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Determination of microscopic dissociation constants of 3-hydroxy-α-(methylamino)methyl-benzenemethanol by a spectral deconvolution method

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Abstract—Microscopic dissociation constants of 3-hydroxy-α-(methylamino)methyl-benzenemethanol have been calculated from the titration spectrophotomeric data ($c = 3{\cdot}8 \times 10^{-4}$ M. Ionic strength = 0·16; buffer system: H_3BO_3/KOH) by application of a spectral deconvolution method. The results found ($pK_a = 9{\cdot}48$; $pK_b = 9{\cdot}71$; $pK_c = 10{\cdot}12$ and $pK_d = 9{\cdot}88$) are in good concordance with those obtained from the conventional regression linear method ($pK_a = 9{\cdot}45$; $pK_b = 9{\cdot}77$; $pK_c = 10{\cdot}14$ and $pK_d = 9{\cdot}81$).

Phenolic compounds with one amino group in the side chain, for example HO—ψ—R—NH_2^+—R′, may be represented as Z_0^+ when the molecule is in its most highly protonated state. Thus, if the dissociation equilibria are simultaneously established, it could be written:

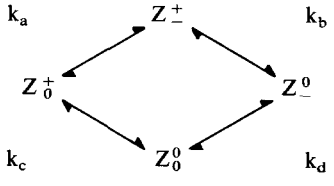
$$Z_0^+ \xleftarrow{K_1} (Z^\pm + Z_0^0 + H^+)$$

$$(Z^\pm + Z_0^0) \xleftarrow{K_2} Z^0 + H^+$$

Scheme 1

Where K_1 and K_2 are the macroscopic constants, Z_0^0 represents the neutral form resulting in amino group dissociation, $Z^\pm$ the dipolar form and Z^0 the completely dissociated form.

Scheme 1 can be described in detail if the dissociation equlibria of each ionizable group are considered



Scheme 2

where k_a, k_b, k_c and k_d are the microdissociation constants.

UV-Vis spectrophotometry has proved to be a useful technique for the determination of microdissociation constants of polyprotic drugs with overlapping dissociations.

The most common procedure for determining k_a, k_b, k_c and k_d is based on the measurement at a particular wavelength of absorbance variations which can be related to the percentage of dissociation of the phenolic group.

Thus, the parameter α is defined as:

$$\alpha = \frac{|Z^\pm| + |Z^0|}{C_T}$$

in which C_T is the total concentration of the analysed compound.

From K_1 (previously evaluated by potentiometry) and α it is possible to obtain the values of the microdissociation constants, by using the following equation

$$\alpha(K_1 + |H^+|) = k_a + K_1K_2\frac{(1-\alpha)}{|H^+|} \quad (1)$$

and the well known relationships between k_a and the other constants (Riegelman et al 1962).

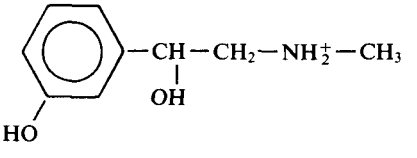
On the other hand, taking into account that, in most instances, an irrelevant absorption of Z^+ and Z_0^0, as well as an identical absorption of $Z^\pm$ and Z^0 ($\varepsilon_{Z^0} = \varepsilon_{Z^\pm}$) may be considered at the wavelength (λ) chosen for the measurements, the values of α can be related to the absorbance as follows

$$\alpha = \frac{A^\lambda}{A^\infty} = \frac{|Z^\pm| + |Z_0^0|}{C_T}$$

in which A^∞ is the absorbance measured at $pH > pK_2 + 2$.

Evidently, when either significant absorbance from Z_0^+ and Z_0^0 is found or the equality $\varepsilon_{Z^0} = \varepsilon_{Z^\pm}$ is not fully achieved, the relation α-absorbance will be altered and, therefore, equation (1) is not useful.

In a present paper, the acid dissociation of 3-hydroxy-α-(methylamino)methyl-benzenemethanol (I) has been studied by means of a spectral deconvolution method (Harris et al 1976) applied to the measurements made over a wide range of wavelengths. The purpose of this study was to determine the microdissociation constants considering the contribution of the four chemical forms involved in the dissociation of I.



(I)

Materials and methods

All the chemicals used were of analytical grade. Compound I was donated by Boehringer Sohn Ingelheim.

The values of macroscopic dissociation constants have been previously obtained (Baena 1987) by potentiometry. A solution of I ($3{\cdot}6 \times 10^{-3}$ M) was titrated with KOH 0·001 M (ionic strength = 0·16 at the midpoint of the titration) and by application of an iterative method (Navarro 1986; Cabeza et al 1988; Talavera et al 1989) to the experimental data the following results were found: $pK_1 = 9{\cdot}28$ and $pK_2 = 10{\cdot}32$.

Spectrophotometric measurements were performed on a Perkin-Elmer Lambda 5 spectrophotometer and pH-measurements on a Radiometer PHM 64 Potentiometer. All measurements were made on freshly prepared solutions. Temperature was controlled with a Termostato Selecta.

Molar absorptivities of I in HCl 0·16 M ($\lambda = 273$ nm; $\varepsilon = 1765$ L mol^{-1} cm^{-1}) and KOH 0·16 M ($\lambda = 291$ nm; $\varepsilon = 2913$ L mol^{-1} cm^{-1}) were determined by the usual procedure.

Determination of the number of absorbent species was carried out by application of Coleman's test (Coleman et al 1970) to the

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measurements made at different wavelengths with solutions of I either in KOH 0·16 M or in a buffered medium (H_3BO_3/KOH) at pH 12·35.

Solutions of I ($3·8 \times 10^{-4}$ M) were used in the spectrophotometric titration. The ionic strength was kept constant at 0·16 by means of KCl.

The absorbances were measured at 2 nm intervals in the regions of 309 nm to 265 nm. The solutions used were maintained at 20°C ± 0·1°C and the pH values of 7·71, 7·92, 8·18, 8·50, 8·88, 9·15, 9·33, 9·46, 9·58, 9·97, 10·10, 10·28 were obtained with H_3BO_3/KOH buffer. However, in the analysis of these experiments only those data in the pH range 9·33–10·28 were used since in this range the four dissociation equilibria are simultaneously established.

The spectral data were analysed by means of the weighted least-square minimization computer program developed by Meztler (Llor et al 1984) and adapted to an Eclipse MV-100000 General Data Computer.

Results

Coleman's test gave straight lines (Fig. 1) with intercepts approaching zero and coefficients of 1·0 in all cases. From this, it is apparent that $Z^0_{\pm}$ is the unique absorbent form at pH = 12·35 or higher.

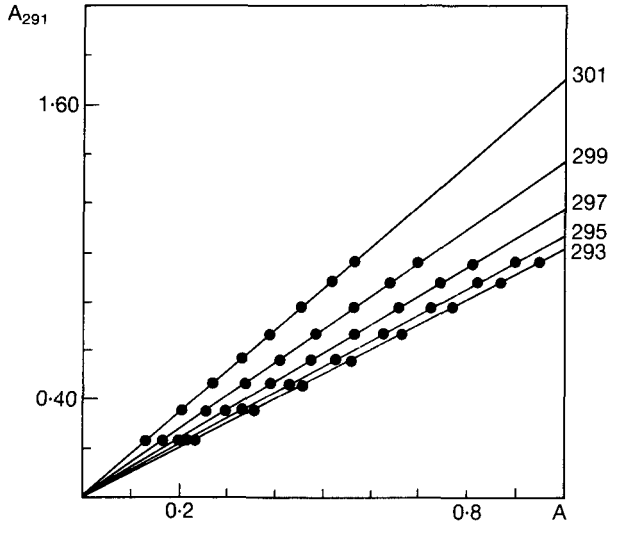

Fig. 1. Plot of the values of absorbance measured at 291 nm vs the values of absorbance measured at 293 nm, 295 nm, 297 nm, 299 nm and 301 nm using buffered solutions of I (pH = 12·35; ionic strength: 0·16).

From the absorbance data recorded in the 309–265 nm wavelength range (Fig. 2) and from the macroscopic constant values $pK_1 = 9·28$ and $pK_2 = 10·32$, spectra of anionic ($Z^0_{\pm}$), cationic (Z^+_0) and uncharged ($Z^{\pm}$, Z^0_0) forms were obtained (Fig. 3) by using the computer method of Metzler.

Furthermore, the spectra were deconvoluted assuming a log normal shape for the component bands. In Fig. 4, as an example, the deconvoluted spectrum of uncharged forms ($Z^{\pm}$, Z^0_0) is shown. It is apparent that there is good agreement when the original curve (experimental data) is compared with the sum of log normal components.

The deconvolution data are summarized in Table 1. These data allow the components of the absorption of the uncharged forms to be separately assigned. Thus, the $Z^{\pm}$ form must have the band I at 34·30 KK and the band II at 38·91 KK since there is an evident structural relation with $Z^0_{\pm}$ whose bands are I: 34·43 KK and II: 38·83 KK. Likewise, the bands located at 36·76 KK (band I) and 36·06 KK (band II) may be attributed to the absorption of the Z^0_0 form due to the similarity with the components of the Z^+_0 absorption (band I = 36·88 KK and band II = 36·02 KK).

These assumptions seem to be supported by the fact that there

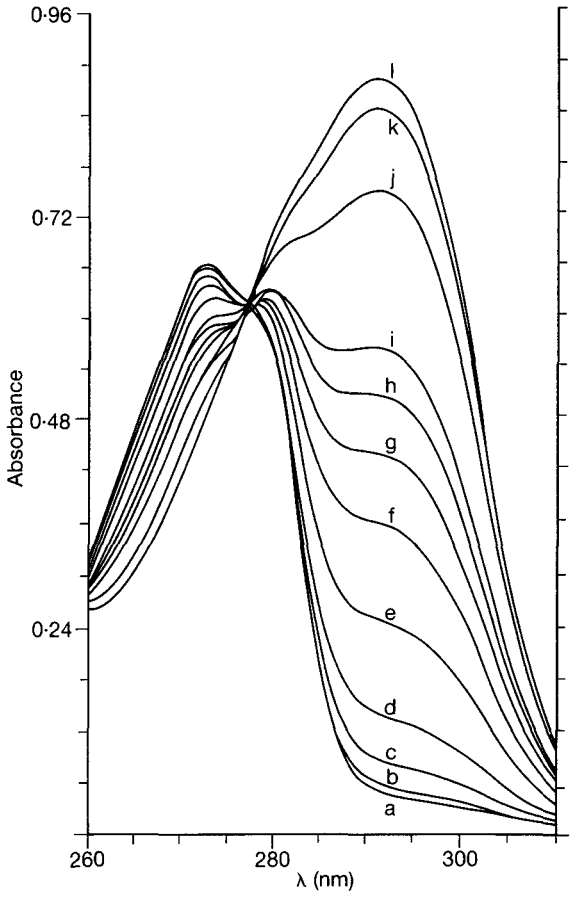

Fig. 2. Absorption spectra of buffered solutions of I ($c = 3·8 \times 10^{-4}$ M; ionic strength: 0·16). (a) pH = 7·71; (b) pH = 7·92; (c) pH = 8·18; (d) pH = 8·50; (e) pH = 8·88; (f) pH = 9·15; (g) pH = 9·33; (h) pH = 9·46; (i) pH = 9·58; (j) pH = 9·97; (k) pH = 10·20; (l) pH = 10·28.

Table 1. Assignments of the data obtained in the spectral deconvolution of anionic ($Z^0_{\pm}$), cationic (Z^+_0) and neutral ($Z^{\pm}$, Z^0_0) forms derived from I.

Ionic forms	Band	$\bar{\nu}_0$ (KK)	ε_0	W (KK)	P	Ii
$Z^0_{\pm}$	1[a]	34·43	2944	3·276	1·415	105
$Z^0_{\pm}$	2[a]	38·83	351	2·114	0·776	8
$Z^{\pm}$, Z^0_0	1[a]	34·30	1913	3·159	1·395	66
$Z^{\pm}$, Z^0_0	2[a]	38·91	292	2·058	0·819	7
$Z^{\pm}$, Z^0_0	3[a]	36·76	477	2·960	1·247	15
$Z^{\pm}$, Z^0_0	4[a]	36·06	195	2·183	2·351	5
Z^+_0	1[a]	36·88	1239	2·980	1·155	40
Z^+_0	2[a]	36·02	622	2·221	2·347	17

Ii represents the integrated Intensity in Mm mol^{-1}.

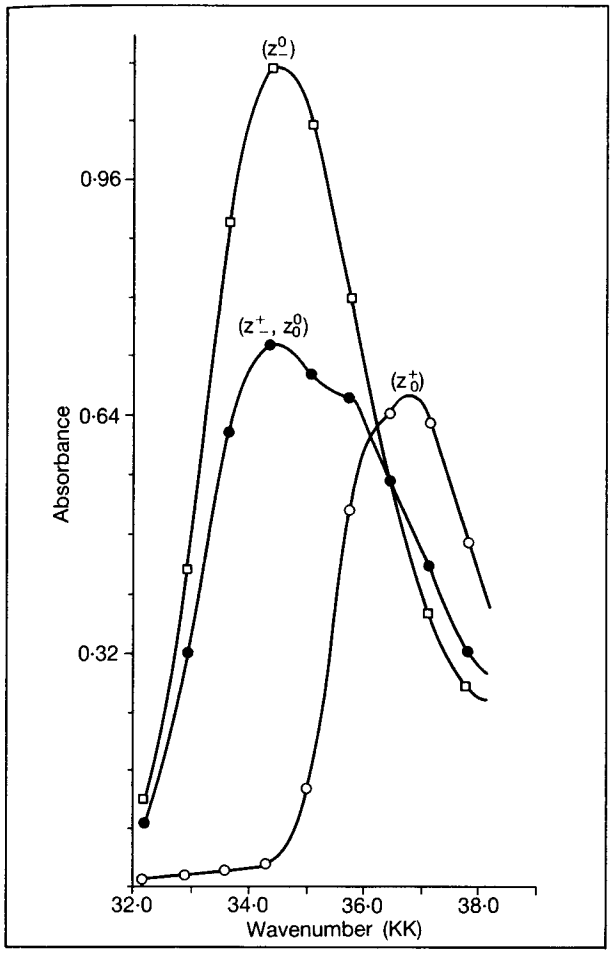

Fig. 3. Spectra of cationic (Z^+_0); anionic (Z^0_-) and neutral (Z^+_-, Z^0_0) forms obtained from the spectrophotometric titration data of I.

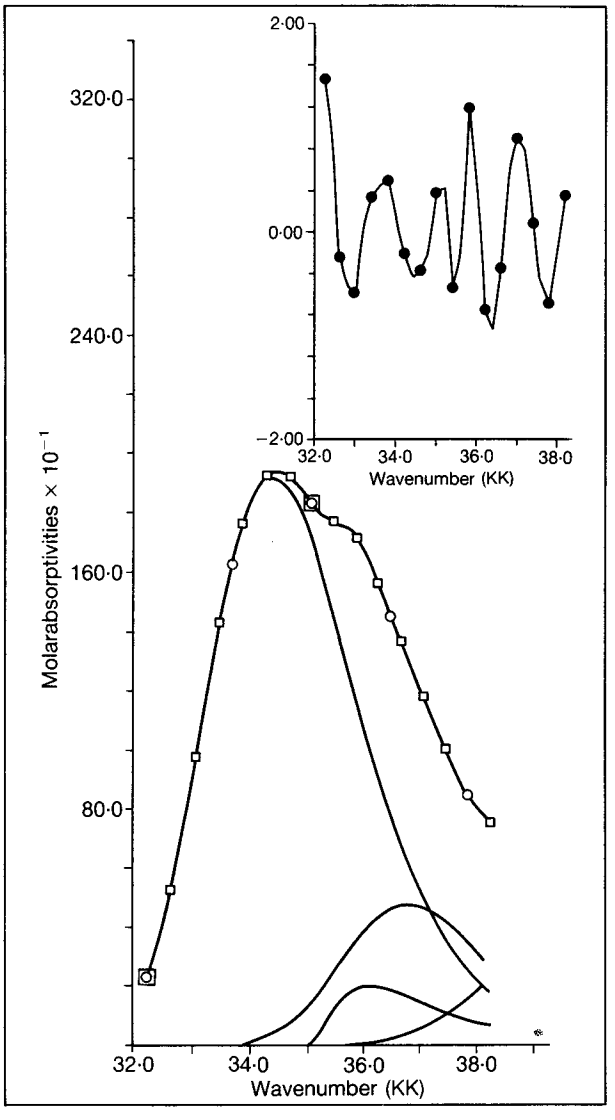

Fig. 4. Deconvolution in log-normal curves of the UV spectrum of neutral forms (Z^+_-, Z^0_0). (——) Log normal curves. (——□——). Sum of log-normal curves. (——○——). Experimental spectrum. 1 KK = 10^3 cm^{-1}. Inset: Differences between experimental spectrum and sum of log-normal curves as a function of percentage of ε_0 in the 1st band.

is a good agreement in the values of width (W) and skewness (P) of the equivalent log normal curves for the Z^+_-/Z^0_- and Z^0_0/Z^+_0 pairs.

On the other hand, A^λ may be expressed by

$$A^\lambda = \varepsilon_{Z^+_0}|Z^+_0| + \varepsilon_{Z^0_-}|Z^0_-| + \varepsilon'(|Z^+_-| + |Z^0_0|) \qquad (1)$$

in which ε' is the molar absorptivity of the uncharged forms.

Moreover, it may also be written that

$$A^\lambda = \varepsilon_{Z^+_0}|Z^+_0| + \varepsilon_{Z^0_-}|Z^0_-| + \varepsilon_{Z^+_-}|Z^+_-| + \varepsilon_{Z^0_0}|Z^0_0| \qquad (2)$$

and it can be easily derived that

$$\varepsilon' = \varepsilon_{Z^+_-}\frac{|Z^+_-|}{|Z^+_-| + |Z^0_0|} + \varepsilon_{Z^0_0}\frac{|Z^0_0|}{|Z^+_-| + |Z^0_0|}$$

and according to the equilibria (schemes 1 and 2) it is possible to obtain that

$$\varepsilon' K_1 = \varepsilon_{Z^+_-} k_a + \varepsilon_{Z^0_0} k_b$$

$$k_a = \frac{\varepsilon' - \varepsilon_{Z^0_0}}{\varepsilon_{Z^+_-} - \varepsilon_{Z^0_0}} \cdot K_1 \qquad (3)$$

The values of $\varepsilon_{Z^0_0}$ and $\varepsilon_{Z^+_-}$ as a function of the wavelength may be calculated from

$$\varepsilon(\bar{\nu}) = \varepsilon_0 \exp -\left\{\frac{\ln 2}{(\ln p)^2} \ln\left[\left(\frac{\bar{\nu}-\bar{\nu}_0}{w}\right)\left(\frac{p^2-1}{p}\right) + 1\right]\right]^2\right\}$$

It is assumed that $\varepsilon_0|Z^0_0| = \varepsilon_0|Z^+_0|$ which appears justified taking into account the practical coincidence between the molar absorptivities of I and its N-acetyl derivative (Quintero et al 1982). Furthermore the identity $\varepsilon_0|Z^+_-| = \varepsilon_0|Z^0_-|$ has also been assumed.

Values of ε' have been calculated by dividing the absorbance of uncharged forms (Z^0_0, Z^+_-) by the total concentration ($3{\cdot}80 \times 10^{-4}$ M).

Thus, utilizing equation (3) and $pK_1 = 9{\cdot}18$, values of k_a have been obtained and subsequently, with $pK_2 = 10{\cdot}32$, the values of the other constants have been attained.

The results found, corresponding to the range from 307 nm (32·6 KK) to 265 nm (37·7 KK), were $pk_a = 9{\cdot}48 \pm 0{\cdot}02$, $pk_b = 9{\cdot}71 \pm 0{\cdot}03$, $pk_c = 10{\cdot}12 \pm 0{\cdot}03$, $pk_d = 9{\cdot}88 \pm 0{\cdot}03$.

These results have been compared to those obtained by the conventional least square method. Evidently equation (1) cannot be used and we obtain from equation (2):

$$\frac{A^\lambda}{A^\infty} = \frac{|Z^0_-|}{C_T} + \frac{\varepsilon_{Z^+_0}}{\varepsilon_{Z^0_-}} \cdot \frac{|Z^+_0|}{C_T} + \frac{\varepsilon_{Z^+_-}}{\varepsilon_{Z^0_-}} \cdot \frac{|Z^+_-|}{C_T} + \frac{\varepsilon_{Z^0_0}}{\varepsilon_{Z^0_-}} \cdot \frac{|Z^0_0|}{C_T}$$

where $\varepsilon_{Z^{\pm}}/\varepsilon_{Z^{0}}$ and $\varepsilon_{Z_0^0}/\varepsilon_{Z^{0}}$ are not available by direct spectrophotometric measurement. However, the structural equivalences allow the acceptance of, in principle, the following approximations.

$$(\varepsilon_{Z^{\pm}}/\varepsilon_{Z^{0}}) \simeq (\varepsilon_{Z^{0}}/\varepsilon_{Z^{0}}) = 1 \quad (4)$$

$$(\varepsilon_{Z_0^0}/\varepsilon_{Z^{0}}) \simeq (\varepsilon_{Z_0^{\pm}}/\varepsilon_{Z^{0}}) = y \quad (5)$$

and, then it can be derived

$$\frac{A^{\lambda}}{A^{\infty}}(|H^+| + K_1) - y|H^+| = (k_a + yk_b) + \frac{K_1K_2}{|H^+|}\left(1 - \frac{A^{\lambda}}{A^{\infty}}\right) \quad (6)$$

The values of y may be calculated as a result of dividing the absorbance at pH 7·71 by the absorbance of the solution in KOH 0·16 M.

Thus, taking $pK_1 = 9{\cdot}28$, from equation (6) the values of k_a obtained were $pk_a = 9{\cdot}45 \pm 0{\cdot}02$, $pk_b = 9{\cdot}77 \pm 0{\cdot}04$, pk_c $10{\cdot}14 \pm 0{\cdot}02$, $pk_d = 9{\cdot}81 \pm 0{\cdot}03$.

The useful range of application of equation (6) was from 301 nm (33·2 KK) to 287 nm (34·8 KK), in which an acceptable fit is attained (correlation coefficient = 0·983).

It seems clear that the above mentioned range corresponds to those wavelengths for which the approximations stated in equations (4) and (5) are more closely accomplished.

On the other hand, the results found from the least square method are in good accordance with those obtained from the procedure of deconvolution which it can be considered in supporting the spectral assignments made in this later method.

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A new approach to prostate cancer

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Abstract—Growth of androgen-dependent human prostatic adenocarcinoma implanted in the nude mouse (Honda tumour), is inhibited by 6-methyleneprogesterone. This steroid is a potent inhibitor of both rat and human prostatic 5α-reductase in-vitro. In-vivo, at the studied dose level, it reduces metabolic conversion of testosterone to dihydrotestosterone with minimal effects upon circulating LH and testosterone. These data support the hypothesis that dihydrotestosterone and not testosterone is the main trophic androgen of the human prostatic neoplasm.

Treatment of prostate cancer is based on the hypothesis of Huggins & Hodges (1941) that the tumour depends on testicular androgen for growth so that treatment should be directed towards androgen ablation. To this end castration, either surgical or medical, represents standard therapy (cf. Schultze et al 1987). Although undoubtedly correct in its basic premise of dependence upon androgen secretion of the testis, there is now an impressive body of experimental evidence to show that, in the prostate, testosterone is reduced to dihydrotestosterone (DHT) by the NADPH-dependent enzyme 5α-reductase, and that DHT is the main trophic androgen of the prostate and, by influence, of the tumour (see Petrow 1986).

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In-vitro studies by Petrow & Lack (1981) have shown that 6-methyleneprogestrone (6-MP) is an irreversible inhibitor of rat prostatic 5α-reductase. Its inhibition of the enzyme in human explants of prostatic tissue has been described by Kadohama et al (1983), who additionally demonstrated suppression of metabolic reduction of testosterone to DHT in this model system. MacIndoe et al (1984) have reported 5α-reductase inhibition by 6-MP in homogenates of MCF-7 human breast cancer cells. More recently, Uilenbroek & Woutersen (1988) have found similar inhibition of rat ovarian 5α-reductase activity using both testosterone and progesterone as substrates. 6-MP does not inhibit steroidal aromatase or 3β-hydroxy-5-ene-oxidoreductase (Robertson et al, in manuscript).

In-vivo, 6-MP shows the characteristic biological properties that result from inhibition of the metabolic conversion of testosterone to DHT. Thus it inhibits growth of the prostate in the castrated rat administered testosterone but *not* DHT (Kendle et al, in manuscript), and enforces marked involution of the prostate in the intact male rate without affecting LH levels