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Synthesis and biochemical evaluation of a range of sulfonated derivatives of 4-hydroxybenzyl imidazole as highly potent inhibitors of rat testicular 17α-hydroxylase/17,20-lyase (P-450$_{17\alpha}$)

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ABSTRACT

We report the synthesis and biochemical evaluation of a range of 4-sulfonated derivatives of 4-hydroxybenzyl imidazole which has been targetted against the two components of 17α-hydroxylase/17,20-lyase (P-450$_{17\alpha}$), namely, 17α-hydroxylase (17α-OHase) and 17,20-lyase (lyase). The results from the biochemical testing suggest that the compounds synthesised are highly potent inhibitors possessing excellent selectivity towards the lyase component.

In the conversion of progestins and pregnanes to the corresponding androgen precursors such as androstenedione (AD) and dehydroepiandrosterone (DHEA) (Fig. 1), 17α-hydroxylase/17,20-lyase (P-450$_{17\alpha}$) is a pivotal enzyme.[1]

This enzyme is of interest as a target in the treatment of androgen-dependent prostate cancer because of its role in the biosynthesis of these androgen precursors, however, 17α-hydroxylase is also involved in the direct biosynthesis of glucocorticoids and mineralocorticoids, the latter two being produced directly from the 17α-hydroxy progestins. The overall enzyme complex is postulated to be a bi-lobed structure with two substrate binding sites, one associated with binding substrate for the hydroxylase reaction and the other with binding for the lyase reaction.[2,3] In particular, the overall conversion of progestins and pregnanes involves two sequential oxidative steps requiring both NADPH and oxygen[4]—the first involves an initial 17α-hydroxylation [catalysed by the 17α-hydroxylase (17α-OHase) component of the enzyme complex] of the C_{21} steroid, followed by the cleavage of the C(17)–C(20) bond of the 17α-hydroxy intermediate [catalysed by the 17,20-lyase (lyase) component], to give the corresponding androgen precursor.

In the inhibition of this enzyme, a number of different compounds has been synthesised and evaluated including ketoconazole (KTZ) and abiraterone acetate (Fig. 2)—this latter compound has been shown to be a selective and potent inhibitor of P450$_{17\alpha}$[5] and has entered Phase II of clinical trials. Indeed, we have recently reported the design and synthesis of a number of benzyl imidazole-based compounds as inhibitors of this enzyme complex.[6–9] In particular, we have shown previously that inhibitors able to bind to the two hydrogen bonding groups present at the active site of P-450$_{17\alpha}$ possess greater inhibitory activity than compounds which only utilise one of the two hydrogen bonding groups[6] [these H-bonding groups are involved in stabilising the enzyme–substrate complex with each H-bonding group binding to the C(3) polar group within each C_{21} steroid, that is, P or 17α-OHP]. Here, we report our continued efforts in the design and synthesis of potent and specific inhibitors of P-450$_{17\alpha}$, in particular, compounds which are able to bind to both H-bonding groups within the active site. As such, we report: the synthesis of a range of sulfonate derivatives of 4-hydroxybenzyl imidazole-based compounds and their biochemical evaluation (in comparison to KTZ) against both components of rat testicular microsomal enzyme.

In the synthesis of the benzyl imidazole-based compounds, the azole functionality was reacted with a benzyl bromide in the presence of a suitable base[6–9] (Scheme 1).

However, in the synthesis of derivatives of 4-hydroxybenzyl imidazole, the reaction outlined in Scheme 1 cannot be used since the labile proton present within the starting material (i.e., where R = OH) would be expected to neutralise the azolyl ion produced if this route was followed. The method of Machin et al.[10] was therefore utilised (Scheme 2, step a), as such, the synthesis of 4-hydroxybenzyl imidazole[11] (**1**) involved heating 4-hydroxybenzyl

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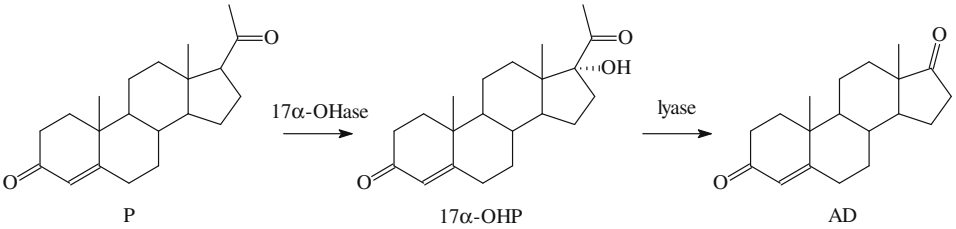


Figure 1. Reaction catalysed by P-450$_{17\alpha}$ in the conversion of progesterone (P) to AD via 17α-hydroxyprogesterone (17α-OHP).

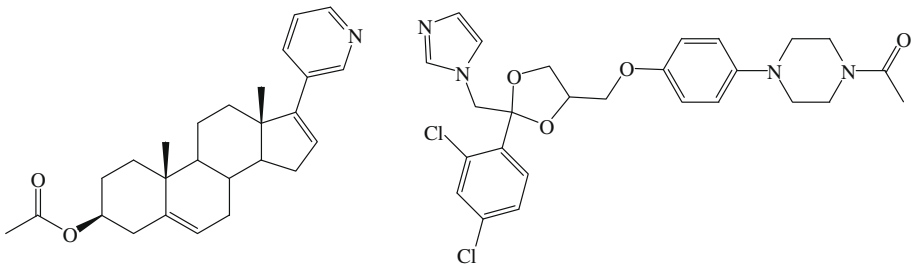
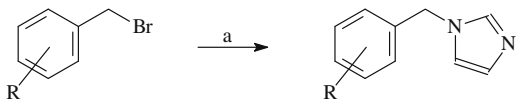
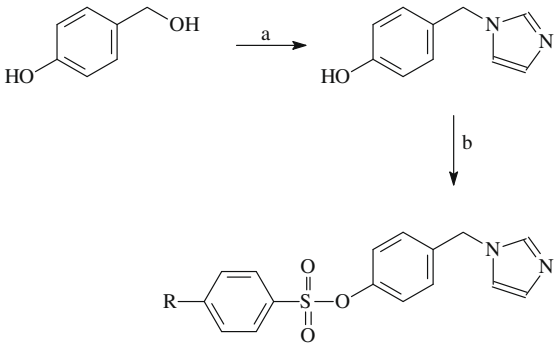


Figure 2. Structures of two known potent inhibitors of P450$_{17\alpha}$.



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



Scheme 2. Synthesis of a number of sulfonate derivatives of 4-hydroxybenzyl imidazole (a = imidazole/Δ; b = 4-substituted phenyl sulfonyl chloride/DCM/Δ; R = various substituents, for example, CH_3, NO_2, F, Cl, and Br).

alcohol in the presence of excess imidazole (in the absence of a solvent), and resulted in the 4-hydroxybenzyl imidazole in very good yield (87%). In the synthesis of the sulfonate derivatives, the appropriate sulfonyl chloride was reacted with **1** in the presence of triethylamine (TEA) and anhydrous dichloromethane (DCM) (Scheme 2, step b)—the synthesis of the *p*-toluenesulfonate derivative (**2**) is given as an example.[12] In general, the reactions proceeded in moderate to good yield (ranging from 60% to 80%) and without any major problems.

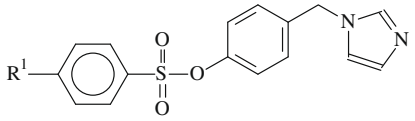
The biochemical evaluation of the synthesised compounds against both 17α-OHase and lyase was undertaken using a literature method.[6–9,13,14] Table 1 shows the IC_{50} values obtained for the compounds considered within the current study against both 17α-OHase and lyase as well as the IC_{50} values obtained for the two standard inhibitors used within our study—it should be noted that we have previously reported 4-iodobenzyl imidazole (**10**) as a potent inhibitor of both 17α-OHase and lyase components, however, we have used the compound as a standard so as to allow us to compare the novel compounds (**2**–**9**) reported here with our previously reported inhibitors.

Initial consideration of the inhibitory data for the compounds shows that all of the benzyl compounds evaluated (**2**–**9**) were considerably weaker inhibitors against 17α-OHase than against lyase—this is suggested to be a beneficial property since it is expected to result in reduced side-effects as the compounds would not be expected to interfere with corticosteroid synthesis. As such, compounds **2**, **7** and **9** are observed to possess an excellent selectivity index value with compounds **3**, **4**, **5** and **8** possessing good selectivity (Table 1). Furthermore, the compounds considered within the study were all found to possess weak inhibitory activity against 17α-OHase when compared to the standard compound, KTZ (IC_{50}=3.76 ± 0.01 μM against 17α-OHase) whilst possessing highly potent inhibitory activity against the lyase component in comparison to KTZ (IC_{50} = 1.66 ± 0.15 μM against lyase).

With regards to our own standard compound (**10**), the compounds within the current study were found to be, in general, equipotent or more potent than **10** against the 17α-OHase component—only compounds **2**, **4** and **9** were weaker. However, all of the compounds (**2**–**9**) were found to be more potent than **10** against the lyase component, indeed, the most potent compound against this component was compound **7** which was found to possess an IC_{50} value of 65 nM. The most potent compound (across both components) within the new range of inhibitors was compound **3** which was found to possess an IC_{50} value of 6.88 μM against 17α-OHase and 85 nM against lyase. Compound **5** was also found to be a potent inhibitor, possessing an IC_{50} value of 6.85 μM against 17α-OHase and 99 nM against lyase. However, in comparison to compounds **2**, **7** and **9**, compounds **3** and **5** were found to possess slightly poorer selectivity.

Due to the microsomal nature of P-450$_{17\alpha}$, the crystal structure of this enzyme has yet to be determined, however, several modelling techniques exist which may be utilised to study the mode of binding of these inhibitors.[2,15–17] These studies have proposed

Table 1
Showing the IC_{50} values ($n = 9$) and the selectivity index (which is the ratio of [IC_{50} (17α-OHase)/IC_{50} (lyase)]) for the compounds considered within the current study against both the 17α-OHase and lyase components of the overall P-$450_{17\alpha}$



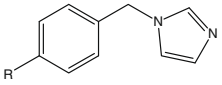
Compound number	R^1	17α-OHase [IC_{50} values (μM)]	Lyase [IC_{50} values (nM)]	Selectivity index
2	CH_3	25.16 ± 1.01	230.2 ± 10.2	109.4
3	NO_2	6.88 ± 0.33	85.0 ± 8.1	80.9
4	F	15.93 ± 0.72	210.4 ± 27.4	75.9
5	Cl	6.85 ± 0.20	98.7 ± 36.2	69.9
6	Br	4.84 ± 0.02	70.4 ± 5.7	34.6
7	I	10.01 ± 0.04	65.1 ± 4.5	100.1
8	OCH_3	6.00 ± 0.01	100.9 ± 13.2	60.0
9	CF_3	64.91 ± 2.12	290.8 ± 43.1	223.8
10	—	10.06 ± 0.66	1580.0 ± 100	6.4
KTZ	—	3.76 ± 0.01	1660.0 ± 150	2.3

the existence of two 'lobes' which are utilised by the two substrates of P-$450_{17\alpha}$. From the consideration of the inhibitory activity observed within this series of compounds, in comparison to previous benzyl azole-based compounds reported by us[6–9], we hypothesise that these compounds are able to utilise additional binding to increase potency. As such, we propose that the potency of the inhibitors is due to two interactions: the 4-substituent on the phenyl ring undergoes polar–polar interaction with one of the hydrogen bonding groups at the active site, whilst the second interaction involves the sulfonate moiety and presumably the S=O groups which are able to interact with the active site. Both interactions (together with the Fe–N dative covalent bond formation) therefore increase the stability of the inhibitor–enzyme complex leading to an increase in the potency of the inhibitor in comparison to compounds which are only able to interact with a single hydrogen bonding group. That this is indeed the rationale for the increased inhibitory activity can be observed within the 4-halogenated compounds, where the potency of the inhibitor increases with a decrease in electronegativity of the halogen.

Furthermore, we observe that compounds which are not able to undergo both interactions with the two hydrogen bonding groups at the same time (i.e., compounds **2**, **8** and **9** which lack groups on the phenyl moiety able to undergo polar–polar interaction) possess poor inhibitory activity in comparison to the other compounds. This therefore adds further support to our previous study where we have shown that the di-halogen derivatives of benzyl imidazole-based compounds possessed greater inhibitory activity than the mono-substituted compounds.[6] That is, the second polar–polar interaction between the inhibitor and the hydrogen bonding group at the active site results in extremely strong binding of the inhibitor to the active site as a result of which the catalytic activity of the overall enzyme complex is greatly reduced.

In conclusion, we have introduced novel compounds which have been shown to be highly potent inhibitors of P-$450_{17\alpha}$, with good selectivity towards the lyase component in comparison to the 17α-OHase component, as such, they appear to be extremely good lead compounds in the continued design and synthesis of potential drug substances against P-$450_{17\alpha}$. However, whilst these compounds have shown extremely favourable selectivity between the two components of P-$450_{17\alpha}$, the compounds would be required to also possess selectivity against other P-450 enzymes, for example, P-450_{11}. In an earlier study, we have shown that related 4-substituted benzyl imidazole compounds possessed highly potent inhibitory activity against aromatase (e.g., compound **13** was found to possess an IC_{50} of 70 nM)[18] (Table 2), as such, the compounds considered within the current study would also be expected to possess inhibitory activity against other P-450 enzymes and therefore may not be suitable drug substances due to their poor selectivity against other P-450 systems. Finally, it should be noted that the current study has utilised rat testicular microsomal enzyme which has been shown to be different to human P-$450_{17\alpha}$, as such, whilst these compounds are highly potent inhibitors, they would be required to be evaluated against human P-$450_{17\alpha}$ prior to further development.

Table 2
Initial screening ([*I*] = 10 μM) and IC_{50} values for the benzyl imidazole-based compounds, including the standard AG18



Compound	R	IC_{50} (μM)
11	NO_2	0.13 ± 0.19
12	F	0.88 ± 0.03
13	Cl	0.07 ± 0.5
14	CN	1.20 ± 0.08
15	CH_3	24.5 ± 0.12
AG	—	70.6 ± 0.1

Acknowledgements

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11. *1-(4-Hydroxy-benzyl)-1H-imidazole* (***1***): Imidazole (13.4 g, 200 mmol) was mixed with 4-hydroxybenzyl alcohol (5 g, 40 mmol). The reaction mixture was then heated at 160 °C for 30 min resulting in a dark brown oil which was added to 500 mL of hot water resulting in a brown solid which was filtered and vacuum dried to give a light brown solid. Column chromatography of the solid gave **1** as a light brown solid (6.08 g, yield 87%); [mp = 209.8–210.6 °C (lit. mp = 212.0–213.0 °C [12])]; R_f = 0.35 [50/50 (diethyl ether/petroleum ether)]. $\nu_{(max)}$ (Film)cm^{-1}: 3421 (OH), 1653 (Im, CH_2–N), 1601 (Ar, C=C); δ_H (400 MHz, DMSO): 9.45 (1H, s, Ph–OH), 7.66 (1H, s, NC*H*N, Im), 7.09 (1H, s, CH_2–NC*H*, Im), 7.06 (2H, d, *J* = 8.79 Hz, Ph–*H*), 6.83 (1H, s, NC*H*, Im), 6.68 (2H, d, *J* = 8.79 Hz, Ph–*H*); 4.99 (2H, s, Ph–CH_2); δ_C (100 MHz, DMSO): 157.55 (Ar, C-OH), 137.65 (NCN), 129.65, 128.51, 115.88 (Ar, C), 129.09, 119.87 (Im, C), 49.67 (Ph–CH_2) ; GC: t_R 9.43 min, LRMS (EI): *m*/*z* 174 (M^+, 18%), 107 (M^+–$C_3H_3N_2$, 100%), 77 (M^+–$C_4H_5N_2O$, 14%); HRMS (EI): Found *m*/*z* 175.0866300 (M^+) $C_{10}H_{11}O_1$, calculated *m*/*z* 175.0866295.
12. *4-(1H-imidazol-1-ylmethylphenyl 4-toluenesulfonate* (***2***): To a mixture of **1** (1.00 g, 5.75 mmol) and triethylamine (0.70 g, 6.89 mmol) in anhydrous DCM, 4-toluene sulfonyl chloride (1.20 g, 6.32 mmol) was added and the reaction mixture refluxed for 12 h. After cooling, the reaction mixture was poured on ice

(100 mL) and washed with sodium carbonate solution (Na_2CO_3) (2 × 50 mL). The organic layer was washed with water (3 × 50 mL) and dried over anhydrous magnesium sulfate ($MgSO_4$), filtered and the DCM removed under vacuum to give a yellow oil which was purified using column chromatography to give **2** as a light yellow coloured oil (0.92 g, yield 63%); R_f = 0.37 [60/30/10 (petroleum ether/diethyl ether/methanol)]. $\nu_{(max)}$ (film)cm^{-1}: 3109 (Ar, C–H), 2303 (Im, C=N), 1596 (Ar, C=C), 1371 (S=O); δ_H (400 MHz, $CDCl_3$): 7.61 (1H, s, NC*H*N, Im), 7.56 (2H, d, *J* = 8.42, Ph–*H*), 7.28 (2H, d, *J* = 8.42 Hz, Ph–*H*), 7.07 (2H, d, *J* = 8.79 Hz, Ph–*H*), 6.97 (1H, s, CH_2–NC*H*, Im), 6.86 (3H; 2H, d, *J* = 8.79 Hz, Ph–*H*, 1H, NC*H*, Im), 5.08 (2H, s, Ph–C*H*$_2$), 2.32 (3H, s, CH_3); δ_C (100 MHz, $CDCl_3$): 149.44, 145.97 (Ar, C), 137.32 (Im, NCN), 136.31, 132.20, 129.72, 128.66, 128.30, 122.55 (Ar, C), 128.14, 119.56 (Im, C), 49.35 (Ph–CH_2), 20.28 (CH_3); GC: t_R 28.65 min; LRMS (EI): *m*/*z* 328 (M^+, 52%), 261 (M^+–$C_3H_3N_2$, 52%), 155 (M^+–$C_{10}H_9N_2O$, 64%), 91 (M^+–$C_{10}H_9N_2O_3S$, 100%); HRMS (EI): found *m*/*z* 329.0955060, $C_{17}H_{17}N_2O_3S$, calculated *m*/*z* 329.0954395.

13. *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 ^3H. 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 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*].
14. *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 ^3H. 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 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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