Study of the binding of jatrophone to *Escherichia coli* s-ribonucleic acid

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The interaction of jatrophone with sRNA from *Escherichia coli* has been investigated through UV, CD, and ^{1}H NMR measurements. The results obtained show that the interaction with jatrophone increases the stability of the polynucleotide. It appears that the optical properties of jatrophone depend upon the jatrophone/nucleotide ratio. The observed behaviour can only be explained by the existence of different types of interaction between jatrophone and sRNA. Even for a jatrophone/nucleotide ratio as low as 0.17 the ^{1}H NMR spectra show a multiplicity of resonances that can only be explained by the simultaneous existence of two different binding modes involving the jatrophone molecules.

Jatrophone *sRNA binding* *Aqueous* *Solution* *UV* *CD* *^{1}H NMR*

1. INTRODUCTION

Jatrophone (fig.1), a macrocyclic diterpene isolated [1] from *Jatropha gossypiifolia* L. (Euphorbiaceae), has received considerable attention [2,3] due to its significant antitumor activity, both in vivo and in vitro [2].

The reaction between jatrophone and the thiol groups present in the side chains of amino acid residues reported for bovine serum albumin and RNA polymerase from *Escherichia coli* [4], in the latter case associated with loss of enzymatic activity, led to the hypothesis that jatrophone selectively alkylates the growth-regulatory biological macromolecules [4].

Previously [5] we obtained evidence in favour of the interaction between jatrophone and DNA, and proposed two possible explanations:

(i) The formation of two types of binding between jatrophone and DNA (e.g., two hydrogen bonds between the C_7 and C_{14} keto groups of jatrophone and the DNA phosphate groups).

(ii) One type of binding between jatrophone and DNA, and jatrophone–jatrophone interactions when these molecules are close to each other on the outer surface of DNA.

To gain further insight in the binding and to investigate whether jatrophone could also interfere at the RNA level we have now investigated its

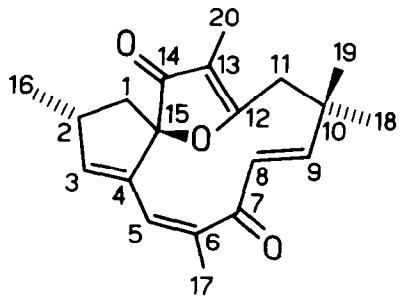

Fig.1. Molecule of jatrophone.

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binding with RNA. The results reported in the present paper provide evidence on the interaction between jatrophone and sRNA, and give a clarifying contribution to the knowledge of the binding mode of jatrophone.

2. MATERIALS AND METHODS

Jatrophone was obtained, purified and checked for purity as in [1,2,5].

The concentration of jatrophone in the jatrophone–sRNA solutions was determined using the molar ellipticity values of jatrophone at 337.5 and 340 nm in 0.01 M Tris–HCl (pH 7.30)–ethanol (90 : 10, v/v) solution ($[\theta]_{337.5} = -15\,130$; $[\theta]_{340} = -14\,152$ deg·cm^2·dmol^{-1}). These molar ellipticity values are not concentration-dependent, nor are they influenced by sRNA binding within the concentration range studied. The probable lack of ethanol may, however, slightly influence the molar ellipticity.

E. coli (strain B) s-ribonucleic acid sodium salt was a product of Calbiochem-Behring (Lucerne). Stock solutions of sRNA freshly prepared in 0.01 M Tris–HCl (pH 7.30) were exhaustively dialysed against the same buffer at 4°C and used immediately, or stored at −20°C. Concentrations of RNA solutions were determined spectrophotometrically, using a value of 7500 l·mol^{-1}·cm^{-1} (mol nucleotide/l) for the molar extinction coefficient at 260 nm [6]. Doubly glass-distilled water was used throughout.

The binding of jatrophone to sRNA was achieved as follows: A solution of jatrophone ($7 \times 10^{-4} - 1 \times 10^{-2}$ M) in ethanol (Uvasol, Merck) was added in portions of few μl to a cooled (0–4°C) sRNA aqueous solution ($2 \times 10^{-4} - 2 \times 10^{-3}$ M). The ethanol content in the solution in each experiment was 4% (v/v). The solution was kept for 100 h with shaking at 2–5°C using a Griffin flask shaker. Lyophilization of the solution followed by resolubilization in 0.01 M Tris–HCl (pH 7.30) and, after 24 h, centrifugation at 10 000 rev/min at 5°C, led to a solution without ethanol.

Absorption spectra were measured with Beckman DK-2A and Cary 219 spectrophotometers. Circular dichroism (CD) registrations were performed at 27°C with a Jasco J-500 A automatic recording spectropolarimeter, using cells of light path 2, 1, 0.2 or 0.1 cm. The instrument was calibrated with androsterone solution as a standard. The calculated curves for both the absorption and CD spectra were worked out by assuming the contribution of jatrophone and sRNA to be additive.

The nuclear magnetic resonance (NMR) spectra were run on a Bruker WH-270 spectrometer at 29°C. Memory blocks of 16 K were typically employed, with sweep widths of the order of 3000 Hz. The reference sample of jatrophone (fig.6A) was prepared by dissolving 1.12 mg/ml of jatrophone in a solvent made of 60% CD_3OD and 40% D_2O; tetramethylsilane (TMS) was used as internal standard. All aqueous samples for the NMR spectra contained 2.772×10^{-3} M sRNA and various amounts of jatrophone, corresponding to jatrophone/nucleotide ratios of ~0.11, ~0.17 and ~1.3 (fig.6, spectra B–D respectively).

D_2O (≥99.95 atom% D) and CD_3OD (≥99.5 atom% D) were from Merck.

All aqueous samples contained a very small amount of 3-trimethylsilyl-1-propanesulfonic acid sodium salt (TSP) for internal reference.

3. RESULTS AND DISCUSSION

The CD spectra of *E. coli* sRNA and jatrophone as well as the absorption spectrum of jatrophone are shown in fig.2. Jatrophone is virtually insoluble in water; its binding with sRNA causes it to become soluble.

Aqueous solutions with various jatrophone/nucleotide ratios in the range ~0.04–1.3 were examined. The observed and calculated absorption spectra of four solutions typical of the jatrophone–sRNA behaviour at low and high ratios are shown in fig.3. The molar concentration of jatrophone in the solutions was: 4.9×10^{-6}, 3.86×10^{-5}, 6.74×10^{-5}, 7.22×10^{-5}, M for curves a–d, respectively. Curves a, b and c show that a significant hyperchromic effect occurs on increasing the jatrophone content, a clear indication of the jatrophone–sRNA interaction. In fact, curve c, which refers to a jatrophone/nucleotide ratio of ~0.48, shows a ~62% hyperchromicity with respect to the calculated curve at 290 nm. Contrary to this result curve d, which refers to a jatrophone/nucleotide ratio of ~0.52, reveals a different behaviour. The observed absorption spectrum is nearly coincident with the calculated one. The decrease in the absorption increment confirms the presence of dif-

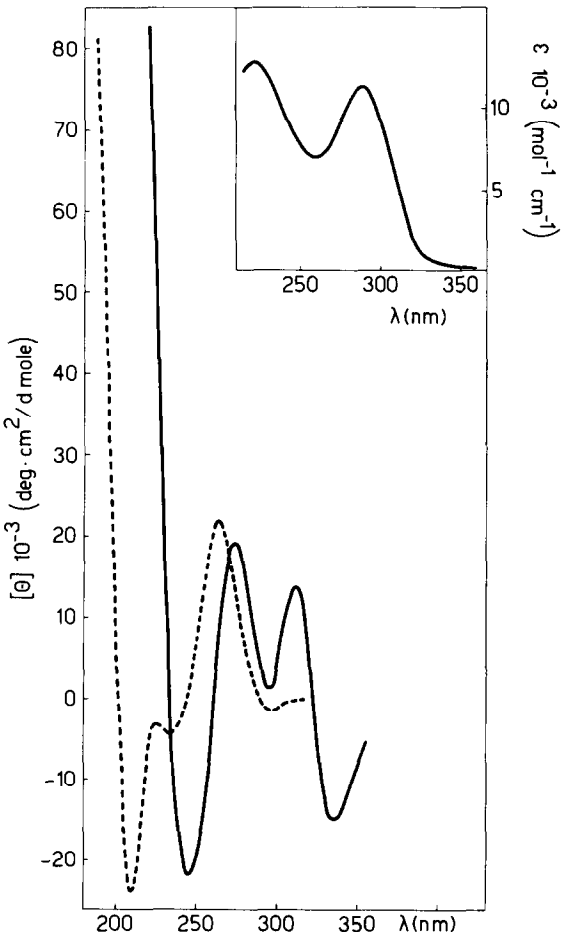

Fig.2. CD spectra of: *E. coli* sRNA, 1.386×10^{-4} M, in 0.01 M Tris–HCl (pH 7.30) (broken line); jatrophone, 5.26×10^{-5} M, in 0.01 M Tris–HCl–ethanol (90 : 10, v/v) solution (pH 7.30) (full line). The absorption spectrum of jatrophone under the same condition is shown (upper right).

ferent types of interaction involving the jatrophone molecules.

The effect of jatrophone on the secondary structure of sRNA was studied by measuring the heat denaturation profile of the jatrophone/nucleic acid solutions with different values of the jatrophone/ nucleotide ratio at 260 nm. The binding of jatrophone to sRNA improves its stability as implied by the increase of the melting temperature (T_m) shown in fig.4, where the hyperchromicity $(A_T - A_{T_0}/\ A_{T_0}) \times 100$ is plotted against temperature. A_T and A_{T_0} are the absorptions of

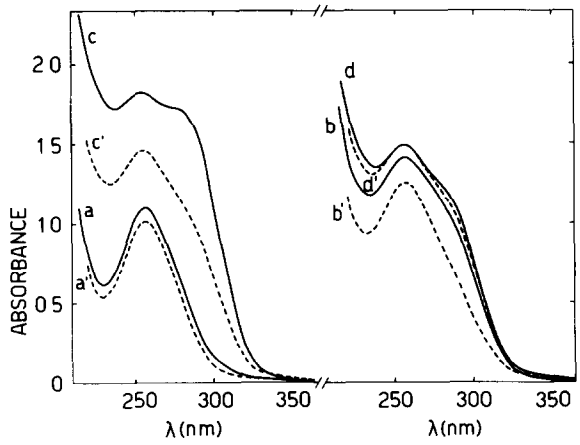

Fig.3. Absorption spectra of jatrophone with sRNA ($c = 1.386 \times 10^{-4}$ M), aqueous solutions (0.01 M Tris–HCl, pH 7.30). The jatrophone/nucleotide ratios are: (a) ~0.04, (b) ~0.28, (c) ~0.48, (d) ~0.52. Observed and calculated spectra are drawn with full and broken lines, respectively. The values are reported for 1 cm optical path.

the solution at a given temperature and at a reference temperature (27°C), respectively. It must be pointed out that, since jatrophone is insoluble in 0.01 M Tris–HCl aqueous solution, its contribution to the total absorbance could not be subtracted. Nevertheless, the A_{260} of jatrophone in ethanol–0.1 M Tris–HCl solution (pH 7.32; 10 : 90, v/v) is constant within 27–78°C, so that its contribution does not affect the result. T_m, obtained as the temperature corresponding to half of the maximum value of the hyperchromicity, increases gradually by increasing the

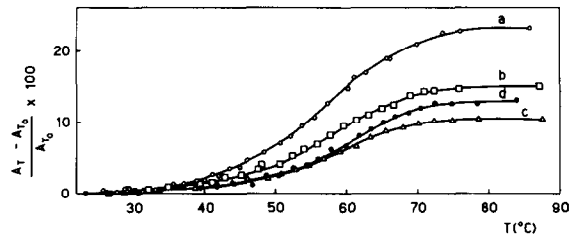

Fig.4. Thermal profiles of jatrophone–sRNA aqueous solutions (0.01 M Tris–HCl, pH 7.30). The sRNA molar concentration is 1.386×10^{-4} M and the jatrophone/nucleotide ratios are 0, ~0.28, ~0.48, and ~0.52 for samples a–d, respectively.

Table 1

270 MHz ^{1}H chemical shifts[a] of jatrophone in different solvent systems

Solvent	C1	C2	C3	C5	C8	C9	C11	C16	C17	C18 C19	C20
CD_3OD (60) D_2O (40)	2.21 1.77	2.97	5.78	5.82	6.01	6.65	3.07 2.51	1.09	1.86	1.36 1.25	1.72
Pyridine	2.13 2.01	2.91	5.86	6.04	6.26	6.75	2.88 2.39	0.93	1.83	1.33 1.20	1.82
D_2O[b]	2.25 1.73	2.99	5.78	5.88	6.06	6.73	3.10 2.49	1.05	1.82	1.33 1.22	1.69

[a] All shifts are in ppm and are referred to TSP (the D_2O solution) and TMS. The headings of the columns report the carbon atoms to which the hydrogens are attached

[b] This solution contains a jatrophone/nucleotide ratio of 0.17

jatrophone/nucleotide ratio (56.2, 56.6, 58.0 and 59.5°C for curves a–d, respectively).

Further evidence supporting the binding of jatrophone to sRNA is given in fig.5, where the observed and calculated CD profiles of four solutions are reported. The CD band at ~312 nm, which is representative of jatrophone since the sRNA contributes only marginally at this wavelength, is strongly dependent on the jatrophone/nucleotide ratio. Indeed curves a and b at jatrophone/nucleotide ratios ~0.04 and ~0.48 respectively, display a remarkable hyperellipticity at 312 nm as compared to the calculated curves. The opposite situation occurs with profiles c and d, at ratios ~0.52 and ~1.11 respectively, the observed ellipticity values being inferior to the calculated ones. All these results indicate that jatrophone forms a complex with sRNA showing many similarities with the complex formed with DNA [5].

In order to clarify the trend of the absorption and CD spectra the jatrophone–sRNA system was studied by ^{1}H-NMR spectroscopy. The effect of sRNA on the spectrum of jatrophone can be seen in fig.6, where the spectra of three solutions at dif-

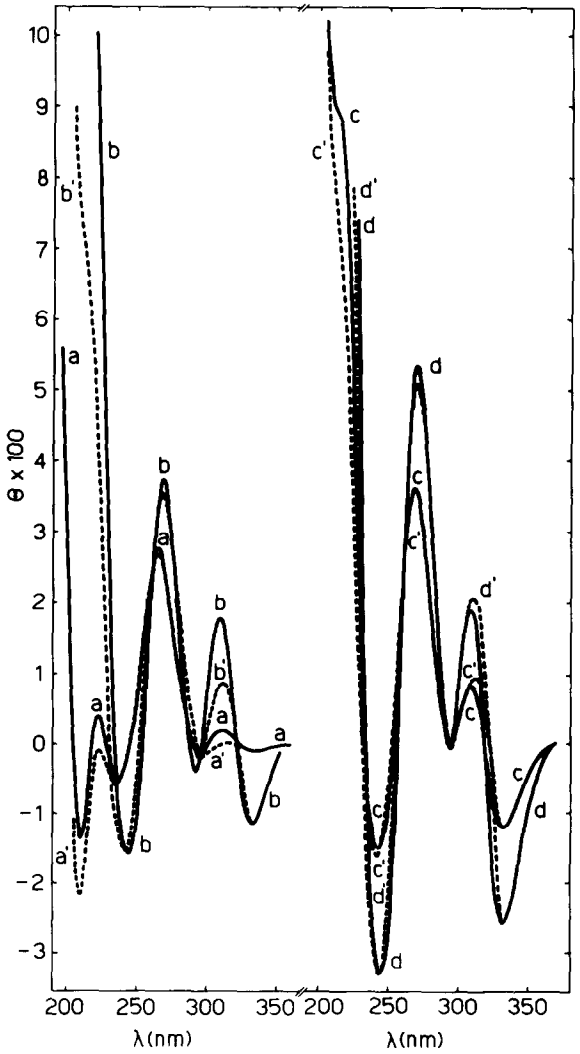

Fig.5. Observed (full lines) and calculated (borken lines) CD spectra of jatrophone with sRNA aqueous solutions (0.01 M Tris–HCl, pH 7.30). The sRNA molar concentration is 1.386×10^{-4} M and the jatrophone/nucleotide ratios are ~0.04, ~0.48, ~0.52, ~1.11 for samples a–d, respectively. The values are reported for 1 cm optical path.

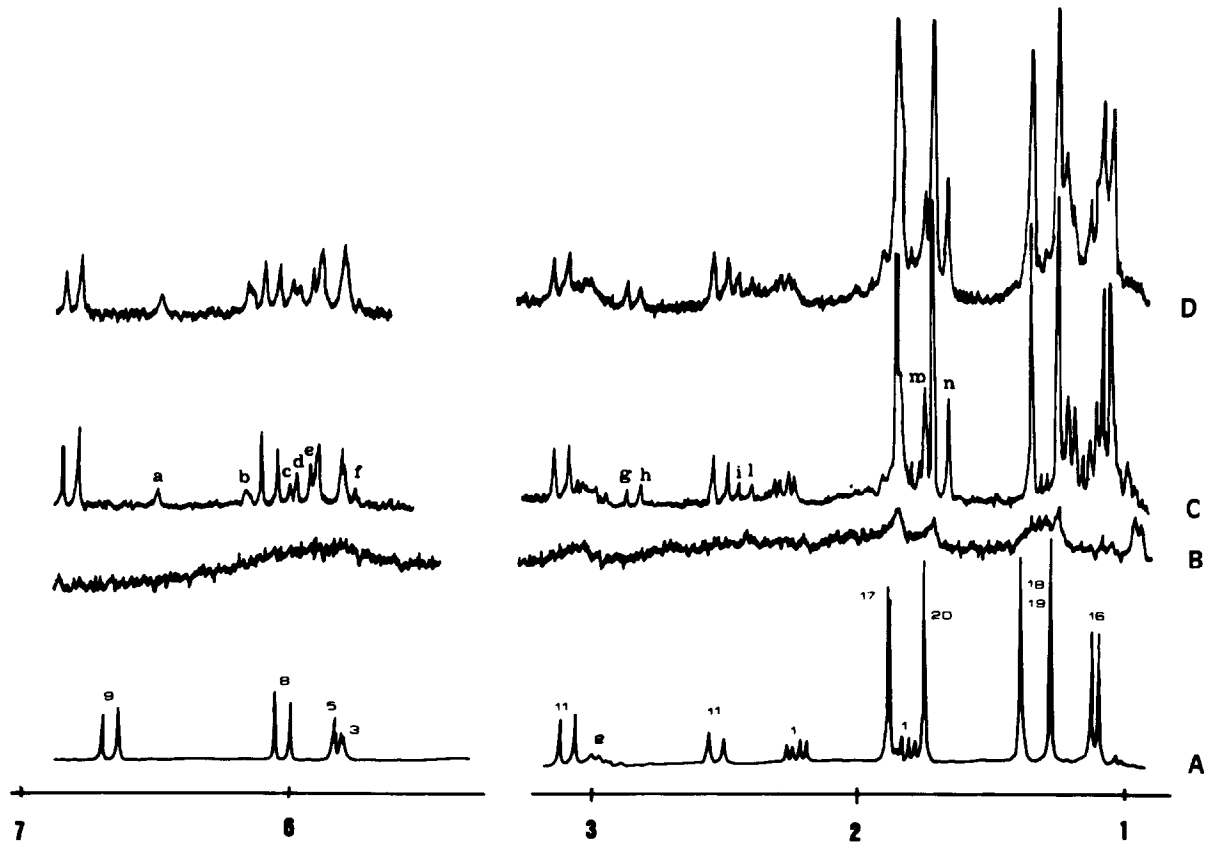

Fig.6. Comparison of the 270 MHz ^{1}H-NMR spectra of: (A) jatrophone in CD_3OD/D_2O (60:40, v/v); and jatrophone–sRNA in D_2O. The sRNA molar concentration is 2.77×10^{-3} M and the jatrophone/nucleotide ratios are ~0.11, ~0.17 and ~1.30 for spectra B–D, respectively. Chemical shifts are in ppm.

ferent jatrophone/nucleotide ratios are compared with the spectrum of jatrophone in a mixture of CD_3OD/D_2O (60:40, v/v). The ^{1}H-NMR spectrum of sRNA at the concentration used in the experiments is simply a distorted baseline; addition of jatrophone in an amount corresponding to a jatrophone/nucleotide ratio of ~0.11 causes the appearance of a few resonances in the aliphatic region (spectrum B). Although it is very likely that they correspond to the methyl groups of jatrophone, a detailed assignment is not justified by the quality of the spectrum. However, a meaningful overall interpretation of the spectrum in terms of interaction with sRNA is possible through a comparison with the spectra of solutions with higher ratios. Fig.6C shows the spectrum of the system with a jatrophone/nucleotide ratio of ~0.17 : the addition of further jatrophone caused dramatic spectral changes. All peaks of jatrophone are present and can be identified with certainty. Some of the chemical shifts are reported in table 1; it can be seen that most are rather close to the values in CD_3OD/D_2O except for those of hydrogens C9, C17, C18 and C19 that are very similar to those reported in pyridine in [2]. In addition to the peaks reported in table 1 many other resonances appear in spectrum C, with smaller intensities (and different shapes in some cases) but in the same regions of chemical shifts. The simplest explanation is that we can observe the coexistence of two different forms of jatrophone linked to sRNA, one corresponding to the main peaks of spectra C and D, the other corresponding to the small peaks labelled a–n in spectrum C. Both the shapes (identical to those of free jatrophone) and the chemical shifts of the resonances of the more populated species point to

a weak and non-specific complexation. In fact the great similarity of the shifts with those of free jatrophone in CD_3OD/D_2O indicates that these molecules are exposed to water to a large extent. That is, it is likely that they lie on the outer surface of sRNA, and are linked to it via hydrogen bonds. The smaller peaks, on the other hand, reflect a much stronger interaction, judging both from the change in the shape of the peaks in the olefinic region (a–f) and from the shifts of the few peaks (g–n) that have been tentatively assigned to protons 11 (the AB quartet from g–l), 17 (m) and 20 (n). All these are shifted well upfield, as if under the influence of the ring currents of the bases of sRNA. Thus it might be proposed that a smaller portion of jatrophone molecules solubilised by sRNA is strongly complexed to it, with the involvement of the C8=C9 double bond and with the region that surrounds this bond affected by the presence of the sRNA bases. The increase of the jatrophone/nucleotide ratio to ~1.3 (spectrum D) induces minor changes in the spectrum. The proportion of strongly bound molecules increases slightly (from 25% in spectrum C to 30% in D) and all peaks broaden to some extent. The only remarkable changes are those in the region between 1.0 and 1.3 ppm, but unfortunately the resolution is not sufficient even for tentative assignments.

Future studies at higher fields and with the aid of 2-dimensional techniques will probably unveil further details of the mechanism of interaction.

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