the exocyclic α-methylene-γ-lactone moiety. This directing action could be due to the binding, possibly hydrogen bonding, of the hydroxyl group to a receptor at the active site of the enzyme. Consequently, the reactivity of the lactone moiety with nucleophilic centers such as thiol groups would be enhanced, thus bringing about a blocking at the active site of the enzyme or a conformational change unfavorable for its activity.

Although the extent of activity is not the same in both instances, it seems worthwhile to point out that the most active inhibitors of the guaianolide and pseudoguaianolide series are also the ones that exhibit the highest molluscicidal activity (Table 1). Their LC_{100} values against the snails of *Biomphalaria glabrata* are 1.0 and 25 ppm for **2** and **8**, respectively. This seems to indicate that molluscicidal activity of the sesquiterpene lactones may be due to reaction of the molluscicides with tiol-containing enzymes in a similar fashion. The action of lactones most likely involves alkylation(s) of essential enzymes of the snail's metabolism.

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