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Synthesis and Biochemical Evaluation of Novel Inhibitors of Aromatase (AR) Using an Enhanced Representation of the Active Site of AR Derived from the Consideration of the Reaction Mechanism

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A novel molecular modeling study, involving inhibitors bound to the iron of cytochrome P450 heme, is described for nonsteroidal inhibitors of aromatase (AR). Study of compounds such as aminoglutethimide (AG) suggests that it utilizes hydrogen bonding group(s) at the active site which would usually H-bond to the steroid C(17) carbonyl group. Interaction between AG's carbonyl groups and the area of the active site corresponding to the substrate C(3)═O group is not possible due to steric interaction. Possible reasons for the difference in activity of enantiomers of alternative inhibitors is also suggested, as well as the mode of action of the new AR inhibitor, Arimidex—whose inhibitory activity previously has not been rationalized. The present study proposes that it is able to use hydrogen bonding groups at the active site corresponding to the steroid C(17)═O *and* C(3)═O area, contradicting a previous study where it is postulated that azole-type compounds only use polar groups at the active site corresponding to the steroid D ring. Using the hypotheses of the modeling study, we designed and synthesized a number of novel (enantiomerically pure) inhibitors, which upon biochemical evaluation were found to be good inhibitors; the *N*-nonyl derivative of the *S*-enantiomer was found to possess 39% inhibition at 100 μM inhibitor concentration (using androstenedione as the substrate), under similar conditions, and AG possessed 20% inhibition.

In the absence of the crystal structure of the cytochrome P450 enzyme Aromatase (AR) [the enzyme responsible for the conversion of C_{19} androgens to the C_{18} estrogens (Fig. 1)], several workers have previously described models for the active site of AR using differing approaches. The first to be produced was that by Banting *et al.* (1)—involving the superimpositioning of several nonsteroidal inhibitors onto the AR substrates androstene-3,17-dione (AD) and testosterone. Although useful at the time, this model soon became redundant as it could not explain, or predict, some potent inhibitory activity obtained by some compounds, such as those studied by Ahmed (2). An alternative group attempted to model the mode of binding of azole-type inhibitors, such as CGS16949A (3) (Fig. 2), with respect to the reversible steroidal inhibitor (19*R*)-10-thiiranylestr-4-ene-3,17-dione. In their study, the authors used previously reported iron-ligand crystallographic data to superimpose the minimized structures of the inhibitors. However, both of these two approaches could not rationalize the inhibitory activity observed for the *R* and the *S* enantiomers of inhibitors such as AG. Recently, homology modeling has also been used to determine the active site, however, this approach is cumbersome and complex.

In an effort to obtain a more realistic model, in particular one which was able to rationalize the enantiomeric inhibitory activity, we argued that the determination of the approximate position of the Fe with respect to the natural substrate was a crucial step. We therefore initiated a line of research which involved the determination of the highest concentration of *inhibiting moieties* of both reversible and irreversible steroidal inhibitors, through the superimpositioning of the inhibitors (and their low energy conformers) onto the substrate [since all these compounds bind to the active site in a similar manner to AD involving hydrogen bond formation with the active site via the steroidal C(3) and C(17) carbonyl groups].

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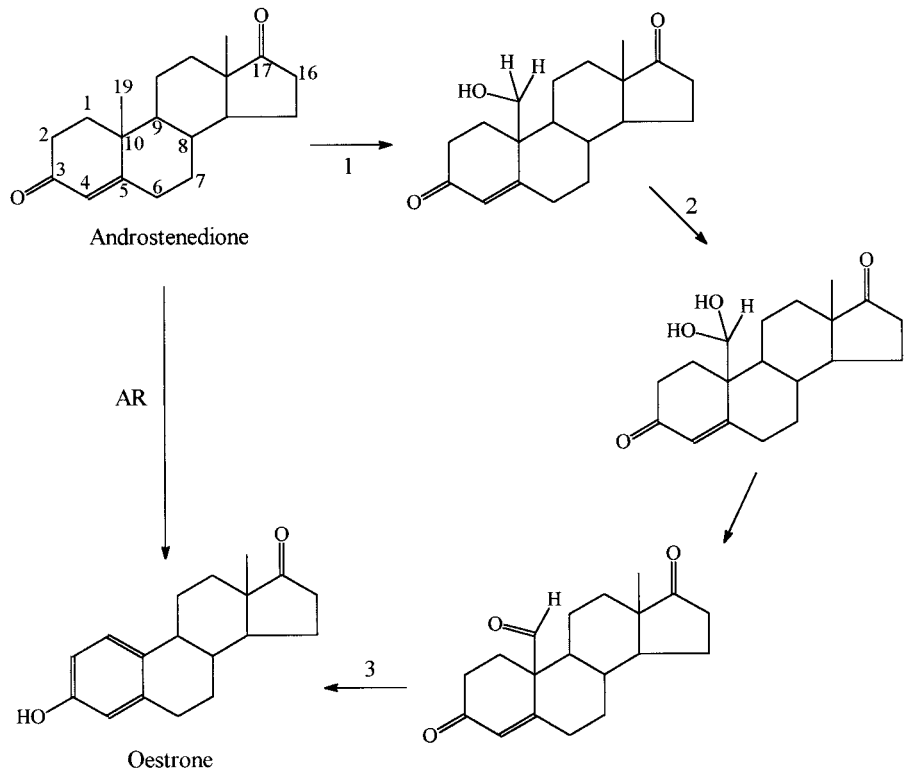


FIG. 1. Aromatization of androgens.

MATERIALS AND METHODS

Using the molecular modeling packages Alchemy III (4), CACHE (5) and the conformational analysis program Powersearch (4), we constructed the structures of the substrate, the heme and the hydrogen bonding groups within CACHE. The potent steroidal and nonsteroidal inhibitors (for the binding studies) were produced within Alchemy [for example (19*R,S*)-10-thiiranylestr-4-ene-3,17-dione, 10β-propargylestr-4-ene-3,17-dione, 10β-vinylestr-4-ene-3,17-dione, (19*R,S*)-10-oxiranylestr-4-ene-3,17-dione and 10β-aziridinylestr-4-ene-3,17-dione, Fig. 2]. An initial conformational analysis was carried out on the flexible parts of the inhibitors. The low energy conformer(s) of the steroidal inhibitors were then fitted onto AD, and each other, by specification of four points, utilizing both the C(3) and C(17) C═O groups of AD. From these fittings, we approximated the position of the heme to be about the C(1) of the steroidal backbone. To determine the three dimensional orientation of the heme about the steroidal plane, we considered the present hypotheses on the mechanism of hydroxylation of the steroid C(19) methyl. On the basis that the mechanism of AR involves a ferroxy oxygen radical (Fe-O $\cdot$) (6), we hypothesized that this atom of the oxygen molecule must be positioned within approximate bonding distance [and angle] to the C(19). Furthermore, we argued that the modeling process should also consider the binding of the dioxygen molecule as this is known to bind *after* the substrate. As such, we attached dioxygen to the heme (peroxy, Fe-O═O) and positioned the substrate (using weak bonds) such that the 1β-H atom could be removed by the ferroxy oxygen (as postulated in the mechanism by Akhtar *et al.*). It should be noted that hydrogen bond donor groups were added to the C(3) and C(17) positions so as to study interactions (if any) between inhibitors and the H-bonding groups. The final complex was then subjected to an initial minimization within CACHE (the structure of the chemical sample was refined by performing an optimize geometry calculation in Mechanics using Augmented MM2 parameters—cycles of 300 iterations were attempted until the gradient dropped below 10^{-3}). The resulting complex was then subjected to a further minimization within ZINDO (using INDO/1 parameters), resulting in the final complex (Fig. 3).

In the development of a novel approach to the modeling of nonsteroidal inhibitors, we reviewed the previous modeling studies and discovered that, although the studies involved modeling compounds onto the steroid backbone, there was an assumption that the initial step in the inhibiting process involved bond formation between the Fe of the heme and the hetero atom lone pair of electrons on the ligand (1). The basis of our study therefore involves the mimicking of this inhibition process by the hetero atom (for example N or S) containing nonsteroidal inhibitors. Also, we postulate that after this initial interaction, inhibitors, containing groups capable of mimicking the steroidal C(3) or C(17) C═O groups, then "search" [due to the rotation of the Fe-hetero atom bond] for the appropriate group(s) at the enzyme active site with which to interact. We therefore modeled this process by bonding the low energy conformers (of non-steroidal inhibitors) directly to the Fe of the heme—resulting in an inhibitor–heme active site complex, an approach which differs from that of Furet *et al.* who fitted inhibitors to a model representing an imidazole and a thiirane ring complexed to a porphyrin nucleus. The Fe-hetero atom bond was then "rotated" so as to study the orientation of the inhibitor within the active-site representation. However, in an effort not to prejudice the result, the inhibitor's orientation within our representation of the active site was obtained involving the attachment hydrogen bonding group (or polar group) of the inhibitor, to the representation of the active hydrogen bonding groups. The resulting inhibitor-heme complex was allowed to minimize—the overall energies of the complexes were then compared

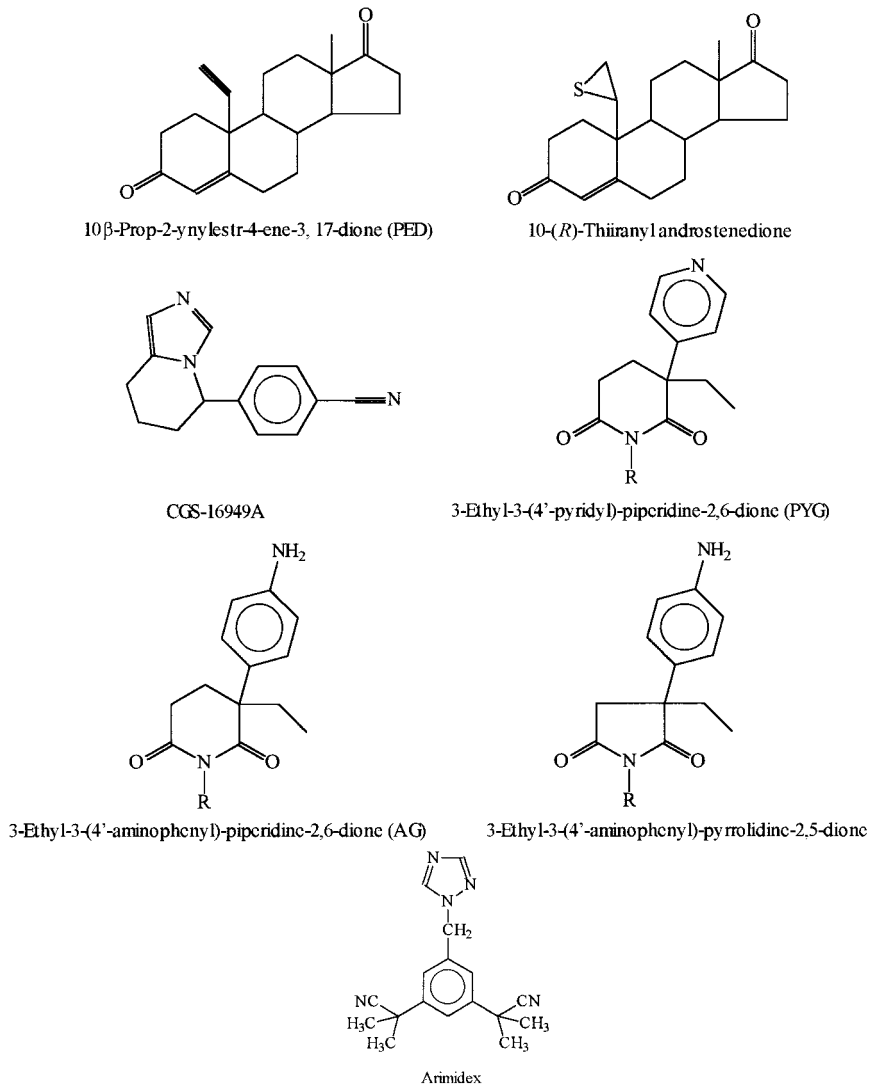


FIG. 2. Diagram to show some of the inhibitors of AR considered within the present study.

[two complexes per structure were obtained as each inhibitor was presumed to be able to undergo interaction, on separate occasions, with both groups corresponding to the steroid C(3) and C(17) carbonyl groups]. We therefore hypothesize that the complex with the lowest overall heat of formation would be the most favored. If, however, the ΔE between the two heats of formation is less than 5 kcal, we propose that both hydrogen bonding groups are favored, thereby enhancing the inhibitory activity.

Chemistry

The novel compounds were all synthesized using Scheme 1. The synthesis of 4(*S*)-4-(4′-aminobenzyl)-2-oxazolinone is given as an example.

*4(S)-(4′-Nitrobenzyl)-2-oxazolidinone (**1**).* (*S*)-4-Benzyl-2-oxazolidinone (1.0 g, 5.6 mmol) was dissolved in dry dichloromethane (DCM) (15 ml). Nitric acid (3.38 ml, 5 M) was added slowly and left to stir at room temperature. After two hours the reaction mixture was quenched in an excess of ice-cold aqueous sodium hydrogen carbonate. The organic layer was separated and the aqueous layer further extracted with DCM (2 × 20 ml). The combined organic layers were dried over magnesium sulfate. The crude product was purified by column chromatography (DCM 80: ethyl acetate 20) to give **1** as a yellow oil (yield, 65%, 0.82 g).

$\nu_{(max)}$ (Film)/cm^{-1}: 3279.6 (NH), 2918.1–2855.6 (CH-aliphatic), 1752 (C═O), 1519 (NO_2), 1347.4 (C═C aromatic), 1182.8–1163.2 (aromatic); δ_H (300 MHz, $CDCl_3$): 8.15–8.18 (2H, d, J = 8 Hz, CH-aromatic), 7.36–7.39 (2H, d, J = 8 Hz, CH-aromatic), 6.26 (NH), 4.44–4.54 (2H, m, O-CH_2), 3.4.11–4.20 (1H, dd, J = 6 Hz, 12 Hz, CH-benzyllic), 2.96–3.06 (1H, dd, J = 6 Hz, 12 Hz, CH-benzyllic); δ_C (75.5 MHz, $CDCl_3$): 41.19 ($\underline{C}H_2$), 53.25 ($\underline{C}H$), 69.34 ($\underline{C}H_2$), 124.18 (CH-aromatic), 130.06 (CH-aromatic), 143.47 (alternative C), 147.27 (alternative C), 159.41 (C═O); LRMS m/z (EI$^+$): 222 (M$^+$ + 1, 20%).

*4(S)-4′(4-Aminobenzyl)-2-oxazolinone (**2**).* (*S*)-4-Nitrobenzyl-2-oxazolidinone (0.2 g, 0.9 mmol) was dissolved in ethanol (7 ml). Saturated ammonium chloride solution (2.5 ml) and indium powder (0.5 g) was added. The reaction mixture was stirred under reflux for four hours. After cooling the reaction mixture was diluted with water (15 ml) and filtered through celite. The aqueous filtrate was adjusted to pH ~ 9 with 4 M sodium hydroxide (NaOH) (2 ml) and extracted

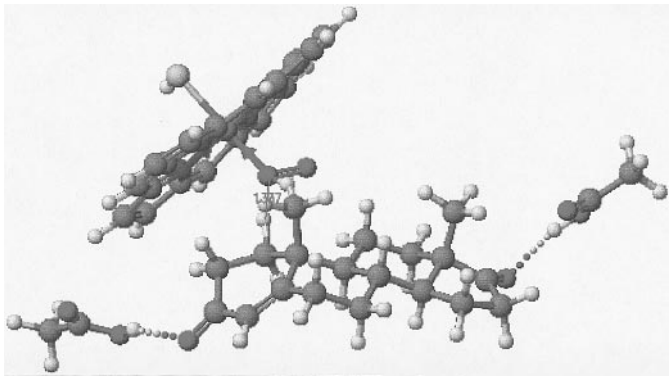


FIG. 3. The overall minimized complex showing the orientation of the substrate with respect to the dioxygen molecules and the P450 heme.

with DCM (3 × 10 ml). The combined organic layers were dried with $MgSO_4$. The crude product was purified by column chromatography (Dichloromethane 80: Ethyl acetate 20) to give **2** as a dark yellow oil (yield 47%, 0.082 g).

ν_{max}(Film)/cm^{-1}: 3348.9 (C—N), 2923.1–2852.6, (CH-aliphatic), 1745.5 (C═O), 1517.7 (C═C), 1408–1023.4 (aromatic); δ_H (300 MHz, $CDCl_3$): 6.86–6.89 (2H, d, J = 8 Hz, CH-aromatic), 6.55–6.58 (2H, d, J = 8 Hz, CH-aromatic), 5.61 (1H, m, NH), 4.32–4.37 (1H, t, J = 8 Hz, CH-benzyllic), 3.89–4.11 (2H, m, O-CH2), 3.63 (2H, b, NH_2), 2.66–2.68 (1H, d, J = 8 Hz, CH-benzyllic); δ_C (75.5 MHz, $CDCl_3$): 40.59 (CH_2), 54.12 (CH), 69.65 (CH_2), 115.55 (CH-aromatic), 125.59 (CH-aromatic), 129.89 (alternative C), 145.53 (alternative C), 159.45 (C═O); HRMS: M^+ = 192.0899 required $C_{10}H_{12}N_2O_2$ M^+ = 192.0899.

Biochemistry

The human placental aromatase enzyme was thawed under cold running water and allowed to warm to 37°C before use. Incubations, in triplicate, were carried out in phosphate buffer (50 mM, pH 7.4) containing NADPH generating system (25 μl), substrate (0.84 μM, 7 μl) (preincubated at room temperature prior to use in the assay) and inhibitor (10 μl, 100 μM final concentration), in a shaking water bath. The incubation mixtures were warmed to 37°C and the reaction started by the addition of placental microsomal protein (50 μl, 0.19 mg/ml). After incubation for 5 min, an aliquot (300 μl) from each assay tube was removed and added to tubes containing activated charcoal (1%, 900 μl) and mercuric chloride (1 mM, 300 μl) and thoroughly mixed. The tubes were allowed to stand on ice for 20 min and then centrifuged at 3600 rpm for 15 min. Aliquots of the supernatant (500 μl) were removed and dispersed in scintillation fluid (5 ml) and the mixture counted for 1 min.

FIG. 4. Stereo diagram to show CGS-20267 bound to enhanced substrate–heme complex.

FIG. 5. Stereo diagram to show the contact between AG and active site group.

RESULTS AND DISCUSSION

Consideration of the new enhanced substrate-heme complex (Fig. 3) shows that the substrate is orientated about the active site such that the C(2) moiety of the steroid backbone is positioned close to the heme. Further consideration of the final complex shows that as a result of the substrate's orientation, steric hindrance (with the heme structure) may be postulated with inhibitors possessing bulky groups—this has been previously observed in inhibition studies where groups larger than ethyl were found to be disfavored (7).

Binding inhibitors to the new complex, we observe that the potent inhibitors produce small steroid C(17) or C(3) carbonyl to inhibitor polar group distances, for example, with CGS-20267, a steroid C(17) to inhibitor C≡N group distance of 1.8 Å is observed (Fig. 4)—the potent inhibition can therefore be attributed to the strong binding ability of the nitrogen lone pair of electrons within the imidazole structure.

With compounds such as aminoglutethimide (AG), we observe that attempts to utilize the C(3) carbonyl results in direct contact between the hydrogen bonding group and the inhibitor (Fig. 5). Consideration of the hydrogen bonding group about the C(17)═O area of the steroid backbone, however, produces an inhibitor C═O to active-site hydrogen bonding group distance of 1.6 Å (Fig. 6)—in comparison to the imidazole based compounds, the amine nitrogen lone pair of electrons is not readily donated, as such, it is a poorer inhibitor than the imidazole based inhibitors although possessing a similar distance to the hydrogen bonding group at the active site.

Finally, the new inhibitor Arimidex has not previously been considered and its inhibitory activity there-

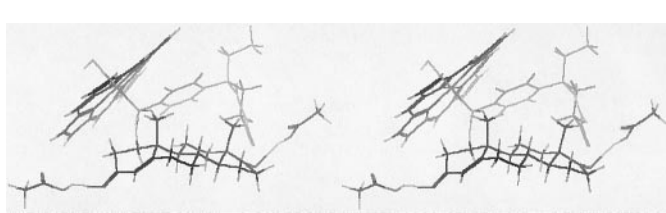


FIG. 6. Stereo diagram to show interaction between AG and the C(17) H bonding group at the active site.

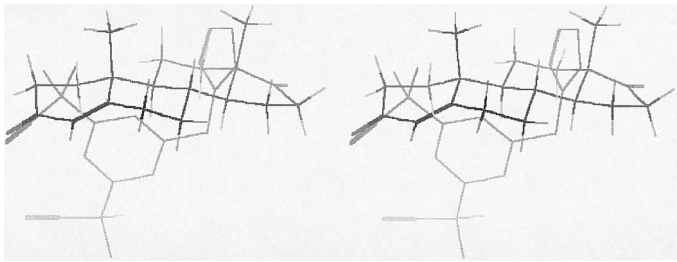


FIG. 7. Stereo diagram to show Arimidex superimposed onto the substrate using the C(3)═O group, as proposed by the model of Banting *et al.*

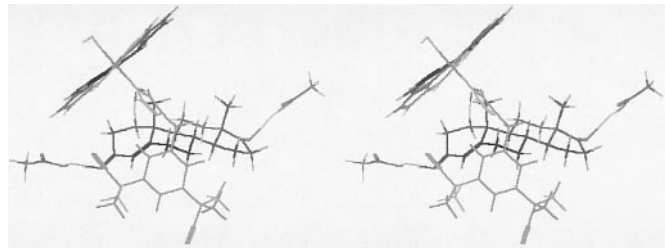


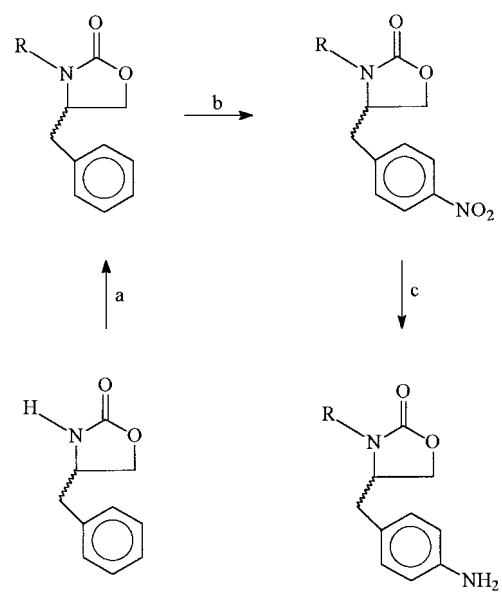
FIG. 9. Stereo diagram to show Arimidex bound to the substrate-heme complex and the interaction with the hydrogen bonding group at the C(3) position of the steroid backbone.

fore has not been rationalized. Attempts to superimpose this inhibitor onto the substrate using the approach of Banting *et al.*, produced a steroid C(19) to inhibitor hetero atom distance of 4.3 Å (Fig. 8). This is considered too large for interaction between the Fe of the heme and the nitrogen atom and suggests that this compound should have a similar inhibitory activity to AG [which has a steroid C(19) to inhibitor hetero atom distance of 4.8 Å]. Arimidex has in fact been shown to be 200 times more potent than AG. Using our inhibitor bound complex, we observe that it is able to undergo interaction with the hydrogen bonding group about the C(17) area of the active site, resulting in an inhibitor C≡N to active-site hydrogen bonding group distance of 1.6 Å (Fig. 7)—this distance therefore allows a strong interaction. Arimidex contains a triazole moiety whose lone pair donation is poorer than that of the imidazole ring; as a result, it is a weaker inhibitor than the imidazole based inhibitors *in vitro*, such as Fadrozole (which is some 180 times more potent than Arimidex). From the results of a previous study into the binding mode of azole based inhibitors and the results obtained above, it may be hypothesized that this new inhibitor utilizes the hydrogen bonding group at the active site which would normally bind the C(17)═O of the substrate. However, upon binding one of the low energy conformers of Arimidex, we find that the steroid C(3) hydrogen bonding group is *also* favored, resulting in an inhibitor C≡N to active-site hydrogen bonding group distance of 1.4 Å (Fig. 9). That is, we hypothesize that Arimidex is able to utilize *either* hydrogen bonding group at the AR active site, contradicting a previous study where it was suggested that the azole type compounds only utilize the steroid C(3) hydrogen bonding group.

In conclusion, we believe that the new enhanced inhibitor–heme complex allows a better understanding of the interactions taking place at the active site of AR, as a result we have been able to rationalize inhibitory activity of compounds not previously considered.

In an attempt to evaluate our hypotheses, we have designed enantiomerically pure inhibitors of AR based upon the "Evans chiral auxiliary" as shown in Scheme 1. The reactions proceeded well and in good yield (the synthesis of the nonsubstituted *S* enantiomer has been given as an example). Upon undertaking biochemical evaluation, the compounds were

FIG. 8. Stereo diagram to show Arimidex bound to the substrate–heme complex and the interaction with the hydrogen bonding group at the C(17) position of the steroid backbone.



SCHEME 1. Synthesis of novel AR inhibitors using the *R* and *S* enantiomers of the "Evans chiral auxiliary" (a, NaH/DMF/Δ; b, fuming HNO_3/0°C; c, NH_4Cl/In) (R = H, Me, Et, Pr to dodecyl).

TABLE 1

Initial Screening Inhibitory Activity for the Novel Compounds (both the *R* and *S* Enantiomers)

Compound (R═)	% Inhibitory activity[a]
H (*R* enantiomer)	14
H (*S* enantiomer)	16
Et (*R* enantiomer)	26
Et (*S* enantiomer)	23
Hex (*R* enantiomer)	31
Hex (*S* enantiomer)	38
Non (*R* enantiomer)	26
Non (*S* enantiomer)	39
Dec (*R* enantiomer)	38
Dec (*S* enantiomer)	28
AG (racemic)	20

[a] The initial screening was undertaken in triplicate ($n = 3$); the data reported are therefore means.

found to be good inhibitors, in particular, we were able to predict the differences in enantiomeric activity using the substrate-heme complex approach. As such, the inhibitory data support the model and the resulting hypotheses (Table 1 shows a table with inhibitory data for some of the synthesized compounds compared to AG).

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