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REACTION OF BIOLOGICAL THIOLS WITH THE TUMOR INHIBITOR JATROPHONE

INHIBITION OF RNA POLYMERASE

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SUMMARY

The plant-derived tumor inhibitor jatrophone has been shown to react with small molecular weight thiols as well as thiol groups on proteins such as bovine serum albumin and RNA polymerase (nucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) from *Escherichia coli*. In the latter case reaction with jatrophone was associated with loss of enzymatic activity. Thus after 11 SH groups per protomer of 400 000 daltons had reacted only approximately 10 % of the activity remained. The substrates only partially protected the enzyme from loss of activity.

INTRODUCTION

A number of naturally occuring α-methylene γ-lactones have been shown to inhibit tumor proliferation *in vitro* and *in vivo*.[1] Comparison of the relative cytoxicities of these sesquiterpene lactones and their derivatives has demonstrated that the α-methylene lactone function is essential to activity[2,3].

Several lines of evidence indicate that the nucleophilic addition of thiols to the tumor inhibitory α,β-unsaturated lactones may be involved in the growth inhibitory properties of these compounds. A study of the reactions of the α-methylene lactones with model biological nucleophiles revealed that thiols were the most reactive of the nucleophiles investigated, and that biological activity ‚decreased, markedly, with successive addition of cystein to bis-unsaturated lactones[2]. Subsequently, the tumor inhibitory α-methylene lactones were shown to inhibit phosphofructokinase[4] and glycogen synthase[5], with concurrent loss of enzyme SH groups. The electrophilicity of the tumor inhibitory quinone methides, taxodone and taxodione[6], and their inhibition of phosphofructokinase upon reaction with its SH groups[4], suggested that these compounds may also act *via* selective alkylation of biologically important macromolecular thiols.

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

Recently, a new tumor inhibitor, jatrophone, has been isolated from the plant *Jatropha gossypiifolia* and its structure (I, Fig. 1) has been elucidated[7]. Jatrophone's novel macrocyclic diterpenoid structure accounted for the readiness with which the molecule underwent two novel nucleophilic transannular conjugate addition reactions to yield the dihydrobromide II. In the present report, we describe the reaction of jatrophone with simple SH compounds, the macromolecular thiol, bovine serum albumin[8], and one SH enzyme involved in nucleic acid synthesis, namely, DNA dependent RNA polymerase (nucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) from *E. coli*[9].

MATERIALS

Enzymes and proteins

DNA dependent RNA polymerase from *E. coli* was a gift from Dr R. Kleppe. It had been prepared after a modified procedure of Chamberlin and Berg[11,12]. The subunit composition and the specific activity were as reported by Burgess[13].

Crystalline bovine serum albumin was obtained from Sigma Chemical Company. The purity was checked by analytical ultracentrifugation.

Nucleic acids and other reagents

T7 DNA was prepared according to published procedures[14]. DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] and all unlabelled nucleoside triphosphates were purchased from the Sigma Chemical Company. ^{14}C- and ^{3}H-labelled nucleoside triphosphates were from New England Nuclear or Amersham Radiochemical Centre. The nucleoside triphosphates were examined for purity by paper chromatography in solvent systems previously described[15]. Jatrophone was prepared according to a previously published procedure[7].

METHODS

Determination of SH groups and proteins

The concentration of free SH groups was determined using the reaction with DTNB[16,17]. The reaction mixture contained 10 mM Tris, pH 8.0, 1 mM DTNB and 10% ethanol. When total SH groups on proteins was determined 5 mM sodium dodecyl sulfate was also present. A small aliquot containing the SH group compounds was mixed with 1 ml of the DTNB solution, left at 20 °C for 10 min, then the absorbance at 412 nm was measured. Protein was estimated according to Lowry *et al.*[18].

Enzyme assay

The assay mixture for RNA polymerase contained 40 mM Tris, pH 8.0, 8 mM $MgCl_2$, 10 mM mercaptoethanol, 0.3 mM each of the four nucleoside triphosphates, 25 μg T7 DNA per ml. The labelled nucleoside triphosphate was [^{3}H]CTP with a spec. act. of 4250 cpm/nmole. The reactions were run for 10 min at 37 °C and the radioactive RNA precipitated on filter paper according to a published procedure[19].

Preparation of jatrophone n-propylthiol adduct (IIIa)

To a solution of I (28 mg) in tetrahydrofuran (0.75 ml) was added 0.5 ml

(pH 9.2) borate buffer[20] and *n*-propylthiol (0.25 ml). The mixture was stirred at room temperature for about 30 min and poured into water (10 ml). The aqueous solution was washed with ether. The ether extract was dried (Na_2SO_4) and evaporated with a stream of N_2. The residue was purified by thin-layer chromatography to afford a chromatographically homogeneous light yellow oil (24 mg): infrared (KBr) 5.72, 6.08 μm; ultraviolet maximum (methanol) 291 nm (ε 12 000); NMR ([^{2}H]chloroform) τ 3.73 (1 H, m), 4.30 (1 H, m), 6.01 (1 H, d, J = 13 Hz), 6.70 (1 H, d, J = 13 Hz), 8.10 (3 H, br s), 8.85 (3 H, s), 8.87 (3 H, d, J = 7 Hz), 8.89 (3 H, s), 9.13 (3 H, d, J = 7 Hz); mass spectrum, M^+ at *m/e* 388.2088 (calcd for $C_{23}H_{32}O_3S$: 388.2071), 360, 345, 318, 313, 312, 297, 289, 285, 279, 197, 196, 175, 165 and 135.

RESULTS

Reaction with small molecular weight thiols

The reaction between jatrophone and *n*-propyl thiol in borate buffer afforded the adduct IIIa, Fig. 1. High resolution mass spectrometry supported the formula $C_{23}H_{32}O_3S$, which substantiated the mono-adduct nature of the product. The absorption maximum at 291 nm (Fig. 2) in the ultraviolet spectrum indicated the presence in IIIa of the C-3–C-7 dienone chromophore. The carbonyl band at 5.72 μm in the infrared spectrum was indicative of the tetrahydrofuran-3-one system of IIIa. The NMR spectrum also supported assignment of structure IIIa, and the doublets at τ 6.01 (J = 13 Hz) and 6.70 (J = 13 Hz) could be assigned to the C-9 and C-8 protons, respectively. The large coupling constant was indicative of a *trans*-relationship of these protons, and defined the β-configuration of the thiol ether group. The ready formation of IIIa is envisioned as a trans-annular conjugate addition involving nucleophilic attack by the SH group at C-9 to form the 8, 12 bond.

The kinetics of the reaction with two other small molecular weight thiols, β-mercaptoethanol and dithiotreitol, are shown in Fig. 3. When plotted in a second order plot the reaction between jatrophone and β-mercaptoethanol gave a straight line indicating second order. The rate constant for this reaction at 25 °C was estimated

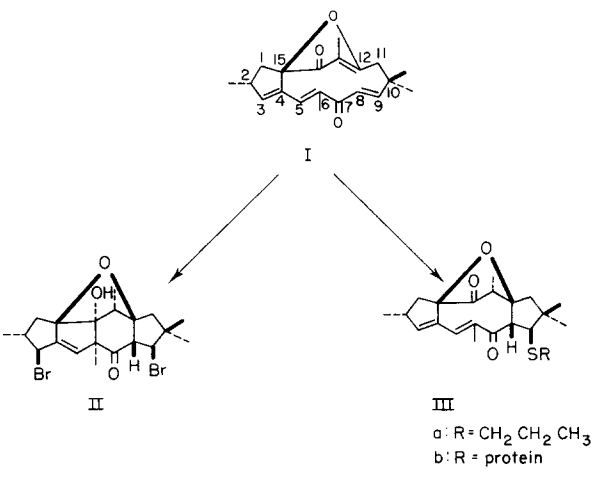

Fig. 1. Structure of jatrophone and addition products.

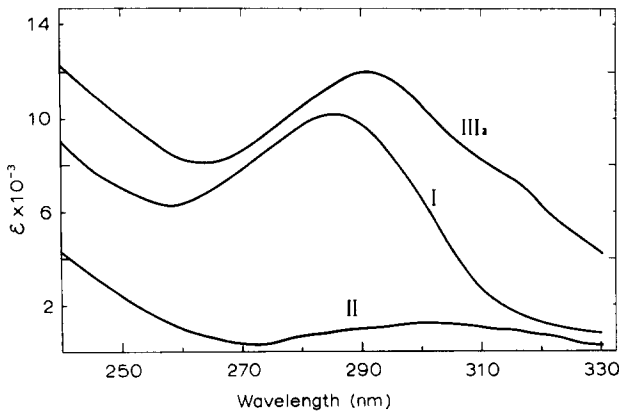

Fig. 2. Molar extinction coefficients in the ultraviolet region for jatrophone and its addition products (see Fig. 1 for structure of I, II and IIIa). The spectra were recorded in ethanol (I and II) and methanol (IIIa).

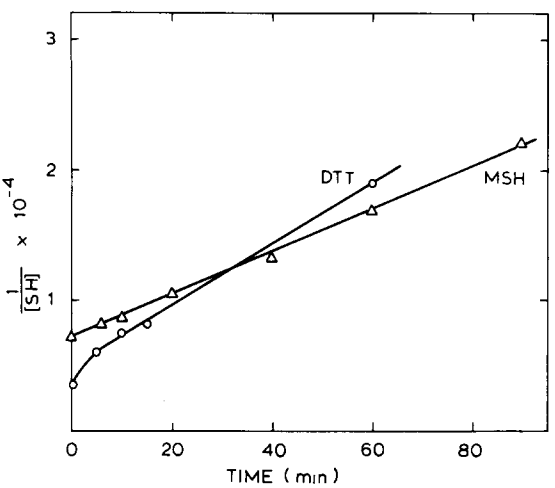

Fig. 3. Kinetics of the reaction between β-mercaptoethanol (MSH), dithiothreitol (DTT) and jatrophone. The reaction mixtures contained 10 mM Tris–HCl (pH 8.9), 10% ethanol, 0.13 mM jatrophone, 0.13 mM dithiothreitol or 0.13 mM β-mercaptoethanol. The reaction temperature was 25 °C and N_2 atmosphere was used. Aliquots were withdrawn at various times and the concentration of SH groups measured with the DTNB reagent as described in Methods. In the case of dithiothreitol a fine precipitate formed during the reaction with jatrophone. This was kept in suspension by bubbling N_2 through the solution. The aliquots withdrawn were pipetted into a solution containing 1 mM DTNB, 10 mM Tris–HCl (pH 8.0) and 70% ethanol where the precipitate dissolved immediately.

to be 160 $l \cdot mole^{-1} \cdot min^{-1}$. The reaction between jatrophone and dithiothreitol was more complex. When equimolar amounts of jatrophone and dithiothreitol were mixed, both SH groups on the dithiothreitol molecule appeared to react. The rate constant for reaction of the first SH groups was found using the half life method, to be 3100 $l \cdot mole^{-1} \cdot min^{-1}$ whereas the rate constant for the second SH group was $2.5 \cdot 10^{-2}$ $\cdot min^{-1}$. The detailed mechanism for the reaction between jatrophone and dithiothreitol is under further investigation and will be reported elsewhere.

Reaction with bovine serum albumin

The data given above suggested that SH groups on proteins also might react with jatrophone. That this indeed is the case is demonstrated in Fig. 4 which shows the kinetics of the reaction of SH groups on bovine serum albumin with jatrophone. After approximately 15 h at 25 °C all the SH groups on the albumin molecule have reacted. In the absence of jatrophone no loss of SH groups was detected in the same period. For comparison the reaction with DTNB was considerably faster than that with jatrophone. When the data were plotted in a semilogaritmic manner two types of SH groups were detected and found in approximately equal amounts. The rate constants with jatrophone for these two different types were estimated to be 18 and 8 $l \cdot mole^{-1} \cdot min^{-1}$. The corresponding rate constants for DTNB were found to be 100 and 86 $l \cdot mole^{-1} \cdot min^{-1}$.

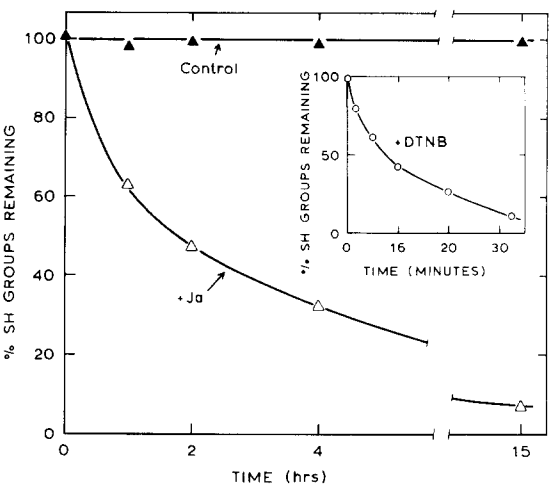

Fig. 4. Time course of the reaction of SH groups on bovine serum albumin and jatrophone. The reaction mixture contained: 10 mg/ml bovine serum albumin, 0.5 mM jatrophone, 20 mM Tris (pH 8.0) and 5% ethanol. N_2 atmosphere was employed and the temperature was 25 °C. The protein had been thoroughly dialyzed against the same buffer prior to the experiments. Aliquots were withdrawn at various times and DTNB and sodium dodecyl sulfate were added to a concentration of 1 mM and 5 mM, respectively. The absorbance was read at 412 nm after 10 min (see Methods). For comparison the reaction with DTNB and bovine serum albumin in the absence of sodium dodecyl sulfate is shown in the inserts. The concentration of DTNB was 0.5 mM, other conditions as described above. The bovine serum albumin contained 0.5 SH groups per monomer of 65 000 daltons.

A sample of bovine serum albumin which had been allowed to react with jatrophone for 15 h was subjected to gel filtration on a column of Sephadex G-25 as shown in Fig. 5. A good separation between protein and excess jatrophone was obtained. The protein from the column contained virtually no free SH groups. The ultraviolet spectrum of the jatrophone-treated bovine serum albumin is shown in Fig. 6 together with an untreated sample. The spectrum is in agreement with a monoadduct product of Type IIIb, (Fig. 2) (*cf.* the ultraviolet maximum for IIIa at 291 nm (ε 12 000) *vs* the ultraviolet maximum for II[7] at 300 nm (ε 1200) and 232 nm (ε 4700)). A portion of the jatrophone-treated sample was precipitated with 7% trichloroacetic acid. No

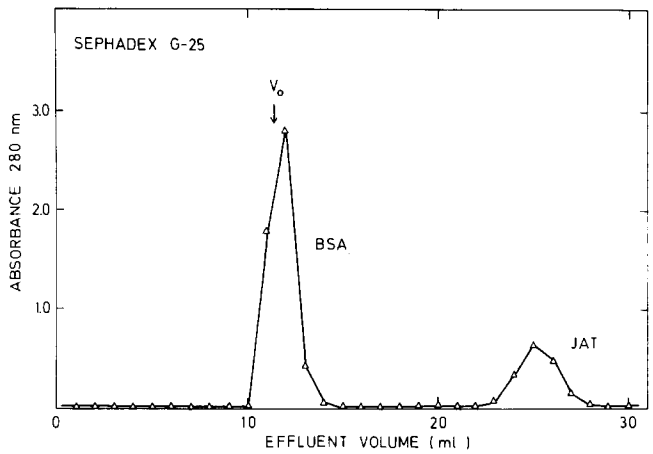

Fig. 5. Gel filtration of a reaction mixture of bovine serum albumin (BSA) and jatrophone (JAT). An aliquot of 1 ml from the reaction mixture described in the legend to Fig. 4 which had been allowed to react for 15 h was subjected to gel filtration on a column of Sephadex G-25 (1.2 cm × 30 cm) equilibrated with 20 mM Tris (pH 8.0). The column was run at 4 °C. Fractions of 1 ml were collected and the absorbance at 280 nm read. V_0, void volume.

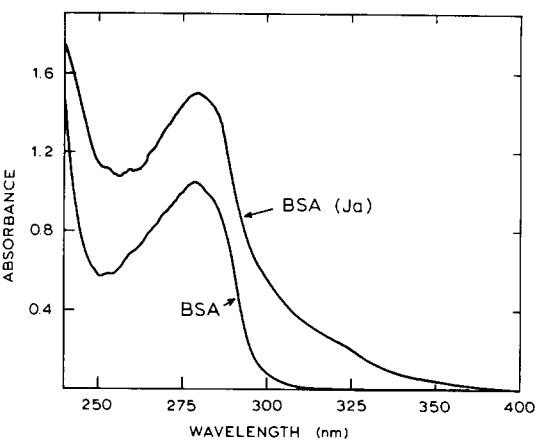

Fig. 6. Ultraviolet spectrum of jatrophone-treated bovine serum albumin BSA(Ja). The spectrum of the untreated protein is also shown. The concentration was 1.85 mg per ml and the buffer 20 mM Tris (pH 8.0).

ultraviolet absorbing material was detected in the supernatant, suggesting that the jatrophone attached to the bovine serum albumin is covalently linked.

Inhibition of DNA dependent RNA polymerase

The reaction between jatrophone and SH groups on proteins was further investigated with DNA dependent RNA polymerase from *E. coli*. As shown in Fig. 7 the enzyme is inactivated by jatrophone. Thus after 4 h at 25 °C approximately 50% of the activity had been destroyed and at the same time approximately 8 SH groups on the enzyme had reacted with jatrophone. Further incubation up to 22 h resulted in almost complete loss of activity. At that point 11 SH groups of the enzyme had

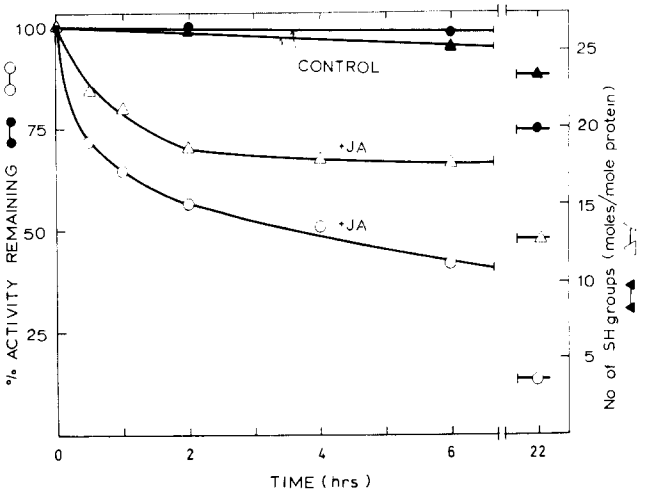

Fig. 7. Time course of the reaction between jatrophone (JA) and RNA polymerase. The reaction mixture contained 1 mg/ml RNA polymerase, 0.5 mM jatrophone, 40 mM Tris (pH 8.0), 0.1 mM EDTA and 5% ethanol. Temperature 25 °C and N_2 atmosphere was employed. The RNA polymerase had been thoroughly dialyzed against the same buffer under N_2 atmosphere prior to the experiment. Aliquots were withdrawn at various times and activity and remaining SH groups determined as described in Methods and in legend to Fig. 4. Closed symbols, control.

reacted. Only approximately half of the total number of SH groups of 28 on the enzyme appeared to be available for the reaction with jatrophone. The presence of nucleoside triphosphates or DNA protected the enzyme somewhat during the initial stages of the reaction as shown on Fig. 8. The degree of inhibition of RNA poly-

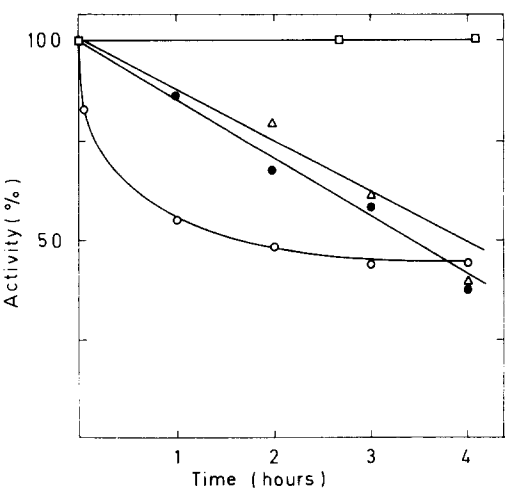

Fig. 8. Effect of nucleoside triphosphates and DNA on the inactivation of RNA polymerase by jatrophone. The incubation mixture contained: 40 mM Tris (pH 8.0), 0.1 mM EDTA, 0.7 mM jatrophone, 10% ethanol, 0.28 mg RNA polymerase/ml, 0.5 mM each of the four nucleoside triphosphates when added and 25 μg T7 DNA when added. Temperature 25 °C and N_2 atmosphere was used. Other conditions as described in the legend to Fig. 7. □—□, control (no jatrophone); ●—●, *plus* nucleoside triphosphates; △—△, *plus* DNA; ○—○, jatrophone only.

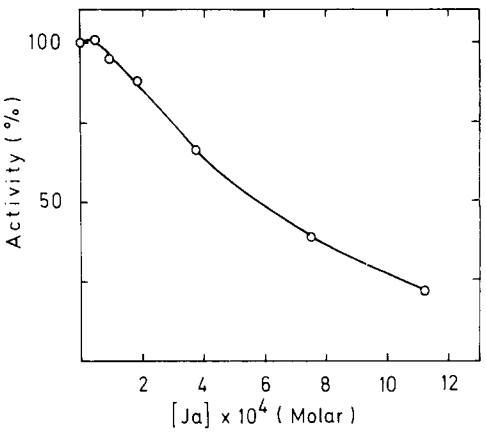

Fig. 9. Effect of concentration of jatrophone (Ja) on the inactivation of RNA polymerase. The conditions were as described in the legend to Fig. 8 except that the concentration of jatrophone varied as shown. The time of incubation was 4.5 h at 25 °C.

merase was dependent on the concentration of jatrophone (Fig. 9). In the presence of 1 mM jatrophone only approximately 25% of the activity remained after 4 h incubation. The rate constant for the reaction with SH groups on RNA polymerase was of the same order of magnitude as given for serum albumin.

A sample of RNA polymerase was allowed to react with jatrophone for 4 h, *i.e.* until approximately 50% of the activity remained. It was then subjected to gel filtration as described for bovine serum albumin. The spectrum of the jatrophone-treated RNA polymerase was similar to that for the bovine serum albumin which had reacted with jatrophone (Fig. 6). The partially inactivated RNA polymerase was also tested for sensitivity towards rifampicin and streptolydigin[21]. The first compound is known to inhibit initiation of RNA synthesis and the second elongation of RNA chains. The jatrophone-treated RNA polymerase was still sensitive to both of these inhibitors.

DISCUSSION

From the present work it is clear that jatrophone resembles the sesquiterpene tumor inhibitors with regard to reactivity towards SH groups. These compounds add SH groups to carbon–carbon double bonds by the Michael type mechanism. The chemical data given suggest that similar nucleophilic addition reactions also occur in the case of jatrophone and the structure of the adduct is shown in Fig. 1, III. SH groups on proteins can also react with jatrophone in a manner similar to that for small molecular weight thiols. Enzymes or proteins which are dependent on free SH groups for activity therefore might become inactivated by jatrophone. Reaction with SH groups on proteins was demonstrated in the present work both with bovine serum albumin and RNA polymerase from *E. coli*. In the case of RNA polymerase only 11 SH groups out of a total of approximately 28 had reacted in 22 h resulting in almost complete loss of activity. This observation is in good agreement with the finding of Sumegi *et al.*[22] who showed that the enzyme was completely inactivated when 12 SH

groups had reacted with cystamin. The SH groups on the enzyme which did not react with jatrophone are probably so-called buried SH groups. When SDS was added to unfold the peptide chains almost all the SH groups reacted readily with jatrophone. The mechanism of inhibition of RNA polymerase is probably similar to that described for inactivation of phosphofructokinase[4] and glycogen synthase[5] by certain sesquiterpene tumor inhibitors.

The inhibition of tumor growth by jatrophone may therefore be due to selective alkylation of the SH groups of key enzymes concerned with growth regulation. A similar mechanism has been postulated for the sesquiterpene lactones[2,4,5] and other plant-derived electrophilic tumor inhibitors[4,23].

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