J. Enzyme Inhibition, 1997, Vol. 12, pp. 59–70
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THE MECHANISM OF A P-450 ENZYME-AROMATASE; A MOLECULAR MODELLING PERSPECTIVE FOR THE REMOVAL OF THE C(19) METHYL AND AROMATISATION OF THE STEROID A RING

SABBIR AHMED

School of Applied Chemistry, Kingston University,
Penrhyn Road, Kingston upon Thames, Surrey, KT1 2EE, UK

(Received 22 November 1995; in final form 9 October 1996)

The three possible mechanisms for the final step of aromatisation, as proposed by Wright and Akhtar, are studied using a molecular modelling approach utilising the 'substrate-heme complex' previously reported. The study considers the three mechanisms from a geometric point of view and concludes that only a ferroxy radical is involved in *all* three steps of aromatase action and not the mixture of both ferroxy and peroxy as previously suggested. The study also suggests that an alternative peroxy-based mechanism is unlikely due to the distances between reacting species. An alternative theoretical mechanism, which circumvents the production of the CHO• radical (regarded to be too small to be retained within the active site) for the final step of aromatisation is suggested involving the concerted breakup of the iron-formate complex together with hydrogen abstraction from C(1) of 3-hydroxy-estra-2,4-diene-17-one, resulting in oestrone and formic acid.

Keywords: Aromatase; substrate-heme complex; mechanism; modelling.

INTRODUCTION

In the treatment of hormone-dependent breast cancer, numerous studies have shown that endocrine therapy plays an important rôle and that hormonal manipulation, through the inhibition of enzymes within the steroidal cascade, may be one route to controlling these hormone-dependent cancers. As such, cytochrome P-450 enzymes have been greatly studied. One cytochrome P-450 enzyme of particular interest in the treatment of hormone-dependent breast cancer is Aromatase (AR).[1]

AR is an enzyme complex which mediates the conversion of androgens [androstene-3,17-dione (AD) and testosterone (T)] to the estrogens via the loss of the C(19) methyl group and the formation of a benzoid A-ring (aromatisation)

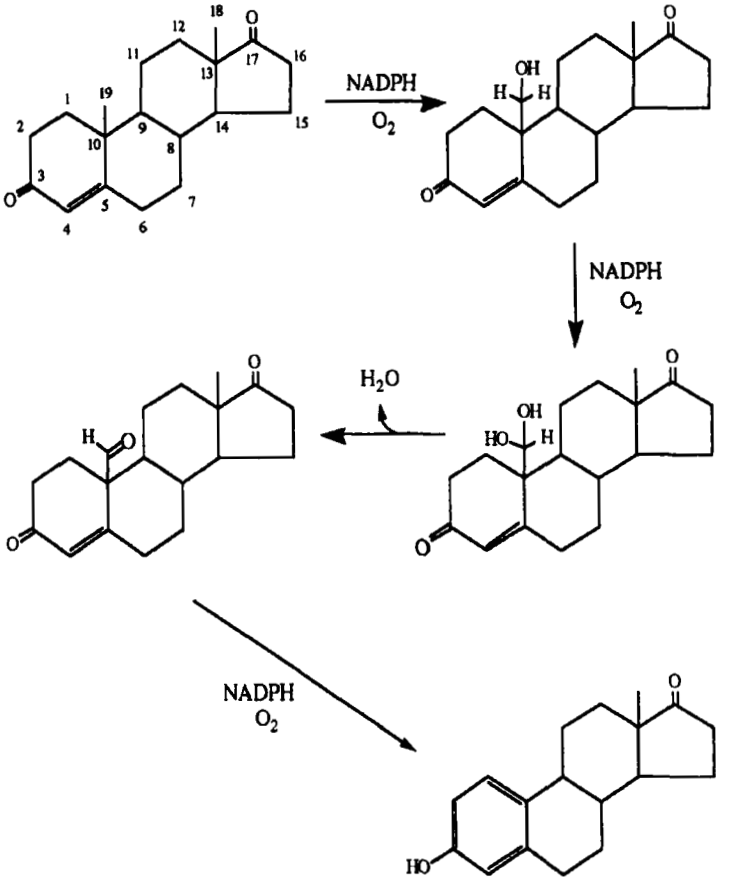


FIGURE 1 Aromatisation of AD to oestrone.

(Figure 1).[2] It involves a NADPH-Cytochrome c and a P-450 moiety.[3] Aromatisation is thus the last step in the biosynthetic progression from cholesterol to the estrogens and has been the target of exhaustive research for some time.

The overall mechanism of AR is known to involve three sequential oxidation steps (Figure 1), each one requiring a mol of O_2 and NADPH.[3] The initial step of aromatisation has been shown to involve the hydroxylation of the C(19)-methyl group of both AD and T, followed by a second hydroxylation of the C(19) to give the C(19) gem diol. This then undergoes rearrangement with the loss of a mol of water to the C(19) aldehyde which is finally removed as formic acid in the third and

final step.[2] Several workers[4,5] have attempted to formalise a detailed mechanism for each of the steps based on experimental observations, and until recently, the type of reaction (polar versus radical) had not been clearly established.[6]

Akhtar *et al.*[2,4,6] carried out extensive work based on the uptake of labelled O_2 and have proposed that radical mechanisms are probably involved due to the energetics of polar reactions. Although radical mechanisms have been postulated, the nature of the attacking group of the cytochrome P-450 has not yet been established, although three possibilities have been suggested[6] – a ferroxy radical (Fe-O•); a peroxy radical (Fe-O-O•) or; a mixture of both (Figures 2(a) to 2(c)).

The first two hydroxylation steps of aromatisation have been 'rationalised' by Akhtar *et al.* using a ferroxy attacking group. That is, the ferroxy radical is thought to abstract a hydrogen atom from the C(19) methyl, resulting in Fe^{IV}-OH and the C(19) methylene radical. The C(19) radical is then neutralised by reacting with the Fe^{IV}-OH resulting in the formation of Fe^{III} and C(19) hydroxylated steroid. This process is then repeated resulting in the formation of the C(19) gem diol. The third and final step involving attack on the C(19) aldehydic intermediate however, has proved to be difficult to elucidate due to the lack of knowledge concerning the attacking group, as such, and several mechanisms have been proposed. The most recent is that by Wright and Akhtar who have reviewed the literature and suggested that three possible mechanisms exist – one involving peroxy attack (Figure 2(a)), and the remaining two involving ferroxy attack on the C(19) aldehydic intermediate (Figures 2(b) and 2(c)) – the mechanism involving peroxy radical being preferred by the authors. In recent studies on the related P-450 enzyme, 17α-Hydroxylase/17,20-Lyase (P-450$_{17\alpha}$),[7] Akhtar *et al.* have again considered the ferroxy versus peroxy question and have favoured the latter mechanism as this appears to be applicable to the P-450 family of enzymes, however, the authors did not totally discount the ferroxy mechanism.

The majority of studies on the mechanism of AR have revolved around chemical (and theoretical) points of view and the exact rôle (and nature) of the AR active site has not generally been considered, since an 'accurate' representation of the active site, or the crystal structure of AR, has not been available. We have recently reported a novel molecular modelling study[8] where we produced a representation of the AR active site involving a 'substrate-heme complex' (Figure 3) which we subsquently used to successfully discuss the mode of action of several steroidal and non-steroidal inhibitors (both reversible and irreversible). We now seek to apply the substrate-heme complex and molecular modelling approach to provide a new perspective on the mechanism of AR. It should be noted that the theoretical study reported within the present report has taken note only of the interactions between the substrate and the porphyrin whilst 'ignoring' the remainder of the flexible 60 kDa protien moiety.

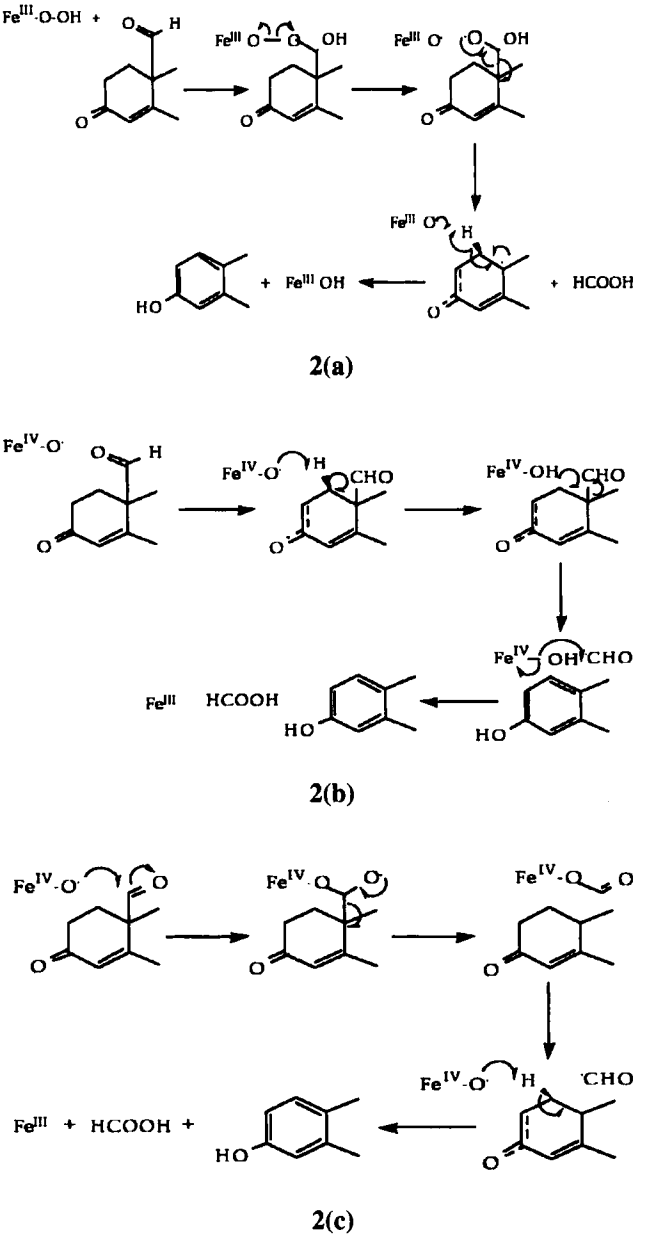
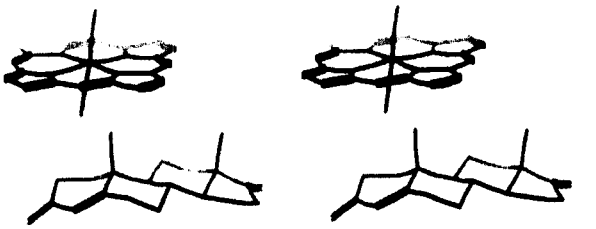
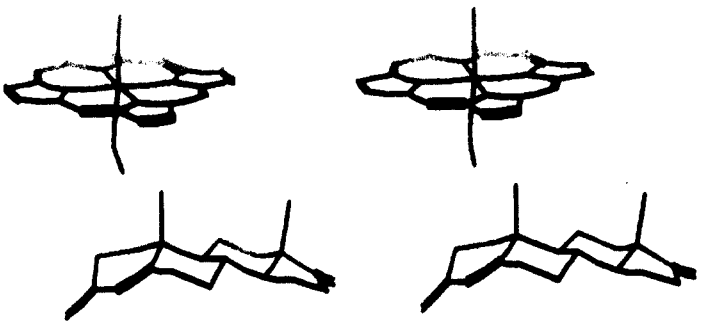


FIGURE 2 (a) Peroxy radical attacking C(19) aldehydic intermediate (only A ring shown). (b) Ferroxy radical attacking C(19) aldehydic intermediate (only A ring shown). (c) Alternative mechanism for ferroxy radical attack on C(19) aldehydic intermediate.



3(a)



3(b)

FIGURE 3 (a) Ferroxy based substrate-heme complex (stereoplot). (b) Peroxy based substrate-heme complex (stereoplot) (see colour plate at rear).

EXPERIMENTAL

The structures of the porphyrin, androstenedione, testosterone, and the presumed intermediates (as proposed by Wright and Akhtar) 3-hydroxy-estra-2,4-diene-17-one and 3-hydroxy-androst-2,4-diene-17-one (Figure 2a to 2c) were all built within Alchemy III[9] molecular modelling software, using suitable starting fragments from the fragment library, on a P90 Intel Pentium microprocessor based IBM compatible microcomputer. For example, in the case of androstenedione, a search of the Alchemy III fragment library resulted in the discovery of cyclohexene (A ring). The cyclohexene ring was modified so as to produce the required 3-keto-cyclohexene. The B and C rings were then 'fused' onto 3-keto-cyclohexene using chair forms of cyclohexane. The cyclopentanone ring was then built using sp^3 and sp^2 carbon atoms followed by the addition of two sp^3 hybridised carbon atoms as C(18) and

C(19) onto the steroid backbone. Finally, hydrogens were added to the whole molecule, resulting in androstenedione – the stereochemistry at the C(10) and C(13) positions were checked using the chirality labelling utility within Alchemy III. The completed structures were then subjected to an initial minimisation using the conjugate-gradient algorithm available within Alchemy III. The minimisations were carried out, in general, in excess of 300 iterations and automatically terminated when the RMS gradient was less than 0.0001. Conformational analysis on the C(10)–C(19) and C(13)–C(18) bonds was carried out using Powersearch[10] using the systematic method with bond rotations of 15° and energy constraints of 10 kcal/mol. The lowest energy conformer was then utilised for further studies.

The rationale and construction of the substrate-heme complexes have been described elsewhere and thus have not been reported here.

RESULTS AND DISCUSSION

Key Step in the Proposed Mechanisms

In their proposed mechanisms of the third step of aromatisation, Wright and Akhtar suggest that hydrogen abstraction from C(1) of the proposed intermediate, leading to the final carbon-carbon double bond, is common to all three mechanisms. Thus, the basis of our present study involves the determination of the feasibility of this crucial step from a geometric point of view. We therefore considered the distance of the C(1)-βH bond with respect to the attacking oxygen in each proposed mechanism, the rationale being that for this step to occur the two reacting species must be close to each other. Also, Wright and Akhtar in their mechanism consider the protonation of the C(3) carbonyl whilst the C(2) to C(3) carbon-carbon double bond is being formed. We hypothesise that as a result of this, hydrogen bonding interaction with the active site via the C(3) carbonyl group is lost, due to the carbonyl oxygen atom gaining a proton from the active site, and that the remaining C(17) carbonyl group of AD (or C(17) hydroxy group in testosterone) becomes important in binding the intermediates to the active site and keeping them within the active site. Thus, in fitting the intermediates to the substrate-heme complexes, points on the D-ring of AD (more specifically the C(17)=O group) were utilised.

Peroxy, Ferroxy or a Mixture?

From the consideration of both the peroxy and ferroxy substrate-heme complexes, we observed that, as would be expected, the 'extra' oxygen within the peroxy substrate-heme complex caused a downward movement of the steroid backbone.

Fitting the two complexes (using points on the heme moiety) we discovered that the steroid backbone of the ferroxy complex was 1.3 Å higher than the steroid backbone of the peroxy complex. From this observation it would seem unlikely that *both* ferroxy and peroxy radicals are involved in aromatisation, as this would require the intermediates of the reaction to reposition themselves for the final bond cleavage step. Also, if repositioning did occur, we would need to presume the existence of numerous hydrogen bonding sites about the active site such that the intermediates could be maintained in a position so that the C(19) was near the heme. We would therefore need to presume the 'movement' of these highly reactive radical intermediates from one hydrogen bonding site to another. The movement required from ferroxy to peroxy would need to be in the vertical plane, as such a 'ladder arrangement' of hydrogen bonding groups would be required. Since AR is thought to undertake reactions which are believed to be common amongst the P-450 family of enzymes, a similar ladder arrangement of hydrogen bonding groups would also be expected in related enzymes, an observation which has not been reported in the literature for either AR or other P-450 enzymes.

If numerous hydrogen bonding sites did exist, most compounds which have been observed to possess low inhibitory activity, could be expected to be more potent as they could utilise these other favourable hydrogen bonding interactions and so further increase their binding to the active site, thereby increasing their inhibitory potency. Indeed, utilising the assumption that only two hydrogen bonding sites are available to androstenedione, or testosterone, corresponding to the steroid C(3) and C(17) carbonyl groups, we have successfully explained the observed inhibitory activity of inhibitors such as 3-ethyl-3-(4′-pyridyl) piperidine-2,6-dione (PYG), aminoglutethimide (AG), 10-thiiranylestr-4-ene-3,17-dione and other known inhibitors of AR. We therefore conclude that aromatisation involving a mixture of two ferroxy radicals (for the hydroxylation steps) and a peroxy radical (for the carbon-carbon bond cleavage step) is unlikely. Indeed, we have applied a similar approach to the enzyme complex 17α-Hydroxylase/17,20-Lyase (P-450$_{17\alpha}$) and concluded that this enzyme also probably uses a single attacking species and not a mixture of ferroxy and peroxy radicals.[11]

Peroxy Radical Attack Mechanism

Although we have rejected the involvement of a mixture of ferroxy and peroxy radicals in aromatisation, we still have the possibility of the involvement of either a purely peroxy or ferroxy mechanism.

In their mechanism for the final step involving the peroxy radical (Figure 2(a)), Wright and Akhtar considered the hydrogen abstraction to be carried out by the oxygen atom directly bonded to the iron atom of the P-450 heme. We considered

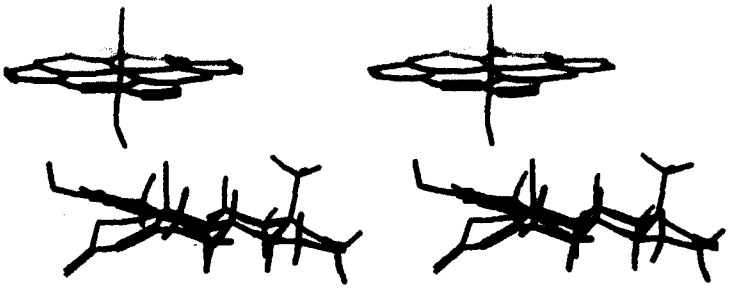


FIGURE 4 3-Hydroxy-estra-2,4-diene-17-one radical fitted onto peroxy based substrate-heme complex (stereoplot) (see colour plate at rear).

the possibility of such an attack on the C(1)-βH by fitting the intermediate radical 3-hydroxy-estra-2,4-diene-17-one onto our peroxy substrate-heme complex. We discovered that due to the formation of the C(10) radical, there was a pronounced upward movement of the C(1) carbon from below the plane of the steroid in an attempt to produce the planar pyramidal C(10) radical, resulting in the equatorial hydrogen now becoming axial, i.e. the C(1) hydrogen in 3-hydroxy-estra-2,4-diene-17-one becomes axial. However, when the distance was considered, the C(1)-βH bond was found to be 2.5 Å away from the appropriate oxygen atom for βH abstraction to occur (Figure 4), i.e. the peroxy oxygen appears to be approximately two and a half bond lengths away from the βH. As we have mentioned above, the hydrogen abstraction from the C(1) position of the intermediate appears to be the most important step and as such the distance obtained would give an indication as to the feasibility of this step. In this case the C(1) hydrogen is presumed to be too far away for abstraction to occur and therefore the mechanism involving the peroxy radical in the carbon-carbon bond cleavage seems unlikely from a geometric point of view.

Should the third step in the AR catalysed conversion occur via a peroxy species, then substantial conformational changes in the enzyme or substrate would need to occur in the final reaction of Figure 2(a) so as to allow the Fe^{III}-O• species to approach the C(1)-βH. However, this line of argument cannot be accepted since it would lead to the conclusion that the process of rational drug design is futile and non-productive. The basis of the molecular modelling studies undertaken to rationalise the inhibitory activity of AR inhibitors, such as that of Banting *et al.*,[12] was that the Type II inhibitors bound to the haem whilst undergoing hydrogen bonding (or polar-polar interaction) with the active site (involving the group at the active site which would normally bind the substrate C(3)=O group). In this and

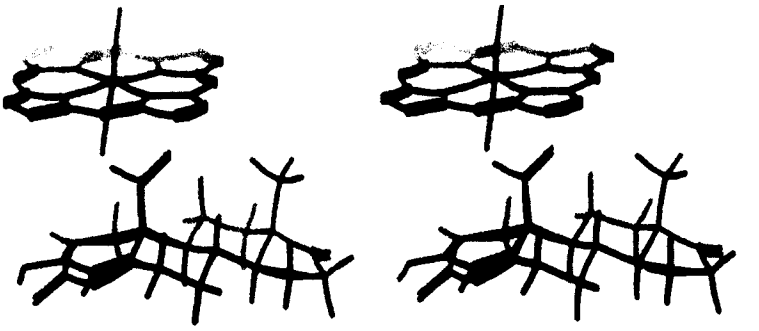


FIGURE 5 3-Hydroxy-androst-2,4-diene-10-al-17-one radical fitted onto ferroxy based substrate-heme complex (stereoplot) (see colour plate at rear).

other related reports, the steroid substrate C(19) to inhibitor hetero atom (e.g. N or S) distance was used to give an indication of the potency of inhibitory activity of the compounds studied, therefore these modelling studies would be incorrect if flexing of the active site or substrate/inhibitor is considered to be large enough to accommodate such distances.

Ferroxy Radical Attack Mechanism

Wright and Akhtar considered the second mechanism involving attack on the C(19) aldehyde group by a ferroxy radical to be unlikely due to the formation of the •CHO radical, which they considered too small to be contained within the active site for conversion to formate at a later stage of the reaction. Although the present study is not able to comment on the particular postulate of Wright and Akhtar, we do agree with the conclusion that this mechanism is unlikely, but for alternative reasons. That is, the second mechanism proposed involves H abstraction from the C(1) atom of the aldehydic intermediate (Figure 2(b)). When the intermediate 3-hydroxy-androst-2,4-diene-10-al-17-one was fitted onto the ferroxy based substrate-heme complex (Figure 5) using points on the D-rings of both steroid backbones and the distances considered, we discovered that the C(1)-βH was 2.4 Å from the ferroxy oxygen atom, i.e. this distance is also presumed to be too large for H abstraction, similar to the situation seen earlier for the peroxy mechanism.

Thus, from a consideration of the distances between reacting species for the different mechanisms in the last step of aromatisation, we have been able to determine that of the three mechanisms, two considered thus far appear improbable.

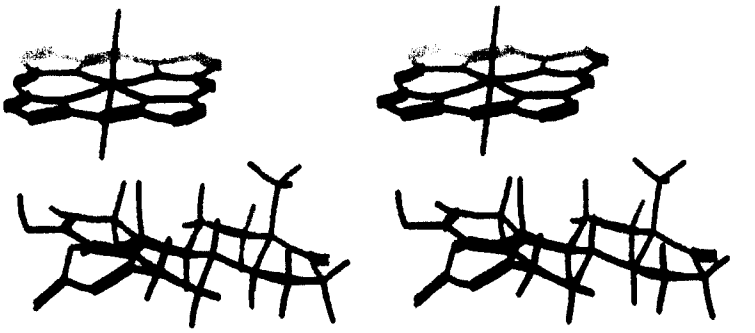


FIGURE 6 3-Hydroxy-estra-2,4-diene-17-one radical fitted onto ferroxy based substrate-heme complex (stereoplot) (see colour plate at rear).

Considering the third and final mechanism proposed by Wright and Akhtar, we observe that this also involves hydrogen atom abstraction from C(l), although the intermediate involved is presumed to be a C(10) radical (also proposed for the peroxy mechanism). As observed previously, the C(10) radical is a planar structure, as a result there is a pronounced upward movement of the C(1) atom (Figure 6) from below the plane of the steroid. The result of this is to place the βH only 1.1 Å away from the ferroxy radical, i.e. C(1)βH is now approximately a bond length away from the oxygen radical, therefore hydrogen abstraction from this intermediate by the ferroxy radical would appear to be possible. We therefore conclude that, from a consideration of the three proposed mechanisms, the ferroxy radical attack on the C(19) aldehydic group resulting in a C(10) radical would appear to be the most probable.

New Mechanism

A detailed consideration of mechanism 3 put forward by Wright and Akhtar shows that it too relies upon the formation of •CHO, which was considered for an earlier mechanism to be unlikely. We suggest that an alternative 'route' exists for the loss of the C(19) without the formation of a •CHO radical (Figure 7) and propose that the 'new mechanism' involves a concerted break-up of the iron-formate complex together with hydrogen abstraction from C(1) of 3-hydroxy-estra-2,4-diene-17-one, resulting in oestrone and formic acid (Figure 7). Whilst we have no direct experimental evidence for our proposed mechanism, it does appear to be consistent with previous experimental results obtained by Akhtar *et al.* and other workers within this field.

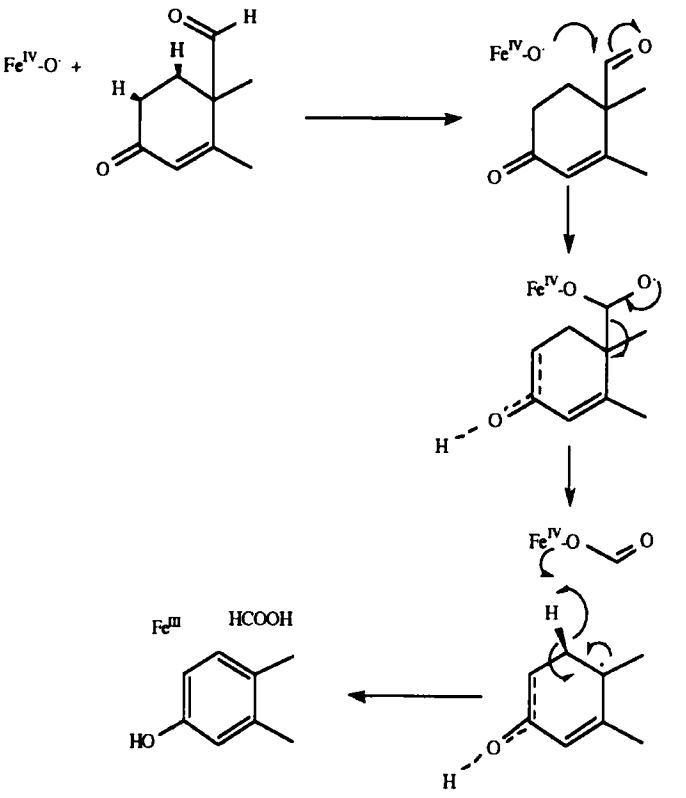


FIGURE 7 Alternative mechanism for the third step of aromatisation.

CONCLUSION

In conclusion, the present study has allowed us to consider the three possible mechanisms for the lyase of the C(10)–C(19) bond in the final step of aromatisation as proposed by Wright and Akhtar. From the present modelling study, we conclude that the mechanism of AR involves only ferroxy radicals during the three steps of aromatisation, and in particular, the last step is hypothesised to be carried out by a ferroxy radical. That is, from a consideration of the distances involved between reacting species in the final step of aromatisation, hydrogen abstraction from C(1) of the C(10) radical (3-hydroxy-estra-2,4-diene-17-one) is considered to be carried out by a ferroxy radical, leading to the formation of the third and final carbon-carbon double bond, due to the C(1)-βH closely approaching the ferroxy

oxygen. Thus, alternative mechanisms involving the combined action of two ferroxy radicals (undertaking hydroxylation steps) and a peroxy radical (involved in the carbon-carbon bond cleavage step) appear to be unlikely due to the reorientation required by the intermediates, the requirement of numerous hydrogen bonding sites within the active site and more importantly, the distances between reacting species. The involvement of a purely perroxy radical mechanism is also thought to be unlikely also due to the large distance between the C(1)-βH and the ferroxy oxygen atom.

Although the present study has strongly suggested that the use of a peroxy attacking species in the aromatisation reaction appears to be unlikely, the situation regarding other cytochrome P-450 enzymes is unclear, where recent studies suggest that the use of the peroxy attacking species is preferred.[13–15] However, the experimental data is unclear and alternative ferroxy-based mechanisms can also be postulated from a consideration of the data. It is believed, however, that experimental data together with techniques such as molecular modelling may aid in the elucidation of the exact nature of the mechanism regarding the cleavage of carbon-carbon bonds by the cytochrome P-450 family of enzymes.

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