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THE EFFECT OF THE SESQUITERPENE LACTONES FROM *GEIGERIA* ON GLYCOLYTIC ENZYMES

A. R. M. D. GASPAR, D. J. J. POTGIETER and N. M. J. VERMEULEN
Department of Biochemistry, University of Pretoria, South Africa

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Abstract—The effect of sesquiterpene lactones isolated from *Geigeria* was tested on three glycolytic enzymes. Phosphofructokinase was inhibited irreversibly by all of the sesquiterpene lactones, with ivalin(III) giving the highest extent of inhibition. Values for the kinetic constants K_i (1.3 mM) and k_p (2.2 min^{-1}) were established. Hexokinase and glyceraldehyde-3-phosphate dehydrogenase were also strongly inhibited at 1 mM and 3 mM concentrations of sesquiterpene lactones, respectively. Pre-incubation of ivalin with dithiothreitol decreased its inhibiting effect on phosphofructokinase, hexokinase and glyceraldehyde-3-phosphate dehydrogenase activities. Phosphofructokinase and hexokinase were protected against inhibition by ivalin by their respective substrates, adenosine-5′-triphosphate and glucose.

Sesquiterpene lactones have been shown to have anti-tumour, cytotoxic, anti-microbial and phytotoxic activity [1]. They are known to poison livestock, to act as insect feeding deterrents and to cause allergic contact dermatitis in humans. Studies on their mechanism of action showed that the α-methylene-γ-lactone and cyclopentenone moieties undergo Michael-type addition with L-cysteine, glutathione, and a number of sulphydryl containing cell enzymes [2–7].

The plant species *Geigeria*, commonly known as "vomiting shrub", is responsible for vomiting disease in sheep [8]. It has been shown that an ethanol extract of *G. aspera* containing the sesquiterpene lactones dihydrogriesenin(I), geigerinin(II) and ivalin(III), produced typical vomiting disease symptoms in sheep. The ethanol extract or the individual purified compounds also produce strychnine-like nervous disorders in guinea-pigs and mice (N. M. J. Vermeulen, unpublished results).

Previous investigations have revealed that the sesquiterpene lactones from *Geigeria* are inhibitors of mitochondrial respiration [9, 10]. The main targets of the sesquiterpene lactones appear to be electron transport complexes I and II as well as the choline dehydrogenase complex of the respiratory chain.

In this report, evidence is presented that these lactones irreversibly inhibit the *in vitro* activity of three key glycolytic enzymes, namely phosphofructokinase, hexokinase and glyceraldehyde-3-phosphate dehydrogenase.

MATERIALS AND METHODS

Materials. Dihydrogriesenin(I), geigerinin(II), ivalin(III) and griesenin(IV) were isolated from *G. aspera* [11–13]. Geigerin(V), vermeerin(VI) and gafrinin(VII) were generous gifts from Dr L. A. P. Anderson (Veterinary Research Institute, Onderstepoort).

The enzymes and substrates were obtained from Boehringer Mannheim. Other reagents were from Merck Chemicals and were of analytical grade. Solutions were made up in double-distilled water.

Assays. The activities of rabbit muscle phosphofructokinase [ATP: D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11 (PFK)], yeast cells hexokinase [ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1 (HK)], and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate: NAD oxidoreductase, EC 1.2.1.12 (GAPDH)] were determined by means of slightly modified coupled enzyme assays, as described by Bergmeyer [14]. PFK activity was established in a coupled assay with rabbit muscle pyruvate kinase [ATP: Pyruvate-2-O-phosphotransferase, EC 2.7.1.40 (PK)] and rabbit muscle lactate dehydrogenase [L-lactate: NAD oxidoreductase, EC 1.1.1.27 (LDH)], while that of HK was coupled to yeast cells glucose-6-phosphate dehydrogenase [D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49 (G6P-DH)]. GAPDH was linked to yeast cells phosphoglycerate kinase [ATP: 3-phospho-D-glycerate-1-phosphotransferase, EC 2.7.2.3 (PGK)] activity. The activities of the enzymes were measured spectrophotometrically from absorbance changes at 340 nm, using a Beckman Model Acta MVI Spectrophotometer. Determinations were carried out at 25° for PFK and GAPDH and at 37° for HK.

Irreversible inhibition was studied by incubating PFK (5×10^{-7} M) and varying concentrations of lactone at 25° and assaying the remaining activity in aliquots removed after set time intervals. The enzyme concentration was sufficiently below the inhibitor concentration to give apparent first order rates for loss of activity. Assays were done in duplicate and activities are reported as the means of two different experiments. Values agreed within 5% of each other.

The inhibition of HK and GAPDH activities was investigated over a range of lactone concentrations varying from 0.1 to 3.2 mM and various pre-incubation time intervals.

Table 1. Inhibition of the glycolytic enzymes by *Geigeria* lactones

Inhibitor	PFK*			HK†			GAPDH‡		
	[I] (mM)	Reaction rate (μmole/dm³/min)	% Inhibition	[I] (mM)	Reaction rate (μmole/dm³/min)	% Inhibition	[I] (mM)	Reaction rate (μmole/dm³/min)	% Inhibition
Control		16.1	0		32.2	0		13.3	0
Dihydrogriesenin(I)	0.8	5.3	67	1.0	9.0	72	3.1	5.2	61
Geigerinin(II)	0.8	10.8	33	1.0	16.1	50	3.0	4.0	70
Ivalin(III)	0.7	2.9	82	1.0	9.0	72	3.2	5.5	59
Griesenin(IV)	0.8	8.0	50	1.0	12.1	62	3.1	9.5	27
Geigerin(V)	0.8	12.9	20	1.0	32.2	0	3.0	12.2	8
Vermeerin(VI)	0.8	8.0	50	1.0	10.0	69	nd§	nd	nd
Gafrinin(VII)	0.7	8.0	50	1.0	6.0	81	nd	nd	nd

* PFK inhibition was measured after pre-incubation of 7×10^{-9} M PFK with lactones in a volume of 2010 μl of 0.1 M triethanolamine buffer, pH 7.6, for 15 min at 25°.

† Inhibition of 4×10^{-7} M HK was measured after pre-incubation with the lactones in a final volume of 500 μl of 0.05 M triethanolamine buffer, pH 7.6, for 30 min at 37°.

‡ Pre-incubation conditions for GAPDH were the same as used for PFK with the exception that the pre-incubation was carried out for 30 min in a volume of 500 μl.

§ Not determined (nd).

RESULTS

Effect of lactones on glycolytic enzymes

The effect of the various lactones on the activities of PFK, HK and GAPDH can be seen from the results given in Table 1. PFK was inhibited by all the sesquiterpene lactones with ivalin(III) giving the highest (82%) extent of inhibition. Geigerin(V), which lacks an α-methylene group (see Fig. 1 for the comparative sesquiterpene lactone structures) only inhibited the enzyme by 20%, whereas geigerinin(II) inhibited PFK activity by 33%. Dihydrogriesenin(I), griesenin(IV), vermeerin(VI) and gafrinin(VII) inhibited the activity between 50 and 60%.

No inhibition of HK by the lactones was observed at 25°, using 1 mM lactone concentrations (results not shown). At a higher temperature of 37°, inhibition became apparent. Gafrinin(VII), with 81% inhibition was the most effective lactone. Dihydrogriesenin(I), ivalin(III) and vermeerin(VI) were equally effective with an inhibition of approximately 70%. No inhibition by geigerin(V) was observed, whereas geigerinin(II) diminished HK activity by 50%.

GAPDH activity was in general inhibited in a pattern comparable to PFK, except that geigerinin(II) gave the highest (70%) extent of inhibition. Geigerin(V) once again showed no or only slight inhibition, as was also observed for PFK and HK.

It was established in appropriate control experiments (not shown) that the activities of the enzymes used in the coupled assays, namely LDH, PK, PGK and G6P-DH were unaffected by ivalin(III) when tested at concentrations ranging from 0.005 to 1.0 mM for various pre-incubation time intervals.

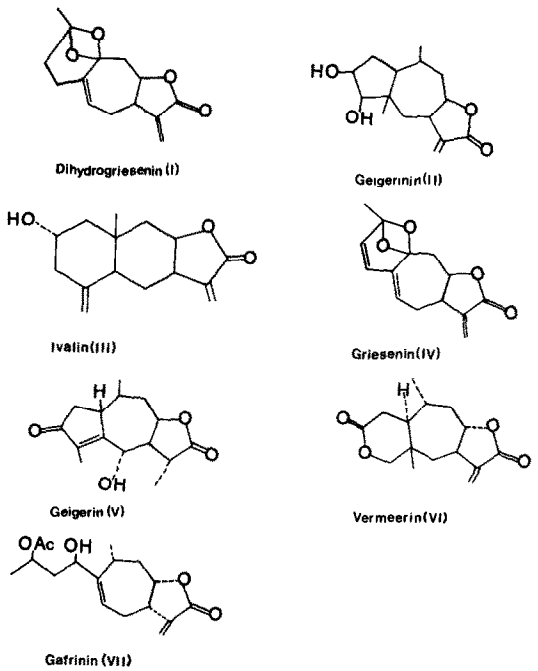

Fig. 1. Structures of the sesquiterpene lactones from *Geigeria*.

Table 2. The effect of DTT on inhibition by ivalin(III)

Enzyme (E)	Treatment						
	Control (E)	Control (E & DTT)		E + I*	E + I + DTT	E + I: then add DTT	I + DTT: then add E
1. *PFK*							
Incubation time (min)	15	15	30	15	30	(15:15)	(15:15)
Reaction rate (μmole/dm^3/min)	16.1	40.8	46.3	6.4	33.8	24.1	48.2
% I	0	0	0	60	27	41	0
2. *HK*							
Incubation time (min)	15	15	30	15	30	(15:15)	(15:15)
Reaction rate (μmole/dm^3/min)	24.1	51.5	45.0	16.1	41.3	24.1	41.3
% I	0	0	0	33	8	53	20
3. *GAPDH*							
Incubation time (min)	30	30	60	30	60	(30:30)	(30:30)
Reaction rate (μmole/dm^3/min)	9.0	28.5	29.4	5.5	16.1	10.8	24.1
% I	0	0	0	39	45	62	15

Pre-incubation conditions for PFK, HK and GAPDH were as described in Table 1 with the exception that 10 mM DTT was included where appropriate.

* Concentration of ivalin in pre-incubation: 0.1 mM (PFK); 1 mM (HK); 3.2 mM (GAPDH).

Effect of dithiothreitol (DTT) on glycolytic enzyme inhibition by ivalin(III)

The results in Table 2 indicate that pretreatment of ivalin with an excess of DTT abolished its inhibiting effect on PFK, HK and GAPDH activities. Addition of DTT after the *Geigeria* inhibitor had been allowed to react with PFK, HK or GAPDH, had little or no effect on the extent of inhibition by ivalin(III). When DTT, ivalin(III) and the enzymes PFK, HK or GAPDH were simultaneously incubated, less inhibition was observed.

Protection of PFK and HK inactivation by ivalin(III) in the presence of the substrates ATP and glucose

Figure 2 shows that PFK is protected against inhibition by ivalin to the extent of about 70% by the substrate ATP (2 mM), but not by the substrate fructose-6-phosphate (F6P) or the product fructose-1,6-diphosphate (FDP). HK was protected completely from inhibition in the presence of 0.3 M glucose (see Fig. 3).

Kinetics of PFK inhibition by ivalin(III)

The rate of inactivation of PFK by ivalin was studied as a function of inhibitor concentration at pH 7.4 and 25° (see Fig. 4a). The results in Table 3 indicate how the first-order rate constant (k_{obs}) for the inactivation of the enzyme varies as a function of inhibitor concentration. Also included in Table 3 is the apparent second-order rate constant for inhibition obtained by dividing the first-order rate constant by the inhibitor concentration.

The data for irreversible inhibition were plotted according to the following equation derived by Dixon and Webb [15].

$$1/k_{obs} = 1/k_p + K_i/k_p[I] \qquad (1)$$

where k_{obs} is the observed first-order rate constant, k_p the first-order rate constant, $[I]$ the inhibitor concentration and K_i the inhibitor constant. Since $1/K_i = 1/[I]$ when $1/k_{obs} = 0$, it could be deduced

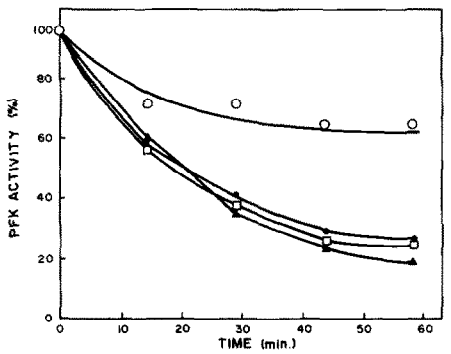

Fig. 2. Influence of MgATP, fructose 6-phosphate (F6P) and fructose 1,6-diphosphate (FDP) on the inhibition of PFK (5×10^{-7} M) activity by ivalin(III). The curve represented by ▲ was obtained with 0.4 mM ivalin in 0.3 ml 0.1 M phosphate buffer, pH 7.4, 25°; ○ represents PFK in the presence of 0.4 mM ivalin, 2 mM ATP plus 8 mM magnesium sulphate; □ represents PFK in the presence of 0.4 mM ivalin and 2 mM F6P and ● represents PFK in the presence of 0.4 mM ivalin and 2 mM FDP.

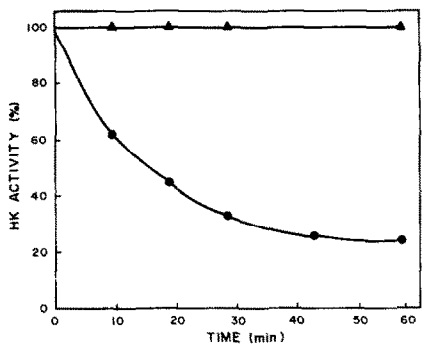

Fig. 3. Influence of glucose on the inhibition of HK by ivalin(III). The curve represented by ● was obtained for HK (4×10^{-7} M) with 1 mM ivalin in 0.475 ml 50 mM triethanolamine buffer, pH 7.6, 37°. In curve represented by ▲, 0.3 M glucose was added.

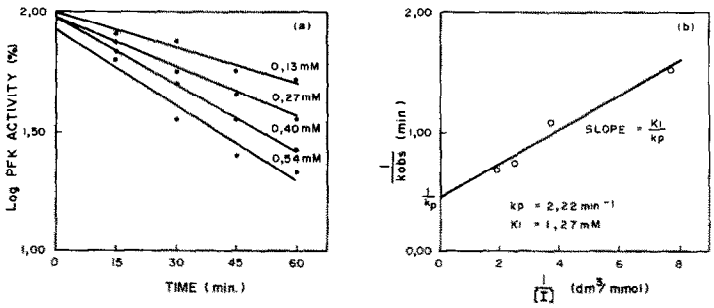

Fig. 4. (a) Kinetics of PFK inhibition by ivalin(III). The observed first-order rate constant (k_{obs}) for inhibition by ivalin was determined from first-order plots of the remaining activity after 5×10^{-7} M PFK had been incubated with different concentrations of ivalin (25°; 0.1 M phosphate; pH 7.4); aliquots with 6 μg of enzyme were assayed for activity. Lines were fitted by linear regression analysis utilizing least squares. (b) K_i and k_p were determined according to equation 1.

Table 3. Inhibition of PFK by ivalin(III)*

Inhibitor concentration (mM)	k_{obs} (min^{-1})	$k_{calc} = k_{obs}/[I]$ (mM^{-1} min^{-1})
0.13	0.7	6.6
0.27	0.9	3.4
0.40	1.3	3.3
0.54	1.4	2.6

* Various concentrations of ivalin(III) were pre-incubated at 25° pH 7.4, with 5×10^{-7} M PFK in a total volume of 360 μl of 0.1 M phosphate buffer. After set time intervals, 30 μl aliquots were removed and assayed for residual PFK activity as described in Materials and Methods.

from Fig. 4(b) that K_i is 1.3 mM and k_p is 2.2 min^{-1} for a reaction mechanism which can be represented by

$$E + I \rightleftarrows E\text{——}I \rightarrow E'\text{–}I,$$

where E–I and E′–I represent non-covalent and covalent enzyme–inhibitor complexes, respectively.

DISCUSSION

The results of a study of the reactions of the conjugated α-methylene lactones with model biological nucleophiles lent support to the view that Michael-type addition of sulphydryl-containing components may play a significant role in the mechanisms by which the lactones exert their biological activities [5]. The observation of a rapid alkylation of the sulphydryl group of L-cysteine by dihydrogriesenin(I) (N. M. J. Vermeulen, unpublished results) suggests that the *Geigeria* lactones may also act via alkylation of biologically important sulphydryl groups.

A loss of one enzyme sulphydryl group per molecule of added lactone was observed for the inhibition of PFK by another sesquiterpene lactone, taxodione [16]. The plant derived sesquiterpene lactones acting as tumor inhibitors, vernolepin and eupatorin, inhibited the activity of PFK by 50% and they appeared to be 10-fold stronger inhibitors than iodoacetamide [6, 16]. The observed inhibition of PFK by the *Geigeria* inhibitors reported here apparently proceeds by a similar mechanism. The same conclusion applies to GAPDH, whose activity depends on the presence of free sulphydryl groups in the molecule [17, 18]. By contrast no effect on HK activity at 25° was exerted by methylmercuric nitrate (CH_3HgNO_3), *p*-chloromercuribenzoate or mercury(II)chloride ($HgCl_2$) [19]. However, at 35° (pH 8.0), CH_3HgNO_3 at a concentration of 5×10^{-5} M abolished HK activity. The results obtained for HK inhibition by the sesquiterpene lactones were similar which suggests that the sulphydryl groups (six per molecule) [20] are normally not available to the inhibitors, but apparently become exposed after a time-dependent conformational change above 30° [19].

It is significant that geigerin(V), which lacks an α-CH_2 group, showed only slight inhibition of PFK, while GAPDH and HK were unaffected. The enzymes used in the coupled assays, i.e. PK, LDH, G6P-DH and PGK were not affected by ivalin(III). Studies by Baer *et al.* [21] on the glycolytic metabolism of human lymphocytes showed that the sesquiterpene lactone, hydroxyisonobilin, caused a marked inhibition of PFK and GAPDH as well as PK and LDH, although to a lesser extent for the latter.

If the *Geigeria* inhibitors act by alkylating the functionally essential sulphydryl groups of PFK, HK and GAPDH, then pretreatment of the inhibitors with an excess of DTT should render them ineffective. The results in Table 2 show that this was indeed the case for ivalin(III). When DTT was added after the inhibitor had been incubated with enzyme, activity was increased only slightly over that with the inhibitor alone, suggesting that inhibition occurs rapidly and irreversibly.

The higher reaction rates observed for the controls in the presence of DTT in contrast to the controls without DTT could possibly be that DTT protects the enzyme sulphydryl groups from oxidation by acting as a scavenger of whatever oxidizing agent that may be present.

It is generally accepted that if an irreversible inhibitor acts by combining with a group at the active site of an enzyme, the presence of the substrate or competitive inhibitor will protect against inhibition by slowing down the rate at which inhibition occurs

[15]. Experiments conducted by Hanson *et al.* [6] showed that the substrates ATP and fructose-6-phosphate protected PFK from inhibition by the sesquiterpene lactones vernolepin, taxodione, taxodone, eupacunin and euparotin acetate. The results in our study indicate that only ATP protected against PFK inhibition by ivalin(III) suggesting a possible common binding site for ATP and ivalin. This may be due to common features in the structure of the ribonucleotide and the sesquiterpene lactones (cf. Fig. 1). Hexokinase was completely protected from the action of NEM in the presence of glucose [20]. Similarly, our results showed complete protection by glucose against ivalin inhibition.

If the inhibition of the glycolytic enzymes were dependent only on the α-methylene-γ-lactone moiety of their structure, then different *Geigeria* inhibitors at the same concentration should display the same extent of inhibition. This was not the case (Table 1). It is therefore concluded that although the presence of an α-methylene group may be the principal prerequisite for inhibition, the rest of the structure is also important, probably in conferring binding affinity. Since the calculated second-order rate constants decrease with increasing inhibitor (Table 3), the following mechanism may be valid for the inhibition of the glycolytic enzymes by the lactones (I—CH=CH_2).

$$\begin{array}{l} E + S \rightleftharpoons E\cdots S \rightarrow E + P \\ + \\ I{-}CH{=}CH_2 \\ k_{-1} \;\; \downuparrows \;\; k_1 \\ \ddot{E}\cdots I{-}CH{=}CH_2 \xrightarrow{k_p} E{-}CH_2CH_2{-}I \end{array}$$

Scheme 1

It is instructive to compare the inhibition by sesquiterpene lactones with that of other alkylating inhibitors. Erythrocyte acetylcholinesterase deactivation by di-isopropyl phosphofluoridate (DIPF) has a K_i value of 1.17 mM and k_p of 40.7 min^{-1} [22]. K_i and k_p values for the alkylation of bovine trypsin with lysine chloromethyl ketone (LCK) were found to be 1.30 mM and 0.50 min^{-1}, respectively [23]. It can therefore be stated from the K_i (1.3 mM) value for PFK inhibition by ivalin(III) that the affinity of the inhibitor for PFK is comparable to that of DIPF and LCK for their respective enzymes. From the k_p (2.2 min^{-1}) value for PFK inhibition it is evident that the E·I (non-covalent enzyme–inhibitor complex) breaks down to alkylated enzyme 4-fold more rapidly that in the case of trypsin inhibition by LCK, but is 18-fold slower than acetylcholinesterase inactivation by DIPF.

The first symptoms to be observed in *Geigeria* affected animals are apparently all related to a general disturbance of the energy metabolism of the animal [8, 24]. *In vitro*, it has now been shown that both mitochondrial respiration [9, 10] and glycolysis are inhibited by these lactones. Although a K_i of ~1 mM may appear high from a physiological point of view, the irreversible nature of the inhibition may, over an extended period of time, suffice to cause interference with *in vivo* energy production at appreciably lower inhibitor concentrations.

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