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Resolution of racemic gossypol and interaction of individual enantiomers with serum albumins and model peptides

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Racemic gossypol has been resolved by HPLC separation of diastereomeric (−) norepinephrine adducts on a reverse-phase column. The binding constants for the interaction of the three gossypol forms (+, − and ±) with human and bovine serum albumins have been determined by fluorescence quenching studies. The K_D values demonstrate that all three forms bind equally effectively to the two proteins, suggesting an absence of chiral discrimination in albumin-gossypol interactions. Circular dichroism studies of (+)-gossypol binding to the model dibasic peptides, Boc-Lys-Pro-Aib-Lys-NHMe and gramicidin S, suggest that distortions of binaphthyl geometry may occur only for specific orientations of interacting residues at the receptor site.

Introduction

Gossypol (**I**), a polyphenolic bissesquiterpene, has been isolated in racemic (±) form from cottonseed [1] and in the optically active (+) form from *Thespesia populnea* [2,3] and *Montezuma speciosissima* [4]. The (−) form has so far not been isolated directly from any natural source. The existence of enantiomeric forms of gossypol is a consequence of restricted rotation about the C–C bond linking the two naphthyl rings [5]. Recent interest in the chemistry and biology of gossypol has been stimulated by the extraordinary efficacy of the (±) form as a male antifertility agent [5–7]. The demonstration of the potential utility of gossypol as an antimalarial drug [8], its reported antitumour properties [9,10] and its possible application in the treatment of herpes virus infections [11] and Chagas disease [12] have further served to focus interest on the properties of this remarkable molecule. Recent reports that the (+) form lacks antifertility activity [13] suggest that the male contraceptive properties of racemic gossypol are derived from the (−) enantiomer. While chiral 'biological receptor sites' would be expected, in principle, to discriminate between the enantiomeric gossypol forms, almost equal affinities for binding of (±)- and (+)-gossypol to serum albumins has been reported [14]. We describe in this communication a rapid procedure for the optical resolution of gossypol as diastereomeric (−)-norepinephrine complexes (**II**) and compare the interaction of the isolated enantiomers with serum albumins. Circular dichroism studies with model dibasic binding sites are also reported. The choice of dilysyl or diornithyl peptides is relevant in view of reports that gossypol interactions with proteins may involve Schiff's base formation, between ε-amino groups of lysine residues and the aldehyde functions on gossypol [15].

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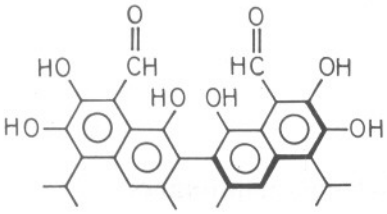
Abbreviations: Boc-, *t*-butoxycarbonyl-; Aib, α-aminoisobutyric acid; NHMe, *N*-methylamide.



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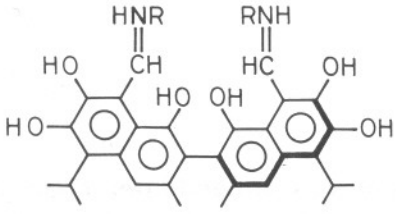

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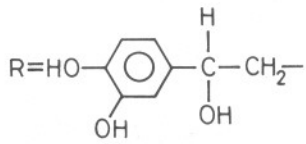

Materials and Methods

(±)-Gossypol – acetic acid was obtained as a gift from the National Institute of Child Health (WHO special program). (+)-Gossypol was isolated from the bark of *Thespesia populnea* [2]. Boc-Lys-Pro-Aib-Lys-NHMe was synthesized by standard solution phase procedures [16] and fully characterized by 80 MHz ^{1}H-NMR and shown to be homogeneous by TLC on silica gel. All other chemicals were from Sigma Chemical Co. or were of the highest available grade.

Optical resolution of (±)-gossypol was achieved by separation of diastereomeric (–)-norepinephrine complexes on a reverse-phase Lichrosorb RP-18 column (4 × 250 mm, 10 μm) using methanol-water gradient elution (62–72% methanol, 10 min, 72–82% methanol, 30 min; flow, 0.8 ml/min; detection at 226 or 365 nm). The complexes were obtained by allowing a (±)-gossypol solution in methanol to stand in contact with solid (–)-norepinephrine for 8–10 h, in the dark. (–)-Norepinephrine is practically insoluble in methanol and the solution contains only the gossypol adducts. Quantitative conversion of free gossypol to the adducts was established by HPLC and NMR. Free (+)- and (–)-gossypol were obtained by hydrolysis of the isolated complexes with 2 M HCl for 2 h, followed by extraction into chloroform. Characterization of the enantiomers was accomplished by HPLC, circular dichroism spectra (450–200 nm, see Fig. 1) and optical rotation ($[\alpha]_D^{25}$ ((+) form) = +388° (c = 0.027, $CHCl_3$); $[\alpha]_D^{25}$ ((–) form) = –379° (c = 0.0197, $CHCl_3$)).

The binding of gossypol to the proteins was monitored by following the quenching of the intrinsic protein fluorescence upon addition of gossypol. Measurements were made in phosphate buffer (1 mM; pH 7.4) using a Hitachi 650-60 fluorescence spectrophotometer. For all fluorescence quenching titrations, $\lambda_{excitation}$ = 284 nm, $\lambda_{emission}$ = 346 nm. Data for the binding of gossypol were analyzed by the method of Halfman and Nishida [17] after correcting for inner filter effects [18].

Circular dichroism spectra were recorded on a JASCO J-20 spectropolarimeter while ^{1}H (270 MHz) and ^{13}C (67.89 MHz) NMR spectra were recorded on a Bruker WH-270 spectrometer at the Sophisticated Instruments Facility, Bangalore.

Results and Discussion

Fig. 1a shows the resolution achieved in the HPLC separation of the (–)-norepinephrine adducts of gossypol. This procedure is simpler and more efficient than the method described by Zheng et al. [19], where optically pure enantiomers could be obtained only from early fractions in column chromatography of gossypol-1-methyl-2-phenylethylamine adducts. In this procedure, isolation of both gossypol enantiomers requires two independent chromatographic separations involving the *R*- and *S*-amines. Another recent report uses HPLC separation on chiral bonded phases [20]. The circular dichroism spectra of the isolated adducts are illustrated in Fig. 1b. The identity of **II**, as the bis-Schiff's base was established by ^{13}C-NMR, which showed the absence of the aldehyde CO group (199.2 ppm) and the appearance of the aldimine carbon resonance (172.7 ppm). The diastereomeric adducts were further characterized by ^{1}H-NMR. Adduct formation results in a shift of the aldehyde proton (11.31 ppm) to high field in the Schiff base (9.79–9.80 ppm). Fig. 2 shows

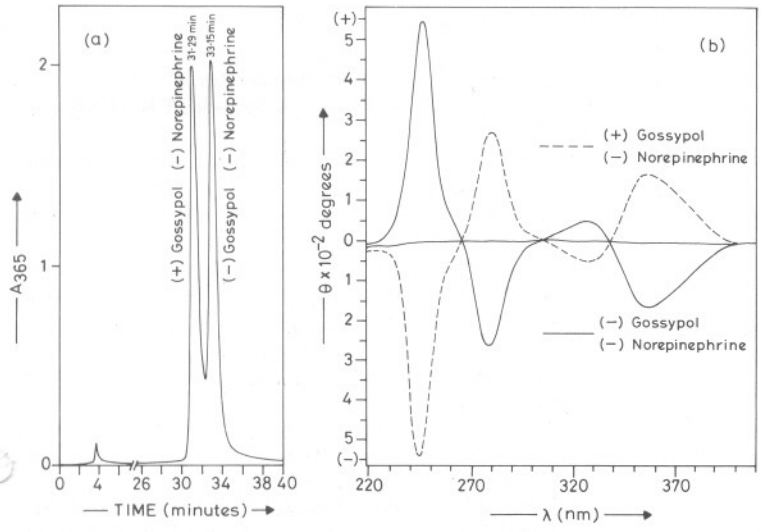

Fig. 1. (a) HPLC trace of the (±)-gossypol – (−)-norepinephrine adduct. Retention time is indicated against the peaks. (b) Circular dichroism spectra of the isolated adducts in 80% methanol-water.

the low field resonances in **II** and in the separated diastereomers. The two forms give rise to distinct aldimine – CH= resonances, while the aromatic signals (7.55 ppm) overlap.

The binding affinities of the three forms of gossypol (+, − and ±) to human and bovine serum albumins were determined by fluorescence quenching studies. Linear Scatchard plots yielded a single high-affinity binding site. K_D values of $(5.43 \pm 3.49) \cdot 10^{-8}$ M (+), $7.98 \pm 2.45 \cdot 10^{-8}$ M (−) and $7.55 \pm 1.84 \cdot 10^{-8}$ M (±) were detrmined for bovine serum albumin. Values of $6.63 \pm 2.07 \cdot 10^{-8}$ M (+), $7.38 \pm 2.44 \cdot 10^{-8}$ M (−) and $4.78 \pm 0.42 \cdot 10^{-8}$ (±) were obtained for human serum albumin. The results establish that all three forms of gossypol bind with almost equal affinities to both proteins, suggesting that these binding sites are not capable of chiral discrimination of gossypol. This is in agreement with our earlier report that the albumins bind the (+) and (±) forms of gossypol with almost similar affinities [14]. The absence of differential effects of the two enantiomers has also been noted in various in vitro assays. These include inhibition of testosterone release by Leydig cells [21], spermicidal effects [22], inhibition of proacrosin conversion to acrosin, inhibition of oocyte penetration by human spermatozoa [23] and inhibition of the testicular mitochondrial respiratory chain [24].

Two model dibasic peptides have been investigated for their ability to interact with gossypol. In the presence of Boc-Lys-Pro-Aib-Lys-NHMe, a weak induced circular dichroism band at approx. 370 nm (negative) was observed for (±)-gossypol, suggesting differential effects on the two enantiomeric forms. Fig. 3 illustrates the long wavelength circular dichroism band of (+)-gossypol in the presence of this peptide. A dramatic enhancement of the 370 nm band, relative to free gossypol, is observed. In the presence of gramicidin S

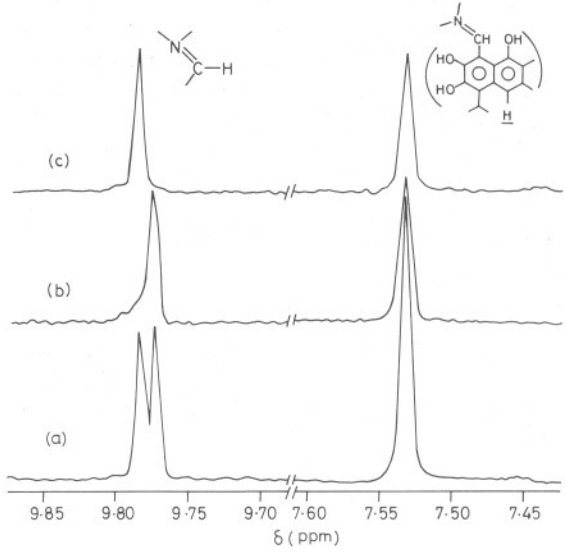

Fig. 2. Low field resonances in the 270 MHz ^{1}H-NMR spectra (C^2H_3OH) of gossypol-norepinephrine adducts. a, (±)-gossypol; b, (−)-gossypol; c, (+)-gossypol.

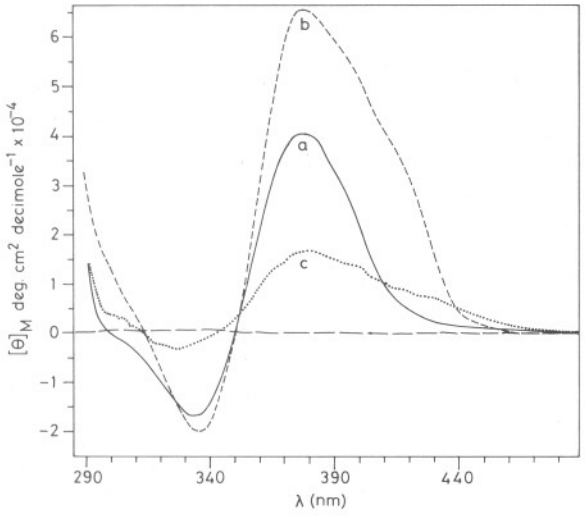

Fig. 3. Partial circular dichroism spectra of aqueous solutions of (+)-gossypol ($8 \cdot 10^{-6}$ M). a, (+)-gossypol; b, (+)-gossypol + Bos-Lys-Pro-Aib-Lys-NHMe ($1 \cdot 10^{-4}$ M); c, (+)-gossypol + gramicidin S ($2 \cdot 10^{-4}$ M).

(cyclo(Leu-D-Phe-Pro-Val-Orn)$_2$) both long wavelength bands show a large decrease in intensity (Fig. 3c). These results suggest that interactions of gossypol with certain diamine receptors can result in significant changes in binaphthyl geometry. If such stereochemical changes are necessary for binding, chiral discrimination may occur as a consequence. Presumably, in the proteins investigated the orientation of the interacting residues does not require major changes in binaphthyl geometry for accommodation at the binding site. For example, in interactions with albumins no significant change has been observed in the long wavelength circular dichroism band of (+)-gossypol [14].

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