Design and Action of Steroidal Aromatase Inhibitors

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ABSTRACT: The biosynthesis of estrogens involves three sequential hydroxylations, progressing from cholesterol, that are mediated by an enzyme complex referred to as aromatase. The last steps in this sequence involve aromatization of the A ring of the steroid nucleus. Compounds that inhibit aromatase have potential applications in the treatment of advanced estrogen-dependent mammary carcinoma and prostatic hyperplasia. The enzyme aromatase is currently a priority target for the development of active-site directed inhibitors. A number of steroid inhibitors may inactivate aromatase by diverse interactions with the enzyme and include competitive inhibitors, affinity labelling agents, and mechanism-based inhibitors ("suicide substrates"). By designing steroidal analogs with substituents at various positions on the steroid nucleus, information has been obtained on the structural requirements needed for favorable interactions with the enzymatic sites.
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KEY WORDS: Action, aromatase, design, inhibition, inhibitor, inhibitors, steroid, steroidal.

Aromatase is the enzyme responsible for catalyzing the conversion of androgens to estrogens in the last step of estrogen biosynthesis (Fig. 1). It was first isolated by Ryan in 1959 from the microsomal fraction of fresh human placental tissue (1). Aromatase is a cytochrome P-450 hemoprotein with about 30% homology to other cytochrome P-450 enzymes. The region of greatest homology among all steroidogenic P-450 enzymes is the heme binding region (2). The term "aromatase" usually refers either to Thompson's crude, insoluble preparation (3) or to Vickery's solubilized preparation (4) rather than to the unstable, purified isozymes 1 and 2 (5). Aromatase is a 55-kDa protein of 503 amino acids. An x-ray structure of cytochrome P-450 aromatase is not available, due to the difficulty of purification and crystallization of this membrane-bound enzyme. However, modeling studies with a variety of computational programs to predict the three-dimensional structure of aromatase have given us insights into the enzyme structure and its action (6).

Because of the importance of estrogen in mammalian metabolism and reproductive processes, aromatase has received much attention, and considerable progress has been made in understanding the structure, biochemical mechanism, and inhibition of the enzyme. The inhibition of aromatase is an important and specific route to control estrogen levels. Inhibitors of aromatase have found application in the control of such estrogen-dependent states as contraception, maintenance of pregnancy, gynecomatsia, endometriosis, and estrogen-dependent breast cancer (7–10).

In the treatment of estrogen-dependent breast cancer, surgical procedures, such as ovariectomy and adrenalectomy (removal of the major organs that produce estrogens and their precursors), only lower estrogen levels temporarily. Peripheral tissues eventually take over estrogen production (11). Traditionally, antiestrogens (e.g., Tamoxifen), which interact with estrogen receptors in tumor cells to compete with estrogen binding, have been used to treat postmenopausal breast cancer patients after surgical removal of major estrogen-producing organs (12). Tamoxifen is a weak antagonist. In spite of the antitumor function of this antiestrogen, breast tumor cells will eventually develop resistance to this drug with resulting disease recurrence.

Aromatase inhibitors usually don't have estrogenic activity because they control the estrogen level through a totally different mechanism that involves blockage of the biosynthesis of estrogens. Aromatase inhibitors, alone or in combination with Tamoxifen, could improve the treatment of breast cancer patients and other estrogen-dependent diseases. Aminoglutethimide was the first aromatase inhibitor used for this purpose. However, its lack of enzyme specificity and significant side effects limit its usefulness. The more specific steroidal aromatase inhibitor 4-hydroxyandrostenedione (4-OHA) has been approved recently for use in the United Kingdom and likely will be approved in other countries as well. Several other steroidal and nonsteroidal aromatase inhibitors are currently undergoing clinical evaluation. The steroidal inhibitors 10- (2-propenyl)estr-4-ene-3,17-dione (MDL 18962), 1-methylandrosta-1,4-diene-3,17-dione (SH-489), and 6-methyleneandrosta-1,4-diene-3,17-dione (FCE 24304) are currently in phase-1 trials (13–15).

BIOCHEMICAL MECHANISM OF AROMATASE

The biochemical mechanism of aromatase has been studied extensively, and considerable progress has been made in understanding the important reactions (Fig. 2) catalyzed by this enzyme. Aromatase converts androgens to estrogens by oxi-

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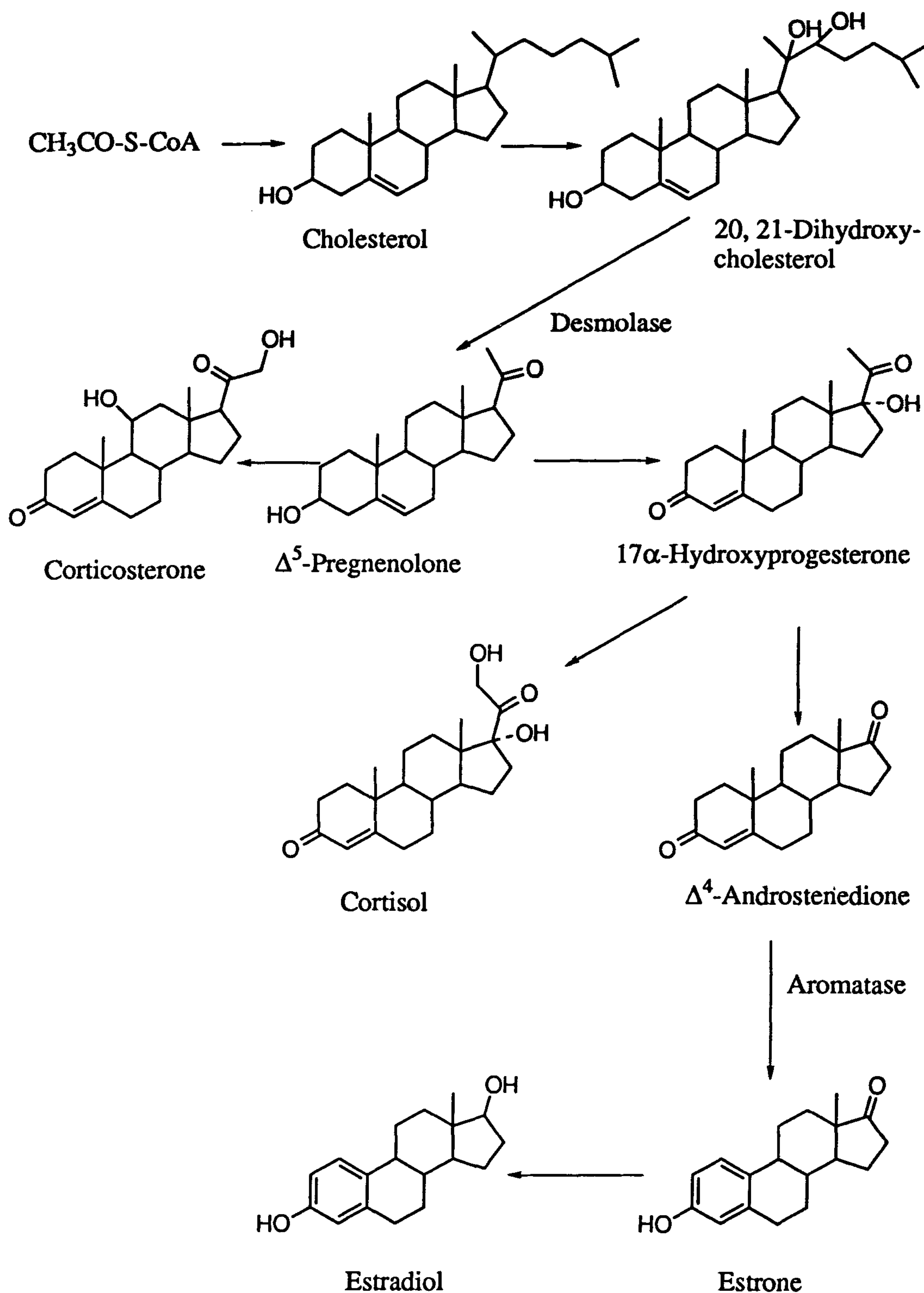

FIG. 1. Biosynthesis of steroid hormones.

dizing the C_{19} methyl group and aromatizing the steroid A-ring. It has been shown that three molecular oxygens and six reducing equivalents from NADPH are consumed during estrogen formation (16). During incubation with 19-^{14}C-testosterone, almost all C_{19} carbon is incorporated in the released formate; some radioactivity was found in CO_2 and H_2CO (17). The fate of the three C_{19} hydrogen atoms was determined by incubating deuterium- and tritium-labeled precur-

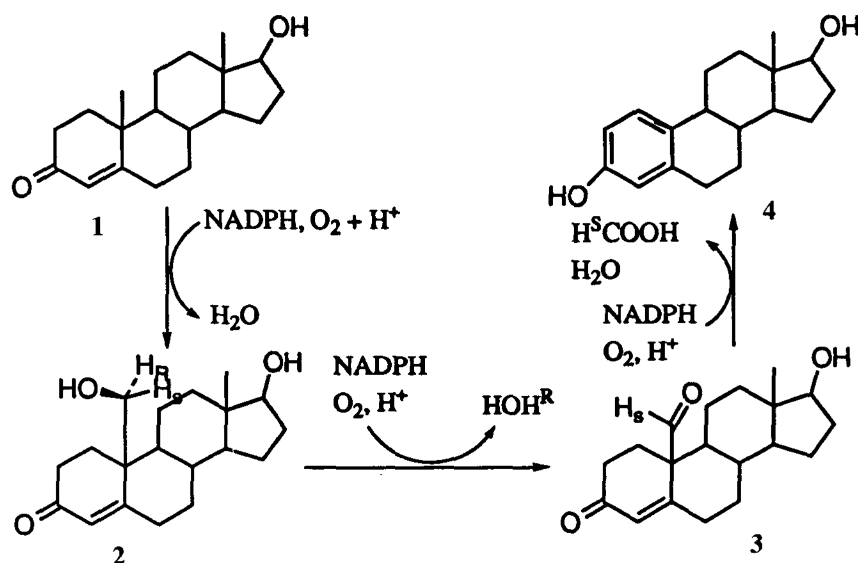

FIG. 2. Conversion of androgens to estrogens by aromatase.

sors with the enzyme. The 19-pro-*R*-hydrogen atom was lost preferentially in aromatization of the 19-hydroxy intermediate (18). In addition to the 19-pro-*R*-hydrogen, aromatase stereospecifically eliminated the 1β and 2β hydrogen atoms. The 1α-H, 2α-H, 4-H, and 6β-hydrogen atoms were retained in the product estrogen (19–22). Oxygen labelling shows that oxygen atoms 1 and 3 are removed as formic acid. The second oxidation yields an intermediate gem diol, and the oxygen atom eventually appears in the water released in the process of formation of an aldehyde. The oxygen labeling experiment also showed that the C_3 and C_{17} oxygens are retained in the process of aromatization (23,24). Based on these labeling studies and enzyme kinetic data, it is generally accepted that aromatase converts androgens to estrogens by three sequential oxidation steps. The androgen is first oxidized to the C_{19}-hydroxy androgens. Then, loss of the 19-pro-R-hydrogen of the 19-hydroxy intermediate to water and retention of the 19-oxygen atom in the intermediate results in the C_{19} aldehyde (25). The explanation for the second step either invokes stereospecific dehydration of the presumed 19-gem-diol intermediate (Fig. 3, path a) or by direct hydrogen abstraction from a 19-carbinol radical species (Fig. 3, path b). A glutamate residue from the enzyme is believed to participate in the first two steps at the oxidation site. In the above two steps, aromatase performs the typical cytochrome P-450-type hydroxylation (with retention of configuration), each requiring one equivalent of molecular oxygen and NADPH (26,27).

The mechanism of the third step in aromatase action is even less clear. Chemically, there are three positions where oxidation would occur readily: the C_{19} aldehyde, the [C_2βH] enolizable position, and the [$C_{4,5}$] alkene (Fig. 4). Several hypotheses have been advanced for the mechanism of the third oxidation step. These proposed mechanisms could explain some of the experimental observations. Akhtar and colleagues' peroxy intermediate model is the most accepted hypothesis (Fig. 5) (28).

Akhtar proposed that the third oxidation step involves a nucleophilic attack of a ferric peroxy intermediate on the aldehyde group of the 19-oxo intermediate to give a peroxide species. The ferric peroxy intermediate undergoes a Baeyer-Villager type rearrangement to yield estrogen and formic acid. The theory developed by Soonsin and Robinson (29) proposes that the fragmentation of the ferric peroxy intermediate proceeds *via* a six-membered ring transition state with a proton transfer mechanism. Following this fragmentation, estrogen and formic acid are released, and the catalytic cycle is completed. This modification could explain the stereospecific 2β-proton loss during the A-ring aromatization. Based on the irreversible inactivation by inhibitors bearing leaving groups on C_4 and C_6, Covey *et al.* (30,31) propose a fragmentation that involves enzymatic nucleophilic attack at C_4 and the $C_{4,5}$ double-bond shift to $C_{5,10}$ concommitant with C_{19} leaving as formic acid (Fig. 6). Subsequent elimination of 1β-H and enolization of the 3-keto group complete the aromatization process (30,31).

Hahn and Fishman (32) suggested that the third oxidative step may occur at the C_2 position (Fig. 7) (32). Oxidation of the 2,3-enol ketone yields 2β-hydroxy 3-ketone. 2β-Hydroxy-19-oxo-4-androstene-3,7-dione is the third oxidation product of aromatase. This intermediate will cyclize to form a hemiacetal intermediate, which yields estrogen elimination of formic acid. This theory is supported by two observations. Immunological probes for 2β-hydroxylated-19-oxygenated androgen indicated the presence of such intermediates in microsomal fractions during aromatization. Incubation of microsomal placental aromatase with [1α-^{3}H]-androst-4-ene-3,17-dione, followed by reduction of the intermediate and isolation of the product, yielded 0.13% of compound 10.

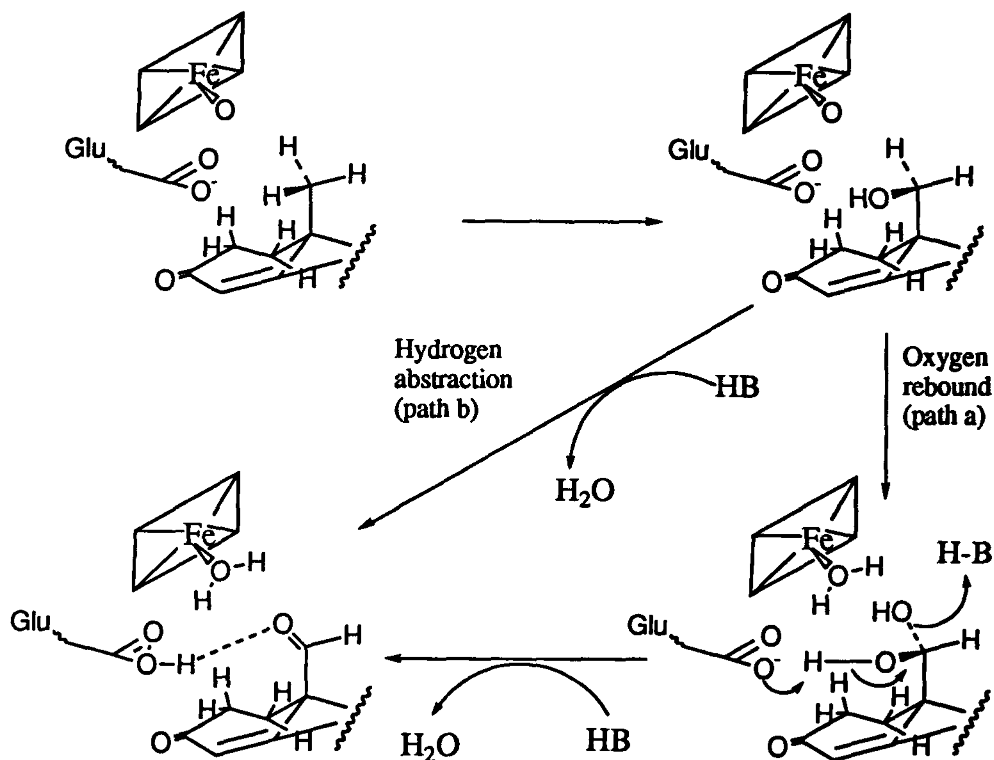

FIG. 3. The first and second oxidative steps in aromatase action.

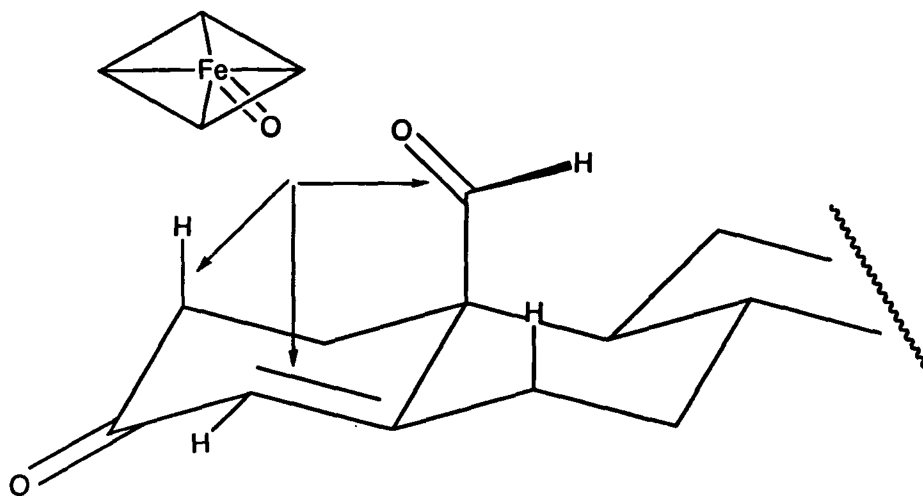

FIG. 4. Possible sites of the third oxidative step of aromatase.

In 1975, Morand and colleagues (33–37) postulated that the third oxidation in the biosynthesis of estrone proceeds *via* 19-oxo-4,5-epoxy-androstane-3,17-dione (Fig. 8). Chemically, the epoxide 13, its 4α,5α-isomer, and C_{19} oxidized analogues, such as 19-carboxy-4,5-epoxyandrostane-3,17-dione, can readily rearrange to estrone under various conditions. The mechanism in Figure 8 would account for the stereochemistry and regiochemistry observed in aromatase. Formation of a stabilized carbocation or an enzyme-assisted epoxide cleavage will yield 15, which undergoes further rearrangement to estrogen.

AROMATASE INHIBITION

Although the active site and the biochemical mechanism of aromatase are not fully understood, the unique features in the

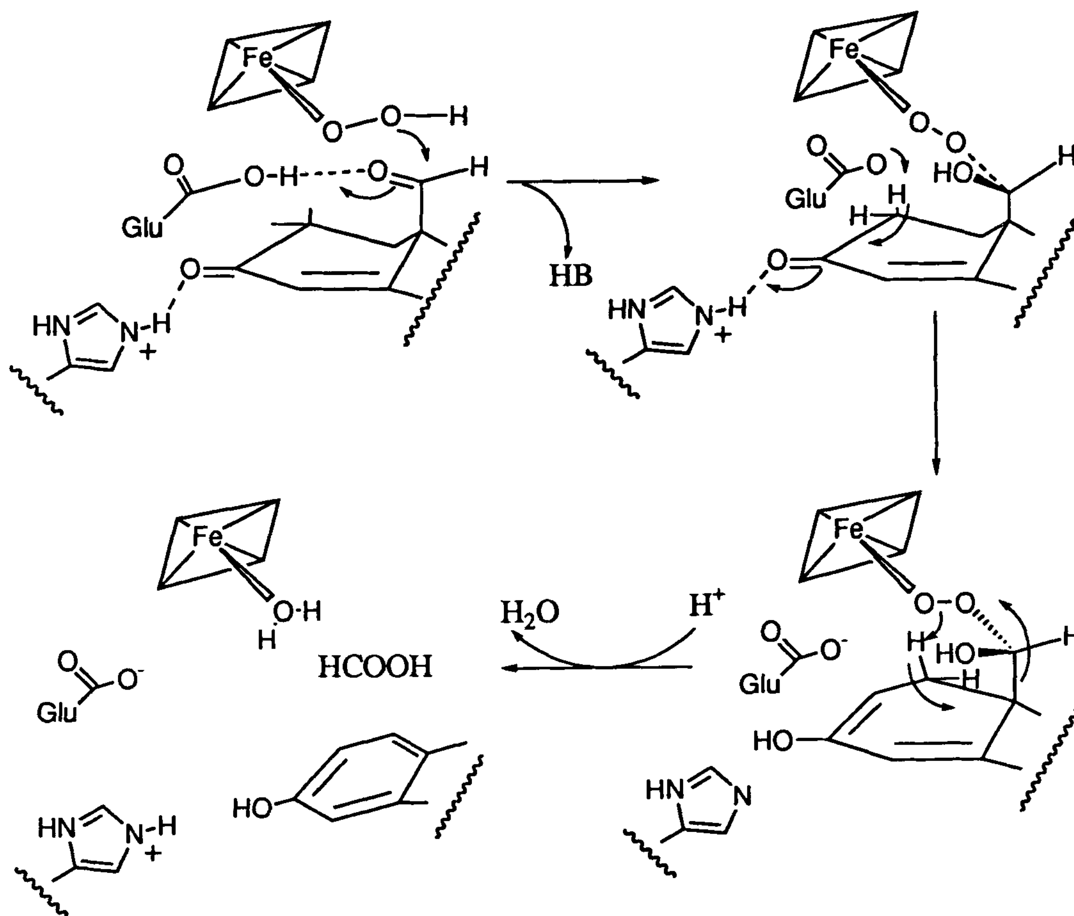

FIG. 5. Akhtar–Robinson Model of the third oxidative step in aromatase action.

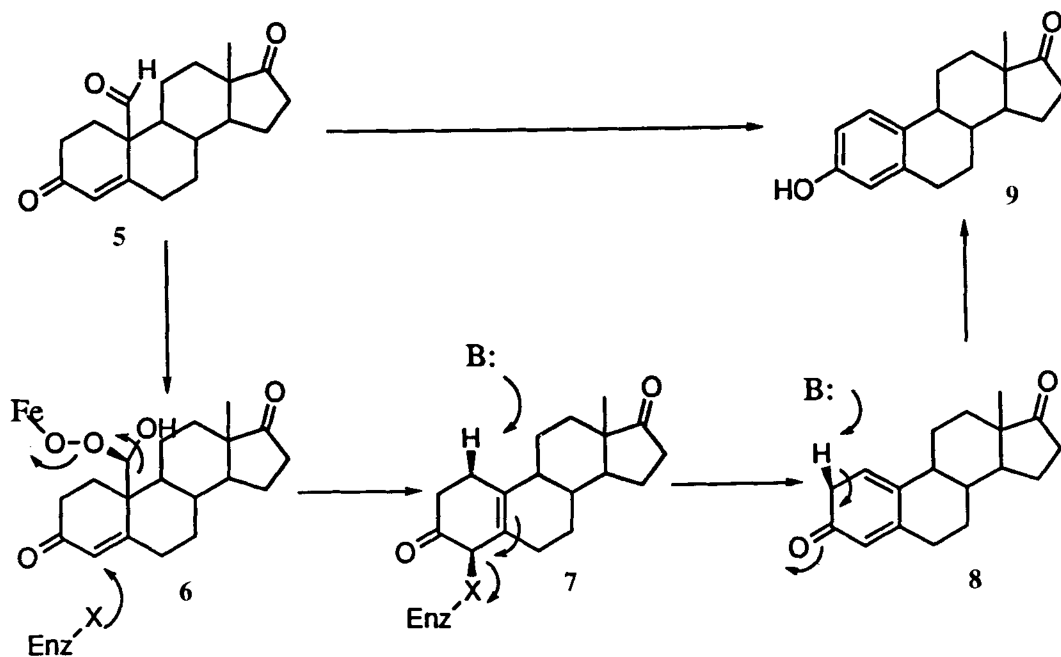

FIG. 6. Akhtar–Covey Model of the third oxidative step in aromatase action.

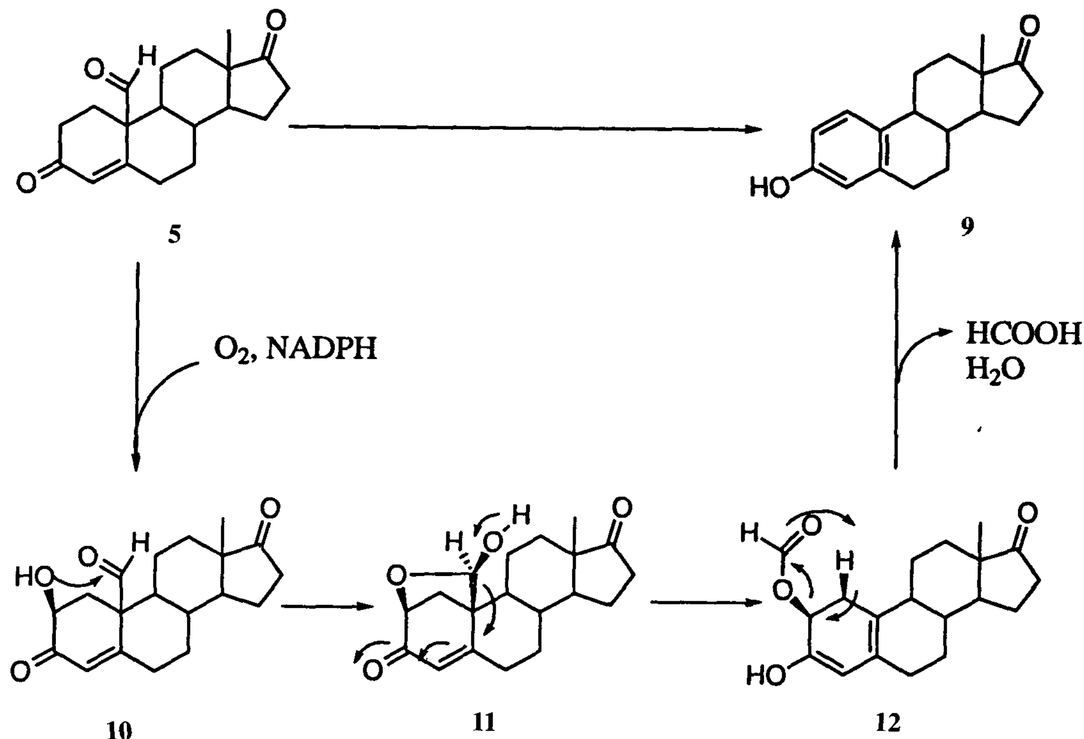

FIG. 7. Fishman Model of the third oxidative step in aromatase action.

aromatization process discussed above provide a rationale for the design of selective aromatase inhibitors from substrate analogs. Because aromatization of androgens is the last step in the biosynthetic sequence of estrogen production, the selective blockage of aromatization will not directly interfere with the production of such other steroids as adrenal corticoids. For this reason, aromatase is a particularly suitable enzyme target for the control of estrogen biosynthesis.

Aromatase inhibitors reported in the literature generally may be classified into three types, according to their general structural resemblances: (i) aminoglutethimides, (ii) imidazoles, and (iii) steroidal substrate analogs. The first two types of nonsteroidal inhibitors are mainly heme binders, which inhibit aromatase by coordinating to the heme iron *via* the inhibitor's heteroatom (38). Steroidal aromatase inhibitors have been studied extensively. A variety of substrate analogs with other functional groups on C_{19}, C_1, C_2, C_4, and C_6 have been synthesized and shown to be good inhibitors of aromatase (39). Mechanistically, steroidal inhibitors could be classified as competitive and noncompetitive. Almost all of the noncompetitive inhibitors are mechanism-based inhibitors (MBI).

The first aromatase inhibitor studied and used clinically in the treatment of postmenopausal breast cancer patients was aminoglutethimide (40), which was originally developed for the treatment of epilepsy and later found to inhibit cytochrome P-450 aromatase. Aminoglutethimides (Fig. 9) affect aromatase inhibition through the aniline amino group coordinating with the heme. There are many aminoglutethimide derivatives with different *N*-alkyl and C_3 alkyl substituents that have been synthesized and studied as aromatase inhibitors (Fig. 9). They show moderate to good inhibition of aromatase (41,42). However, aminoglutethimides also inhibit other cytochrome P-450 (heme protein) enzymes, including desmolase, which blocks the biosynthesis of hydrocortisone. The IC_{50} value of aminoglutethimide for the inhibition of estrogen biosynthesis is 30 μM, which is the same as for its inhibition of progesterone biosynthesis. Consequently, patients treated with aminoglutethimides also have to be given supplemental hydrocortisone. The lack of specificity and potency and the significant side effects of aminoglutethimide inhibitors limit their usefulness (43).

Imidazole derivatives are the other class of nonsteroidal aromatase inhibitors that have been investigated (Fig. 10). Unlike aminoglutethimide, imidazole inhibitors have been shown to be quite selective as well as potent aromatase inibitors (44). Such compounds include fadrozale 25 [4-(5,6,7,8-tetrahydroimidazo-[1,5α]-pyridin-5-yl)-benzonitrile monohydrochloride](CGS 16949A) and (3αR)-*trans*-1-(3α,4,5,6-hexahydro-1 H-phenalin-2-yl)-methyl1]-1 H-imidazole HCl (ORG 33201). Fadrozale inhibits aromatase selectivity with an IC_{50} value of 0.03 μM by contrast with a 120 μM value for progesterone synthase (45,46).

In addition to imidazoles, the structurally related *N*-substituted triazoles have also been found to inhibit aromatase specifically (Fig. 10). These compounds are competitive, re-

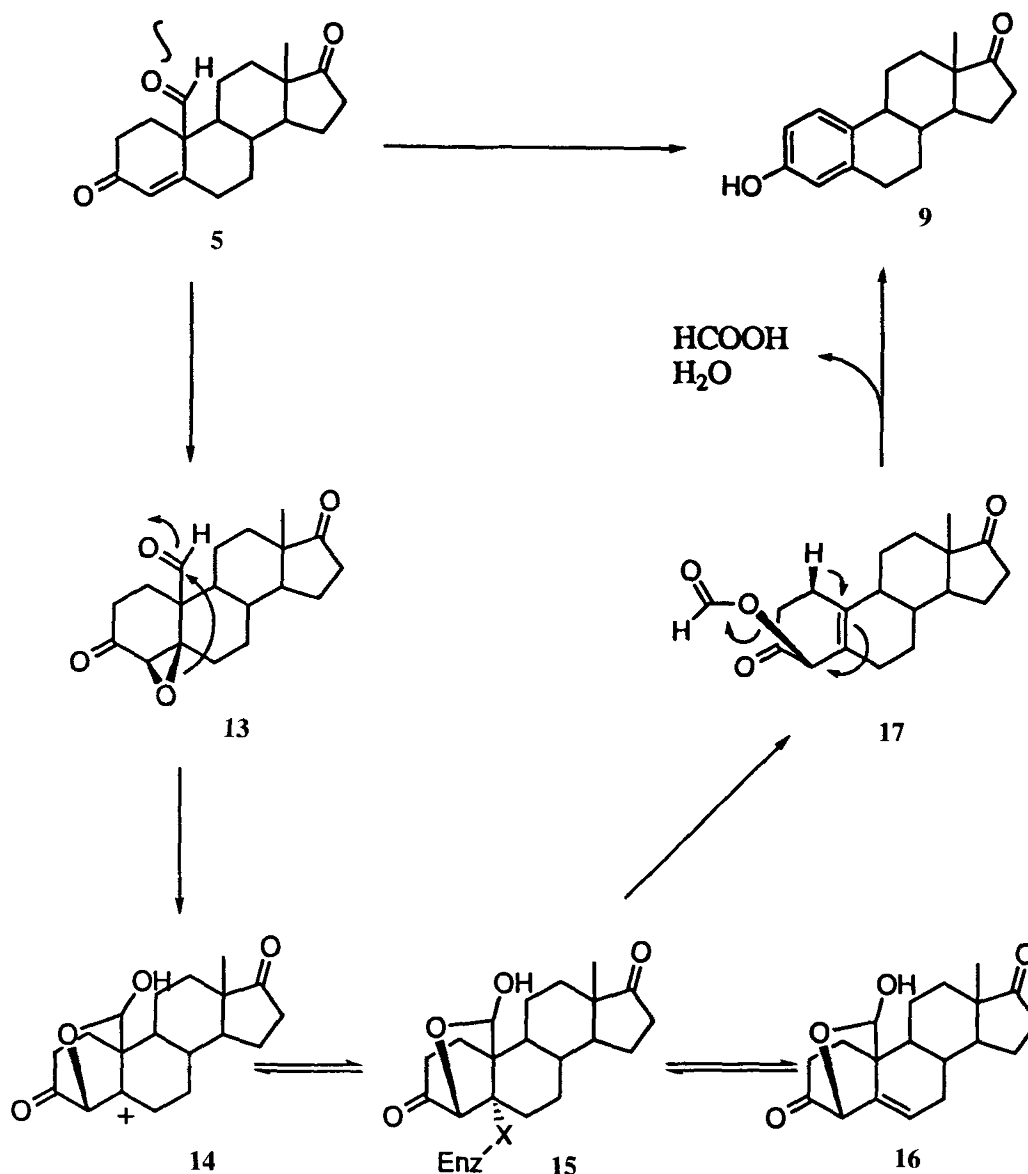

FIG. 8. Morand Model of the third oxidative step in aromatase action.

versible aromatase inhibitors (47). The inhibition potency of imidazoles arises from the coordination of nitrogen atoms on the heterocyclic ring with the heme iron and thus competes with the binding of oxygen. Greater selectivity for aromatase might result from partial overlap with the steroid binding site of the enzyme (Fig. 11). Studies on the Interaction of R76713 (vorozole) [6-(4-chlorophenyl) (1H-1,2,3,-triazol-1-yl) methyl-1-H-benzotriazole] with aromatase have shown that this triazole derivative coordinates with the heme iron and that its *N*-1 substituent projects into a hydrophobic pocket of cytochrome P-450 aromatase. This hydrophobic pocket is not occupied by the natural substrate and extends from the $C_7\alpha$ position of the steroid (Fig. 4) (48).

In addition to R-76713, two other triazole derivatives now in clinical trials are potent and selective aromatase inhibitors (49,50). They are Arimidex, 2,2′[5-(1 H-1,2,-triazol-1-yl-methyl)-1,3-phenylene]bis-(2-methyl propiononitrile (ZD 1033) and letrozole, 4-(-cyano-phenyl)-1-(1,2,4-triazolyl) methyl] benzonitrile (CGS 20267). Letrozole is 10 times more potent *in vivo* in rats than fadrazole hydrochloride, while they have the same inhibitory activity toward human placental aromatase *in vitro*. In humans, letrozole is about 100 times more potent than fadrazole in reducing serum estradiol levels. This difference in potency is thought to be due to the significantly longer half-life of letrozole *in vivo* (51).

In 1973, Schwarzel *et al.* (52) published their pioneering work on steroidal aromatase inhibitors. They evaluated the relative inhibitory activities of over 100 readily available steroids and nonsteroids for human placental aromatase. The initial structure-activities relationships for aromatase inhibi-

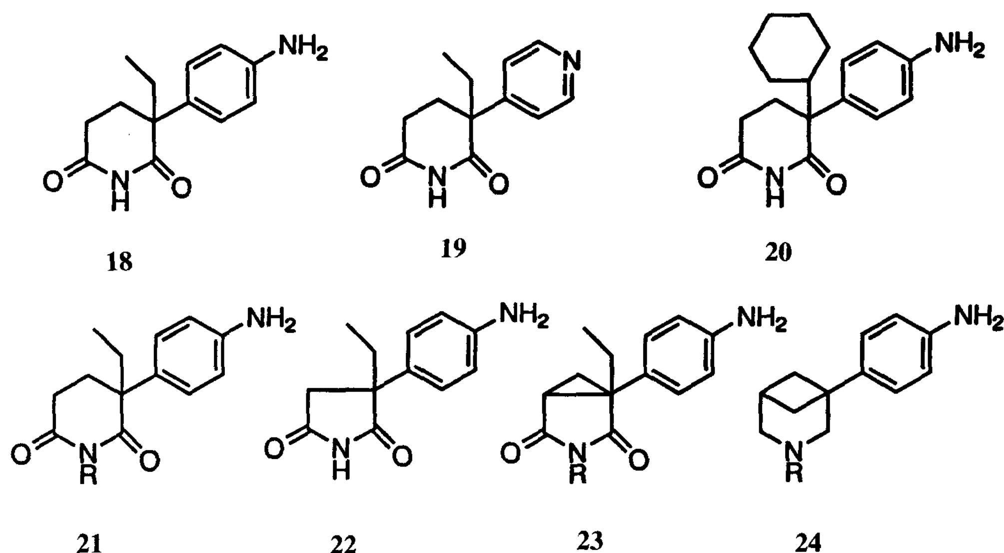

FIG. 9. Aminoglutethimide-type aromatase inhibitors.

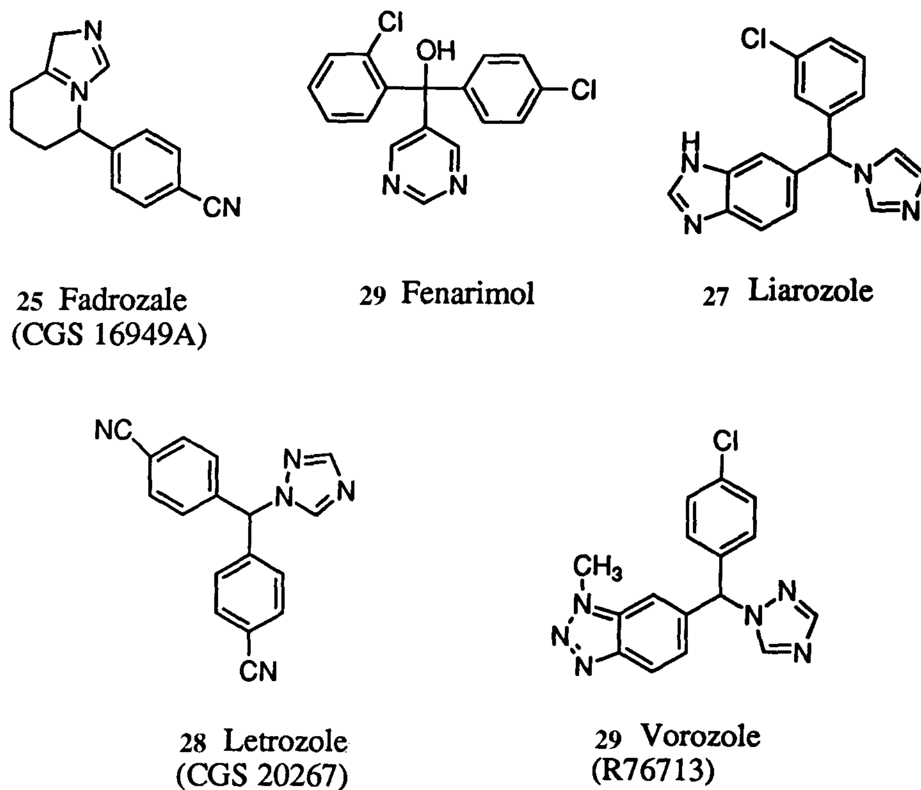

FIG. 10. Imidazole- and triazole-type aromatase inhibitors.

tion were established and subsequently confirmed by other investigators of these compounds. They found that the most effective inhibitors share the following common structural features: (i) a C_{19} steroid nucleus with an A/B *trans* ring junction; (ii) keto functionality at C_3, and (iii) 17-keto or 17β-hydroxy substituents are essential structural requirements. Extended linear conjugation on the androstenedione nucleus (e.g., 4,6-diene-3-one, 1,4,6-triene-3-one, and 4-ene-3,6-dione) usually increases the inhibitory activity. Steroidal inhibitors that have been developed to date build upon this basic androstenedione nucleus and incorporate a variety of substituents on the steroid A and B rings. In summary, the pub-

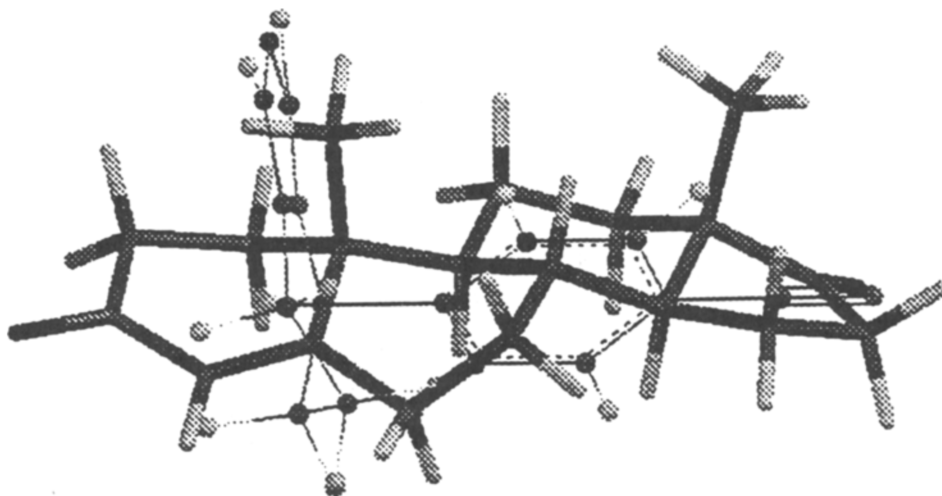

FIG. 11. The partial overlap of Fadrozale and androgen.

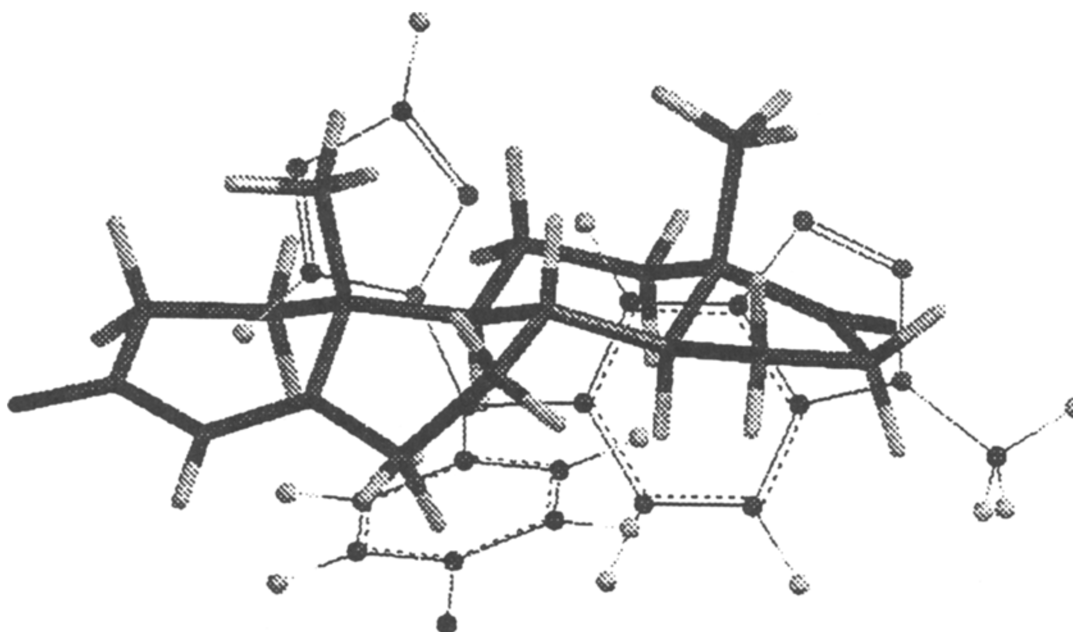

FIG. 12. The N-1 *P*-chloro phenyl group of Vorozole projects into the 7α-hydrophobic pocket.

lished steroidal aromatase inhibitors developed to date can be structurally classified into five categories: compounds with substituents on the A ring, B ring, 19-methyl group, A B ring-bridged and A ring 19-methyl group-bridged compounds. Some compounds are potent and selective aromatase inhibitors and have found application in the treatment of postmenopausal breast cancer and other estrogen-dependent states (15).

Modification of the A ring of the androstenedione molecule initially focused on substituents at C_4 (Fig. 13). The steroids 4-hydroxy-4-androstene-3,17-dione (4-OHA) and 4-acetoxy-4-androstene-3,17-dione are effective inhibitors *in vitro* with reported Ki values of 2 and 10 μM, respectively, and they produce enzyme-mediated inactivation (53,54). The mechanism possibly involves catalytic conversion to a reactive intermediate, which then causes active-site inactivation. Covey invoked enzyme-assisted addition-elimination at C_4 to explain the irreversible inhibition caused by 4-OHA and other 4-substituted androstenediones (Fig. 14). Lombardi (55) and collaborators have synthesized 4-aminoandrogens that are also mechanism-based irreversible inhibitors (55). Further modifications with extended conjugation through Δ 1,4 or Δ

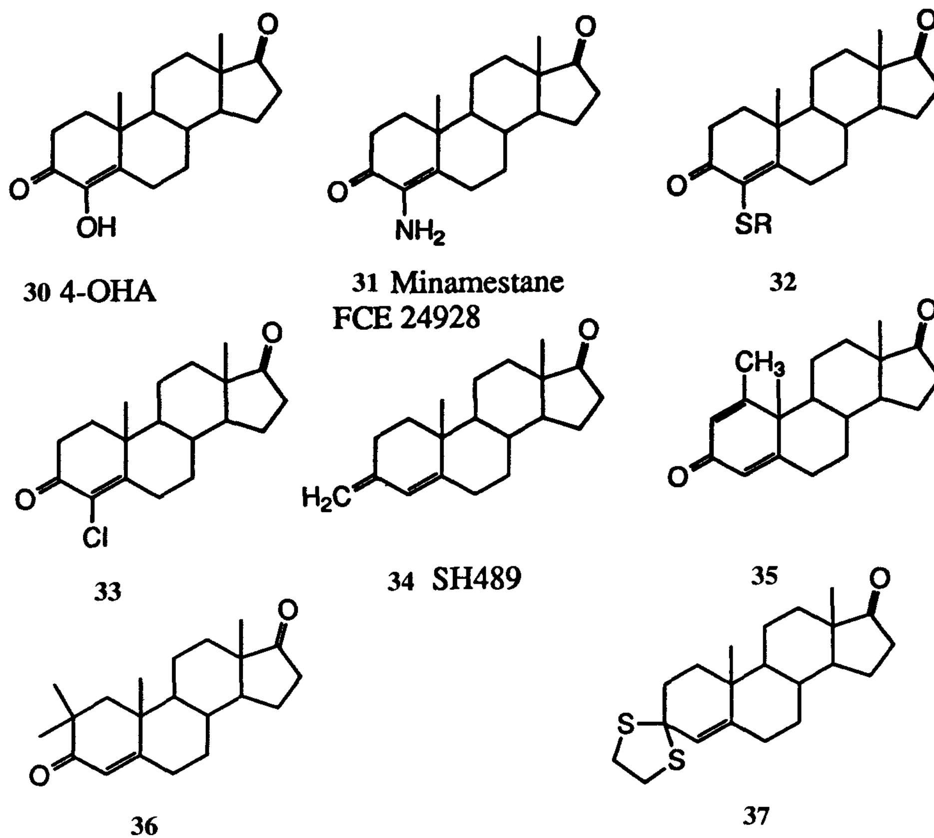

FIG. 13. A-ring substituted steroidal aromatase inhibitors.

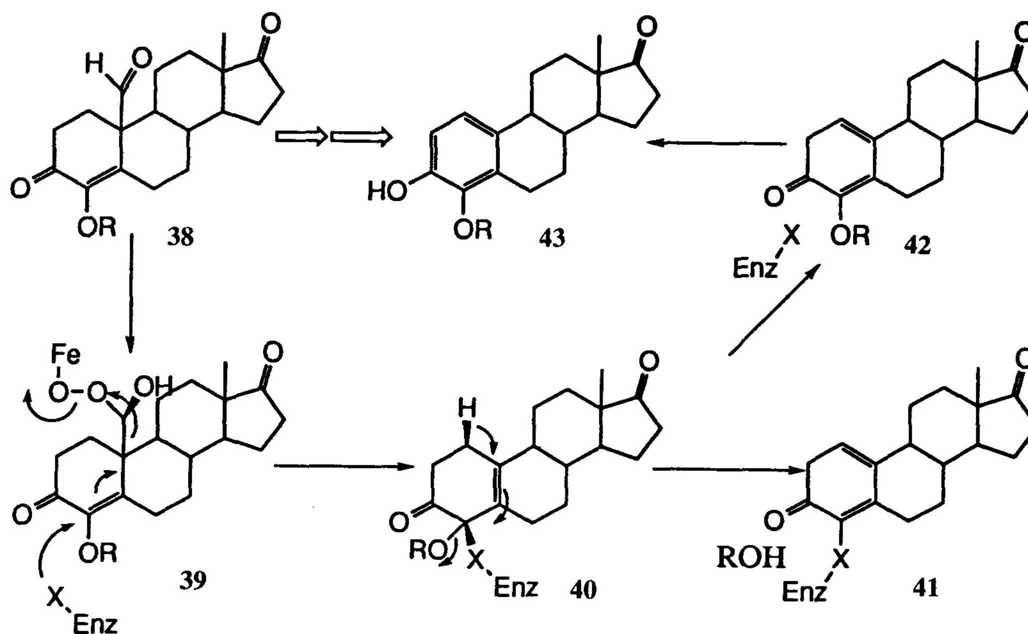

FIG. 14. The mechanism of inactivation at C-4.

4,6 double bonds enhance the inhibitory activity. Marsh and colleagues (56,57) found that the inhibitory activities of sulfur derivatives 4-SH and 4-Sac are lower than those of corresponding oxygen analogs. Evaluation of a number of 4-thioethers by Abuj-Haij (57) indicated that an alkyl chain of up to three carbons fits into the active site. The straight-chain derivatives with 4-S-C_4, 4-S-C_5, and the benzyl 4-SCH_2Ph exhibit little inhibition ($IC_{18\%}$, $IC_{8\%}$, $IC_{8\%}$ = 1.5 μM, respectively). The aromatic 4-S-Ph and 4-S-Ph-*p*-OCH_3, -*p*-NH2, -*p*-Br, and -*p*-NO_2 cause no inhibition at 1.5 μM. These results indicated existence of a hydrophobic pocket-accommodating substituent at the C_4 position, which is 4.3 Å in width and 5.5 Å in length. These results also suggest that there is no correlation between activity and electronic factors of substituents, although the low activity of inhibitors with such substituents may be due to their steric inaccessibility. The most valuable inhibitor in this series 4-OHA was found to inhibit estrogen reproductive processes and is used clinically in the treatment of advanced breast cancer in postmenopausal women. A limited number of inhibitors with substituents at other positions on the A ring have been reported. 1-Methyl-1,4-androstadiene-3,17-dione and 2,2-dimethyl 4-androstenedione are potent inhibitors of aromatase *in vitro* (58). 1-Methyl-1,4-androstadiene-3,17-dione suppresses gonadal and peripheral aromatization of androgens in juvenile rats, and its application in the treatment of benign prostatiac hypertrophy (BPH) is now being explored (59). However, derivatives with bulky substituents at the 1α position are poor inhibitors (60). At the C_3 position, replacement of the ketone with a methylene and thioketal provides effective inhibitors (61). In general, the spatial requirements of the A ring for binding of the steroidal inhibitors to aromatase are rather restrictive and permit few structural modifications to be made. Incorporation of the polar hydroxy group at C_4 enhances inhibitory activity.

Effective aromatase inhibitors derived from B-ring modification are mainly C_6 and $C_{7\alpha}$ substituted derivatives of 4-androstenedione (Fig. 15). The 6-ketoxime derivatives of 4-androstenediones are potent aromatase inhibitors (62). Although the 6-keto derivative is one of the most common clinical inhibitors in use, it exhibits relatively poor affinity. Its competitive and pseudo-first-order kinetics of the enzyme inhibition result from an enzyme-generated intermediate (63). Proposed mechanisms of inhibition have invoked delocalization of the charge toward the C_6 instead of the C_4 position. Other C_6 substituent inhibitors are 6-methyleneandrosta-14-diene-3,17-dione (FCE 27985) analogs. FCE 24304 is now undergoing phase-1 clinical trials (64,65). The nucleophile addition–elimination mechanism would explain the inhibition

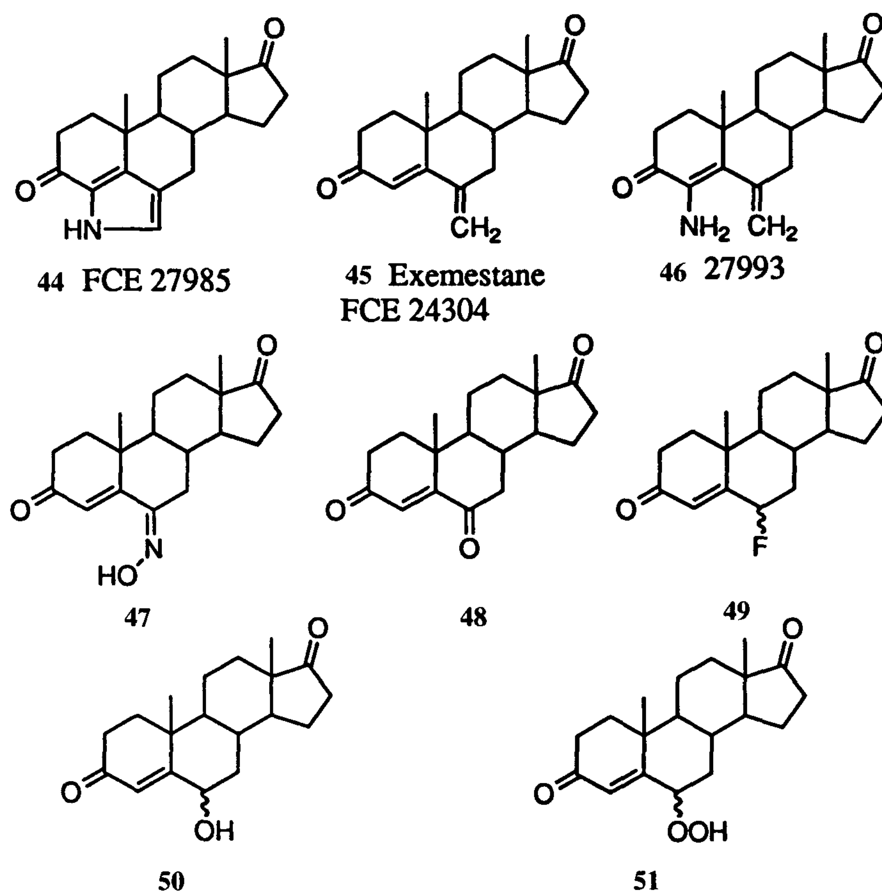

FIG. 15. The C-6 substituted steroidal aromatase inhibitors.

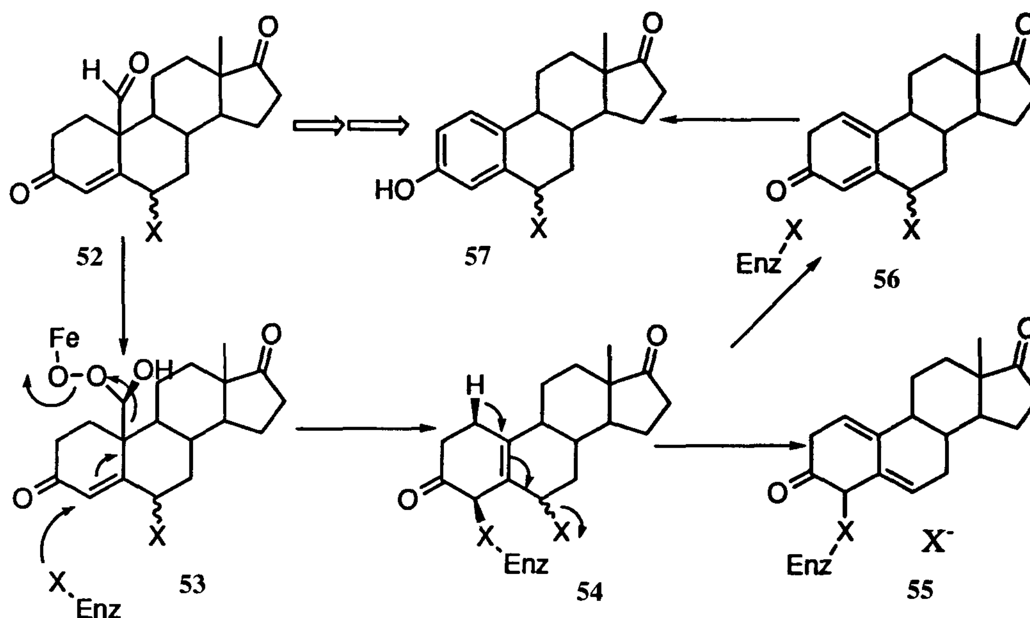

FIG. 16. The inhibitory mechanism of androstenedione with C-6 leaving groups.

by androgens that bear leaving groups on C_6 (Fig. 16). Derivatives with 6-fluoro, -chloro-, hydroxy, or -acetoxy groups showed good activity in the inhibition of human placental aromatase *in vitro* (66,67). Tan and Petit (68) modified these competitive inhibitors to make more reactive epimers of 6-hydroperoxyandrostenedione. These are NADPH-independent irreversible inhibitors and do not require enzymic activation. It is not unreasonable to postulate that inhibition occurs as a result of oxidation of the active center cysteine by C_6 hydroperoxides.

As with restrictive modification of the A ring with bulky groups, in contrast to A-ring modification with restrictive bulky groups, bulky substituents at the $C_{7\alpha}$ position of the B ring have provided several potent aromatase inhibitors. Brueggemeier and Li (69) reported 7α-APTA to be an effective competitive inhibitor, with an apparent K_i of 18 nM. This inhibitor has also demonstrated effectiveness in inhibiting aromatase in cell cultures and in treating hormone-dependent rat mammary tumors. Evaluation of various substituted aromatic analogs of 7α-APTA provided no correlation between the electronic character of the substituents and inhibitory activity. A study with radioiodinated 7α-(4′-iodo) phenyl thio-1,4-androstadienedione demonstrated that the radioactive inhibitor is covalently bound to a lipophilic region of aromatase. An effective mechanism-based inhibitor 7α-(4′amino)-phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD), with an apparent K_i of 9.9 nM, was also reported by his research group and rapidly inactivates the enzyme (69). Results with various 7-substituted 4,6-androstadiene-3, 17-dione derivatives suggest that only those derivatives that can project the 7-aryl substituents into the 7α hydrophobic pocket are effective inhibitors. Recently, Bruggemeier reported that 7α-arylaliphatic-substituted androst-4-ene-3,17-diones possess the same extents of inhibitory activity as the thioaryl analogs, which indicates that the thioether of 7α-APTA is not important as an inhibitor of aromatase (70).

Panzeri *et al.* (71) described another novel mechanism-based B-ring-modified inhibitor, androsta-4,6,8,(9)-triene-3,17-dione (FCE 24918), in which the triene moiety acts as a latent alkylating group. This inhibitor behaves as a suicide substrate for aromatase. It is believed that, after the third oxidation at C_{19} and the nucleophilic attack at C_4, the inhibitor B ring instead of the A ring is aromatized. As a result, the inhibitor binds to the enzyme covalently, and the enzyme is inactived (Fig. 17).

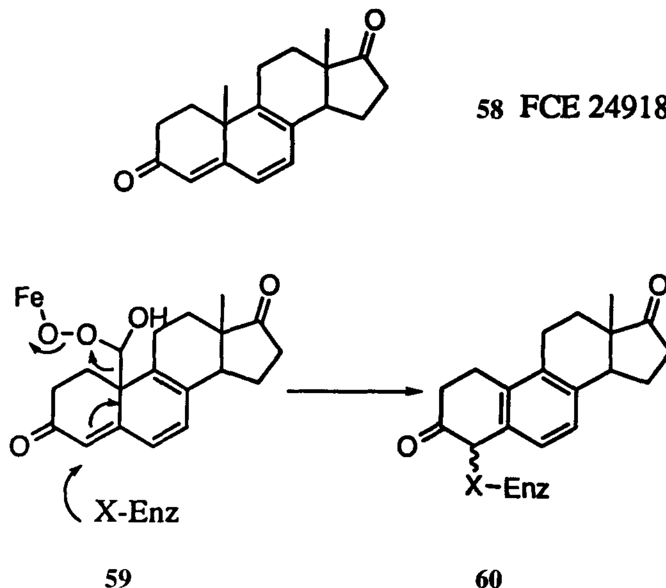

FIG. 17. The inhibitory mechanism of androsta-4,6,8(9)-triene-3,17-dione.

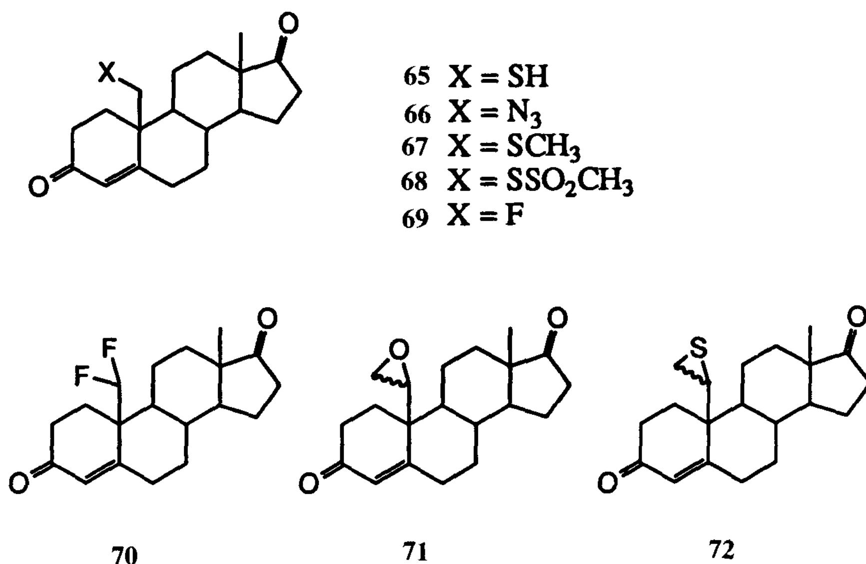

FIG. 18. The C-19 substituted steroidal aromatase inhibitors.

Derivatives with heteroatoms at C_{19} are obvious substrate analogues of aromatase, and many of them compete for the enzyme active site (Fig. 18). The heteroatom at C_{19} could coordinate to the heme iron as the sixth ligand and compete with oxygen binding. Several of these types of inhibitors are modified as mechanism-based inhibitors, presumably undergoing enzyme-mediated oxidation at the C_{19} carbon to uncover the alkylating group. 10-Propargyl (PED, MDL 18962) and allen-4-estren-3,17-dione are extremely potent enzyme-activated inhibitors of aromatase (72–75). PED was synthesized and studied independently by three research groups (76–78). The extent of the inhibition by PED has been disputed due to inconsistent K_i values reported, although it remains one of the most potent inhibitors. PED is now undergoing clinical trials for breast cancer in England. The mechanism of inactivation is believed to involve oxidation in the active center to generate an oxirene or allene oxide species. The generated reactive epoxides could alkylate the enzyme and cause the inactivation (Fig. 19).

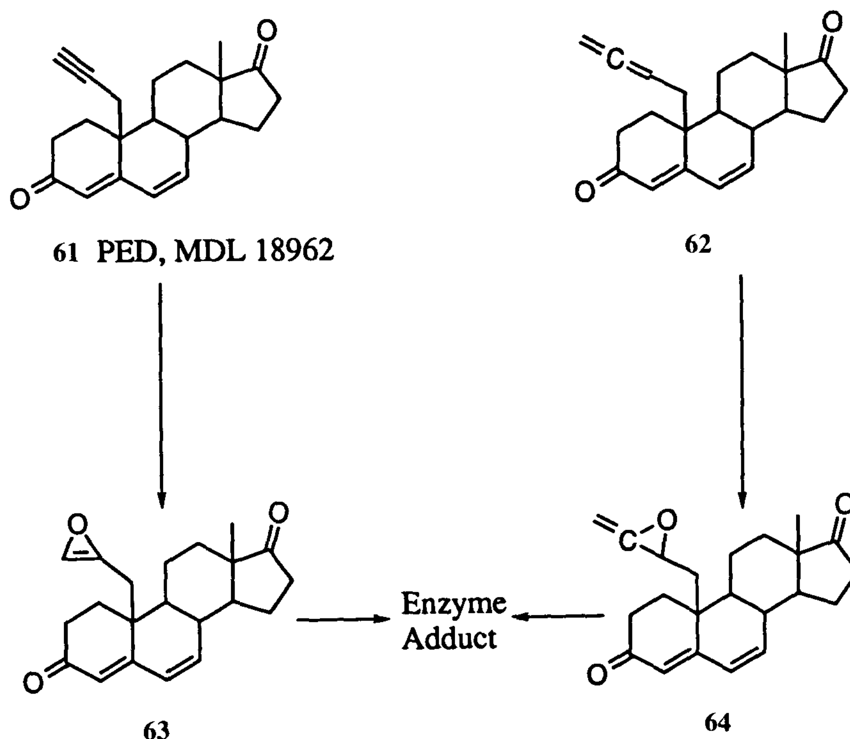

FIG. 19. The inhibitory mechanism of 10-propargyl and allen-4-estren-3,17-dione.

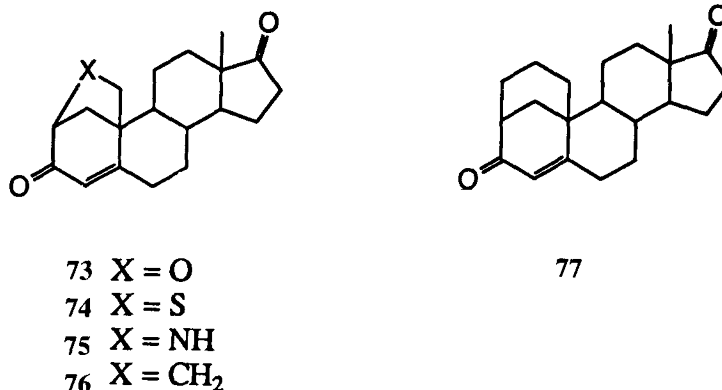

FIG. 20. The C-2, C-19 bridged steroidal aromatase inhibitors.

Marcotte and Robinson (78) synthesized and tested 19-fluorinated androstenediones (79). Whereas the monofluorinated derivative is aromatized, the difluorinated androstenedione acts as a mechanism-based inhibitor, possibly *via* an acyl fluoride. Time-dependent release of tritium from C_{19} labelled ^3H CF_2 indicates that the mechanism probably involves 19-HO CF_2, which quickly rearranges to the rective acyl fluoride in solution. Mann and Pietrzak (80) combined the 19-F_2 and the 4-OH pharmacophores in one inhibitor. This combination leads to a slight loss of activity. The less-reactive C_{19} thirane and oxirane are also potent competitive reversible inhibitors. The stereoisomers have shown differential inhibitory activity, with 36- to 80-fold higher activity observed for the (19*R*)-isomers (81). Spectral analysis confirms that these inhibitors bind to the heme iron (54). The tighter coordination of the 19*R* derivatives has been interpreted to favor a heme iron binding, which is located above C_1 and C_2 in the A-ring. Johnston *et al.* (82) further explored the favorable binding position by studying 2,19-bridged androstenediones (Fig. 20). The methylene-, ethylene-, oxygen-, sulfur-, and nitrogen-bridged derivatives were found to be potent aromatase inhibitors with K_i of 2 (ethylene), 35 (methylene), 7(oxygen), and 20 μM (sulfur). When the bridge is an NH group, the inhibitory kinetics changed from competitive to noncompetitive, which implies that the enzyme-substrate inhibitor complex formed caused the enzyme inactivation. This type of inhibitor may find application in the treatment of breast cancers of premenopausal women where estrogen production is conducted in high concentrations of androgens. These A-ring-bridged androstenedione analogs represent a novel series of potent steroidal aromatase inhibitors. The restrained A-ring bridges that contain C, O, S, and N atoms could effectively coordinate with the heme iron of the P-450 aromatase due to the favorable position (Fig. 20).

MOLECULAR MODELING FOR HUMAN AROMATASE ACTIVE CENTER

The rational design of new aromatase inhibitors is hindered by the lack of knowledge of the enzyme's three-dimensional structure. Most eukaryotic P-450 enzymes are membrane-binding enzymes and insoluble, which makes purification and crystallization difficult. The only cytochrome P-450 for which an X-ray structure has been reported is P-450_{cam} from *Pseudomonas putida.* The degree of homology between P-450_{cam} and mammalian P-450 is generally low. However, the three-dimensional structure of the cytochrome P-450 enzyme family is well conserved. The membrane-binding region of the eukaryotic enzymes, absent in P-450_{cam}, is confined to the *N*-terminal region of the proteins (83–85). These findings serve as a basis for the three-dimensional structure prediction of cytochrome P-450 aromatase by computer modeling. Combined with the results of site-directed mutagenesis studies and the structures of substrates and inhibitors, a number of attempts have been made to predict the structure of this enzyme by a variety of computational programs (86–89). Figure 21 represents the overall active center shape predicted by Loughton *et al.* (90). The long axis of the steroid is inclined at an angle of about 35° to the plane of the heme. The distances from the heme-iron atoms C_1 and C_{19} of the steroid, the oxidation sites, are both about 4.9 Å. The important active site residues include Glu^{302} at the 2β-position, His^{475} at the C_3 keto position, Phe^{234} and Phe^{235} at the $C_{7\alpha}$ position, and Asp^{309} at the C_{17} position. These predictions fit quite well

FIG. 21. The predicted active-site structure for aromatase.

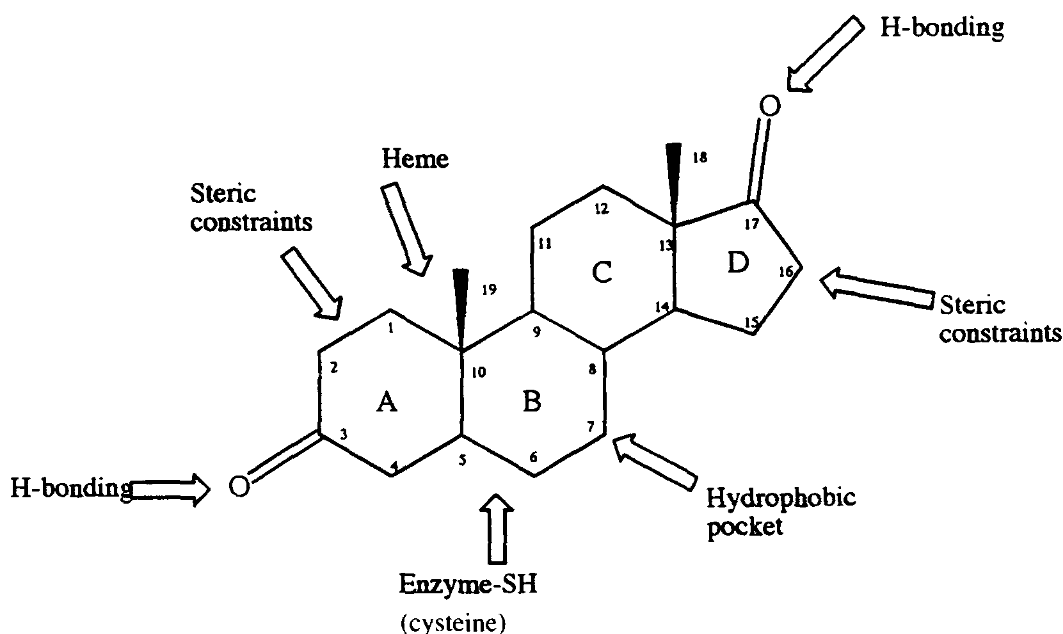

FIG. 22. Elements of the active-site structure of aromatase.

with the required elements of the active-site structure of aromatase as deduced from the experimental data on substrates and inhibitors (Fig. 22).

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