onds, the I-waves of the PR after 3 ± 1 minutes, and the D-wave after 4.5 ± 1.5 minutes of ischemia (Fig. 1). After the clamps were released the brains in all instances were immediately recirculated with blood, and cerebral pulsations were visible. There was also an immediate color difference between the pial veins and arteries, indicating the oxygen uptake of the brain. An impairment of the recirculation could effectively be avoided by simply raising the systemic blood pressure to 140 mm-Hg by intravenous infusion of noradrenaline after the clamps had been released.

The recovery process of neuronal function was relatively independent of the duration of ischemia and started surprisingly fast after the blood flow was restored (Fig. 1). After total ischemia of up to 1 hour, the latency of recovery of the D-wave was less than 10 minutes, and that of the I-wave was less than 30 minutes. Only the recovery of the EEG showed a certain relationship to the duration of ischemia. After ischemia of less than 30 minutes the EEG recovered within 1 hour, whereas in the longer experiments only short-lasting bursts could be elicited by a strong cortical stimulation (Fig. 1). A recovery of neuronal function after ischemia of more than 1 hour's duration was no longer predictable. In one case, however, a transient recovery of the D-wave of the PR occurred even after 2 hours of total ischemia.

Information about the permanence of recovery is still limited, since this investigation was performed in acute experiments. After ischemia of less than 40 minutes a secondary suppression of neuronal activity was not observed, although the animals were kept alive up to 24 hours. In some of the experiments in which ischemia was longer than 40 minutes, recovery was only transient and a secondary suppression which was not due to an impairment of the cerebral blood flow occurred 4 to 6 hours later. At present we have no explanation for this phenomenon.

The mechanism of the recovery process remains to be clarified. The prerequisite for any recovery, of course, is an adequate blood recirculation. However, noradrenaline which has been used to control the systemic blood pressure may have a direct effect on the cortical neurons, although such an action has been refuted in normal animals (*6*).

The fact that unequivocal signs of neuronal recovery can be detected after complete ischemia of more than 1 hour's duration raises serious questions about the reliability of criteria currently used for the determination of brain death. Furthermore, our experiments suggest that concrete chances exist for the successful treatment of prolonged cerebral ischemia.

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Reactions of Alpha Methylene Lactone Tumor Inhibitors with Model Biological Nucleophiles

Abstract. *Thiols are the most reactive nucleophilic reagents among the biological models investigated. They undergo "Michael-type" addition to the polyfunctional sesquiterpene lactones. The rapid rates of reaction with* L-*cysteine were measured and the reaction products were characterized. Each addition of thiol successively decreased the cytotoxicity of the adducts formed.*

The search for tumor inhibitors of plant origin has led to the isolation and characterization of a number of sesquiterpene lactones (*1*) that exhibit growth inhibitory activity in vivo against animal tumor systems and in vitro against cells derived from human carcinoma of the nasopharynx (KB). Many of these biologically active lactones are α-methylene γ-lactones that have other functional groups, such as epoxide, chlorohydrin, unsaturated ester, unsaturated lactone, and unsaturated ketone groups. Little is known about the relation between structure and activity in these compounds (*2*), but the demonstrated reactivity of unsaturated lactones toward thiols (*3*) and amines (*4*) and the presence of other reactive functional groups suggested that the cytotoxicity of these compounds may result from alkylation of nucleophilic centers in a biological system. Therefore the chemical reactivity of some of the cytotoxic lactones toward model biological nucleophiles is being studied and we now describe the reactions of elephantopin (**1**, *5*), eupatundin (**2**, *6*), and vernolepin (**3**, *7*) with aqueous solutions of L-cysteine at *p*H 7.4.

Second-order rate constants (Table 1) for the reactions of the lactones with cysteine were determined spectrophotometrically with a Beckman DK-2A spectrophotometer equipped with a thermostated cell compartment. To $10^{-4}M$ cysteine in $0.067M$ phosphate buffer (*p*H 7.4), prepared in a 1-cm quartz cell (volume, 3.60 ml), $10^{-2}M$ lactone in tetrahydrofuran (36 μl) was added, and the resultant solution was mixed rapidly. After an appropriate reaction time, the sulfhydryl content was measured by quenching the reaction with an excess of a tetrahydrofuran solution of 2,2′-dipyrydyl disulfide, which reacts with cysteine to give 2-thiopyridone (*8*) ($\varepsilon = 7780$ at 343 nm). The rate of addition of the second mole of cysteine to **4** was determined by allowing the reaction of an equimolar mixture of **1** and cysteine to reach completion and then measuring the rate of reaction of this preformed adduct with an added mole of cysteine. Eupatundin and vernolepin also reacted with a second mole of cysteine.

Comparison of the rate constants of the foregoing reaction with reported values for the reactions of *N*-ethylmaleimide and iodoacetate with cysteine showed that the lactones have about the same order of reactivity toward cysteine as iodoacetate does. In contrast to the reactivity of lactones **1**, **2**, and **3** toward sulfhydryl groups under these conditions, their reaction with amino groups appears to be very slow. When a solution equimolar in lysine and vernolepin (**3**, $10^{-2}M$ in 50 percent aqueous tetrahydrofuran) at *p*H 7.4 was allowed to stand for 6 days at 25°C, 75 percent of the original lactone could be recovered. Guanine also proved unreactive toward either vernolepin or elephantopin.

The cysteine addition products were isolated from a solution of $10^{-2}M$ lactone in aqueous tetrahydrofuran (*p*H

7.4). The ninhydrin-sensitive products were detected by thin-layer chromatography on silica gel HF_{254} (1-butanol, water, acetic acid, 3 : 1 : 1) and cellulose F (30 percent aqueous acetone) and were isolated by preparative layer chromatography on Avicel with the use of aqueous acetone solvent systems (30 to 55 percent).

The reaction of eupatundin (**2**) with an equimolar amount of cysteine was complete in 0.5 hour, yielding a mixture containing a component with an R_F higher than that of cysteine and a trace of material with an R_F lower than that of cysteine in both thin-layer systems. The material with the higher R_F was separated by Avicel chromatography and was freed from inorganic salts by repeated (3 times) chromatography on a Bio-Gel p-2 (100 to 200 mesh) column in water to give **5** as a colorless, water-soluble powder, m.p. (with decomposition) 161° to 164°C (found: C, 54.90; H, 6.21; and N, 2.81 percent). The nuclear magnetic resonance (NMR) spectrum of **5** in deuterium oxide displayed resonances at τ (ppm) 6.10 (t, 1H) for the α-proton of the amino acid, 3.80 (m, 1H), 8.05 (d, $J = 7$ hz, 3H) and 8.15 (s, 3H), characteristic of the angelate ester side chain, and at 6.58 (s, 1H) and 8.35 (s, 3H), characteristic of the epoxide grouping. The absence of signals for a conjugated exocyclic methylene group provided evidence that the reaction of **2** with cysteine was at the α-methylene adjacent to the γ-lactone carbonyl group.

Treatment of **2** with an excess of cysteine for 48 hours resulted in a mixture enriched in the lower R_F component. This material was isolated in crude form, but because of its instability could not be further purified. However, the absence of the resonances characteristic of the angelate group and the appearance of signals at τ 8.68 (d, $J = 7$ hz, 3H) and 8.85 (d, $J = 7$ hz, 3H) indicated that reaction with a second mole of cysteine had occurred with the angelate ester side chain.

In a similar manner, the monocysteine adduct (**4**) of elephantopin was prepared and isolated as a monohydrate, m.p. (with decomposition) 235° to 240°C (found: C, 53.00; H, 6.10; N, 3.15; S, 6.68 percent). The NMR spectrum contained signals at τ 1.93 (s, 1H) and 4.45 (broad, 1H) for the endocyclic unsaturated lactone group and at 3.80 (broad, 1H), 4.18 (broad, 1H), and 8.05 (s, 3H), for the methacrylate ester side chain; there were no signals for an exocyclic methylene group, in support of structure **4** for the adduct.

Treatment of **1** with an excess of cysteine yielded a bis adduct, which was separated by Avicel chromatography. Attempts to further purify this material led only to decomposition. The presence of signals characteristic of the endocyclic unsaturated lactone group and the absence of signals for the methacrylate vinyl protons in the NMR spectrum indicated that the second mole of cysteine had added to the unsaturated ester side chain.

Table 1. Second-order rate constants at 25°C for reaction with L-cysteine at *p*H 7.4.

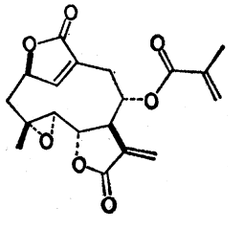
Compound	Rate constant (liter mole^{-1} min^{-1})
1	2600
4	100
2	About 2500*
3	About 12,000*
Iodoacetate	1500 (*9*)
N-Ethylmaleimide	225,000 (*10*)

* The second-order kinetics for the addition of cysteine to eupatundin (**2**) and vernolepin (**3**) were nonlinear. The rate constants were calculated from initial rates.

Treatment of vernolepin with 1 meq of cysteine rapidly yielded a complex mixture, consisting of unreacted vernolepin, two very similar compounds whose R_F was higher than that of cysteine (probably monoadducts), and the vernolepin bis adduct, of lower R_F. Treatment of vernolepin with 2 meq of cysteine led to rapid formation of the unstable bis adduct which exhibited no signals for exocyclic methylene protons. This adduct resisted attempts at purification, but on the basis of its properties and the known structure of the bis-1-propanethiol adduct (*11*), structure **6** was proposed for this compound.

The median effective dose, ED_{50}, values for lactones **1**, **2**, and **3** and their cysteine derivatives against KB cell cultures (*12*) are (in μg/ml): **1**, 0.32; **4**, 1.7; **1** bis adduct, > 100 (inactive); **2**, 0.39; **5**, 3.2; **2** bis adduct, > 100 (inactive); **3**, 2.0; **6** > 100 (inactive).

The monocysteine adducts **4** and **5** still showed appreciable cytotoxicity, but the bis cysteine adducts were essentially devoid of growth inhibitory activity. The results support the view that the reactions of α-methylene γ-lactones and other conjugated systems with biologically important sulfhydryl groups may play a significant role in the mechanisms by which these compounds exert their biological activities.



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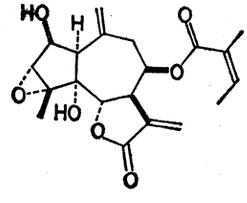

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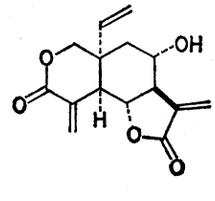

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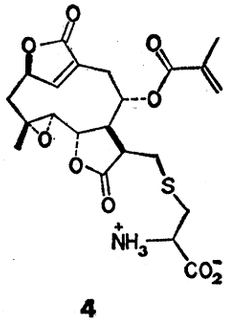

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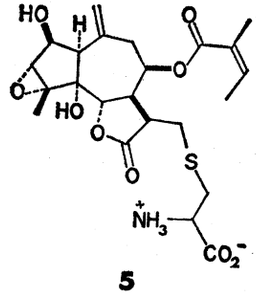

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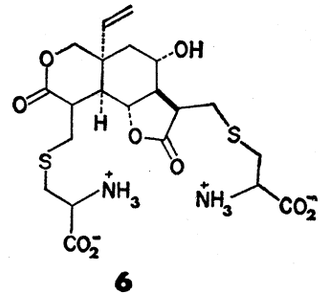

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Inhibition of Phosphofructokinase by Quinone Methide and α-Methylene Lactone Tumor Inhibitors

Abstract. *The plant-derived tumor inhibitors taxodone, taxodione, vernolepin, eupacunin, and euparotin acetate each inhibit the sulfhydryl enzyme, phosphofructokinase. The substrates, fructose-6-phosphate and adenosine triphosphate, protect the enzyme from this inhibition as does the addition of dithiothreitol to the inhibitors. Incubation of taxodione with phosphofructokinase is associated with the loss of about one sulfhydryl group per inhibitor molecule, and the substrates protect six sulfhydryl groups per protomer of 93,000 daltons.*

The conjugated α-methylene lactones and quinone methides taxodone, taxodione, vernolepin, euparotin acetate, and eupacunin have been isolated from plants; they inhibit growth in vivo of the Walker intramuscular carcinosarcoma 256 in rats and inhibit growth in vitro of cells derived from human carcinoma of the nasopharynx (KB) (*1*).

The results of a study of the reactions of conjugated α-methylene lactones with model biological nucleophiles lent support to the view that Michael-type addition of sulfhydryl-bearing compounds may play a significant role in the mechanisms by which the lactones exert their biological activities (*2*). Furthermore, the well-known sensitivity of quinone methides to nucleophilic addition (*3*) and the observation of the rapid addition of the sulfhydryl group of glutathione to taxodone and taxodione (*4*) suggested that these compounds may also act via alkylation of biologically important sulfhydryl groups.

To test the possibility that these inhibitors may be reacting with sulfhydryl enzymes, we have studied their effects on phosphofructokinase, which contains 16 to 18 sulfhydryl groups per protomer of 93,200 daltons (*5, 6*). In their study of the reactivity of these groups with Ellman's reagent at *p*H 7, Kemp and Forest (*5*) found that four of the sulfhydryls titrate more rapidly in the native than in the denatured state and that two sulfhydryls essential for enzymatic activity can be protected by fructose-6-phosphate or adenine nucleotides. Five more sulfhydryl groups become available as the *p*H is increased from 7 to 9. Similarly, Younathan, Paetkau, and Lardy (*6*) found that, at *p*H 8, six sulfhydryl groups react immediately with Ellman's reagent and that two of these can be protected by the addition of fructose-6-phosphate. Younathan *et al.* also found that 60 percent of the enzyme's activity is lost by alkylation of six sulfhydryl groups with iodoacetamide, and that fructose-6-phosphate and magnesium adenosine triphosphate (ATP) protect against this loss of activity.

We now show that the tumor inhibitors inhibit phosphofrucktokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) and present evidence that the inhibition results from their reaction with the sulfhydryl groups of this enzyme.

Crystalline phosphofructokinase from rabbit skeletal muscle was prepared by the method of Ling *et al.* (*7*). The crystalline enzyme suspension was dialyzed against a 27 m*M* borate (sodium)–0.03 m*M* EDTA buffer system at *p*H 8. Protein concentrations were determined assuming an optical density of 1.09 mg^{-1} ml^{-1} cm^{-1} in 0.1*N* NaOH (*8*).

The *p*H stat assay for phosphofructokinase was used (*9*). The basic system contained, in a volume of 4.00 ml at *p*H 7 and 30°C: 80 m*M* KCl, 2 m*M* sodium fructose-6-phosphate, 1.2 m*M* *o*-phenanthroline, 5 m*M* sodium ATP, and 5 m*M* $MgSO_4$. Ordinarily, dithiothreitol is used in the assay to protect the sulfhydryls of the enzyme, but *o*-phenanthroline can be substituted for dithiothreitol (*10*). The dialyzed enzyme was diluted about tenfold into 0.1*M* potassium phosphate, 1.2 m*M* *o*-phenanthroline, *p*H 8; and 0.01 ml of this solution was added to the assay system. The NaOH was standardized with potassium biphthalate.

Table 1. The assay system is described in the text. Incubations were at 30°C, *p*H 7.2 to 7.5. In each assay 10.2 μg of phosphofructokinase were used. When there were no additions to system, KCl, *o*-phenanthroline, and inhibitor were incubated for 5 minutes, then phosphofructokinase was added. After 5 minutes, the enzymatic reaction was initiated by addition of ATP, fructose-6-phosphate, and $MgSO_4$. When dithiothreitol was added before phosphofructokinase, KCl, *o*-phenanthroline, inhibitor, and dithiothreitol (4 or 20 μmole in the case of iodoacetamide) were incubated for 5 minutes (or for 10 minutes in the case of iodoacetamide); then phosphofructokinase was added. After 5 minutes the enzymatic reaction was initiated by addition of ATP, fructose-6-phosphate, and $MgSO_4$. When dithiothreitol was added after phosphofructokinase, KCl, *o*-phenanthroline, inhibitor, and phosphofructokinase were incubated for 5 minutes; then dithiothreitol (4 or 20 μmole in the case of iodoacetamide) was added. After 5 minutes (or 10 minutes in the case of iodoacetamide) the enzymatic reaction was initiated by addition of ATP, fructose-6-phosphate, and $MgSO_4$. (The large amount of iodoacetamide used required a larger amount of dithiothreitol to be added and required incubation for a longer time.)

System		Reaction rate with:		
Inhibitor	Amount (μg)	No addition	Dithiothreitol	
			Before PFK	After PFK
None		0.795	0.968	
Taxodone	3.76	0.168	1.15	0.308
Taxodione	0.5	0.78	1.08	0.214
Vernolepin	200	0.165	0.968	0.250
Euparotin acetate	200	0.239	1.09	0.469
Eupacunin	200	0.242	1.00	0.466
None		0.760	1.00	
N-Ethylmaleimide	2	0.101	1.04	0.054
Iodoacetamide	3×10^3	0.187	0.676	0.104
Ellman's reagent	1	0.137	0.987	1.030