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Synthesis and biochemical evaluation of a range of potent benzyl imidazole-based compounds as potential inhibitors of the enzyme complex 17α-hydroxylase/17,20-lyase ($P450_{17\alpha}$)

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Abstract—The cytochrome P-450 enzyme, 17α-hydroxylase/17,20-lyase ($P450_{17\alpha}$), is a potential target in hormone-dependent cancers. Here, we report the synthesis and biochemical evaluation of a range of benzyl imidazole-based compounds which have been targeted against the two components of this enzyme, that is, 17α-hydroxylase (17α-OHase) and 17,20-lyase (lyase). The results from the biochemical testing suggest that the compounds synthesised are good inhibitors, with *N*-4-iodobenzyl imidazole (**5**) (IC_{50} = 10.06 μM against 17α-OHase and IC_{50} = 1.58 μM against lyase) showing equipotent activity against lyase compared to the standard compound, ketoconazole (KTZ) (IC_{50} = 3.76 ± 0.01 μM against 17α-OHase and IC_{50} = 1.66 ± 0.15 μM against lyase). Furthermore, the compounds tested are less potent towards the 17α-OHase component, a desirable property in the development of novel inhibitors of $P450_{17\alpha}$.

The enzyme complex 17α-hydroxylase/17,20-lyase ($P450_{17\alpha}$) is a pivotal enzyme in the conversion of progestins and pregnanes to androgen precursors such as androstenedione and dehydroepiandrosterone.[1] It is a cytochrome P450 enzyme, requiring both NADPH and oxygen for the sequential oxidative steps,[2] which initially involves 17α-hydroxylation [via 17α-hydroxylase (17α-OHase)] followed by the subsequent cleavage of the C(17)–C(20) bond [via 17,20-lyase (lyase)] (Fig. 1). $P450_{17\alpha}$ is responsible for the second step in the steroidal cascade, leading to the biosynthesis of the sex hormones, glucocorticoids and mineralocorticoids, the latter two being produced directly from the 17α-hydroxy progestins.

$P450_{17\alpha}$ is a membrane-bound enzyme and therefore no crystal structure for it has been reported. Homology modelling studies have suggested that the enzyme possesses an active site consisting of a bilobed structure with two substrate binding sites, one associated with binding substrate for the hydroxylase reaction and the other with binding for the lyase reaction.[3,4] However, a more recent molecular modelling study suggests only a single substrate-binding pocket is present.[5]

This enzyme is of interest as a target in the treatment of androgen-dependent prostate cancer because of its role in the biosynthesis of androgen precursors. However, ideally, potential inhibitors of this enzyme should preferentially inhibit the lyase reaction and have minimal effect on the hydroxylase reaction and therefore corticosteroid biosynthesis.[6] Ketoconazole (KTZ), a known azole-based $P450_{17\alpha}$ inhibitor, has been used in the treatment of prostate cancer, however, was withdrawn for this use due to serious adverse effects. Here, we report: the synthesis of a range of imidazole-based compounds and their biochemical evaluation (in comparison to KTZ) against both components of this enzyme.

In the synthesis of the proposed inhibitors, the azole functionality was reacted with a phenyl alkyl halide in

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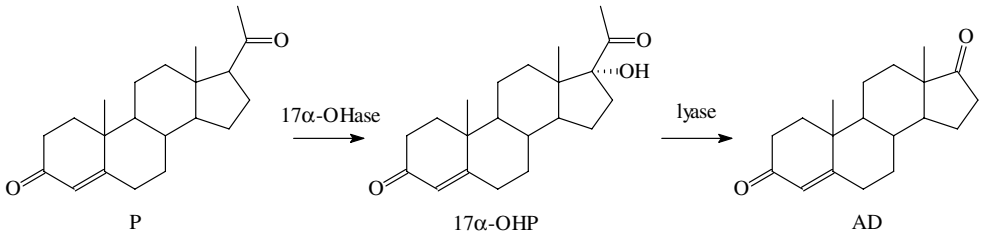


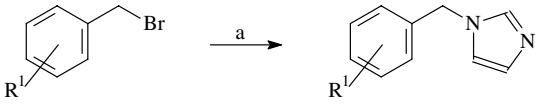
Figure 1. Reaction catalysed by $P450_{17\alpha}$ in the conversion of progesterone (P) to androstenedione (AD) via 17α-hydroxyprogesterone (17α-OHP).

the presence of a suitable base (Scheme 1)—the synthesis of *N*-1-benzyl-imidazole (**1**) is given as an example.[7] In general, the reactions proceeded in moderate to good yield (ranging from 25% to 70%) and without any major problems. However, in the synthesis of 4-hydroxybenzyl imidazole, the method of Machin et al.[8] was used due to the labile proton present within the starting material (which would be expected to neutralise any azolyl ion produced). This involved heating 4-hydroxybenzyl alcohol in the presence of excess imidazole (in the absence of solvent), and resulted in the 4-hydroxybenzyl imidazole in very good yield (80%).

The biochemical evaluation of the synthesised compounds against both 17α-OHase and lyase was undertaken using a modified literature method of Li et al.[9] involving the use of an alternative mobile phase in the separation and identification of the radiolabelled substrate from the incubation mixture.[10,11] Tables 1a and 1b show the IC_{50} values obtained for the compounds under consideration against both 17α-OHase and lyase.

Initial consideration of the inhibitory data for the compounds shows that all of the benzyl compounds tested were weaker inhibitors against 17α-OHase than against lyase, and, when compared to the standard compound, KTZ, they were also found to possess weaker inhibitory activity. The most potent compound was found to be 4-iodobenzyl imidazole (**5**), which had an IC_{50} = 10.06 ± 0.96 μM against 17α-OHase and an IC_{50} value of 1.58 ± 0.17 μM against lyase, compared to KTZ (IC_{50} = 3.76 ± 0.01 μM against 17α-OHase and IC_{50}=1.66 ± 0.15 μM against lyase).

Detailed consideration of the mono- and di-substituted compounds does not show a clear structure–activity relationship, other than that all the substituted inhibitors (except for **10**) show greater potency against both 17α-OHase and lyase in comparison to the non-substituted derivative. For example, the unsubstituted derivative, **1** (IC_{50} = 214.58 ± 19.67 μM against 17α-OHase and IC_{50} = 39.06 ± 1.22 μM against lyase), is found to be ~2.5 and ~2 times weaker, respectively, than the least active mono-substituted derivative, **2**



Scheme 1. Synthesis of azole-based derivatives (a) imidazole/K_2CO_3/THF/Δ; R^1 = various substituents).

(IC_{50} = 86.58 ± 5.21 μM against 17α-OHase and IC_{50} = 18.44 ± 0.17 μM against lyase). Considering the 4-substituted series of compounds alone, we do not see a clear structure–activity relationship.

Consideration of the di-substituted compounds shows that these inhibitors are, in general, more potent than the 4-substituted mono-derivatives against both components of $P450_{17\alpha}$. For example, the mono-fluoro derivative (**2**) is weaker than both disubstituted derivatives, **11** (IC_{50} = 83.10 ± 7.05 μM against 17α-OHase and IC_{50} = 11.80 ± 0.41 μM against lyase) and **12** (IC_{50} = 70.66 ± 6.72 μM against 17α-OHase and IC_{50} = 9.60 ± 0.14 μM against lyase), with greater potency being seen when the substitution is in the *meta*- and *para*-positions. This is also observed for the chloro derivatives, where the 3,4-dichloro derivative (**14**) is the most potent against both 17α-OHase and lyase. The modelling of the di-substituted compounds using the substrate–haem complex approach[12] shows that the di-substituted compounds are able to increase the interaction they undergo with the active site in comparison to the 4-substituted mono-derivatives, which are only able to undergo a single interaction with the active site. For example, when **14** is modelled within the combined substrate–haem complex as a representation of the overall $P450_{17\alpha}$ active site, it is found that this compound is able to undergo interaction with the hydrogen bonding groups within both components of the active site, whereas the mono-substituted compound is only able to undergo interaction with one of the components (Figs. 2a and b). That is, from the modelling study, we propose that the disubstituted (in particular, the 3,4-disubstituted) compounds are able to utilise two differently situated hydrogen bonding groups within the active site which would be utilised by the C(3) hydrogen bonding groups of the two natural substrates in the overall reaction (e.g., progesterone and 17α-hydroxyprogesterone) to bind to the active site. As such, this is the first study to suggest that inhibitors may be designed which are able to utilise both hydrogen bonding groups, and the results of the current study would appear to support the bilobed nature of the $P450_{17\alpha}$ active site.

In summary, whilst the compounds within the current study have been shown, in general, to possess weaker inhibitory activity against the lyase components of the overall enzyme complex of $P450_{17\alpha}$ in comparison to the standard compound KTZ, all are significantly weaker inhibitors against the 17α-OHase component and therefore would be expected to have less of an effect on corticosteroid biosynthesis. The greatest selectivity is seen

Table 1a. Showing the IC_{50} values ($n = 9$) of the mono-substituted derivatives of benzyl imidazole against both the 17α-OHase and lyase component of the overall P-$450_{17\alpha}$

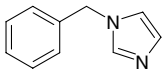
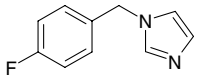
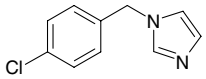
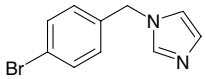
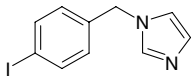
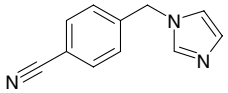
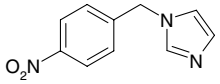
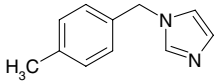
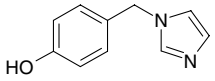
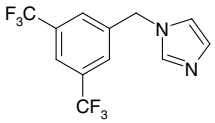
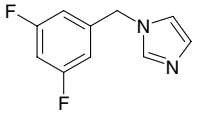
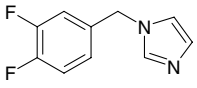
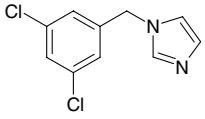
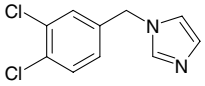
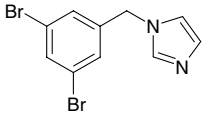
Compound	Structure	17α-OHase [IC_{50} values (μM)]	Lyase [IC_{50} values (μM)]
1		214.58 ± 19.67	39.06 ± 1.22
2		86.58 ± 5.21	18.44 ± 0.17
3		31.63 ± 3.86	2.81 ± 0.27
4		53.40 ± 4.40	6.76 ± 0.36
5		10.06 ± 0.96	1.58 ± 0.17
6		40.26 ± 3.49	7.67 ± 0.05
7		25.38 ± 1.65	7.17 ± 0.13
8		50.56 ± 8.01	8.81 ± 0.43
9		72.05 ± 3.95	8.22 ± 0.97

Table 1b. Showing the IC_{50} values ($n = 9$) of the di-substituted derivatives of benzyl imidazole against both the 17α-OHase and lyase component of the overall P-$450_{17\alpha}$

Compound	Structure	17α-OHase [IC_{50} values (μM)]	17,20-lyase [IC_{50} values (μM)]
10		244.85 ± 24.45	19.46 ± 0.75
11		83.10 ± 7.05	11.80 ± 0.41
12		70.66 ± 6.72	9.60 ± 0.14
13		22.56 ± 0.34	3.34 ± 0.11
14		12.22 ± 0.88	2.07 ± 0.07
15		25.95 ± 0.91	3.16 ± 0.11
	KTZ	3.76 ± 0.01	1.66 ± 0.15

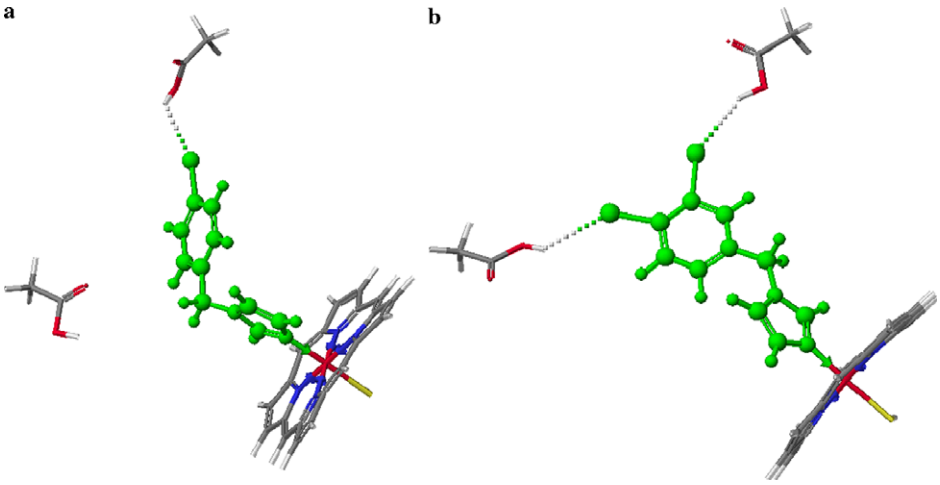


Figure 2. (a) Binding of **3** (in green) into the substrate–haem complex for the overall enzyme complex. (b) Binding of **14** (in green) into the substrate–haem complex for the overall enzyme complex.

for compounds **3** and **10** which show over 10-fold difference in activity between the two components. They are thus good lead compounds in the design and synthesis of more potent inhibitors of this biochemical target.

Acknowledgments

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7. *N*-1-Benzyl-imidazole (**1**): Imidazole (2 g, 29.4 mmol) was added to anhydrous potassium carbonate (K_2CO_3) (1.02 g, 7.34 mmol) and anhydrous tetrahydrofuran (THF) (50 mL). The mixture was stirred at room temperature for 10 min prior to the addition of benzyl bromide (2.51 g, 14.7 mmol). The mixture was then stirred under reflux for 24 h. After filtration, the THF was removed under vacuum to leave a yellow solid which was dissolved in dichloromethane (DCM) (40 mL) and washed with water (3× 50 mL). The organic layer was then extracted using hydrochloric acid (HCl) (2 M, 3× 30 mL) followed by water (2× 50 mL). The combined acid layer was neutralised with solid saturated sodium bicarbonate ($NaHCO_3$) and then extracted into DCM (2× 40 mL). The combined DCM layer was washed with water (3× 50 mL), dried over anhydrous magnesium sulfate ($MgSO_4$) and filtered. Removal of DCM under vacuum gave **1** as a yellow solid (0.93 g, yield 40%) (mp 71.5–72.5 °C). $\nu_{(max)}$ (film) cm $^{-1}$: 3387.3 (NCN imidazole), 2938.9 (CH aliphatic); δ_H ($CDCl_3$): 7.46 (1H, s, NC*H*N imidazole), 7.18 (5H, m, Ar*H*), 7.00 (1H, s, NC*H* imidazole), 6.81 (1H, s, NC*H* imidazole), 5.02 (2H, s, PhCH_2); δ_C ($CDCl_3$): 137.43 (N*C*N), 129.78, 128.97, 128.24, 127.26 (Ar*C*), 136.18, 119.30 (Im*C*), 50.76 (Ph*C*H_2); GCMS t_R 8.24 min. *m*/*z* 158 (M^+), 91 (base peak).
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10. 17α-OHase assay using rat microsomal enzyme: Rat testicular microsomal suspension was thawed under cold running water and vortexed. The final incubation assay mixture (1 mL) consisted of sodium phosphate buffer (50 mM, pH 7.4, 905 μL), radiolabelled progesterone as substrate (1.5 μM, 15 μL), NADPH generating system (50 μL) and solution of the inhibitor (10 μM, 20 μL) in absolute ethanol. Tubes were warmed to 37 °C for 5 min and the assay initiated by the addition of microsomal enzyme (final concentration 0.16 mg/mL, 10 μL). The assay mixture was incubated for 15 min. The reaction was quenched by the addition of ether (2 mL), vortexed and placed on ice. The organic layer was then placed into a separate tube. The assay mixture was further extracted with ether (2× 2 mL), and the organic layers combined. The solvent was removed under a stream of nitrogen, acetone (30 μL) was added to each tube and the solution spotted onto silica-based TLC plates along with carrier steroids (progesterone, 17α-hydroxyprogesterone, testosterone and androstenedione, 5 mg/mL). The TLC plates were developed using the mobile phase DCM/ethylacetate (7:3). The separated spots were identified under UV light and each spot cut out and placed into scintillation vials. Acetone (1 mL) and scintillation fluid (Cocktail T) (3 mL) were added to each vial, vortexed and counted for 3 min for ^{3}H. Control samples with no inhibitor were incubated simultaneously. In determining the IC_{50} values for the most potent compounds, the inhibitory activity was determined using the method outlined above, however,

for each compound, five or more inhibitor concentrations were used and the inhibitory activity was determined at each concentration (in triplicate); the IC_{50} was then determined from a graph (using linear regression analysis) of the inhibitory activity versus log[I].

11. Lyase assay using rat microsomal enzyme: Rat testicular microsomal suspension was thawed under cold running water and vortexed. The final incubation assay mixture (1 mL) consisted of sodium phosphate buffer (50 mM, pH 7.4, 905 μL), radiolabelled 17α-hydroxyprogesterone as substrate (1 μM, 10 μL), NADPH generating system (50 μL) and solution of the inhibitor (10 μM, 20 μL) in absolute ethanol. Tubes were warmed to 37 °C for 5 min and the assay initiated by the addition of microsomal enzyme (final concentration 0.23 mg/mL, 15 μL). The assay mixture was incubated for 30 min. The reaction was quenched by the addition of ether (2 mL), vortexed and placed on ice. The organic layer was then removed and placed into a separate tube. The assay mixture was further extracted with ether (2× 2 mL), and the organic layers combined. The solvent was removed under a stream of nitrogen, acetone (30 μL) was added to each tube and the solution spotted onto silica-based TLC plates along with carrier steroids (17α-hydroxyprogesterone, testosterone and androstenedione, 5 mg/mL). The TLC plates were developed using the mobile phase DCM/ethylacetate (4:1). The separated spots were identified under UV light and each spot cut out and placed into scintillation vials. Acetone (1 mL) and scintillation fluid (Cocktail T) (3 mL) were added to each vial, vortexed and counted for 3 min for ^{3}H. Control samples with no inhibitor were incubated simultaneously. In determining the IC_{50} values for the most potent compounds, the inhibitory activity was determined using the method outlined above, however, for each compound, five or more inhibitor concentrations were used and the inhibitory activity was determined at each concentration (in triplicate); the IC_{50} was then determined from a graph (using linear regression analysis) of the inhibitory activity versus log[I].
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