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Perspective

Mechanism and Inhibition of Cytochrome P-450 Aromatase

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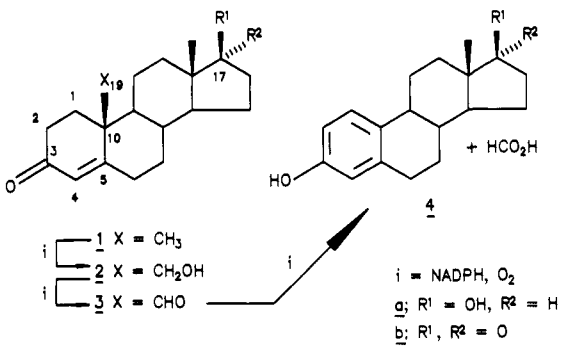
The role of estrogen blockade in the treatment of certain neoplastic disorders has been of wide interest for a number of years. It has long been known that specific tumors, particularly breast cancer in women, can grow more rapidly in proportion to the surrounding levels of estrogen.[1] Recent advances in the areas of molecular and cellular biology have led to a more detailed understanding of estrogen action. The estrogen receptor gene has been cloned, sequenced, and expressed.[2] The estrogen receptor protein seems to be composed of two nonoverlapping functional domains: the first domain has a high affinity for estrogen and the second has a high affinity for DNA. Upon binding of estrogen to the first domain, a conformational change is apparently triggered in the second domain, allowing it to productively interact with a specific sequence of DNA nucleotides known as a transcriptional enhancer region. The result of this interaction is the greatly increased production of mRNA from select genes, one or more of which is probably essential for efficient tumor cell growth.[2]

One can envision that there are many levels at which medical intervention could lead to the inhibition of estrogen action. For practical reasons, most of the research effort has been concentrated on two approaches. The first is the development of estrogen receptor antagonists. The most well known of these compounds is the stilbene derivative tamoxifen. In 1988, tamoxifen ranked second in sales in the U.S. among drugs used to treat cancer, and it accounted for 75% of the market for drugs used to treat breast cancer.[3] The second approach, which is the main topic of this review, is directed at lowering estrogen levels by inhibiting estrogen biosynthesis. Because estrogen synthetase (aromatase) catalyzes the final step in estrogen production in humans (the conversion of steroidal androgens **1** to steroidal estrogens **4**; see Scheme I), it has been the principal target of inhibition.

Scheme I



Aromatase Mechanism

Before discussing aromatase inhibition, it seems important to outline current knowledge of aromatase mechanism. Human placental aromatase, the most extensively studied form, is a cytochrome P-450 enzyme[4-8] that

(1) (a) Brodie, A. M. H. *Biochem. Pharmacol.* **1985**, *34*, 3213. (b) Santen, R. J.; Misbin, R. I. *Pharmacotherapy* **1981**, *1*, 95. (c) Van Wauwe, J. P.; Janssen, P. A. J. *J. Med. Chem.* **1989**, *32*, 2231. (d) Johnston, J. O.; Metcalf, B. W. In *Approaches to Cancer Chemotherapy*; Prasad, S. S., Ed.; Academic Press: New York, 1984; p 307.

(2) (a) Webster, N. J. G.; Green, S.; Tasset, D.; Ponglikitmongkol, M.; Chambon, P. *EMBO J.* **1989**, *8*, 1441. (b) Green, S.; Chambon, P. *Nature* **1987**, *325*, 75. (c) Walter, P.; Green, S.; Greene, G.; Krust, A.; Bornert, J. M.; Jeltsch, J. M.; Staub, A.; Jensen, E.; Scrace, G.; Waterfield, M.; Chambon, P. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7889.

(3) (a) Personal communication from the ICI Pharmaceuticals group. (b) Kolata, G. *Science* **1985**, *229*, 1378.

(4) (a) Sligar, S. G.; Murray, R. I. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986; p 429. (b) Poulos, T. L. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986; p 505.

(5) Waxman, D. J. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986; p 525.

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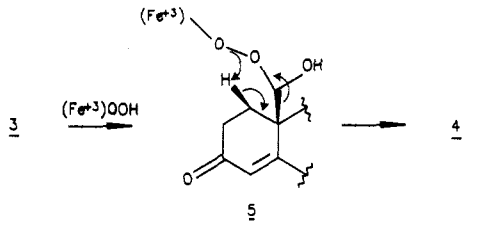
functions in association with an NADPH-dependent reductase.

The overall process of androgen to estrogen conversion is comprised of three steps, each apparently requiring 1 molar equiv of NADPH and O_2.[9] The first two steps are relatively well-understood. In the first step, the androgen (androstenedione (**1b**), testosterone (1a)) is hydroxylated at C-19 to afford the 19-hydroxy intermediate **2**.[10] The reaction occurs with retention of configuration, and is accompanied by a significant kinetic isotope effect.[11] It is considered to be a "classical" cytochrome P-450 hydroxylation. In the second step the 19-hydroxy intermediate **2** is oxidized to the 19-oxo compound **3**. This reaction involves stereospecific removal of the C-19 *pro-R* hydrogen,[12] with no isotope effect.[13] It has also been shown that the aldehyde oxygen atom is derived solely from the first mole of oxygen consumed.[14] These results imply that there is either a second "classical" hydroxylation leading to the *gem*-diol, followed by stereospecific dehydration, or a direct dehydrogenation of the 19-alcohol group. The former mechanism is probably correct, however, on the basis of the elegant work of Beusen and Covey with artificial substrates.[15]

In the mysterious third step, the bond between C-10 and C-19 is oxidatively cleaved to afford estrogen (estradiol (4a), estrone (**4b**)) and formic acid. Oxygen atoms from each of the first and third moles of oxygen consumed,[14] as well as one of the original C-19 hydrogens, are incorporated into the formic acid. On the basis of the early work of Fishman and of Brodie, it has been thought for many years that the androgen 1β- and 2β-hydrogens are lost stereospecifically to the aqueous medium.[16] There is now thought to be a substrate-dependent variation in stereospecificity at C-2.[17] In the case of testosterone (1a), there is significant 2α-H loss. Since it has been shown that placental microsomes readily interconvert testosterone (**1a**) and androstenedione (**1b**) in the presence of NADPH, the classical tritium release aromatase activity assay of Thompson and Siiteri[9] (measurement of the rate of 1β,2β-^{3}H incorporation from substrate into water) may not be completely accurate. The recent shift to the use of [1β-^{3}H]androstenedione in assays is therefore a necessary change.

Scheme II

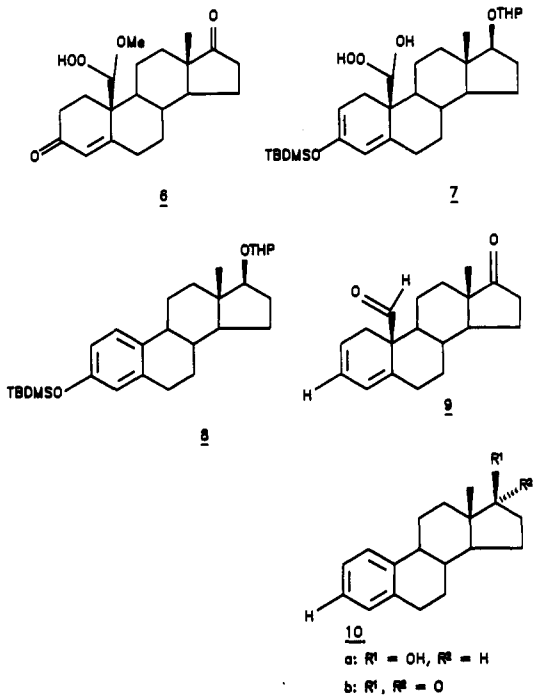


As far as is known, all of the aromatase transformations occur in the same active site of the same protein molecule. The recent cloning of the human placental aromatase cDNA and its transfection into COS cells has led to expression of the complete enzymic activity.[18] This provides the strongest evidence that one protein molecule is responsible for all three steps. Furthermore, there is presently no evidence that more than one aromatase gene exists in human tissues.[18] The hypothesis that one active site is responsible for all three oxidative transformations is likely, but may not be rigorously established until a crystallographic structure is obtained.

Several mechanistic theories for the third step have been shown experimentally to be unlikely. These include 2β-hydroxylation,[19] 4,5-epoxidation,[20] Baeyer–Villiger oxidation at C-19,[14] 10β-hydroxyestr-4-ene-3,17-dione formation,[21] and any process that involves water attack on the 19-aldehyde group. One theory that has remained consistent with all known aromatase data and cytochrome P-450 theory was proposed by Akhtar.[14] It features attack of an enzyme-bound ferric peroxide on the 19-aldehyde group, to produce the geminal hydroxyferric peroxide **5** (Scheme II). This unstable intermediate could collapse to estrogen and formic acid by hydride shift,[14] proton transfer (as shown),[22] or free-radical[23] pathways. A key point is that the high electrophilicity of the aldehyde leads to circumvention of normal ferric peroxide breakdown and alters the normal hydroxylation cycle. While present technology prevents a rigorous evaluation of this concept, analogues of the proposed Akhtar intermediate (e.g. **6**)[24] as well as a 2,3-enolized version (e.g. **7**)[25] have been studied.

(6) McMurry, T. J.; Groves, J. T. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986; p 1.
(7) (a) Nelson, D. R.; Strobel, H. W. *Biochemistry* **1989**, *28*, 656. (b) Nebert, D. W.; Gonzalez, F. J. *Annu. Rev. Biochem.* **1987**, *56*, 945.
(8) White, R. E.; Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315.
(9) (a) Thompson, E. A.; Siiteri, P. K. *J. Biol. Chem.* **1974**, *249*, 5364. (b) Siiteri, P. K.; Thompson, E. A. *J. Steroid Biochem.* **1975**, *6*, 317.
(10) (a) Meyer, A. S. *Biochim. Biophys. Acta* **1955**, *17*, 441. (b) Meyer, A. S. *Experientia* **1955**, *11*, 99.
(11) Caspi, E.; Arunachalam, T.; Nelson, P. A. *J. Am. Chem. Soc.* **1986**, *108*, 1847.
(12) (a) Osawa, Y.; Shibata, K.; Rohrer, D.; Weeks, C.; Duax, W. L. *J. Am. Chem. Soc.* **1975**, *97*, 4400. (b) Arigoni, D.; Battaglia, R.; Akhtar, M.; Smith, T. *J. Chem. Soc., Chem. Commun.* **1975**, 185.
(13) Miyairi, S.; Fishman, J. *J. Biol. Chem.* **1985**, *260*, 320.
(14) Akhtar, M.; Calder, M. R.; Corina, D. L.; Wright, J. N. *Biochem. J.* **1982**, *201*, 569.
(15) Beusen, D. D.; Carrell, H. L.; Covey, D. F. *Biochemistry* **1987**, *26*, 7833.
(16) (a) Brodie, H. J.; Kripalani, K. J.; Possanza, G. *J. Am. Chem. Soc.* **1969**, *91*, 1241. (b) Fishman, J.; Guzik, H. *J. Am. Chem. Soc.* **1969**, *91*, 2805. (c) Fishman, J.; Guzik, H.; Dixon, D. *Biochemistry* **1969**, *8*, 4304.
(17) (a) Fronckowiak, M. D.; Osawa, Y. The Endocrine Society Meeting, New Orleans, June 8–11, 1988 (Abstract 430). (b) Cole, P. A.; Robinson, C. H. *Biochem. J.* **1990**, *268*, 553.
(18) (a) Corbin, C. J.; Graham-Lorence, S.; McPhaul, M.; Mason, J. I.; Mendelson, C. R.; Simpson, E. R. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8948. (b) Means, G. D.; Mahendroo, M. S.; Corbin, C. J.; Mathis, J. M.; Powell, F. E.; Mendelson, C. R.; Simpson, E. R. *J. Biol. Chem.* **1989**, *264*, 19385.
(19) (a) Hosoda, H.; Fishman, J. *J. Am. Chem. Soc.* **1974**, *96*, 7325. (b) Goto, J.; Fishman, J. *Science (Washington, D.C.)* **1977**, *195*, 80. (c) Fishman, J.; Raju, M. S. *J. Biol. Chem.* **1981**, *256*, 4472. (d) Hahn, E. F.; Fishman, J. *J. Biol. Chem.* **1984**, *259*, 1689. (e) Caspi, E.; Wicha, J.; Arunachalam, T.; Nelson, P.; Spiteller, G. *J. Am. Chem. Soc.* **1984**, *106*, 7282.
(20) (a) Morand, P.; Williamson, D. G.; Layne, D. S.; Lompa-Krzymien, L.; Salvador, J. *Biochemistry* **1975**, *14*, 635. (b) Mastalerz, H.; Morand, P. *J. Chem. Soc., Perkin Trans. 1* **1982**, 2611.
(21) (a) Covey, D. F.; Hood, W. R.; Beusen, D. D.; Carrell, H. L. *Biochemistry* **1984**, *23*, 5398. (b) Townsley, J. D.; Possanza, G. J.; Brodie, H. J. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1966**, *25*, 282 (Abstract 520). (c) Caspi, E.; Dharmaratne, H. R. W.; Shackleton, C. *J. Chem. Soc., Chem. Commun.* **1989**, 1699.
(22) Stevenson, D. E.; Wright, J. N.; Akhtar, M. *J. Chem. Soc., Chem. Commun.* **1985**, 1078.
(23) Stevenson, D. E.; Wright, J. N.; Akhtar, M. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2043.
(24) (a) Cole, P. A.; Robinson, C. H. *J. Chem. Soc., Chem. Commun.* **1986**, 1651. (b) Cole, P. A.; Robinson, C. H. *J. Chem. Soc., Perkin Trans. 1* **1990**, 2119.
(25) Cole, P. A.; Robinson, C. H. *J. Am. Chem. Soc.* **1988**, *110*, 1284.

No detectable aromatization was found to occur in any of the 3-keto analogues under several conditions. In contrast, the 2,3-enol 19-hydroxy 19-peroxide analogue **7** smoothly aromatized in high yield to afford **8** under very mild conditions, thus providing a "model reaction" for Akhtar's theory. These model studies led us to examine the importance of enolization to the actual enzymatic reaction. Recent work demonstrating substrate-dependent stereospecificity for hydrogen removal at C-2 for **3a** vs **3b** provides evidence that aromatase does mediate hydrogen removal at this position.[17b]



Another set of experiments that supports this proposal involved the study of 3-desoxy-2,4-diene-19-oxo androgen analogue **9**.[26] Compound **9** was viewed as an analogue of the 2,4-dien-3-ol tautomer of **3b**. It was found that **9** was a nonenzymatic "model reaction" substrate that reacted with HOOH (via the intermediate 19-hydroxy 19-hydroperoxide) to form 3-desoxyestrone (**10b**). Furthermore, it was shown that compound **9** was a potent competitive inhibitor of aromatase, as expected for an analogue of a reaction intermediate. Finally, in an unprecedented event, it was demonstrated that **9** was converted by aromatase, in an NADPH-dependent fashion, to 3-desoxyestrogen **10** at an enzymatically significant rate. These results implicate enzyme-induced enolization as an important aspect of aromatization.

Future aspects of mechanistic exploration may not only involve continuing and refining studies related to the above-mentioned topics but may branch out into new areas. One exciting development has been the cloning of the aromatase gene.[18] If the potential for overexpression and overproduction of aromatase in easily harvested cells is realized, many physical, biochemical, and spectroscopic studies that have heretofore been unrealistic should become feasible. In addition, the molecular biology techniques of site-directed mutagenesis, coupled with new improvements in molecular modeling and structural analysis, may provide insights into the key active site amino acids that result in the unique chemical reactions catalyzed by aromatase. In particular, it will be of great interest to understand this carbon–carbon bond cleavage reaction in the context of the class of cytochrome P-450 lyases operative in steroid biosynthesis. As recently reviewed by Akhtar, there are several lyases that may utilize a similar mechanism to aromatase.[23] These include 14α-demethylase,[27] 17,20-lyase,[28] and the 16,17-ene-producing enzyme.[29] All can be formulated as involving nucleophilic attacks of ferric peroxide species on carbonyl groups, followed by fragmentation of the resultant hydroxy peroxide species. A more detailed study of this group of lyases may lead to unifying principles of enzyme mechanism and inhibition. To date, no metalloporphyrin models have been shown to perform this class of reactions and this question, too, could be worthy of investigation.

Aromatase Inhibition

As a cytochrome P-450 protein and a steroid-processing enzyme, aromatase is an exciting and multifaceted target for inhibition. Many steroids and non-steroids have been designed, synthesized, and tested as aromatase inhibitors. An encyclopedic listing and discussion of all aromatase inhibitors would not be wise because of space limitations, nor would it be particularly helpful since it would dilute the important developments. For these reasons we have chosen to describe trends in inhibitor design, with particular emphasis on rational design. Attention will be focused on those compounds where mechanism of action has at least been partly explored, those that show exceptional inhibitory properties, and those that may show clinical promise. There is now a very large number of steroidal inhibitors of aromatase, and it should be pointed out that a pioneering paper by the Brodie laboratory in 1973 led the way in this field.[30] It should be stated that the aromatase inhibition assays are performed differently in various laboratories and that a range of K_m values for androstenedione have been obtained. Unless otherwise mentioned, the inhibitory data are based on assays with human placental aromatase.

Type I Competitive Inhibitors

Compounds that bind reversibly to the active site of aromatase as steroid substrate analogues, and fail to turn over very rapidly may be useful inhibitors. If these compounds bind as normal androgen substrates, they may induce analogous Type I changes in the UV-absorption spectrum Soret band of the enzyme-bound heme. This phenomenon is a general characteristic for cytochrome P-450 enzymes. Type I binders induce a shift in the Soret band maximum from about 420 nm to about 390 nm.

There are many compounds that have either been demonstrated or are presumed to act as Type I inhibitors. Several of the most impressive in this category include 2,2-dimethylandrostenedione (**11**),[31] 7α-[(4-iodophenyl)-thio]androstenedione (**12**),[32] 3-*exo*-methyleneandrost-4-en-17-one (**13**),[33] 10β-vinylestr-4-ene-3,17-dione (**14**),[34] and the 10β-ethylestr-4-ene-3,17-dione (**15**).[34] The K_i values for these compounds represent nearly the best that have

(26) Cole, P. A.; Bean, J. M.; Robinson, C. H. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2999.

(27) Akhtar, M.; Alexander, K.; Boar, R. B.; McGhie, J. F.; Barton, D. H. R. *Biochem. J.* **1978**, *169*, 449.

(28) Kominami, S.; Shinzawa, K.; Takemori, S. *Biochem. Biophys. Res. Commun.* **1982**, *109*, 916.

(29) Shimizu, K. *Biochim. Biophys. Acta* **1979**, *575*, 37.

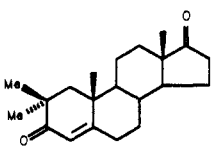
(30) Schwarzel, W. C.; Krugel, W. G.; Brodie, H. J. *Endocrinology* **1973**, *92*, 866.

(31) Furth, P. S., Rosenberger, J. G., Marcotte, P. A.; Robinson, C. H. *J. Enzyme Inhib.* 1990, in press.

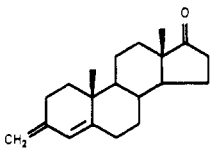
(32) Brueggemeier, R. W.; Li, P.-K.; Snider, C. E.; Darby, M. V.; Katlic, N. E. *Steroids* **1987**, *50*, 163.

(33) Miyairi, S.; Fishman, J. *J. Biol. Chem.* **1986**, *261*, 6772.

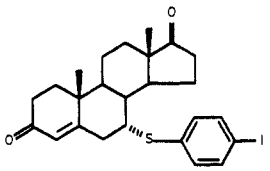
(34) Marcotte, P. A.; Robinson, C. H. *Steroids* **1982**, *39*, 325.



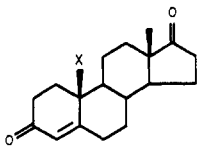
11
Ki=2.3 nM



13
Ki=4.7 nM



12
Ki=12 nM



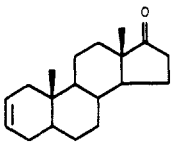
14 X = vinyl
Ki=3 nM (from IC_{50})

15 X = ethyl
Ki=9 nM (from IC_{50})

16 X = CH_3CHOH

17 X = propyl
Ki=3000 nM (from IC_{50})

18 X = $CH_2{=}CH_2CH_2$
Ki=56 nM (from IC_{50})



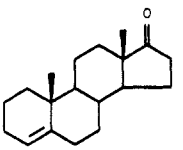
19

been achieved for aromatase inhibitors (K_m for androstenedione **1b** about 30 nM). Furthermore, it is interesting that the best inhibitors all have similar K_i values despite their different structures. It is possible that these values may be near the lower limit. There are few examples in studies of small molecule–large molecule interactions where dissociation constants below 0.1 nM are found. While transition-state analogues in some enzymes can have dissociation constants on the order of 10^5 times lower than the K_m, it is not clear that cytochrome P-450 reactions achieve accelerations by binding the transition state more tightly. Rate constants for P-450 type reactions catalyzed by porphyrin model compounds are not much lower than the fastest rates in the actual P-450 enzyme reactions. For example, an iron tetramesitylporphyrin analogue epoxidizes styrene with a turnover number of 8.6 s^{-1} at 20 °C.[35] Reconstituted camphor hydroxylase, one of the most catalytically active P-450 enzymes, has a turnover number of 17 s^{-1} at 25 °C.[4a] For comparison, reconstituted aromatase has a turnover number of about 5 min^{-1} at 37 °C.[36] Furthermore, the 19-hydroxy **2** and 19-oxo **3** compounds, which may be considered "high-energy intermediates" for aromatase, actually have higher K_m values than does the 10β-methyl starting material **1**.[36]

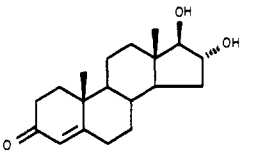
Another important point is that a K_i of 1 nM shown by a seemingly powerful aromatase inhibitor is only a factor of 30 (about 2 kcal/mol) lower than the Michaelis constant (K_m) for androstenedione (**1b**). Aromatase has evolved to have a rather sluggish k_{cat} (5 min^{-1}) but a very low K_m. Thus it would seem that mechanism-based inactivators (k_{cat} inhibitors) might be better candidates for in vivo inhibition (vide infra). Still, if adequate drug levels can be achieved in patients without producing toxic side effects, competitive inhibitors may still be a pharmacologic option.

Little metabolic work has been reported on the above compounds. The 10β-ethyl substituent of **15** was shown not to be converted to the corresponding 10β-hydroxyethyl **16** in placental microsomes.[15] The 3-*exo*-methylene compound **13** does not apparently get oxidized at C-19.[33] Thus the mode of binding of these competitive inhibitors may be slightly altered compared to normal substrates, and therefore the former are not processed rapidly.

The varied structures of the above inhibitors make it difficult to deduce key active site features. One clear trend appears to be that hydrophobic functionalities lead to increased affinities. The optimum placement of these functional groups is hard to predict, however. For example, simply increasing the 10β-ethyl chain length by a CH_2 group (as seen in **17**)[34] leads to an increase in the binding constant by a factor of 300 (3.4 kcal/mol). Inserting a CH_2 group into the C-10–C-19 bond of 10β-vinyl compound **14** (as in **18**)[34] increases the K_i by a factor of 20 (1.8 kcal/mol). Furthermore, A-ring conformation seems to play a critical role, as 5α-androst-2-en-17-one (**19**) is a mediocre binder,[30] whereas androst-4-en-17-one (**20**) is a potent inhibitor (K_i = 37 nM).[37]



20
Ki=37 nM



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Another important aspect of competitive inhibitor design that has received attention recently concerns the conformation of and substitution in the D ring. It is known that the three substrates androstenedione (**1b**), testosterone (**1a**), and 16α-hydroxytestosterone (**21**) have similar k_{cat} values for aromatase but widely different K_m values. According to Kellis and Vickery the K_m values for **1b**, 1a, and **21** are 60, 210, and 58000 nM, respectively, with purified aromatase.[36] This suggests that the best steroidal competitive inhibitors might be expected to be 17-ketones. As will be discussed in the time-dependent inactivator section, D-ring modifications have also been found to be important in inactivator binding and inactivation rate.

Bathochromic Shift (Type II) Competitive Inhibitors

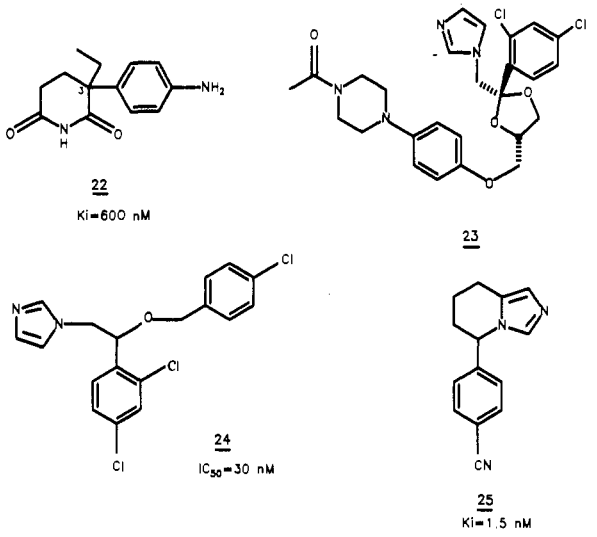
There are some organic compounds containing suitably positioned heteroatoms that are capable of binding to cytochrome P-450 enzymes such that their heteroatoms coordinate to the heme iron. This special type of binding is reflected in Soret band changes (usually bathochromic with respect to Type I binders). Furthermore, the precise Soret band displacement is often diagnostic of the heteroatom type (N, S, O, S^-). When these inhibitors coordinate to the heme iron via a nitrogen atom, they are often referred to as Type II inhibitors. For convenience, we will use the term Type II to refer to all the Fe-coordinating inhibitors, regardless of the nature of the heteroatom.

There are several examples of non-steroidal Type II binding aromatase inhibitors. One of the earliest nonsteroidal aromatase inhibitors is aminoglutethimide (**22**), which has been used clinically to treat breast cancer.[38] It

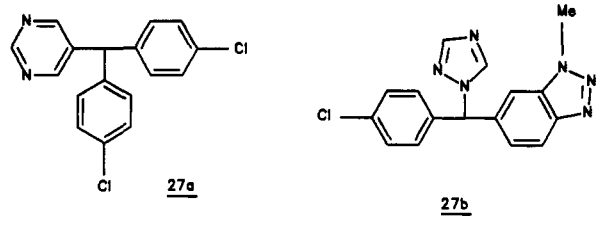
(35) Naruta, Y.; Maruyama, K. *Tetrahedron Lett.* **1987**, *28*, 4553.
(36) Kellis, J. T., Jr.; Vickery, L. E. *J. Biol. Chem.* **1987**, *262*, 4413.
(37) Numazawa, M.; Mutsumi, A.; Hoshi, K.; Koike, R. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 1009.
(38) (a) Santen, R. J.; Worgul, W. J.; Samojlik, E.; Interrante, A.; Boucher, A. E.; Lipton, A.; Harvey, H. A.; White, D. S.; Smart, E.; Cox, C.; Wells, S. *New Engl. J. Med.* **1981**, *305*, 545. (b) Shaw, M. A.; Nicholls, P. J.; Smith, H. J. *J. Steroid Biochem.* **1988**, *31*, 137.

is a moderately potent aromatase inhibitor (K_i = 600 nM) previously known for its role in adrenocortical steroidogenesis blockade and treatment of Cushing's syndrome. The aminophenyl nitrogen appears to be key for binding although it is not certain which atom is actually coordinated to the iron.[39] Compounds with increased size of the 3-alkyl side chain also bind more tightly.[40] Interestingly, the *d* enantiomer binds about 40 times as efficiently as the *l* enantiomer.[41]

Several of the imidazole antimycotics have been tested for aromatase inhibition. These compounds have been shown to block ergosterol biosynthesis in yeast by inhibiting 14α-methyl demethylase, a P-450 lyase mentioned above. Ketoconazole (**23**), for example, is a mildly effective aromatase inhibitor (IC_{50} = 65 μM, using 1.5 μM androstenedione substrate).[42] Another member of this class, econazole (**24**), has a very potent IC_{50} (0.03 μM, using 1.5 μM androstenedione substrate).[42]

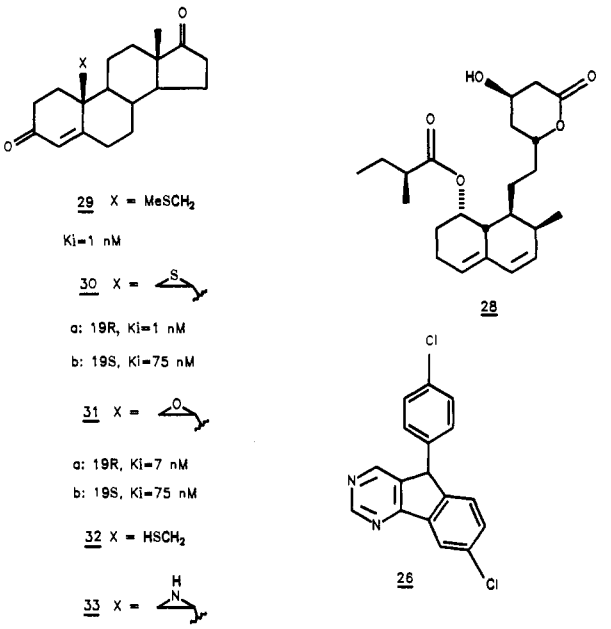


Other promising compounds include CGS 16949a (**25**),[43] LY 113174 (**26**) (IC_{50} = 24 nM, using 0.1 μM androstenedione substrate and rat ovarian microsomal aromatase),[44a] and the related pyrimidine LY56110 (**27a**) (IC_{50} = 29 nM, using 0.1 μM androstenedione substrate and rat ovarian microsomal aromatase).[44a] The recently reported triazole derivative **27b** also shows very potent aromatase inhibition



(IC_{50} = 3 nM, using 1 μM androstenedione substrate and a rat granulosa cell aromatase assay).[44b] The Ciba compound **25** has appeared very impressive in rat tumor model studies and phase I clinical trials.[45] CGS 16949a (**25**) appears to be 500 times more effective than aminoglutethimide on a per milligram basis at lowering estrogen levels in breast cancer patients.[45]

Good inhibitors appear to contain two domains: an Fe-coordinating domain and a hydrophobic domain. The structural requirements of the hydrophobic domain appear to be critical, but the chemical details of the specificity are unknown. This sort of dual interaction is comparable to the interaction with and inhibition of HMG CoA reductase by compactin (**28**). Abeles has proposed that the decalin moiety of compactin serves as a hydrophobic anchor and the lactonic portion as a mevalonate analogue.[46] The entropic gain for linking these two portions in compactin was estimated to correspond to about 50 000-fold decrease in K_d.

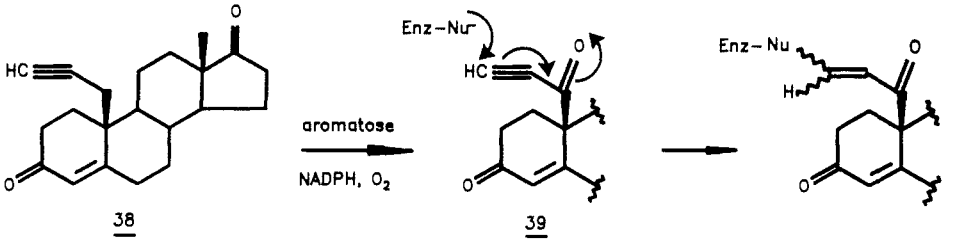


It is tempting to speculate that the hydrophobic domain of the non-steroidal Type II binders is binding in the pocket normally occupied by the steroid. This suggests another approach to aromatase inhibitor design. That is, steroids containing heteroatoms appropriately placed near the iron might be expected to behave as Type II competitive inhibitors. Such an approach has been successfully accomplished previously with inhibitors for the cholesterol side-chain cleavage cytochrome P-450 by Vickery and coworkers.[47] Recently, several tight-binding compounds of this type have also been reported for aromatase.

This class of compounds now includes the 10β-$MeSCH_2$ **29**,[48] 10β-thiirane **30**,[49] 10β-oxirane **31**,[49] and 10β-$HSCH_2$

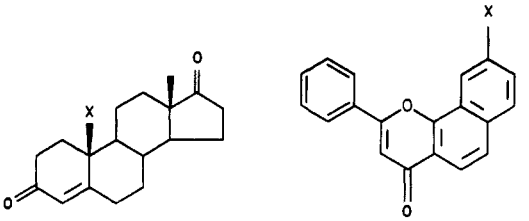
(39) Foster, A. B.; Jarman, M.; Leung, C. S.; Rowlands, M. G.; Taylor, G. N.; Plevey, R. G.; Sampson, P. *J. Med. Chem.* **1985**, *28*, 200.
(40) Hartmann, R. W.; Batzl, C. *J. Med. Chem.* **1986**, *29*, 1362.
(41) Graves, P. E.; Salhanick, H. A. *Endocrinology* **1979**, *105*, 52.
(42) Mason, J. I.; Carr, B. R.; Murry, B. A. *Steroids* **1987**, *50*, 179.
(43) Steele, R. E.; Mellor, L. B.; Sawyer, W. K.; Wasvary, J. M.; Browne, L. J. *Steroids* **1987**, *50*, 147.
(44) (a) Hirsch, K. S.; Jones, C. D.; Lindstrom, T. D.; Stamm, N. B.; Sutton, G. P.; Taylor, H. M.; Weaver, D. E. *Steroids* **1987**, *50*, 201. (b) Wouters, W.; De Coster, R.; Krekels, M.; van Dun, J.; Beerens, D.; Haelterman, C.; Raeymaekers, A.; Freyne, E.; Van Gelder, J.; Venet, M.; Janssen, P. A. J. *J. Steroid Biochem.* **1989**, *32*, 781.
(45) (a) Lipton, A.; Harvey, H. A.; Demers, L. M.; Hanagan, J.; Mulagha, M.; Sanders, S.; Fitzsimmons, S.; Santen, R. J. 13th Congress of the European Society for Medical Oncology, Lugano, Switzerland, October 30, 1988. (b) Lipton, A.; Harvey, H. A.; Demers, L. M.; Hanagan, J. R.; Mulagha, M. T.; Kochak, G. M.; Fitzsimmons, S.; Sanders, S. I.; Santen, R. J. *Cancer* **1990**, *65*, 1279. (c) Stein, R. C.; Dowsett, M.; Davenport, J.; Hedley, A.; Ford, H. T.; Gazet, J. C.; Coombes, R. C. *Cancer Res.* **1990**, *50*, 1381.
(46) Abeles, R. H. *Drug Dev. Res.* **1987**, *10*, 221.
(47) (a) Sheets, J. J.; Vickery, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5773. (b) Sheets, J. J.; Vickery, L. E. *J. Biol. Chem.* **1983**, *258*, 1720. (c) Sheets, J. J.; Vickery, L. E. *J. Biol. Chem.* **1983**, *258*, 11446.

Scheme III



32[50] derivatives of estr-4-ene-3,17-dione. Most recently, the 10β-aziridine diastereomers 33[53] have also been synthesized and preliminarily evaluated. All of these compounds are potent competitive inhibitors of aromatase and show UV-absorption spectra that suggest Fe coordination. Furthermore, the bathochromic Soret band shifts observed are consistent with the type of heteroatom involved. Binding of the thioether compounds **29**, **30** to aromatase caused the Soret maxima to shift to 425 nm in each case, the epoxide **31** to 411 nm, and the thiol **32** to 474 nm (indicative of a thiolate anion). The placement of the heteroatoms on the steroids suggests that the steroid is binding in a manner similar to the natural androgen substrate and that the heme iron is located near the androgen 19-carbon as expected for a direct attack of the iron-oxo species on the methyl. Incidentally it is interesting to note that neither the 19-OH **2b** nor the 19-OMe **34** compounds coordinate to heme iron, as evidenced by the Type I spectrum observed when added to substrate-free enzyme.[51] Furthermore, the 19(*R*)-epoxide **31a** and 19(*R*)-thiirane **30a** coordinate to iron and are better inhibitors than the corresponding 19(*S*)-epoxide **31b** and 19(*S*)-thiirane **30b**, which coordinate marginally under the experimental conditions.[49c] Thus there are still subtleties about the nature of the enzyme-active site-inhibitor interactions that may only be understood when larger quantities of pure enzyme are available for physical study. An interesting point is that the crystal structure of 19-hydroxyandrostenedione **2b** shows that the 19-oxygen atom lies directly over the A ring. In the 19(*R*)-epoxide **31a** the 19-oxygen is also positioned over the A ring whereas in the 19(*S*)-epoxide the 19-oxygen sits over the B ring.[49a] The 19(*R*)-epoxide **31a** and 19(*R*)-thiirane **30a** have been shown to be potent in vivo inhibitors in a nude mouse tumor model.[52]

An interesting prodrug **35**, which has the 19-thiol grouping derivatized as a disulfide, has entered Phase I clinical trials.[54] Presumably this derivatization allows for improved stability extracellularly, while intracellular reduction of the disulfide would produce the potent 19-thiol **32**.



34 X = $MeOCH_2$ **36** X = H

35 X = $EtSSCH_2$ **37** X = OH

Flavones, a class of plant natural products, and some of their in vivo metabolites have also been tested as aromatase inhibitors.[55] α-Naphthoflavone (**36**) (7,8-benzoflavone) is a potent competitive inhibitor of aromatase (K_i = 20 nM).[55a] Furthermore, a structure-activity study of flavone derivatives revealed that 9-hydroxy-7,8-benzoflavone (**37**) was an even more potent competitive inhibitor (K_i = 5 nM).[55b] These compounds also appeared to act as Type II binders, suggesting that a heteroatom coordinates to the heme iron as a sixth ligand. Whether this heteroatom is an oxygen from the inhibitor molecule or comes from water or an amino acid residue is still unknown. This question may not be answerable until further structural elucidation of the aromatase active site is achieved.

Suicide Inactivators

Because aromatase has a relatively low K_m, high dosage levels may be needed even for competitive inhibitors with apparently low K_i. In the search for increased efficacy, as well as specificity, a number of groups have developed suicide or mechanism-based inhibitors. Such compounds that contain a latent electrophilic group, intended to be activated by the target enzyme, can provide high specificity. The irreversible nature of the enzyme-inactivation process due to covalent modification at the active site provides several advantages. One lies in the expectation that lower amounts of inhibitor will be needed. Furthermore, the destruction of an enzyme that has a low turnover rate means that the inhibitor's effect can persist after its clearance from the system. Finally, such compounds can serve as valuable mechanistic probes and as active site mapping agents. Unfortunately, there have been no reports as yet on the successful use of such inhibitors to label purified aromatase. In the absence of such labeling, the details of their mechanism cannot be established.

(48) (a) Wright, J. N.; van Leersum, P. T.; Chamberlin, S. G.; Akhtar, M. *J. Chem. Soc., Perkin Trans. 1* **1989**, 1647. (b) Wright, J. N.; Calder, M. R.; Akhtar, M. *J. Chem. Soc., Chem. Commun.* **1985**, 1733.

(49) (a) Shih, M.-J.; Carrell, M. H.; Carrell, H. L.; Wright, C. L.; Johnston, J. O.; Robinson, C. H. *J. Chem. Soc., Chem. Commun.* **1987**, 213. (b) Childers, W. E.; Robinson, C. H. *J. Chem. Soc., Chem. Commun.* **1987**, 320. (c) Kellis, J. T.; Childers, W. E.; Robinson, C. H.; Vickery, L. E. *J. Biol. Chem.* **1987**, *262*, 4421. (d) Childers, W. E.; Shih, M.-J.; Furth, P. S.; Robinson, C. H. *Steroids* **1987**, *50*, 121.

(50) Bednarski, P. J.; Nelson, S. D. *J. Med. Chem.* **1989**, *32*, 203.

(51) Personal communication from L. E. Vickery, University of California, Irvine.

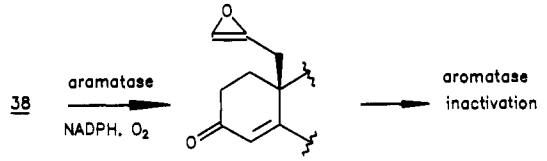
(52) Unpublished work in the laboratory of J. O. Johnston, Merrell Dow.

(53) Njar, V.; Safi, E.; Robinson, C. H. *Abstracts of Papers*, 199th National Meeting of the American Chemical Society, Division of Organic Chemistry, Boston, MA, Spring 1990; American Chemical Society: Washington, DC, 1990; Abstract 44.

(54) Organon company communication, March 1988.

(55) (a) Kellis, J. T., Jr.; Vickery, L. E. *Science* **1984**, *225*, 1032. (b) Kellis, J. T., Jr.; Vickery, L. E. *Biochem. Pharmacol.* **1986**, *35*, 2887.

Scheme IV



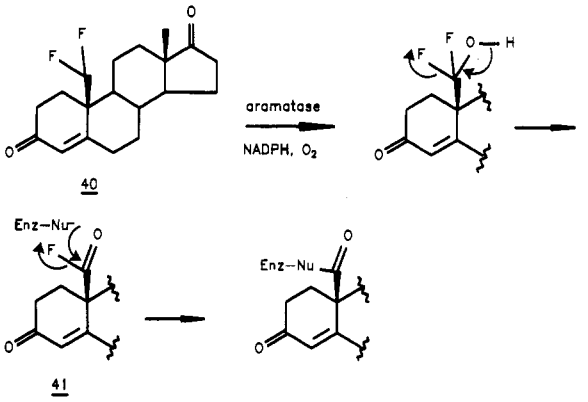
The 10β-propargyl compound **38**, synthesized independently in three laboratories, was shown to be a potent suicide inactivator.[56] Inactivation is dependent upon NADPH. The K_i of competitive inhibition (68 nM) was found to be in rough agreement with the K_i extrapolated from a Kitz-Wilson analysis (23 nM) suggesting that the inactivation process occurs at the active site.[56a] Two major classes of mechanisms have been postulated to account for the inactivation. The first mechanistic proposal (Scheme III) postulates that the 19-methylene group is oxidized to a 19-carbonyl, and the resulting α,β-acetylenic ketone can then react as an electrophilic 1,4 acceptor of enzyme active site nucleophiles.[56a] While the acetylenic ketone **39** actually proved to be a time-dependent inactivator of aromatase, its rate of inactivation (k_{inact} = 5.35 × 10^{-4} s^{-1}) was somewhat smaller than that shown by the parent propargyl compound (k_{inact} = 1.11 × 10^{-3} s^{-1}).[56a] Furthermore, Covey has shown that 10β-ethyl compound **15** is not hydroxylated at C-19 by aromatase.[15] This casts doubt on the likelihood of aromatase-catalyzed hydroxylation of the propargyl methylene group. Johnston and co-workers have found that there is no detectable isotope effect associated with the inactivation process for 19-deuterium-labeled propargyl compound.[56b] These results taken together argue against the acetylenic ketone mechanism.

The second mechanistic proposal of inactivation involves iron-oxo attack on the acetylenic grouping to afford an oxirene species (Scheme IV).[56b] The unstable oxirene could then directly, or after rearrangement, react with an active site grouping. Such a mechanism is analogous to those proposed by Ortiz de Montellano for inactivation of liver microsomal cytochrome P-450 enzymes by terminal acetylenic compounds.[58] While the oxirene mechanism is interesting, more supporting evidence is needed to assess its validity, in the case of aromatase.

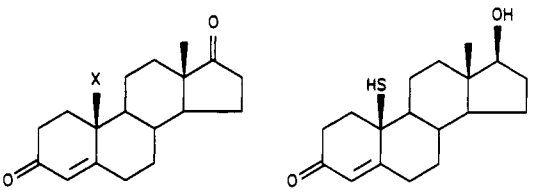
The 10β-propargyl compound has shown excellent success in many in vivo settings including oral activity in a nude mice study in which the mice harbored aromatase-producing human choriocarcinoma. Compound **38** showed IC_{50} = 3 mg/kg for the inhibition of aromatase activity, 6 h after oral administration of drug.[57] This should be compared to the clinically used aromatase inhibitor aminoglutethimide (**22**), which has IC_{50} = 250 mg/kg under the same conditions.[57] The 10β-propargyl compound **38** is presently undergoing Phase I clinical trials in the U.S.

Another, and even closer structural analogue of androstenedione (**1b**) is 19,19-difluoroandrost-4-ene-3,17-dione (**40**), which was designed as a mechanism-based inactivator of aromatase.[34] Enzymatic hydroxylation at the 19-carbon would give a geminal fluorohydrin, which should

Scheme V



spontaneously lose HF, yielding acyl fluoride **41** (Scheme V). The latter might react covalently with an active site nucleophile and thus inactivate the enzyme.



40 X = CHF_2

41 X = FC=O

42 X = CH_2F

44

In fact, the difluoro compound **40** was found to be a competitive inhibitor of aromatase.[34] It also caused time-dependent irreversible inactivation of human placental aromatase. (K_i = 1.0 μM; k_{inact} = 0.023 min^{-1}).[59] The inactivation process was NADPH-dependent. Although the postulated acyl fluoride intermediate **41** was synthesized and was shown to cause inactivation of the enzyme, the instability of the compound under the experimental conditions did not provide conclusive evidence about its involvement.[59] Addition of scavenging nucleophiles such as dithiothreitol or ethanolamine to incubations of the difluoro compound with aromatase had no effect on the enzyme inactivation rates. This excludes the possibility that an electrophilic intermediate is released into solution, followed by rebinding to the enzyme and inactivation.[59]

Indirect evidence for enzymatic attack on C-19 came from studies[59] with 19-fluoroandrost-4-ene-3,17-dione (**42**). This *mono*fluoro analogue proved to be an alternate substrate for aromatase, and was converted by human placental microsomes in high yield (52%) to estrone, presumably via the aldehyde intermediate **3b**. Compound **42** was found not to inactivate aromatase. Presumably the rate of production of 19-aldehyde **3b** is more rapid than a second hydroxylation at C-19 which would lead to acyl fluoride **41**.

Direct evidence for attack by aromatase at the 19-carbon of 19,19-difluoroandrostenedione (**40**) came from studies[60] using tritium labeling at C-19. When [19-^{3}H]-19,19-difluoroandrostenedione was incubated with human placental aromatase, NADPH-dependent release of tritium into solution occurred. It was also observed that the deuteriated analogue, [19-^{2}H]-19,19-difluoro-

(56) (a) Covey, D. F.; Hood, W. F.; Parikh, V. D. *J. Biol. Chem.* **1981**, *256*, 1076. (b) Metcalf, B. W.; Wright, C. L.; Burkhart, J. P.; Johnston, J. O. *J. Am. Chem. Soc.* **1981**, *103*, 3221. (c) Marcotte, P. A.; Robinson, C. H. *Fed. Proc.* **1981**, *40*, 1867.

(57) Johnston, J. O. *Steroids* **1987**, *50*, 105.

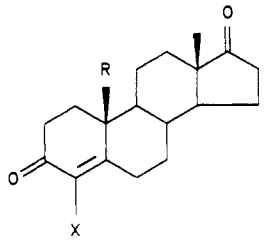
(58) (a) Ortiz de Montellano, P. R.; Kunze, K. L.; Beilan, H. S.; Wheeler, C. *Biochemistry* **1982**, *21*, 1331. (b) Kunze, K. L.; Mangold, B. L. K.; Wheeler, C.; Beilan, H. S.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1983**, *258*, 4202. (c) Ortiz de Montellano, P. R.; Komives, E. A. *J. Biol. Chem.* **1985**, *260*, 3330.

(59) Marcotte, P. A.; Robinson, C. H. *Biochemistry* **1982**, *21*, 2773.

(60) Furth, P. S.; Robinson, C. H. *Biochemistry* **1989**, *28*, 1254.

androstenedione, inactivated the enzyme at the same rate as the nondeuteriated parent.[60] Therefore cleavage of the C19–H bond is not the rate-limiting step in the inactivation process caused by the 19,19-difluoro compound.

The 4-hydroxy analogue **43** has been synthesized.[61] It is reported to be an inhibitor of placental aromatase (IC_{50} = 3.3 μM) although no experimental details are provided.



43 X = OH, R = CHF_2
45 X = OH, R = CH_3
46 X = OH, R = H
47 X = OH, R = CHO
48 X = OH, R = CH_2OH
49 X = OAc, R = CH_3
50 X = OMe, R = CH_3
51 X = Cl, R = CH_3
52 X = SH, R = CH_3
53 X = NH_2, R = CH_3

Another class of time-dependent inactivators is the thio-substituted compounds represented by 10β-mercapto **44** (K_i = 106 nM, k_{inact} = 0.0032 s^{-1}) and 19-mercapto compounds **32** (K_i = 34 nM, k_{inact} = 0.0012 s^{-1}).[62] Bednarski and Nelson found that these compounds obeyed many of the classical criteria of suicide inactivators. It is also very interesting that the 19-thiol **32** is capable of coordinating to the heme iron via sulfur (vide supra). Such a complex would presumably be unrelated to the time-dependent inactivation, since its formation does not require NADPH.

The compound 4-hydroxyandrostenedione (**45**) (4-OHA) was originally described as a competitive inhibitor of human placental aromatase by the Brodies in 1977.[63] It was later shown that 4-OHA (**45**) causes time-dependent inactivation ($t_{1/2}$ = ca. 2 min, K_i = ca. 50 nM).[64] The time-dependent inactivation required NADPH. The inactivation was irreversible as demonstrated by examining activity after prolonged dialysis to remove the inhibitor. The inactivation rate was slowed when substrate (androstenedione) was included in the preincubation step, evidence for an active site process. Covey and Hood showed that 19-nor-4-hydroxyandrostenedione (**46**), while capable of binding to the enzyme, did not bring about aromatase inactivation.[64b] This finding led them to suggest that 19-oxygenated intermediates could be the inactivating species. On the other hand, 4-hydroxy-19-oxoandrostenedione (**47**) has been found to be a poor inactivator relative to 4-OHA (**45**), which argues against their idea.[65] No studies have been reported with the 19-hydroxy analogue **48**.

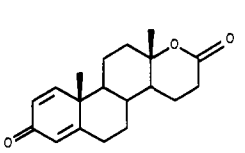
The 4-*O*-acetyl derivative **49** of 4-OHA (**45**) was also demonstrated to be a time-dependent inactivator of aromatase.[64] It should be emphasized that it is likely that this compound is hydrolyzed to 4-OHA (**45**) before inactivation occurs. It has been shown that human placental microsomes possess ample esterase activity that can convert 4-*O*-acetyl derivative **49** to the 4-OH compound **45**.[64b] Furthermore, the 4-OMe **50** and 4-Cl **51** compounds are not time-dependent inactivators, underlining the importance of the free O–H.[66] The 4-substituted thio compounds (e.g. **52**) also have been synthesized, but details of time-dependent inactivation experiments have not yet been reported.[67] Although 4-aminoandrostenedione (**53**) was shown to be a time-dependent inactivator ($t_{1/2}$ = 31 min, K_i = 37 nM),[68] its mechanism of inactivation and that of 4-OHA (**45**) have not been satisfactorily accounted for.

In clinical trials in Britain, 4-OHA (**45**) has shown promise in promoting tumor regression in patients with advanced breast cancer.[69] A clinical response was seen in 34% of patients receiving therapy. Side effects were less frequent than with aminoglutethimide. A problem that exists is rapid glucuronidation, which may need to be addressed for optimal dosing.

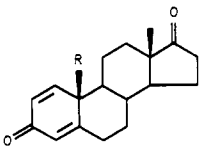
The class of inhibitors containing extra units of unsaturation in the A and B rings has expanded rapidly since a report on the aromatase inhibitory properties of testolactone (**54**) appeared in 1979.[70] It was subsequently shown that androsta-1,4-diene-3,17-dione (**55**) ($t_{1/2}$ = 12.7 min, K_i = 320 nM) as well as testolactone (**54**) ($t_{1/2}$ = 32 min, K_i = 35 μM) behave as time-dependent aromatase inactivators (inactivation dependent upon NADPH), due to the presence of the 1,2-double bond.[71] It is known that androsta-1,4-dien-3-one (**55**) is converted to estrone **4b** by placental microsomes, but this could presumably occur without enzymic assistance by a retro-aldol type reaction after formation of the 19-hydroxy dienone **56**.[72] A recently proposed mechanism of inactivation suggests that the 1,2-double bond is oxidized to a radical cation, which is then intercepted by an active site grouping.[73] There are little published data to support this theory, however. The more highly conjugated androsta-1,4,6-triene-3,17-dione (**57**) is also a time-dependent inactivator ($t_{1/2}$ = 10.5 min, K_i = ca. 180 nM)[74] whereas the androsta-4,6-diene-3,17-

(61) Mann, J.; Pietrzak, B. *J. Chem. Soc., Perkin Trans. 1* **1987**, 385.
(62) Bednarski, P. J.; Porubek, D. J.; Nelson, S. D. *J. Med. Chem.* **1985**, *28*, 775.
(63) Brodie, A. M. H.; Schwarzel, W. C.; Shaikh, A. A.; Brodie, H. J. *Endocrinology* **1977**, *100*, 1684.
(64) (a) Brodie, A. M. H.; Garrett, W. M.; Hendrickson, J. R.; Tsai-Morris, C.; Marcotte, P. A.; Robinson, C. H. *Steroids* **1981**, *38*, 693. (b) Covey, D. F.; Hood, W. F. *Mol. Pharmacol.* **1982**, *21*, 173.
(65) Newitt, J. A.; Robinson, C. H. *Fed. Proc.* **1986**, *45*, 1885 (Abstract 2362).
(66) Marsh, D. A.; Brodie, H. J.; Garrett, W.; Tsai-Morris, C.-H.; Brodie, A. M. H. *J. Med. Chem.* **1985**, *28*, 788.
(67) (a) Abul-Hajj, Y. J. *J. Med. Chem.* **1986**, *29*, 582. (b) Abul-Hajj, Y. *J. Steroid Biochem.* **1990**, *35*, 139.
(68) di Salle, E.; Giudici, D.; Ornati, G.; Briatico, G.; Lombardi, P. Conference on Aromatase: Future Perspectives, Miami, FL, March 4, 1987 (Abstract 18).
(69) (a) Coombes, R. C.; Goss, P. E.; Dowsett, M.; Hutchinson, G.; Cunningham, D.; Jarman, M.; Brodie, A. M. H. *Steroids* **1987**, *50*, 245. (b) Reed, M. J.; Lai, L. C.; Owen, A. M.; Singh, A.; Coldham, N. G.; Purohit, A.; Ghilchik, M. W.; Shaikh, N. A.; James, V. H. T. *Cancer Res.* **1990**, *50*, 193.
(70) Barone, R. M.; Shamonki, I. M.; Siiteri, P. K.; Judd, H. L. *J. Clin. Endocrinol. Metab.* **1979**, *49*, 672.
(71) Covey, D. F.; Hood, W. F. *Cancer Res. (Suppl.)* **1982**, *42*, 3327.
(72) (a) Ryan, K. J. *J. Biol. Chem.* **1959**, *221*, 268. (b) Gual, C.; Morato, T.; Hyano, M.; Gut, M.; Dorfman, R. I. *Endocrinology* **1962**, *71*, 920.
(73) Covey, D. F. In *Sterol Biosynthesis Inhibitors: Pharmaceutical and Agrochemical Aspects*; Berg, D., Plempel, M., Eds.; Ellis Horwood, Ltd.: Chichester, England, 1988; p 534.

dione (**58**) (K_i = 42 nM) is not.[71]

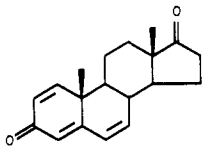


54



55 R = CH_3

56 R = CH_2OH



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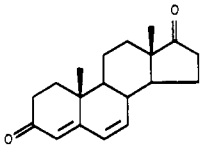

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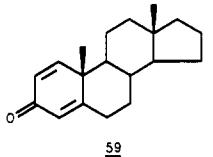

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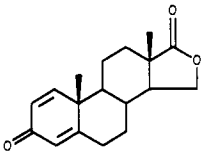


60

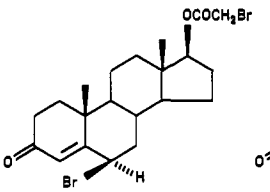
More highly substituted 1,4-dienone compounds have been synthesized and evaluated as aromatase inhibitors. Substitutions include 4-hydroxy,[66] 7α-[(*p*-aminophenyl]-thio],[75] 1-methyl,[76] 6-*exo*-methylene,[77] and 4-amino.[68] There have also have been similar types of substitutions attempted on the 1,4,6-triene systems. While some of these compounds display potent aromatase inhibition, their properties do not shed much light on the basic mechanism of inactivation.

Recently, Covey and co-workers have synthesized modified D-ring analogues of androsta-1,4-dienones.[78] They found that the reduced compound 17-methylene **59** (K_i = 1.1 μM, $t_{1/2}$ = 15.2 min) showed less than a 4-fold decrease in inactivation rate or binding affinity relative to **55**. Similarly, the corresponding D-ring butyrolactone **60** (K_i = 390 nM, $t_{1/2}$ = 6.2 min) was still a potent suicide inactivator. In contrast, analogous inhibitors with open D rings were poor inhibitors and in general were very poor inactivators. Covey inferred that the open D-ring chain may sterically interfere with binding of the inhibitor to the enzyme, but that the precise substitution of the intact D ring does not appear to be critical for binding or enzyme processing of these inhibitors.[78]

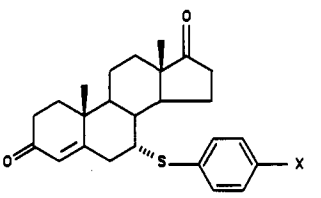
Affinity Labels

Such compounds are expected to play an important role in mapping out the active site of aromatase. Ideally these labels should show low K_i and high k_{inact} values (although a high k_{inact} is the more important) and require the use of purified enzyme. Denatured enzyme, even though pure, would clearly be unsuitable.

A number of affinity labels have been synthesized and evaluated as time-dependent inactivators of aromatase, but no reports of their use with purified enzyme have appeared, as yet. Osawa and co-workers have reported[79] that the 2α-, 6α-, and 19-bromoacetoxy derivatives of androstenedione induce time-dependent inactivation of aromatase at micromolar levels, but K_i values were not recorded. In addition, testosterone 17β-bromoacetate and its 6β-methoxy, 6β-bromo, and 6-keto derivatives were found[79] to cause time-dependent loss of activity. One of the best of the above derivatives was 6β-bromotestosterone 17β-bromoacetate (**61**), which had a poor K_i (25 μM) but a good k_{inact} (0.0274 min^{-1}). There are no reports of the use of these reagents for labeling of aromatase preparations.



61



62 X = $HNCOCH_2Br$

63 X = N_3

Finally, Snider and Brueggemeier have synthesized a bromoacetamido affinity label, **62**, which has a poor K_i (33 μM) but a good k_{inact} of 0.148 min^{-1}.[80] The ^{14}C-labeled compound was shown to bind irreversibly to microsomal placental aromatase, although specific binding was not obtained.

Photoaffinity Label

Brueggemeier and co-workers have described[81] a 7α-[(4′-azidophenyl)thio] derivative, **63**, of androstenedione, and have shown that, in the dark, the compound is a powerful competitive inhibitor of aromatase (K_i = 1.3 nM). When **63** is incubated with placental microsomes under UV irradiation, time-dependent inactivation of aromatase is observed (K_i = 0.89 μM; k_{inact} = 0.0993 min^{-1}). This inactivation process, presumably involving a nitrene intermediate, is slowed in the presence of androstenedione. The use of this label with purified aromatase has yet to be described.

Future Directions

The rational design of a new generation of highly specific mechanism-based inhibitors will require detailed understanding of the mechanism of action both of aromatase and of the present group of inhibitors.

In more general terms, given the relatively large number of available inhibitors with K_i values in the nanomolar range, potency is not a major issue. Instead, major questions are oral effectiveness and enzyme and tissue specificity in vivo, as well as stability toward metabolic inactivation. Potential problems with steroidal inhibitors of aromatase are illustrated by the possibility of interaction with androgen-transforming enzymes (e.g. 5α-reductase) and with androgen receptors, or on the other hand with cytochrome P-450 enzymes of the adrenal.

In addition, steroidal aromatase inhibitors with 17-keto groups may also prove to be substrates for 17β-hydroxysteroid dehydrogenases. The resulting 17β-ols would be expected to be less effective inhibitors of aromatase than

(74) Covey, D. F.; Hood, W. F. *Endocrinology* **1981**, *108*, 1597.
(75) Snider, C. E.; Brueggemeier, R. W. *J. Biol. Chem.* **1987**, *262*, 8685.
(76) Henderson, D.; Norbisrath, G.; Kerb, U. *J. Steroid Biochem.* **1986**, *24*, 303.
(77) Giudici, D.; Ornati, G.; Briatico, G.; Buzzetti, F.; Lombardi, P.; di Salle, E. 8th International Symposium of the Journal of Steroid Biochemistry, Paris, France, 1987 (Abstract 53P).
(78) Sherwin, P. F.; McMullan, P. C.; Covey, D. F. *J. Med. Chem.* **1989**, *32*, 651.
(79) Numazawa, M.; Tsuji, M.; Osawa, Y. *Steroids* **1986**, *48*, 347.
(80) Snider, C. E.; Brueggemeier, R. W. *J. Steroid Biochem.* **1985**, *22*, 325.
(81) Brueggemeier, R. W.; Snider, C. E.; Kimball, J. G. *Steroids* **1982**, *40*, 679.

the parent 17-ketones, and they also could be conjugated and excreted as water-soluble 17-conjugates. Covey et al.[78] have already drawn attention to possible ways of circumventing this problem (cf. the use of *D*-secosteroids).

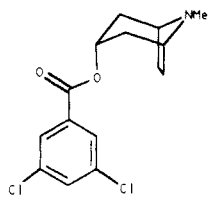
Acknowledgment. We thank the National Institute of Child Health and Human Development (Grant HD-11840 to C.H.R.) and the Medical Scientist Training Program of the National Institute of General Medical Sciences (Grant GM-07309 for P.A.C.) for financial support.

Registry No. Cytochrome P450, 9035-51-2; aromatase, 9039-48-9.

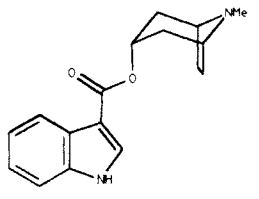
Communications to the Editor

Benzotriazinones as "Virtual Ring" Mimics of *o*-Methoxybenzamides: Novel and Potent 5-HT_3 Receptor Antagonists

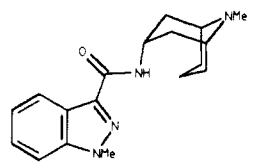
The majority of 5-HT_3 receptor antagonists can be regarded as falling into three structural classes. In the first are the benzoate esters in which the carbonyl is directly attached to the 6-membered aromatic ring and is typified by MDL 72222 (**1**).[1] In the second are the 6,5-heterobicyclic esters and amides in which the carbonyl is connected to the 6-membered aromatic ring via an sp^2 hybridized N or C atom, for example, ICS 205-930 (**2**),[2] granisetron (**3**),[3] and indoline 4.[4] In the third class are the carbazoles such as ondansetron (**5**) in which the basic side chain nitrogen is provided by an aromatic imidazole.[5] Included in the first group are the *o*-methoxybenzamides such as zacopride (**6**)[6] and BRL 24682 (**7**).[3] A particular characteristic of these latter benzamides is the possible intramolecular hydrogen bond between the amide and the methoxy group which holds the carbonyl group both in plane, forming a "virtual ring", and in a particular orientation with respect to the other substituents on the benzene ring. Despite the proposal that this "virtual ring" is important for the activity of certain of these compounds as either gastric motility stimulants[7] or dopamine receptor antagonists,[8] little convincing evidence has been published demonstrating that compounds in which the "virtual ring" has been replaced by an actual ring retain either of these activities. We have recently suggested that it is this H bonding which is, in part, responsible for the exceptional potency of certain o-methoxybenzamides as 5-HT_3 receptor antagonists.[9] In this communication we show that the H-bonded system can be replaced by the cyclic aromatic system benzotriazinone with retention of 5-HT_3 receptor antagonist activity. A similar structure-activity relationship is also observed with respect to aromatic substitution between the two series.



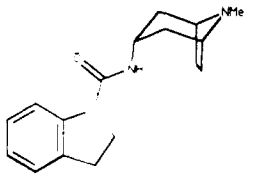
(1) MDL 72222



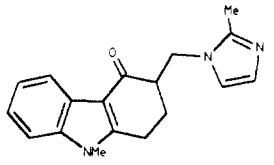
(2) ICS 205-930



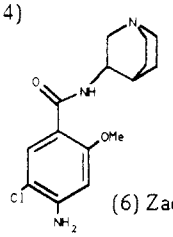
(3) Granisetron



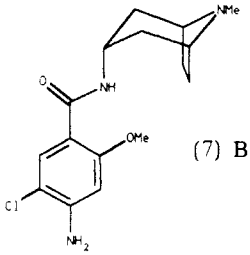
(4)



(5) Ondansetron

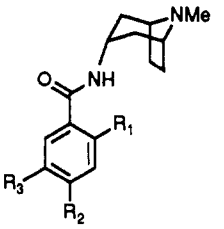


(6) Zacopride



(7) BRL 24682

Table I. Structure and Activity of Benzamides **8a-c**



no.	R_1	R_2	R_3	antagonism of B-J reflex: ID_{50}, μg/kg iv	antagonism of B-J reflex: no. of rats
1		MDL 72222		35.0 ± 0.1	4
2		ICS 205-930		1.4 ± 0.4	5
7	OMe	NH_2	Cl	0.8 ± 0.2	5
8a	H	NH_2	Cl	36.0 ± 5.0	3
8b	OMe	NH_2	H	16.0 ± 3.0	3
8c	OMe	H	Cl	15.0 ± 3.0	3

(1) Fozard, J. R. *Arch. Pharmacol.* **1984**, *326*, 36.
(2) Richardson, B. P.; Engel, G.; Donatsch, P.; Stadler, P. A. *Nature* **1985**, *316*, 126.
(3) Fake, C. S.; King, F. D.; Sanger, G. J. *Br. J. Pharmacol.* **1987**, *91*, 335P.
(4) Bermudez, J.; Joiner, K. A.; King, F. D.; Sanger, G. J. *J. Med. Chem.* **1990**, *33*, 1929.
(5) Butler, A.; Hill, J. M.; Ireland, S. J.; Jordon, C. C.; Tyers, M. B. *Br. J. Pharmacol.* **1988**, *94*, 397.
(6) Smith, W. W.; Sancilio, L. F.; Owera-Atepo, J. B.; Naylor, R. J.; Lambert, L. J. *J. Pharm. Pharmacol.* **1988**, *40*, 301.
(7) Sanger, G. J.; King, F. D. *Drug Des. Del.* **1988**, *3*, 273.
(8) Van der Waterbeemd, H.; Testa, B. *J. Med. Chem.* **1983**, *26*, 203.
(9) Bermudez, J.; Fake, C. S.; Joiner, G. F.; Joiner, K. A.; King, F. D.; Miner, W. D.; Sanger, G. J. *J. Med. Chem.* **1990**, *33*, 1924.

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