



## Review

## A review of mechanistic studies on aromatase (CYP19) and 17 $\alpha$ -hydroxylase-17,20-lyase (CYP17)

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## ABSTRACT

In the conventional P-450 dependent hydroxylation reaction, the Fe<sup>III</sup> resting state of the enzyme, by a single electron transfer, is reduced to Fe<sup>II</sup>, which reacts with O<sub>2</sub> to produce a Fe<sup>III</sup>-O-O<sup>\*</sup> intermediate. The latter following the transfer of another electron furnishes a ferric-peroxyanion, Fe<sup>III</sup>-O-O<sup>-</sup>, which after protonation leads to the fission of the O-O bond resulting in the formation of Fe<sup>V</sup>=O, the key player in the hydroxylation process. Certain members of the P-450 family, including CYP17 and CYP19, catalyze, at the same active site, not only the hydroxylation process but also an acyl-carbon bond cleavage reaction which has been interpreted to involve the nucleophilic attack of the ferric-peroxyanion, Fe<sup>III</sup>-O-O<sup>-</sup>, on the acyl carbon to furnish a tetrahedral intermediate which fragments, leading to acyl-carbon cleavage. Evidence is presented to show that in the case of CYP17 the attack of Fe<sup>III</sup>-O-O<sup>-</sup> on the target carbon is promoted by cytochrome b<sub>5</sub>, which acts as a conformational regulator of CYP17. It is this regulation of CYP17 that provides a safety mechanism which ensures that during corticoid biosynthesis, which involves 17 $\alpha$ -hydroxylation by CYP17, androgen formation is avoided. Finally, a brief account is presented of the inhibitors, of the two enzymes, which have been designed on the basis of their mechanism of action.

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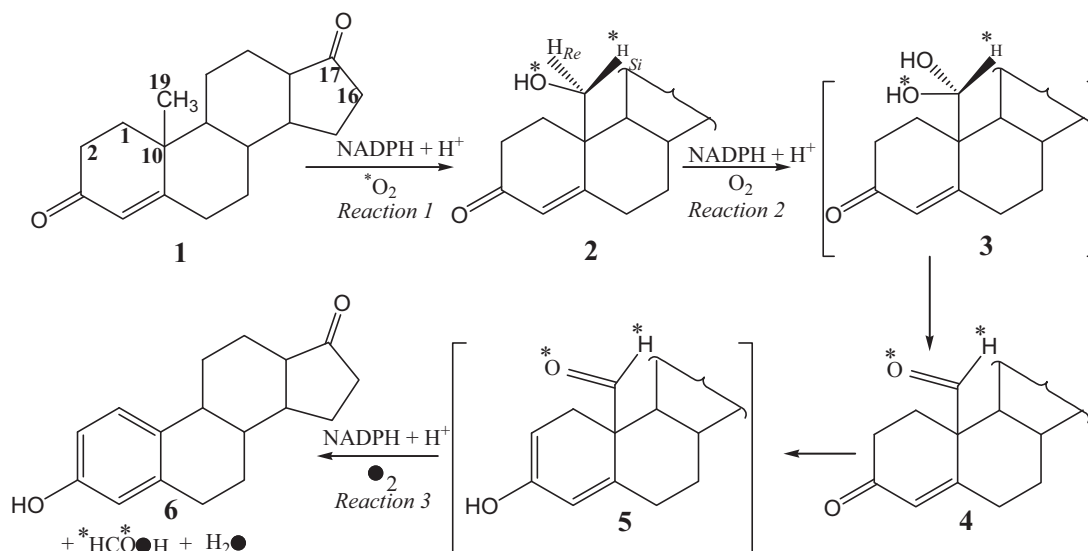
### 1. Aromatase (CYP19)

#### 1.1. Historic background

That androgens (of the type **1**, Scheme 1) are the precursor of estrogens (**6**, estrone; 17 oxygen replaced by 17 $\alpha$ -H, 17 $\beta$ -OH is estradiol) was postulated by Nathanson et al. in 1939 [1]. Chemical consideration had suggested that the removal of

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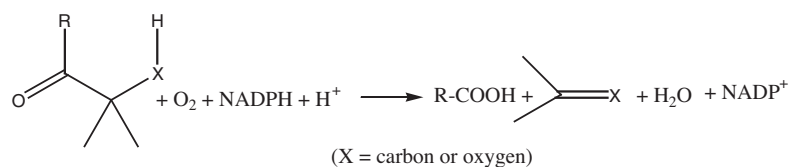
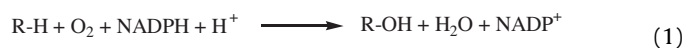
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**Scheme 1.** Sequence of reactions catalysed by aromatase. The fate of oxygen in *Reactions 1 and 3* is shown by asterisk and filled atoms, respectively. The key feature of the scheme is that  $^*H$  and  $^*O$  atoms in structure **2** are transferred to formic acid expelled in *Reaction 3*.

19-methyl group, in the process generating the aromatic ring A of estrogens (**6**), would be facilitated if C-19 was first functionalized. This was supported by the formation of estrone from 19-hydroxyandrostenedione (**2**) [2,3]. Subsequent progress in the field owes a great deal to the pioneering observation of Ryan that human placental microsomes contain the complete enzymic machinery for the aromatization process [4]. Over the years the placental enzyme, now known as aromatase (CYP19) and belonging to cytochrome P-450 class of proteins, has been isolated with an increasing degree of purity [5–8], enabling its crystallization and X-ray structure determination [7].

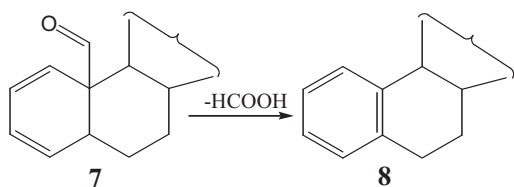
Aromatase is a single chain protein of 419 amino acids ( $M_r$ , 48629; Accession: AAA35556) and catalyses three different generic reactions at the same active site, as shown in the sequence of *Scheme 1* (**1** → **2**; **2** → **4** and **4** → **6**) [2,9–11, also see 15 for cumulative experimental details]. Three androgenic steroids, androstenedione (**1**), testosterone (**1**, 17 oxygen replaced by 17 $\alpha$ -H, 17 $\beta$ -OH) and 16 $\alpha$ -hydroxyandrostenedione, are the substrates for aromatase, however, kinetic parameters are most favourable for androstenedione. In all three cases, the first step is the conventional hydroxylation reaction (Eq. (1)), characteristic of most P-450s, producing a 19-hydroxy androgen (**2**). Aromatase was probably the first enzyme for which it was found that the conversion of a hydroxyl into an aldehydic group, does not occur through a dehydrogenase type of reaction but involves a process using NADPH and  $O_2$  [2]. This requirement for the conversion of an alcohol into an aldehydic group (**2** → **4**) was rationalized by assuming the involvement of a second hydroxylation reaction producing a gem diol intermediate (**3**). In the latter conversion the new hydroxyl group was inserted into the  $H_{Re}$  position and then stereospecifically removed in the next dehydration reaction producing **4** [9,12–15].



The earlier expectation was that if an aldehyde, of the type **4**, were to be an intermediate in the aromatization process, it would

be converted into a carboxylic acid and then release  $CO_2$ , during the fission of the C-10–C-19 bond. This, however, turned out not to be the case. Instead the aldehydic carbon was expelled as formic acid [9,15,16]. The mode through which the expulsion occurs has aroused keen interest and lively debate, even though the scope of possible mechanisms for the process (**4** → **6**) had been severely restricted by our isotopic experiments involving  $^{18}O$  [15,17]. The key features of these experiments are that the original aldehydic oxygen atom is transferred to formic acid and that the second oxygen (filled oxygen, *Scheme 1*) is derived from  $O_2$  used in the third oxidative stage of the sequence. To accommodate these features two earlier suggestions were modified. The first assumed [18] that the third oxidative reaction produces 1 $\beta$ -hydroxy intermediate which facilitates the C-10–C-19 bond cleavage in such a way that the hydroxyl oxygen is transferred to the released formic acid. The second mechanism assumes the formation of a 2 $\beta$ -hydroxy steroid which participates in an intramolecular attack on the C-19 aldehyde group, to produce a hemiacetal which fragments to a 2 $\beta$ -formyl derivative [19]. The latter then undergoes an elimination reaction to produce the aromatic ring A. Despite a wealth of evidence against the latter mechanism it has survived in some respectable publications. Two pieces of concrete experiments against the mechanism are (a) the demonstration by Caspi et al. that the conversion of [2- $^{18}O$ ]2 $\beta$ -hydroxy-19-oxo-androstenedione into estrogen did not show the incorporation of the 2 $\beta$ -hydroxyl oxygen into the released formic acid [20] and (b), the work of Cole et al. showed that the diene aldehyde (**7**, *Scheme 2*) could act as a substrate producing 3-deoxyestrogen (**8**) [21]. In the latter conversion there is no provision for the formation of a 2 $\beta$ -hydroxy derivative thus the aromatization of **7** must occur without involving a 2 $\beta$  hydroxylation.

The possibility that the gem diol (**3**), itself, may act as a substrate for the third step, in the aromatase catalysed process, has been considered [22] but is eliminated by two sets of observations. The first is the kinetic study using 19-hydroxy androgen (**2**) which clearly



**Scheme 2.** Conversion of an analogue of the aldehyde (**4**, Scheme 1) into deoxyestron.

showed that the accumulation of the aldehyde (**4**) preceded the formation of estrogen (Fig. 2 in [15]). Such a temporal profile is broadly in agreement with the earlier careful work of Thompson and Siiteri [10], and recent study [23] in which the aromatization of androstenedione was attended by a stepwise appearance of 19-hydroxy androgen (**2**) first, then of the aldehyde (**4**) before the formation of estrogen (**6**).<sup>1</sup> The second observation is based on <sup>18</sup>O<sub>2</sub> experiments described above. Furthermore, if the third step involved the direct cleavage of the gem diol (**3**), the aromatization of the aldehyde (**4**) (the best of the three substrates **1**, **2** or **4**) for the enzyme could only occur following its hydration. In which case, the expelled formate would not contain an <sup>18</sup>O atom from <sup>18</sup>O<sub>2</sub> used in the third step, as has been shown repeatedly [15–17].<sup>2</sup>

### 1.2. Mechanism of the third step in the reaction catalysed by aromatase

We regard the third step in estrogen biosynthesis to belong to a new generic reaction, conforming to the stoichiometry of Eq. (2), in which an acyl–carbon bond cleavage occurs. The comparison of this reaction, Eq. (2), with that involved in classical hydroxylation process (Eq. (1)), would suggest that the P-450 centre of aromatase is endowed with the property of catalyzing two entirely different generic reactions (Eqs. (1) and (2)) at the same active site. How is this possible? To examine this proposition we consider the precise chemical nature of the iron–oxygen species known to be involved in the hydroxylation reaction. A systematic account of our deliberations, directed to this issue, is described elsewhere [24] and the salient features of which are as follows.

The resting state of aromatase, like those of other P-450s, contains the iron of the heme prosthetic group in the Fe<sup>III</sup> form (**9**) which through one electron reduction, catalysed by NADPH–P-450 reductase is converted into Fe<sup>II</sup>; Refs. [25,26] contain comprehensive coverage of the chemistry and biochemistry of P-450s. Next, the latter species reacts with oxygen producing a covalent adduct whose spectroscopic and magnetic properties are similar to those of oxyhemoglobin [27]. Although, the seminal observation that oxygen binding to hemoglobin is attended by the conversion of a high spin iron into a low spin iron was made by Pauling and Coryell in 1936 [28], the oxidation state of the iron and precise bond-structure of the ligands around it, in hemoglobin remained undefined. Reviving an earlier proposal of Weiss [29,30] we advocated that oxyhemoglobin should be formulated as a hex-

acoordinated complex of Fe<sup>III</sup> in which a superoxide anion is one of the ligands [24]. This formulation is gaining gradual acceptance and the equivalent one-electron reduced intermediate in P-450 has structure **10** (Scheme 3).

The transfer of a second electron, from the NADPH–P-450 reductase system, converts the superoxide ligand to a peroxide dianion (peroxy-ferric intermediate). Which upon protonation [31], followed by the scission of the O–O bond, produces the oxo-derivative (**12**) [32–34] that is involved in the first two hydroxylation reactions catalysed by aromatase. During the normal cycle, the peroxy-dianion (**11**) is rapidly processed to the oxo-derivative but we argued that when the target C-19 atom is in the form of an aldehyde, embedded at the active site, the Fe<sup>III</sup>–O–O<sup>2-</sup> species is trapped by nucleophilic attack to produce the adduct **14** [15–17,35]; also see [36]. This proposal originally made in 1976 [16] and bolstered by later studies [15,17,35,36], has received strong endorsement by recent EPR analysis [37]. There are several routes through which the latter may fragment to expel formic acid. The first is a Baeyer–Villiger rearrangement giving a 10β-formyl derivative which undergoes an elimination reaction to set the stage for the formation of ring A of estrogens. This possibility has been ruled out by an unambiguous preparation of the 10β-formyl derivative which was shown not to be a substrate for aromatase [15,35].

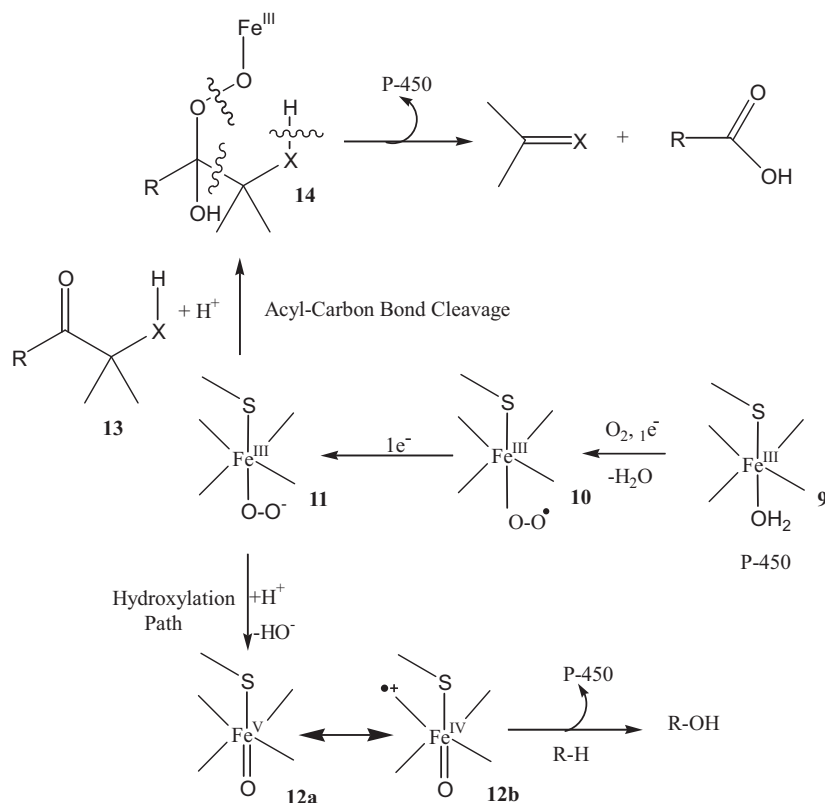
Consequently, our favourite option is the one in which the crucial C10–C19 bond cleavage, by aromatase, is promoted by the fragmentation of the peroxy adduct (**15**, Scheme 4), which can occur by heterolytic, homolytic or a cyclic pathway; the choice between these options being dictated by the geometric considerations together with the acidity of the C–H bond [24]. In the case of aromatase catalysed reaction, **4** → **6**, when the C-1β-hydrogen cleaved in the process will be expected to have a high pK<sub>a</sub>, a cyclic mechanism or one operating via radical intermediates seems most appropriate. The latter process is initiated by a homolytic cleavage of the peroxy bond in **15** to furnish Fe<sup>III</sup>–O• and an alkoxy radical (**16**). There are numerous precedents to suggest that a favourable course for the decomposition of such an alkoxy radical is the fragmentation reaction to produce formate and a tertiary carbon radical. The latter is quenched by a disproportionation process, creating the 1,10-ene system of estrogen (**17** → **6**, Scheme 4). With respect to the stage at which the 2,3-double bond of the aromatic ring is created, we have argued that this occurs before the fragmentation process since a preexisting double bond in the C-2–C-3 position (as in **15** → **17**) will aid the abstraction of the 1β-hydrogen [38]. Support for this conjecture is provided by experiments which showed that 3-deoxy-2,4-diene system of the aldehyde **7** was converted by aromatase into deoxyestrogen (**8**) [21]. Recent findings, however, suggest that the presence C-2–C-3 double bond for the abstraction of the C-1 hydrogen, though desirable, may not be mandatory since 3-deoxyandrogens – where enolisation is not possible – can act as substrates for aromatase [39].

In 2009 a 2.9 Å resolution X-ray crystal structure of aromatase was published [7]. This was remarkable for being of the full length native protein purified from human placenta and shows the enzyme in complex with its natural substrate, androstenedione (**1**). Examination of the positioning of the substrate in the active site enabled the authors to assign individual amino acids to catalytic roles predicted by the chemical studies on the mechanism (and supported by site-specific mutagenesis studies) [40,41].

The structure shows that Asp 309 is positioned so that, in its protonated form, it can hydrogen bond to the 3-keto oxygen of androstenedione. This would not only be to aid substrate binding but would also promote enolisation of the ketone to form the 2,3 double bond as discussed above. To direct the enolisation it would be desirable to have some groups to aid the removal of the 2β hydrogen. It appears that the carbonyl of Ala 306 with participation of the hydroxyl of Thr 310 and possibly a catalytic water, is

<sup>1</sup> Whenever the same enzyme catalyses consecutive steps in a pathway and *in vitro* experiments show the accumulation of intermediates in the medium, a question remains. That is, whether *in vivo*, when the quantity of the initial substrate in comparison to the enzyme is low, the intermediates dissociate and re-associate to the enzyme (distributive reaction course) or remain bound to it through out their journey to the final product (processive reaction course). Recent work shows the enzyme to be a distributive rather than a processive enzyme [23]. From a chemical mechanistic view point we regard it a peripheral issue, and focus on the importance of the electrophilicity of the carbonyl group of **4** to trap the peroxide anion, irrespective of how **4** reaches the active site.

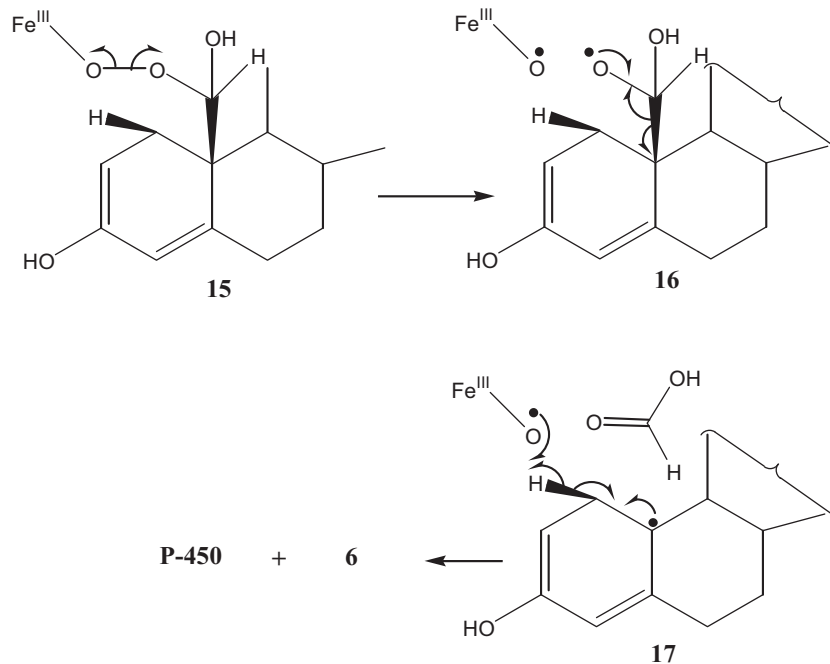
<sup>2</sup> Recently, in the case of 14α-demethylase, the view in [22] has been revised in favour of the peroxy mechanism in Scheme 3 [92].



**Scheme 3.** Dual pathways for multifunctional P-450. The resting state of the P-450 (**9**) is converted via **10** into **11**, by two single-electron reduction and reaction with  $\text{O}_2$ . The latter (**11**) then either follows the hydroxylation path, or when the target centre contains a carbonyl centre (as in **13**), participates in the formation of the adduct **14** which undergoes a cleavage reaction.

positioned to carry out this role. In the X-ray crystal structures of soluble bacterial P-450 hydroxylases, the equivalent of these two residues have been proposed to take part in the proton relay network that provides the two protons required to break the O–O bond of the iron-bound peroxy species [42,43]. This forms the iron mono-

oxygen species required for hydroxylation. Thus, in aromatase, it is proposed that in the first two oxidative steps, Ala 306 and Thr 310 are involved in the formation of the iron mono-oxygen species and in the final step they participate in the removal of the  $2\beta$  hydrogen.



**Scheme 4.** Mechanism of Reaction 3 (Scheme 1) catalysed by aromatase. The tetrahedral complex **15** is formed by the nucleophilic attack of **11** (Scheme 3) on the aldehydic carbon of **4** (Scheme 1).

In earlier models [44] it has been proposed that Thr 310 participates in promoting the cleavage of the aldehyde (**4**) by hydrogen bonding to the C-19 carbonyl to polarize it for nucleophilic attack. In addition, the proton relay network protonating the distal oxygen of the iron-peroxy anion (**11**) would be disrupted, resulting in the protection of this species against O–O scission and so favouring the acyl–carbon cleavage reaction. Evidence in support of this ‘Threonine switch’ mechanism has been provided by resonance Raman experiments [45] on the carbon monoxide–aromatase complex with the various steroid intermediates bound. The X-ray crystallographic structure does not provide clear evidence for or against this model, validation of which will have to await further structural work on the aldehyde-bound enzyme.

The A ring of androstenedione, in a dienol form (Scheme 4) needs to be aromatised with a stereospecific removal of the 1 $\beta$  hydrogen. It is interesting that no amino acid residue could be identified as participating in this process. This is consistent with our view of the mechanism, in which the removal of the 1 $\beta$  hydrogen occurs concomitantly with the breaking of the C10–C19 bond. In the radical fragmentation mechanism the 1 $\beta$  hydrogen is removed as a hydrogen radical, quenching the oxygen radical left on the heme iron: the participation of any other groups is not required [24].

## 2. 17 $\alpha$ -Hydroxylase-17,20-lyase (CYP 17)

### 2.1. Background

The multifunctional enzyme, cytochrome P-450 17 $\alpha$ -hydroxylase-17,20-lyase (CYP 17) lies at the crossroads of corticoid and androgen biosynthesis. It catalyses the conventional cytochrome – P-450 hydroxylation reaction, at position 17 $\alpha$  – of the pregnene nucleus (**18**), which is common for both the pathways (Scheme 5, **19**  $\rightarrow$  **21**) and an acyl–carbon cleavage required only for androgen formation (**21**  $\rightarrow$  **22**) [46]. In addition, the enzyme promotes the formation of two other steroids (**20a** and **20b**), the former being a precursor to a pheromone in the pig [47] and postulated to have an analogous role in man [48]. The 17 $\alpha$ -hydroxysteroid (**20b**) is the probable precursor to *epitestosterone*, whose physiological function is uncertain [49]. The study on the mechanism of their formation has provided key pointers to our understanding of the detailed mechanism of CYP 17 and related C–C bond cleaving P-450s, aromatase (CYP19) and sterol 14 $\alpha$ -demethylases (CYP51) [24,35,50–52]. Another feature that has facilitated work on CYP17 is the fact that homogeneous preparations of the enzyme can be obtained from neonatal pig testes [53–55] and the human isoform has been expressed in *E. coli* ([56] and references to the previous work of the group cited therein) and purified in amounts necessary for physicochemical studies [56,57].

One of the four reactions, the hydroxylation process (**19**  $\rightarrow$  **21**) requires no comment and we focus attention on experimental probes used to unravel the mechanisms of the other three reactions, which are represented by the conversions, **21**  $\rightarrow$  **22**, **19**  $\rightarrow$  **20a** and **19**  $\rightarrow$  **20b**.

### 2.2. Mechanism of the acyl–carbon bond cleavage reactions catalysed by CYP17

All the three transformations of Scheme 5 (Reactions 2, 3 and 4) may be regarded as improvisations on the mechanism used for the third reaction of the aromatase catalysed process in which the key player is the Fe<sup>III</sup>–O–O<sup>–</sup> species. For androgen biosynthesis the latter reacts with the C-20 carbonyl group of 17 $\alpha$ -hydroxyprogesterone to give the adduct **23** which fragments via **24** and **25** (Scheme 6) to expel the side chain as acetic acid. The latter retains all the original atoms of the acetyl moiety and incorporates an atom of oxygen from molecular oxygen (**21**  $\rightarrow$  **22**,

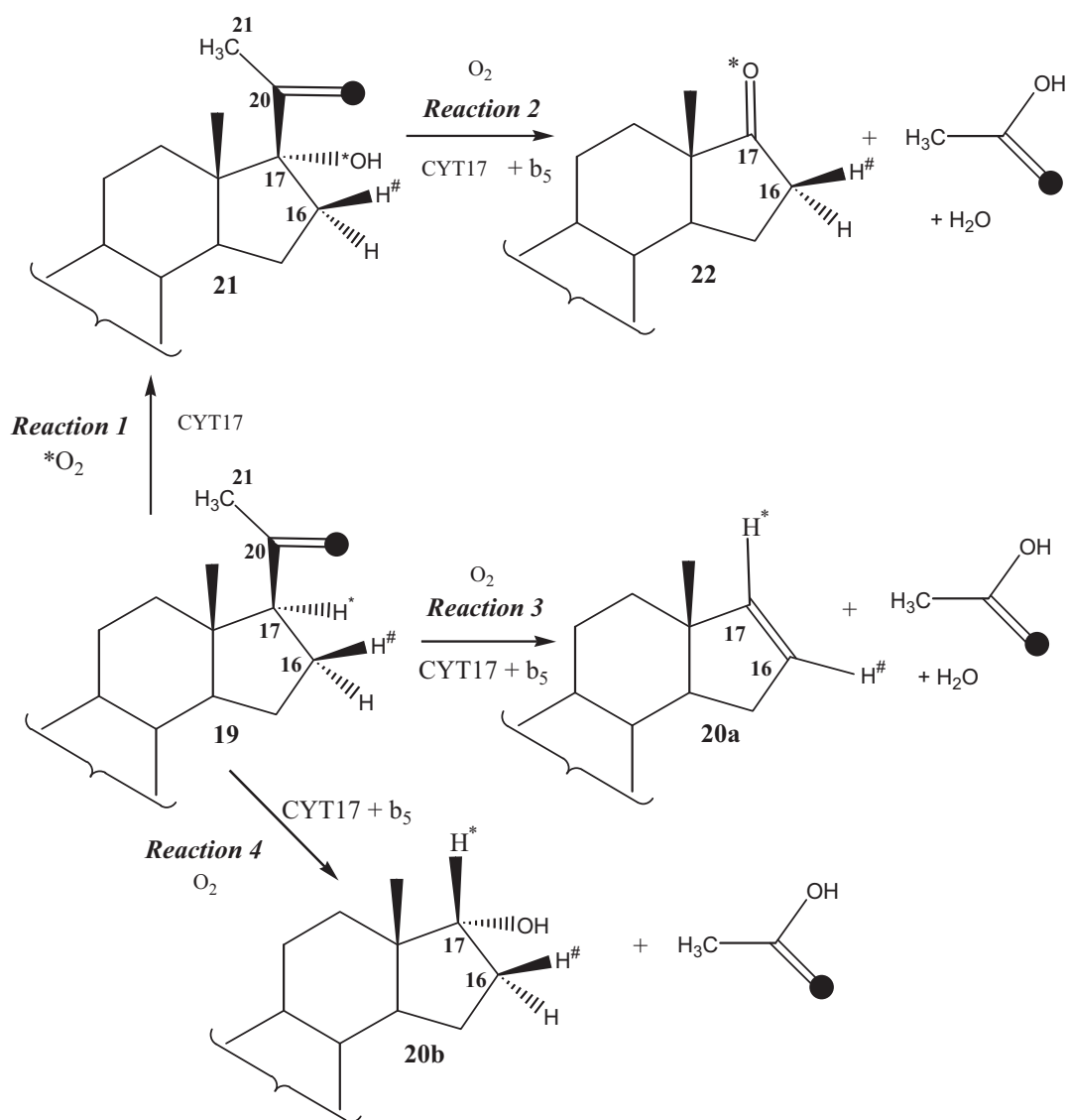
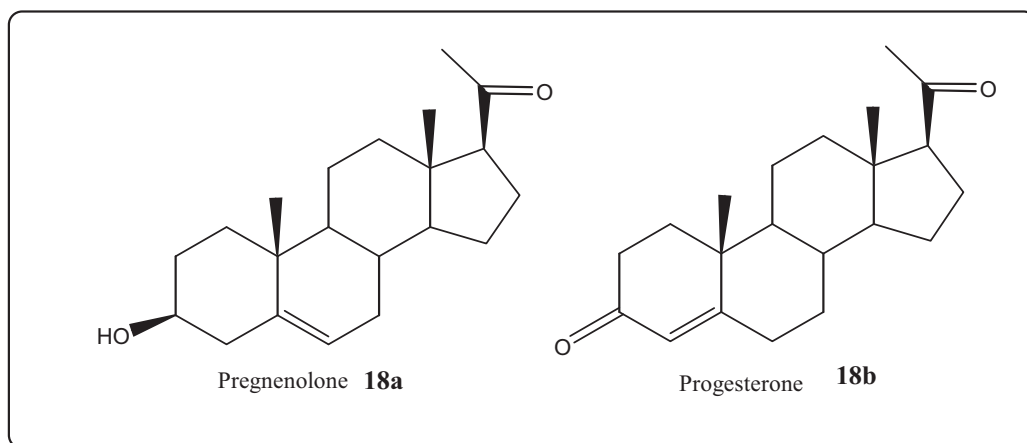
Scheme 5) [58]. For the formation of the 16,17-ene steroid, the Fe<sup>III</sup>–O–O<sup>–</sup> species is trapped by the progestogen, prior to hydroxylation, and the resulting peroxy adduct **26** decomposes to generate a C-17 radical **28** which is neutralized by a disproportionation reaction involving the loss of C-16 $\alpha$ -hydrogen atom (**28**  $\rightarrow$  **20a**, Scheme 6). That the overall process occurs involving *trans* oriented substituents is consistent with a stepwise, rather than an electrocyclic process. The conditions which favour the formation of 16,17-ene (**20a**), invariably lead to the co-production of 17 $\alpha$ -hydroxyandrogen (**20b**). The latter can be envisaged to be formed by the quenching of the C-17 radical (**28**) by the delivery of a hydroxyl radical (path a, Scheme 6). There are, thus, two alternative courses for the neutralization of the C-17 radical, one producing the 16,17-ene and the other, a 17 $\alpha$ -hydroxy steroid, the precursor of 17 $\alpha$ -hydroxytestosterone (*epitestosterone*). It is to be emphasized that the formation of 17 $\alpha$ -hydroxy steroid is attended by an inversion of configuration at C-17 and occurs at the expense of the cleavage of a single bond in the steroid side chain, between C-17 and C-20. These features underpinning the formation of **20a** and **20b**, in which an acyl–carbon bond from the  $\beta$ -face of the steroid nucleus is cleaved and an activated oxygen species approaches the substrate from its  $\alpha$ -face, restrict the mechanism of their genesis to a single process of the type shown in Scheme 6 [52,55]. The multiplicity of acyl–carbon cleavage reactions catalysed by CYP17 have thus provided a testing ground for the mechanism of the third step of aromatase reaction and the equivalent conversion involved in the process catalysed by CYP51 (steroid 14 $\alpha$ -demethylase) [35,59].

### 2.3. Modulation of the acyl–carbon bond cleavage activity of CYP17 by cytochrome b<sub>5</sub>

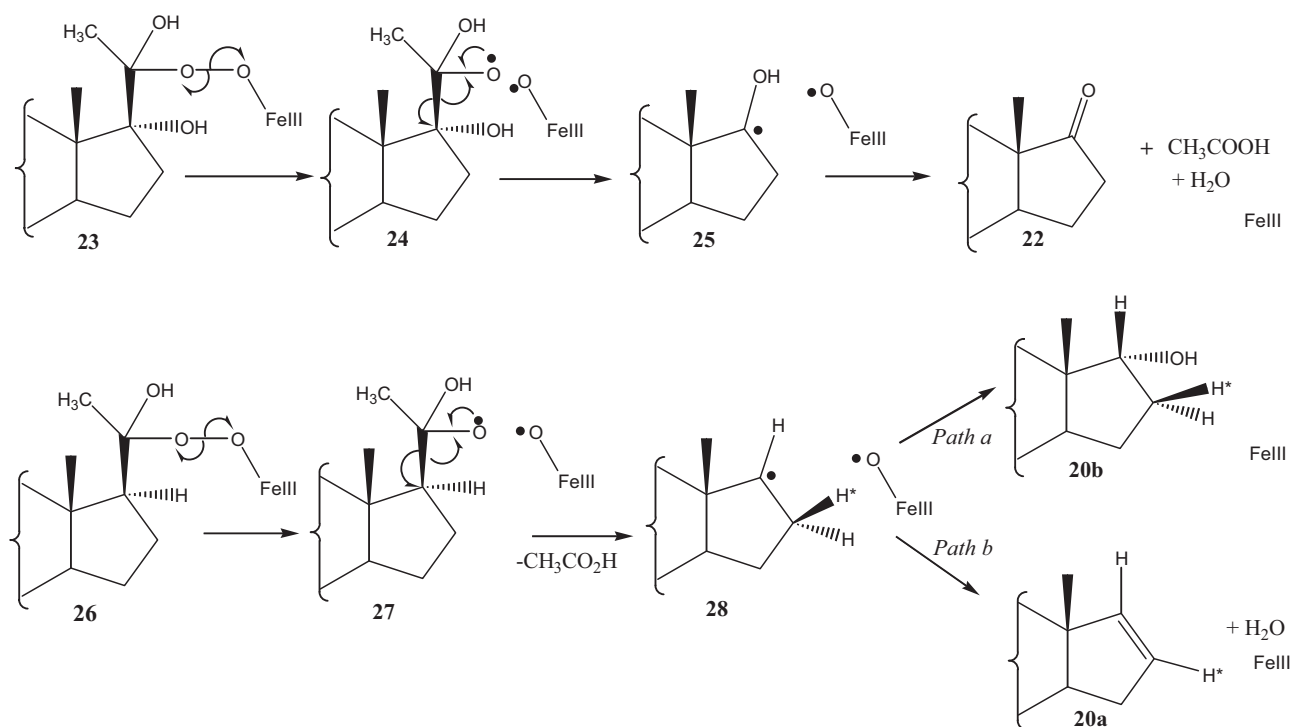
During the course of studies on the elucidation of the chemical mechanism of the dual-function P450s, it was discovered that for the human isoform of CYP17, the androgen producing lyase reaction was heavily dependent on the presence of the membrane-bound form of cytochrome b<sub>5</sub> [56,57,60]. Table 1 summarises the essential data, in terms of  $k_{cat}/K_m$  values, showing that for the hydroxylation of pregnenolone (**18a**) the parameter increased, barely, 2-fold in the presence cytochrome b<sub>5</sub>, whereas the two side chain cleavage reactions, from being non-existent, in the absence of cytochrome b<sub>5</sub>, reached a respectable level, in its presence. Interestingly, with the  $\Delta^4$ -steroid, progesterone (**18b**), as substrate, the direct cleavage represented by Reaction 3 was undetectable, while, the cleavage of the 17 $\alpha$ -hydroxylated progesterone (Reaction 2) was totally dependent on cytochrome b<sub>5</sub>.

The proposed role of cytochrome b<sub>5</sub>, in regulating the acyl–carbon bond cleavage activity of human CYP17, gains further support from immunohistochemical studies. The adrenal zona reticularis, the adrenal cortex tissue layer that excretes androgens, was shown to express CYP17 and cytochrome b<sub>5</sub>, whereas the zona fasciculata, that excretes cortisol but not androgens, expresses CYP17 only [61,62]. In addition, in the human adrenal zona fasciculata and zona reticularis, the expression of CYP17 remains reasonably constant, as a function of age, where as the expression of cytochrome b<sub>5</sub> in the zona reticularis, at the onset of adrenarache, increases along with the acyl–carbon bond cleavage activity of CYP17 [63,64]. Furthermore, high levels of cytochrome b<sub>5</sub> have been reported in human testes and in the adenomas of patients suffering from Cushing’s syndrome that produce excessive androgens [62,65–69].

To explore the nature of the interaction of CYP17 with cytochrome b<sub>5</sub>, we performed mutagenesis of certain basic amino acid residues of CYP17 which we anticipated may form electrostatic interactions with acidic residues of cytochrome b<sub>5</sub>. The basic residues targeted, were selected from an amino acid linear sequence alignment of CYP17 with P-450<sub>BM-3</sub>, being in or immedi-



**Scheme 5.** Reactions catalysed by CYP17. The box contains the two key progestogens, pregnenolone and progesterone. The hydroxylation *Reaction 1* and cleavage by *Reaction 2* occur with both the substrates. However, with the human CYP17 the cleavage *Reactions 3 and 4* are observed only with the pregnenolone skeleton.



**Scheme 6.** Pathways for the formation of three cleavage products from peroxy adducts **23** and **26**.

ately adjacent to the P-450<sub>BM-3</sub>-redox protein binding region [70]. Initially, residues targeted for mutation were changed to alanine, however, during the course of the work, single amino acid mutations, Arg<sup>347</sup> → His and Arg<sup>358</sup> → Gln, had been reported to result in the loss of the acyl–carbon cleavage activity of CYP17 and to cause sexual phenotype changes in 46XY human patients [71]—these disease state mutants were then also constructed.

The mutant proteins were purified to homogeneity and detailed kinetic studies performed [72–74]. The two disease-state proteins and their alanine equivalents, as well as another mutant, Arg<sup>449</sup> → Ala, were found to possess the hydroxylation activity but be completely devoid of any of the cytochrome b<sub>5</sub>-dependent acyl–carbon cleavage activities (Fig. 1A, B and C, respectively). These mutants thus had been converted from a multifunctional enzyme into a conventional hydroxylase. The mutational experiments also show that the interaction between CYP17 and cytochrome b<sub>5</sub> involves the cationic residues of the former, in particular Arg<sup>347</sup>, Arg<sup>358</sup> and possibly Arg<sup>349</sup>. The anionic residues of cytochrome b<sub>5</sub> which pair with the three, aforementioned, cationic sites of CYP17 are not known, but a subsequent study, on the mutation of cytochrome b<sub>5</sub>, had shown that the substitution of Glu<sup>48</sup>

and Glu<sup>49</sup> with Gly gave mutants which showed decreased activity to stimulate the acyl–carbon bond cleaving activity of CYP17 [75].

The role of cytochrome b<sub>5</sub> in regulating the various activities of cytochrome P-450s has been well documented but the molecular basis of these affects is not fully understood [76–79]. It has been argued that cytochrome b<sub>5</sub> may enhance the efficiency of electron transfer to a key iron–oxygen species involved in the catalytic P-450 cycle [78,80,81]. Evidence against this was provided by Guengerich's laboratory [82] but this was countered by a subsequent study [83]. Our results with an aldehyde analogue, lacking the 21-methyl group (structure **18a**, the 21-methyl group replaced by H), provided a forceful argument against the direct involvement of cytochrome b<sub>5</sub> in the electron transfer process during the side chain cleavage catalysed by CYP17 [74]. We had shown that the deformylation of the aldehyde analogue occurred through the same chemical mechanism as the side chain cleavage of the two physiological substrates [55]. However, this deformylation occurred at a rate 4-fold faster than any physiological transformation catalysed by CYP17 yet did not require, nor was enhanced by, cytochrome b<sub>5</sub>. Furthermore, the mutant CYP17 proteins, that had lost their physiological side chain cleav-

**Table 1**  
Kinetic parameters of purified human CYP17.

	Kinetic parameters					
	Without cytochrome b <sub>5</sub>			With cytochrome b <sub>5</sub>		
	K <sub>m</sub>	V <sub>max</sub>	k <sub>cat</sub> /K <sub>m</sub>	K <sub>m</sub>	V <sub>max</sub>	k <sub>cat</sub> /K <sub>m</sub>
$\Delta^5$ -steroid						
Pregnenolone <b>18a</b> → 17 $\alpha$ -hydroxypregnenolone (of the type <b>21</b> )	0.65	2.20	5.6 × 10 <sup>4</sup>	0.65	3.90	1.0 × 10 <sup>5</sup>
Pregnenolone <b>18a</b> → 5,16-diene (of the type <b>20a</b> )		<0.02	–	0.65	0.39	1.0 × 10 <sup>4</sup>
17 $\alpha$ -Hydroxypregnenolone <b>19a</b> → DHEA (of the type <b>22</b> )		<0.02	–	1.18	2.45	3.5 × 10 <sup>4</sup>
$\Delta^4$ -steroid						
Progesterone <b>18b</b> → 17 $\alpha$ -hydroxyprogesterone (of the type <b>21</b> )	1.00	3.33	5.6 × 10 <sup>4</sup>	1.00	4.66	7.8 × 10 <sup>4</sup>
Progesterone <b>18b</b> → 4,16-diene (of the type <b>20a</b> )		0.00	0.00	–	0.00	0.00
17 $\alpha$ -Hydroxyprogesterone (of the type <b>21</b> ) → androstenedione (of the type <b>22</b> )		0.00	0.00	5.00	1.80	6.0 × 10 <sup>3</sup>

Adapted from [57]. Activities are expressed as nmol of product formed/min/nmol of cytochrome P450, K<sub>m</sub> data are in  $\mu$ M and k<sub>cat</sub>/K<sub>m</sub> units are M<sup>-1</sup> s<sup>-1</sup>.

age activities, were able to cleave the formyl side chain (Fig. 1D). The oxidative cleavage of the acyl-carbon side chain by CYP17, thus, does not depend on the electron transfer properties of cytochrome  $b_5$ . Cumulatively, these findings bolstered our earlier hypothesis that the interaction of cytochrome  $b_5$  with the CYP17-substrate complex causes protein conformational changes which culminate in directing the iron-oxygen ligand of P-450 away from C-17 and towards C-20 (Scheme 7). This facilitates a nucleophilic attack of the peroxide anion on the carbonyl carbon producing a tetrahedral adduct that follows the side chain cleavage path.

Finally, we have shown that the kinetic parameters for the cytochrome  $b_5$ -dependent cleavage of the  $17\alpha$ -hydroxy- $\Delta^5$ -steroid (**18a** containing a hydroxyl group at  $17\alpha$ ) are more favourable than those for the cleavage of the  $17\alpha$ -hydroxy- $\Delta^4$ -steroid (**18b** containing a hydroxyl group at  $17\alpha$ ). When considered with the finding that the zona reticularis does not express  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^{5-4}$  isomerase [84], it seems reasonable to infer, *in vivo*, the preferential cleavage of  $17\alpha$ -hydroxypregnenolone to produce dehydroepiandrosterone, which using the sulphotransferase activity of the adrenal cortex, is secreted as the inactive sulphoconjugate. In addition to the control exerted by cytochrome  $b_5$ , serine phosphorylation of CYP17 has been shown to increase the lyase activity, with the interplay of these two processes adding to the complexity of the physiological regulation [85]. These interrelated and co-ordinated transformations provide the mechanism by which the human adrenal avoids contributing to the unwanted production of active androgens, testosterone and androstenedione; enabling the female of the human species to escape from the physiological ramifications which could be promoted by the male hormone.

### 3. Inhibitors of aromatase (CYP19) and CYP17<sup>3</sup>

#### 3.1. Sixth-ligand inhibitors

Scheme 3, structure **14**, highlights the participation of a hexa-co-ordinated species in a pivotal role in the third step catalysed by aromatase. This feature led us to conjecture that, by the introduction of sulphur or nitrogen containing groups at C-19, it may be possible to produce compounds which not only interact with the steroid-binding site of the enzyme but also provide the sixth-ligand to its haem-iron. We reasoned that such compounds should be strong and specific aromatase inhibitors. Accordingly, two steroids, 19-thiomethyl- and 19-azido-androstenedione (**29** and **30**, Scheme 8) fulfilling these criteria were discovered in 1985 [86,87] and their mode of inhibition established by kinetics and spectroscopic studies [88]. These compounds, 19-thiomethyl- and 19-azido-androstenedione had  $K_i$  values of  $1 \times 10^{-9}$  and  $5 \times 10^{-9}$  M, respectively, and interacted with aromatase as shown in **31** and **32** (Scheme 8). Contemporaneously, other inhibitors based on co-ordination to haem iron, were discovered for aromatase (for example **34** and **35**) and cholesterol side chain cleavage enzyme (P-450<sub>scc</sub>) [89]. Work on the inhibitors of aromatase until 1990 is reviewed by Cole and Robinson [90]. Subsequently, the same reasoning was used to design inhibitors for CYP17 and the most successful example of this is abiraterone (**36**, Scheme 8) [91].

#### 3.2. 'Suicide' inhibitors

Another popular approach for designing inhibitors for aromatase and related enzymes is to search for structures which are

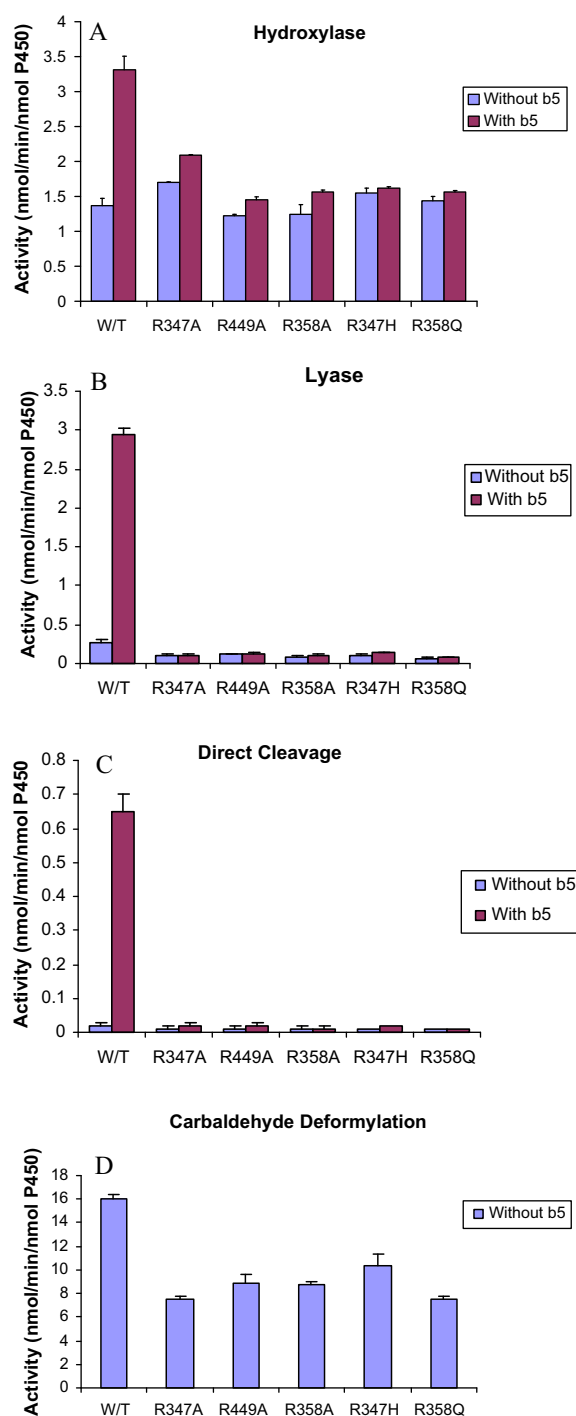
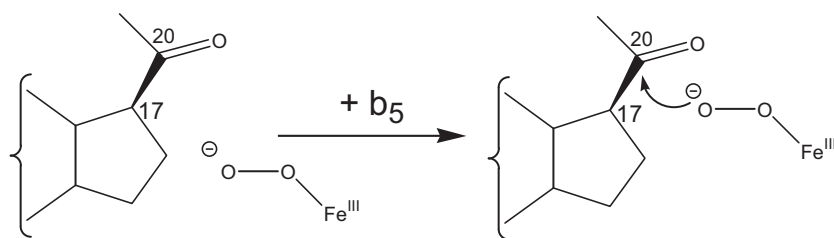


Fig. 1. Native and human CYP17 hydroxylase (Scheme 5, Reaction 1), lyase (Reaction 2), direct cleavage (Reactions 3 and 4) and carbaldehyde deformylation. Adapted from [74]. Data expressed as means  $\pm$  S.E. bars.

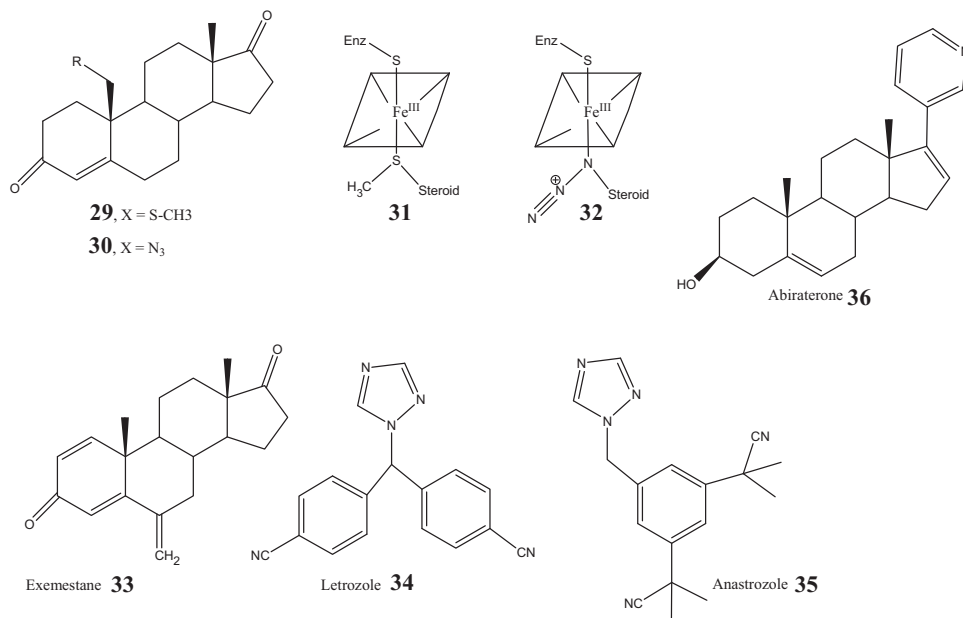
modified by the enzyme to produce a reactive intermediate which may irreversibly bind to the enzyme. Several steroids, for example **33**, when incubated with aromatase in the presence of  $O_2$  and NADPH have been found to inactivate the enzyme in a time dependent fashion as expected from suicide inhibitors [90]. However, in none of the cases has the mandatory requirement of showing the formation of a covalent adduct between the inhibitor and aromatase, been met. Possibility exists that such inhibitors promote the formation of reactive oxygen species, of the type **10** to

<sup>3</sup> This theme is comprehensively considered, in this issue of the journal, by Drs Angela Brodie and Vincent Njar.





**Scheme 7.** Conformational role of cytochrome  $b_5$  in modulating the cleavage activity of CYP17. The cartoon diagram shows that binding of cytochrome  $b_5$  to CYP17 promotes protein conformation change which directs the iron-oxygen species away from C-17 towards the carbonyl carbon at C-20 to allow the formation of a tetrahedral complex.



**Scheme 8.** Sixth-ligand coordination and 'suicide' inhibitors of aromatase and CYP17. The steroids **29** and **30** interact with aromatase as shown in **31** and **32**. Several other steroidal inhibitors also act via the complex of the type **31** and **32** [90]. Non-steroidal aromatase inhibitors, letrozole and anastrozole may form a sixth-ligand coordination complex using one of the nitrogen atoms in the triazole ring. Likewise, abiraterone, inhibits CYP17 using the pyridine N as the sixth-ligand. The aromatase inhibitor, exemestane, inactivates the enzyme in a time-dependent process by a mechanism whose details are not yet known (see text).

**12** (Scheme 3) but that these are not suitably juxtapositioned to the target C-19 and react instead with a neighboring group at the enzyme active site leading to inactivation.

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