Inhibition of Liver Alanine Aminotransferase Activity by Some Benzophenanthridine Alkaloids

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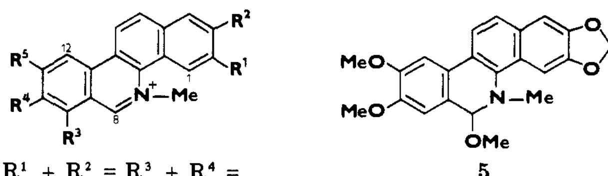
The quaternary benzophenanthridine alkaloids sanguinarine (1) and chelerythrine (2) inhibit rat liver L-alanine-:2-oxoglutarate aminotransferase (EC 2.6.1.2) activity. Nitidine (3) has no inhibitory effect. The inhibitory activity of alkaloids depends on the reactivity of the iminium bond with the nucleophilic reagent, e.g., the thiol group. The stability constants of adduct formation for thioethanol, cysteine, and glutathione with sanguinarine (1) and chelerythrine (2) are given. The mechanism of the inhibition of alanine aminotransferase activity by the alkaloids 1 and 2 is discussed.

The quaternary benzophenanthridine alkaloids sanguinarine (1), chelerythrine (2), and nitidine (3) and their



1, $R^1 + R^2 = R^3 + R^4 = OCH_2O$; $R^5 = H$
2, $R^1 + R^2 = OCH_2O$; $R^3 = R^4 = OMe$; $R^5 = H$
3, $R^1 + R^2 = OCH_2O$; $R^4 = R^5 = OMe$; $R^3 = H$
4, $R^1 = R^2 = OMe$; $R^4 + R^5 = OCH_2O$; $R^3 = H$
6, $R^1 = R^4 = R^5 = OMe$; $R^2 = OH$; $R^3 = H$
7, $R^1 = R^2 = R^4 = R^5 = OMe$; $R^3 = H$

natural or synthetic analogues are among the most systematically studied biologically active compounds.[1-3] In aqueous solutions at the appropriate pH values these alkaloids covalently add hydroxide ions to form the so-called pseudobases.[4] In solution, at a physiological pH value, sanguinarine (1) and chelerythrine (2) are present in the form of a quaternary cation and pseudobase, whereas nitidine (3) is present only in the form of a cation.[5] Sanguinarine (1) and chelerythrine (2) exhibit good antimicrobial activity[6,7] and have strong antiinflammatory effects.[8] They are inactive against the L-1210 and P-388 leukemias in mice, in contrast to nitidine (3) which has antitumor activity.[6] Nitidine (3), allonitidine (4), 8-methoxy-7,8-dihydronitidine (5), fagaronine (6), and O-methylfagaronine (7) inhibit RNA-directed DNA polymerase (reverse transcriptase) activity through binding to specific base pairs, e.g., A-T, of the template–primer.[9,10] Nitidine (3) and some of its derivatives inhibit tRNA O-methyltransferase and catechol O-methyltransferase activities.[11,12] It was found that sanguinarine (1) and benzophenanthridines substituted on ring D in the same position inhibited the cardiac (Na/K)ATPase activity of guinea pigs in vitro.[13-15] It has been suggested that the addition of thiol groups off the (Na/K)ATPase enzyme to the iminium bond probably is the mechanism bringing about inhibition.[16]

The purpose of our work has been to study the effect of the alkaloids 1–3 on the activity of the liver L-alanine-:2-oxoglutarate aminotransferase enzyme (EC 2.6.1.2). Alanine aminotransferase is an enzyme containing 24 to 30 thiol groups, of which 7 to 8 are nonessential for its catalytic activity.[17,18]

Results and Discussion

In assays carried out in vitro with rat liver postmitochondrial supernatant, sanguinarine (1) and chelerythrine (2) were found to possess an inhibitory effect on the activity of the alanine and aspartate aminotransferases. A more pronounced inhibitory effect on the activity of alanine aminotransferase was observed. For the studies of the mechanism of inhibition, a partially purified rat liver alanine aminotransferase enzyme was used.[18]

The measurements of the concentration dependency of the inhibitory effects of sanguinarine (1), chelerythrine (2), and nitidine (3) revealed that alanine aminotransferase activity was inhibited only by alkaloids with substituents in positions 9 and 10 of ring D. Sanguinarine (1) was a more powerful inhibitor than chelerythrine (2) (Figures 1 and 2). Nitidine (3), a structural isomer of chelerythrine (2), had no inhibitory effect even when a tenfold concentration compared to that of compounds 1 and 2 was used. The optimum inhibitory activity was reached after a 30-min preincubation of the alkaloid with the enzyme at room temperature (Figure 3). The ID_{50} values (50% inhibitory

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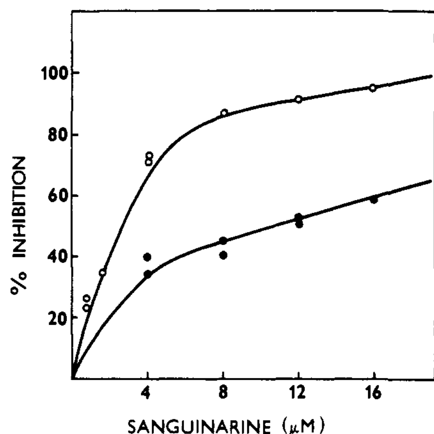

Figure 1. Dependence of the inhibition of alanine aminotransferase activity on the concentration of sanguinarine. The enzyme (6.75 μkat/L) was preincubated with various concentrations of sanguinarine at room temperature at pH 7.4. At zero time (●) and at 30-min intervals (○), the enzyme activity was measured (10-μL samples were added to 300 μL of substrate solution). Concentrations given are those in the final reaction mixture.

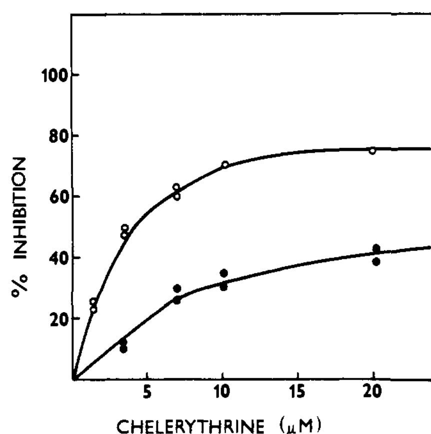

Figure 2. Dependence of the inhibition of alanine aminotransferase activity on the concentration of chelerythrine. The experimental conditions were the same as those in Figure 1, except that various concentrations of chelerythrine were used.

dose) were 3.4 × 10^{-6} M for sanguinarine (1) and 4.0 × 10^{-6} M for chelerythrine (2) when an enzyme solution with an activity of 230 nkat/L was used. The concentrations of sanguinarine (1) ranging above 5 × 10^{-5} M brought about a complete inactivation of the enzyme, whereas an increase in the concentration of chelerythrine (2) above the value of 3 × 10^{-5} M did not lead to an increase in the inhibition of alanine aminotransferase activity. The maximum inhibition of enzyme activity by chelerythrine (2) was ca. 80%. The plotting of the data obtained from the measurements of the enzyme activity comparing the dependence on the concentration of the two studied alkaloids showed that, contrary to sanguinarine (1), chelerythrine (2) behaved like a partial inhibitor (Figure 4).

The interaction of the benzophenanthridine alkaloids with the alanine aminotransferase enzyme is not quite reversible. The dependence of the reaction rate on the enzyme concentration, observed at various concentrations of the alkaloids, was characteristic for the tight-binding inhibitors[19] (Figures 5 and 6). Dialysis of the solution (24 h) containing the enzyme and the corresponding alkaloid led to only partial restoration of the enzyme activity: sanguinarine (1), 0–3%; chelerythrine (2), 20–25%.

The determination of the inhibition type on the basis of kinetic data is difficult because of the two-substrate enzyme reaction and the tight association of the inhibitors

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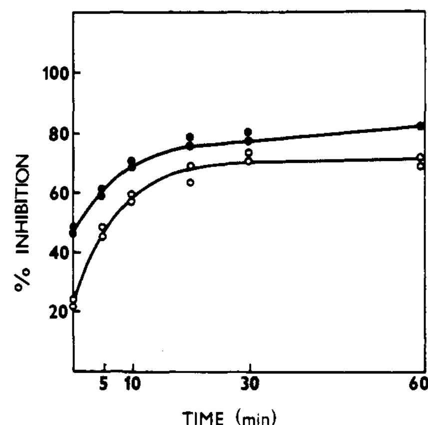

Figure 3. Effect of the preincubation with sanguinarine (●) and chelerythrine (○) on the inhibition of alanine aminotransferase activity. The enzyme (17.5 μkat/L) was preincubated with the alkaloid (6.1 × 10^{-4} M) in a buffered solution of pH 7.4 at room temperature. At various time intervals, the mixture (10 μL) was added to the substrate solution (600 μL). The final concentrations of alkaloids in the reaction mixture were 1 × 10^{-5} M.

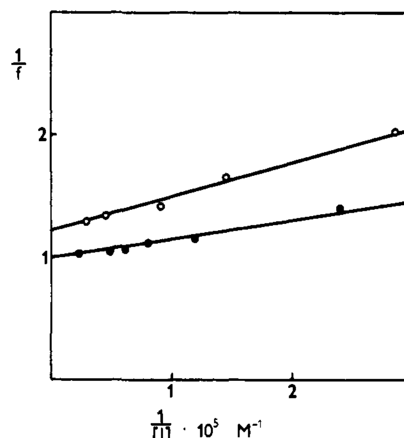

Figure 4. Plots for assessment of the partial inhibition by sanguinarine (●) and chelerythrine (○): $f = 1 - v_i/v_0$; v_i/v_0 = reaction rate in the presence or absence of the inhibitor; [I] = concentration of the inhibitor.

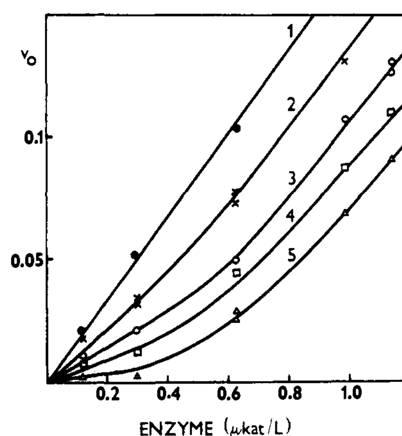

Figure 5. Changes in the reaction rate with increasing concentrations of alanine aminotransferase at various levels of sanguinarine. The experimental conditions were the same as those in Figure 1, except that various concentrations of the enzyme were used. Concentrations of sanguinarine: 1 = 0, 2 = 1.25, 3 = 2.5, 4 = 6.3, and 5 = 11.1 μM.

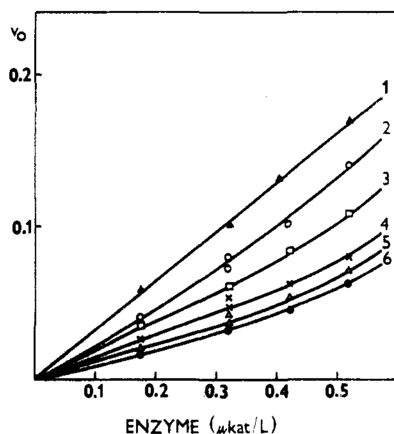

Figure 6. Changes in the reaction rate with increasing concentrations of alanine aminotransferase at various levels of chelerythrine. The experimental conditions were the same as those in Figure 2, except that various concentrations of the enzyme were used. Concentrations of chelerythrine: 1 = 0, 2 = 2.2, 3 = 5.6, 4 = 11.2, 5 = 22.3, and 6 = 33.5 μM.

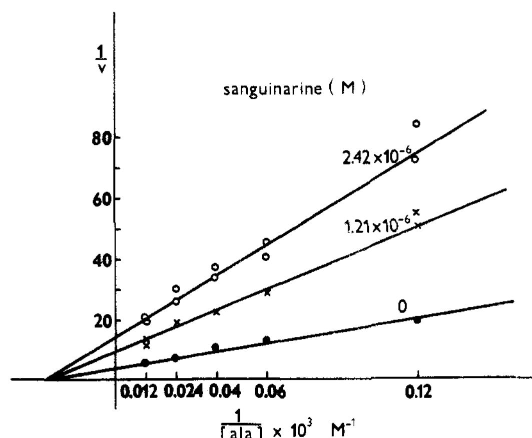

Figure 7. Lineweaver–Burk plots for the effect of sanguinarine on alanine aminotransferase activity. The enzyme (17.5 μkat/L) was preincubated with sanguinarine in a buffered solution of pH 7.4 at room temperature. After 30 min, the mixture (10 μL) was added to the substrate solutions (300 μL) containing various concentrations of L-alanine. Concentrations given are those in the final reaction mixture.

Table I. Effect of Some Thiols on Enzyme Inhibition with Sanguinarine (1) and Chelerythrine (2)[a]

	sanguinarine (10^{-5} M)		chelerythrine (10^{-5} M)	
compd	% inhibn	% protection	% inhibn	% protection
thioethanol	20 ± 3	75 ± 3	23 ± 3	55 ± 3
cysteine	74 ± 3	5 ± 3	40 ± 3	5 ± 3
glutathione	15 ± 2	81 ± 2	13 ± 2	70 ± 2

[a] Inhibition (%) in the absence of thiol: sanguinarine, 78 ± 3; chelerythrine, 42 ± 3.

with the enzyme.[19] The double-reciprocal plot of our kinetic data at variable L-alanine concentrations indicated noncompetitive inhibitions (Figures 7 and 8).

The compounds with a thiol group protect the studied enzyme against the inhibitory effects of the benzophenanthridine alkaloids probably due to the addition of the thiol group to the iminium bond (Table I). For an effective blocking of the inhibition, a comparatively higher concentration of thiols will be required (10^3 higher compared to the concentrations of the alkaloids). Incubation of the enzyme–alkaloid complex with thioethanol and cysteine did not result in a restoration of the enzyme activity. On incubation with only glutathione (10^{-2} M), the activity of the enzyme–sanguinarine complex increased by 13% and the activity of the enzyme–chelerythrine complex increased by 31%. The stability constants (K) of the adduct formation for some thiols with sanguinarine (1) and chelerythrine (2) are given in Table II. Glutathione, which has the highest stability constant, protects the enzyme most effectively against the inhibitory effect of the two alkaloids.

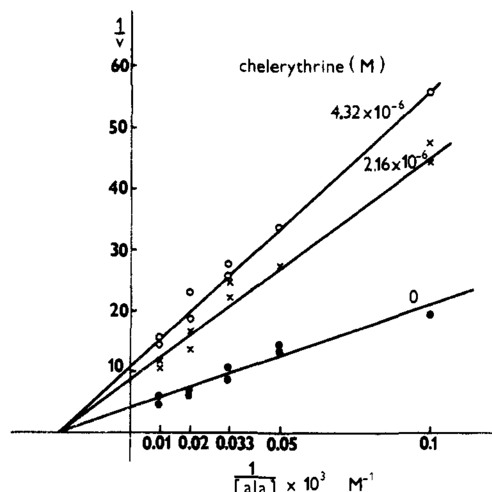

Figure 8. Lineweaver–Burk plots for the effect of chelerythrine on alanine aminotransferase activity. The experimental conditions were the same as those in Figure 7, except that the enzyme was preincubated with chelerythrine.

Table II. Stability Constants of Adduct Formation (Log K) for Some Thiols with Sanguinarine (1) and Chelerythrine (2)[a]

	sanguinarine		chelerythrine	
compd	pH 5.28	pH 5.69	pH 5.68	pH 6.25
thioethanol	3.77 ± 0.11	4.01 ± 0.08	3.46 ± 0.05	4.05 ± 0.02
cysteine	2.97 ± 0.07	3.33 ± 0.05	2.74 ± 0.11	3.09 ± 0.05
glutathione	4.64 ± 0.07	4.79 ± 0.10	4.39 ± 0.02	4.39 ± 0.08

[a] pK_{R^+} values of pseudobase formation (in water): sanguinarine, 7.92 ± 0.08; chelerythrine, 8.77 ± 0.07.[5]

It is assumed that the inhibitory effect of sanguinarine (1) and chelerythrine (2) on the aminotransferase activity may be brought about by formation of an adduct between the thiol groups of the enzyme and the iminium bond of the quaternary cation. The relative susceptibility of the iminium bond to nucleophilic attack is mainly dependent of the position of the electron-donating substituents of ring D.[3] The substituent in position 9 destabilizes the cation via the electronic effect relative to the adduct [sanguinarine (1) and chelerythrine (2)], whereas the substituent in position 11 [nitidine (3)] stabilizes the cation. The partial nature of the inhibition with chelerythrine (2) cannot be interpreted unequivocally. In view of the lower reactivity compared to sanguinarine (1), it may be attributed to a looser association with the enzyme or to the binding to another site of the enzyme molecule.

Experimental Section

Materials. Sanguinarine (1) and chelerythrine (2) were available from the isolation procedure.[20] Nitidine (3) was obtained from Professor M. Cushman, Department of Medical Chemistry

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and Pharmacognosy, Purdue University. Thioethanol, cysteine, and glutathione were purchased from Sigma Chemical Co.

Equilibria Measurements. The stability constants were measured at 20 °C in 10-mm cells on a Perkin-Elmer 552 spectrophotometer, and the pH values were measured on a Radiometer PHM 64. The aqueous solutions contained 4×10^{-5} M alkaloids, 4×10^{-6} to 1.6×10^{-2} M thiols, 4×10^{-3} M hexadecyltrimethylammonium bromide, and phosphate buffers of ionic strength of 0.05.

The stability constant (K) of adduct formation of the thiol (S) with the alkaloid (A) (eq 1) was calculated by logarithmic transformation (eq 2), where ϵ_A and ϵ are the molar absorption

$$K = [AS]/[A][S] \quad (1)$$

$$\log \frac{\epsilon_A c_A - A}{A - \epsilon c_A} = \log K + \log \left(c_S - \frac{\epsilon_A c_A - A}{\epsilon_A - \epsilon} \right) \quad (2)$$

coefficients of the alkaloid and of its adduct with the thiol, c_A is the total concentration of the alkaloid (eq 3) and c_S that of the thiol (eq 4), and A is the absorbance of the solution in a 10-mm cell (eq 5). The measurements were carried out for wavelengths

$$c_A = [AS] + [A] \quad (3)$$

$$c_S = [AS] + [S] \quad (4)$$

$$A = \epsilon_A[A] + \epsilon[AS] \quad (5)$$

where the molar absorption coefficient of the alkaloid differed from that of its adduct with the thiol [sanguinarine (1): 275, 328, and 470 nm; chelerythrine (2): 268, 315, and 400 nm]. A medium of 4×10^{-3} M hexadecyltrimethylammonium bromide was used for protection of the adduct in the solution. At the selected pH, dissociation of the SH groups of thiols in the solution did not occur.[21]

Assay of Aminotransferase Activity. The effect of the alkaloids on L-alanine:2-oxoglutarate aminotransferase and L-aspartate:2-oxoglutarate aminotransferase activities was studied by using the postmitochondrial supernatant of rat liver. For all experiments, male Wistar rats, weighing ca. 200 g, were used. The rats were decapitated, and the liver was removed and homogenized after the addition of 0.1 M, pH 7.4 phosphate buffer (5 mL/g of liver) at 0 °C. The homogenate was centrifuged (15 min at 10000g) at 0 °C. For enzyme assays, the supernatant was diluted 2000× with a 0.1 M, pH 7.4 phosphate buffer. The enzyme assays were performed by the method of Reitman and Frankel.[22] Partially purified L-alanine:2-oxoglutarate aminotransferase was isolated from the liver of corticoid-treated rats.[23] To increase the high levels of the enzyme, prednison in doses of 10 mg/kg (suspended in a solution of tylose, 50 g/L) was orally administered to each rat for 5 days before decapitation. The purification procedure was that as described in ref 18. The purification was terminated after ammonium sulfate fractionation by precipitation. For storage, the enzyme solution was lyophilized. Prior to use, the enzyme was freshly diluted with 0.1 M, pH 7.4 potassium phosphate buffer. For the measurements of the partially purified alanine aminotransferase, the method[22] was modified, i.e., the substrate solution (8.33×10^{-2} M L-alanine; 1.67×10^{-3} M 2-oxoglutarate; 0.1 M, pH 7.4 phosphate buffer) (0.3 mL) was started with 10 μL of an enzyme solution or an enzyme–alkaloid mixture after a 5-min preincubation. All the measurements were carried out at 30 °C, the incubation period was varied to retain optimal levels of absorbancy changes, and then 2,4-dinitrophenylhydrazine reagent (0.25 mL) was added, left standing for 20 min, and made alkaline with 0.4 M NaOH (2.5 mL). After 10 min, the absorbancy at 505 nm was measured. For analysis of the effect of the thiols on enzyme inhibition, substrate solutions were used containing 10^{-2} M of the corresponding thiol.

For dialysis, the enzyme (0.25 mg) was dissolved in a pH 7.4 phosphate buffer (5 mL), containing 0.1 M L-alanine, for prolongation of enzyme stability, and the studied alkaloid was added (3.1×10^{-4} M). After standing for 30 min at room temperature, the solution was dialyzed against the same buffer for a period of 24 h at 4 °C. The enzyme activity and the protein contents were assayed in aliquot parts of sample at the beginning and at the end of the dialysis. For control, the dialysis of only the enzyme was carried out under the same conditions. The proteins were determined by the procedure of Lowry.[24]

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A Structure–Activity Relationship Study of Spirolactones. Contribution of the Cyclopropane Ring to Antimineralocorticoid Activity

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A series of spirolactones containing a cyclopropane ring in the molecule was examined for its effects on the mineralocorticoid receptor. The results were compared with those of a similar series of spirolactones in which the cyclopropane ring was replaced by a double bond. Insertion of a double bond or an α-cyclopropane ring into the 1,2 or the 6,7 position leads to a reduction in the binding affinity. The π-bonding system of the β-cyclopropane ring at C-6 and C-7 does not promote binding to the receptor. The presence of the 6β,7β-cyclopropane ring may deter metabolic inactivation to account for the enhanced in vivo activity.

Aldosterone plays an important role in maintaining the electrolyte balance in the body. Since it promotes the retention of sodium and the excretion of potassium, inhibition of its effects on gene expression would be an appropriate adjunct to the treatment of pathologic conditions which are sodium dependent or which are characterized by edema formation.

In the mid-1950's, Kagawa, Cella, and Van Arman discovered a series of spirolactones that inhibited the effects of aldosterone.[1] One of them, spironolactone (1a), was ultimately marketed. Since the discovery of Kagawa et

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