Synthesis, Biochemical Evaluation and Rationalisation of a Series of 3,5-Dibromo Derivatives of 4-Hydroxyphenyl Ketone-Based Compounds as Probes of the Active Site of Type 3 of 17β-Hydroxysteroid Dehydrogenase (17β-HSD3) and the Role of Hydrogen Bonding Interaction in the Inhibition of 17β-HSD3

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Abstract: We report the synthesis, evaluation and rationalisation of the inhibitory activity of a series of 3,5-dibromo derivatives of 4-hydroxyphenyl ketone as probes of the active site of the type 3 of 17β-hydroxysteroid dehydrogenase (17β-HSD3). The results support the important role of hydrogen bonding interaction in the inhibition of 17β-HSD3.

Keywords: Androgen ablation, Enzyme inhibition, Hydrogen bonding, 17β-hydroxysteroid dehydrogenase, Type 3.

INTRODUCTION

The biosynthesis of the more potent androgens (and estrogens) from less potent steroids is catalysed by the 17β-hydroxysteroid dehydrogenase (17β-HSD) family of enzymes and involves the reduction of the C(17)=O moiety to a C(17)-βOH group. The reduction reaction involves an hydride transfer from the co-factor NADPH to the α-C(17) position of the steroid together with proton transfer between the amino acids at the active site and the C(17)-βO^- resulting in the formation of the β-hydroxy moiety [1] (Fig. **1**). To date, some 15 different types of 17β-HSD have been reported and have been shown to be responsible for a number of redox reactions [2]. For example, the conversion of the weak androgen, androstenedione (AD), to the more potent androgen testosterone (T), the principle precursor to dihydrotestosterone (DHT) (which has been shown to be the major mitogen in the prostate diseases such as benign prostate hyperplasia and prostate cancer [3]) is catalysed by the type 3 isozyme (17β-HSD3) [1]. A number of the types of 17β-HSD family of enzymes also catalyse the oxidation reaction, thereby resulting in a decrease in the potency of the more potent sex steroid, for example, the type 2 form of 17β-HSD family of enzymes has been shown to catalyse the reverse oxidation of the more potent estrogens and androgens, in particular, the oxidation of the C(17)-βOH moiety to the C(17)=O functionality (Fig. **1**) [1]. In the treatment of hormone-dependent cancers such as prostate and breast cancer, a decrease in the biosynthesis of these potent steroids has been shown to lead to a major loss of stimulation of the cancer cells, as such, the 17β-HSD family of enzymes has become a major biochemical target in the treatment of hormone-dependent cancers [4].

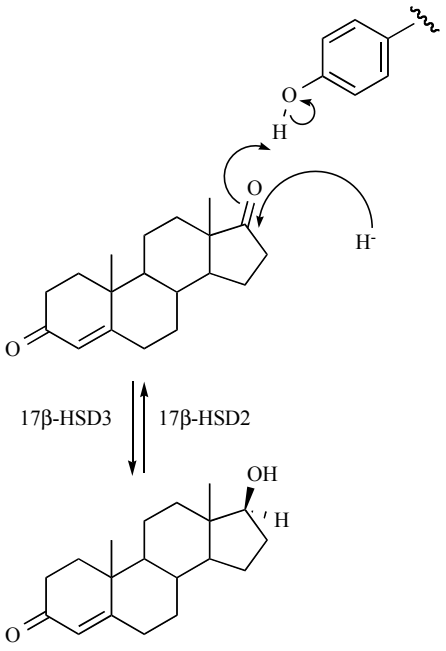

Fig. (1). α-Hydride (from NADPH) attack on the C(17) of AD in the conversion of AD to T catalysed by 17β-HSD3 and type 2 of 17β-HSD (17β-HSD2).

In an effort to aid both the design of novel inhibitors and the rationalisation of the inhibitory activity of known inhibitors of this enzyme, we have previously undertaken the derivation of the transition-state (TS) for type 1 17β-HSD (17β-HSD1) [5], the enzyme which has been shown to catalyse the biosynthesis of the more potent estrogen, namely estradiol (E2), from estrone (E1). Using this technique, we undertook the derivation of the TS for the reaction catalysed by 17β-HSD3 (Fig. **2**) since no crystal structure exists currently for this enzyme. Using the derived TS, we undertook a study where we superimposed a number of known inhibitors of 17β-HSD3 onto the steroid backbone

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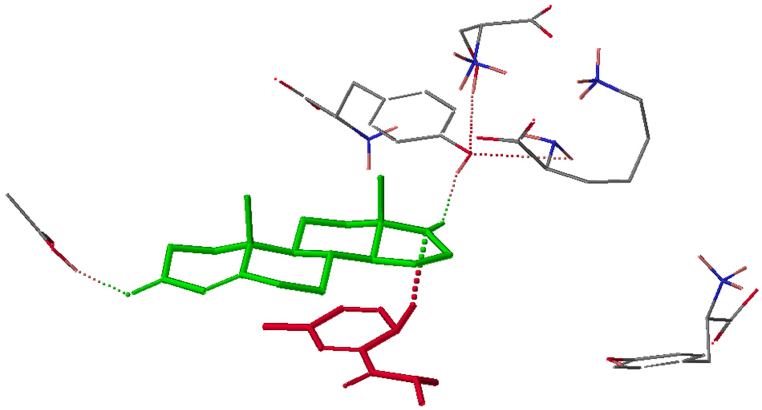
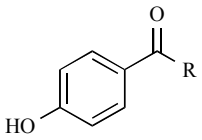
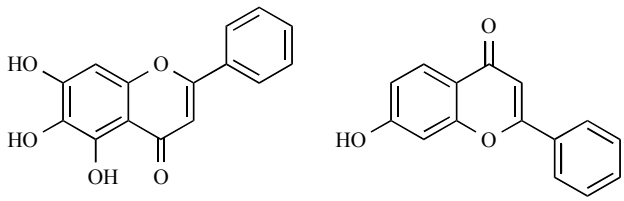


Fig. (2). To show the TS for the reaction catalysed by 17β-HSD3 (substrate in red) [6].

Table 1. Inhibitory Data Obtained for the 4-Hydroxyphenyl Ketone-Based Compounds Previously Reported as Inhibitors of 17β-HSD3 [12]



Compound	R	% Inhibition [I]=100μM
1	-	38.8 ± 1.4
2	-	53.9 ± 1.1
3a	CH_3	36.59 ± 0.52
4a	C_2H_5	53.03 ± 2.18
5a	C_3H_7	60.18 ± 0.77
6a	C_4H_9	61.81 ± 0.89
7a	C_5H_{11}	76.40 ± 0.18
8a	C_6H_{13}	80.26 ± 0.20
9a	C_7H_{15}	82.58 ± 0.49
10a	C_8H_{17}	83.53 ± 0.48
11a	C_9H_{19}	81.39 ± 0.09



Baicalein (**1**) 7-Hydroxyflavone (**2**)

Fig. (3). Structures of the two alternative standards previously used by other workers within the field and within the current study.

[6] and concluded from our study that the carbonyl moiety at the C(17) position of the androgen precursor backbone was an important feature in the mimicking of the substrate and therefore an important feature for potential inhibition of 17β-HSD3 [5, 6]. We therefore modelled a series of non-steroidal compounds based on a backbone containing a phenyl ring and an aldehydic or ketone C=O group, with the carbonyl moiety within the potential inhibitors being used to mimic the C(17)=O of the AD backbone. We therefore designed, synthesised and subsequently evaluated a number of 4-hydroxyphenyl ketone-based compounds and rationalised the inhibitory activity of these compounds [6, 7] (Table **1**). From our study, we also concluded the existence of a hydrogen bonding group at the active site close to the C(15) and C(16) position of the steroid backbone [7]. We hypothesised that it was this additional H-bonding group which allowed the 4-hydroxyphenyl ketone-based compounds to be orientated within the active site such that the phenyl moiety was positioned close to the C(15) and C(16) of the steroid backbone, thereby allowing the OH of the inhibitor and the active site to undergo hydrogen bonding interaction. We also hypothesised that the alkyl chain (or indeed the cycloalkyl moiety) within the inhibitors was involved in mimicking the steroid carbon backbone, thereby allowing the inhibitors to

possess the appropriate hydrophobicity. We also hypothesised from our modelling study that the removal of (or a reduction in the ability of the OH moiety within the 4-hydroxyphenyl ketone based compounds to undergo) this additional H-bonding interaction would potentially result in a reduction in the inhibitory activity within the 4-hydroxyphenyl ketone-based compounds. Here, in an effort to evaluate our hypotheses with regard to the role of interactions within the active site of 17β-HSD3, in particular, the involvement of the hydrogen bonding interaction in the inhibition of this enzyme, we undertook the synthesis of di-brominated derivatives of a range of 4-hydroxyphenyl ketone-based compounds, and their subsequent biochemical evaluation against rat testicular microsomal enzyme using radiolabelled AD as the substrate. In an effort to compare the current range of compounds with our previous study, we utilised two standard and known inhibitors of 17β-HSD3, namely baicalein (**1**) and 7-hydroxyflavone (**2**) (Fig. **3**) which have both been previously shown to possess inhibitory activity against this enzyme by us and other workers within the field [8-10].

EXPERIMENTAL

Methods and Materials

Chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, England), and checked for purity by ^{1}H and $^{13}CNMR$ (JEOL 400MHz and 100MHz respectively) using either $CDCl_3$, or d_6-acetone as a solvent. Infrared spectra were obtained on a Perkin-Elmer Fourier Transform-Paragon 1000 IR. Gas chromatography-mass spectrometry was carried out on a Hewlett Packard 5890 series II GC-MS at a flow rate of 0.58mL/min, and a temperature range increasing from 120-270°C at the rate of 10°C/min. Melting points were uncorrected and were obtained on a BUCHI 512 or a Gallenkamp Instrument. Elemental analysis was undertaken at the School of Pharmacy, London. Ultraviolet spectroscopy was carried out on a CARY 100 Scan UV-visible spectrophotometer. All non-radioactive steroids and laboratory reagents were analar grade; β-nicotinamide adenine dinucleotide phosphate (NADP, mono sodium salt), D-glucose-6-phosphate (mono sodium salt), D-glucose-6-phosphate dehydrogenase (suspension in ammonium sulfate) were obtained from Roche Diagnostics, Lewes, East Sussex whilst **1** and **2** were obtained from Sigma-Aldrich Company, Poole, Dorset. [1,2,6,7-^{3}H]AD was obtained from Amersham Pharmacia Biotech UK Limited, Buckinghamshire. Optiscint HiSafe was obtained from Perkin-Elmer Life and Analytical Sciences, Beaconsfield, Bucks.

Chemistry

1-(4-Hydroxy-phenyl)-ethanone (3a)

Aluminium chloride (4.5g, 33.8mmol) was added to a solution of phenol (1.5g, 16.0mmol) in anhydrous dichloromethane (DCM) (15mL). The slurry was left to stir for 1h before acetyl chloride (1.3mL, 17.6mmol) was added in a dropwise manner. The solution was left to stir for further 14h. The reaction was quenched using an ice-cold solution of aqueous hydrochloric acid (HCl, 1M) (30mL) and extracted into diethyl ether (DEE) (2x50mL). The combined organic layer was extracted into sodium hydroxide (NaOH, 2M) (2x50mL) and then acidified to pH 2 using aqueous HCl (1M, 40mL). The product was extracted into DEE (2x50mL), the organic layer was washed with water (2x50mL) and dried over anhydrous magnesium sulfate ($MgSO_4$). The solvent was removed under vacuum to give a brown solid. Column chromatography of the crude solid gave 3a as a white solid (1.6g, 73.5% yield); m.p.=109.4-110.3°C (lit. m.p.=110.2-110.4°C [11]); R_f=0.35 [diethyl ether (DEE)/petroleum spirit (40-60°C) (50:50)].

$\nu_{(max)}$ (Film) cm^{-1}: 3315.6 (OH), 1661.6 (C=O), 1605.3 (Ar C=C); δ_H (d_6-Acetone): 9.20 (1H, s, O<u>H</u>), 7.89 (2H, dd, *J*=8.9Hz, Ph-<u>H</u>), 6.91 (2H, dd, *J*=8.9Hz, Ph-<u>H</u>), 2.48 (3H, s, C<u>H</u>$_3$); δ_c (d_6-Acetone): 196.36 (C=O), 162.63 (C-O), 131.57, 130.51, 115.97 (Ar, C), 26.34 (CH_3); GC: t_R=5.6min; LRMS (*m/z*): 136 (M^+, 41%), 121 (M^+-CH_3, 100%), 93 (M^+-C_2H_3O, 28%); HRMS (ES): found 137.05971 $C_8H_9O_2$ requires 137.15586; Elemental analysis: found C 70.42%, H 5.88% $C_8H_8O_2$ requires C 70.58%, H 5.92%.

4-Hydroxy-3,5-dibromoacetophenone (3b)

Bromine water was added to a solution of **3a** (1.05g, 7.71mmol) in glacial acetic acid (15mL) and the mixture left to stir for 3h. The mixture was extracted into DEE (2 x 50mL), washed with water (2 x 50mL) and dried over anhydrous $MgSO_4$. The solvent removed under vacuum gave an off-white solid; recrystallisation (from 50% aqueous ethanol) gave **3b** as a white solid (1.47g, 64.4% yield); m.p.=189-191ºC (lit. m.p.=187ºC [12]); R_f=0.60 [DEE/pet spirit 40-60ºC (70/30)].

$\nu_{(max)}$ (Film) cm^{-1}: 3224.3 (Ph-OH), 1663.4 (C=O), 1581.5 (Ar C=C); δ_H (d_6-Acetone): 8.13 (2H, s, Ph-<u>H</u>), 2.56 (3H, s, C<u>H</u>$_3$); δ_C (d_6-Acetone): 194.40 (<u>C</u>O), 154.89 (<u>C</u>O), 133.35 (<u>C</u>Br, Ar), 132.37 (<u>C</u>H, Ar), 110.08 (<u>C</u>, Ar), 26.16 (<u>C</u>H$_3$); GC: t_R=7.39min; LRMS (*m/z*): 294 (M^+, 38%), 279 (M^+-CH_3, 100%), 251 (M^+-C_2H_3O, 11%), 170 (M^+-C_2H_4BrO, 12%); HRMS (ES): found 292.8807 $C_8H_7Br_2O_2$ requires 294.94798.

Preliminary Screening of Compounds for 17β-HSD3 [9]

All incubations were carried out in triplicate at 37°C in a shaking water bath. Incubation mixtures (1mL), containing NADPH-generating system (50μL), inhibitor (100μM, 20μL) and prepared substrate AD (1.5μM final concentration, 15μL), in phosphate buffer (pH 7.4, 905μL), were allowed to warm at 37°C. The testicular microsomes were thawed and warmed to 37°C before addition (0.097mg/mL final concentration, 10μL) to the assay mixture. The solutions were incubated for 30 min at 37°C and the reaction was quenched by the addition of ether (2mL). The solutions were vortexed, then left to stand over ice for 15min. The assay mixture was extracted with further aliquots of ether (2 x 2mL) and the organic layers combined into a clean tube before the solvent was evaporated. Acetone (30μL) was added to each tube and vortexed thoroughly. Aliquots, along with steroid carriers (AD and T, 5mg/mL, approximately 10μL) were spotted onto TLC plates and run, using a mobile phase consisting of dichloromethane (70mL) and ethyl acetate (30mL). After development, the separated steroids were identified, using a UV lamp, cut from the plate

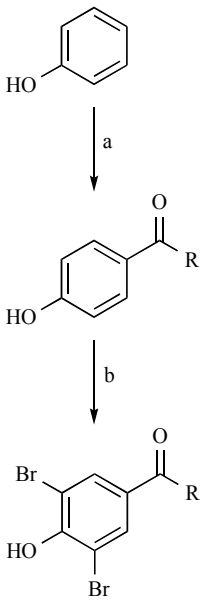
and placed into scintillation vials. Acetone (1mL) was added to each vial in order to dissolve the steroid from the silica plate and then scintillation fluid (Optiscint HiSafe) (3mL) was added. The samples were vortexed and read for tritium for 4 min per tube.

pKa Determination

The compounds (2-4mg) were added to borax buffer (200mL), and the UV spectrum recorded between 200-450nm. The absorbance of the major peak was then adjusted to approximately 1 absorbance unit either by the addition of more buffer or more of the specific compound. After decanting the solution free of any undissolved phenolic compound, 20mL of this stock solution was placed into 3 separate volumetric flasks (25mL) and filled up to the mark with HCl (2M) (Aa), borax buffer pH 9.0 (A), or NaOH (2M) (Ab). The UV spectrum of each solution was recorded and the absorbance of each solution at the wavelength corresponding to the NaOH (2M) (fully dissociated) maxima, was determined. The pK_a was then determined from the calculation of the mole fraction [13].

RESULTS AND DISCUSSION

In the synthesis of the 3,5-dibrominated derivatives of 4-hydroxyphenyl ketone-based compounds (Scheme **1**), the latter series of compounds was initially synthesised according to the literature methodology previously reported by us via Friedal-Craft acylation [11]. Compounds **1a** to **17a** were then derivatised to the target compounds involving the use of bromine and glacial acetic acid as the reaction solvent (Scheme **1**) – the target compounds were therefore obtained in good to excellent yield [ranging from ~53% for compound **8b** (3,5-dibromo-4-hydroxyheptanophenone) to ~73% for compound **5b** (3,5-dibromo-4-hydroxypropiophenone)] and



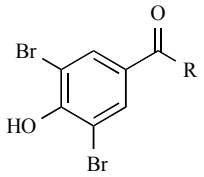
Scheme 1. Synthesis of dibrominated derivatives of 4-hydroxyphenyl ketones as potential inhibitors of 17β-HSD (a=various acid chlorides/$AlCl_3$/DCM; b=Br_2/CH_3COOH/; R=C_1 to C_{12}).

without any major problems. It should be noted that no monobrominated by-product was observed, indeed, the bromine was added in excess so as to prevent the production of any monobrominated product – no other multiple brominated (i.e. tri- or indeed tetra-brominated) derivatives were observed. Although in general no major problems were encountered in the synthesis of the target compounds, the synthesis of **3b** proved to be somewhat difficult and resulted in a number of by-products which were produced in small quantities – the target compound was obtained as a result of an extensive series of column chromatography, however, the synthesis of the remainder of the series proved to be less challenging and without any major problems, presumably due to the increased steric hindrance to the ortho positions within the phenyl ring.

From the consideration of the inhibitory activity data (Tables **1** and **2**), we observe that the dibrominated derivatives of the 4-hydroxyphenyl-based compounds which synthesised within the current study are extremely poor inhibitors of 17β-HSD3, indeed, a number of compounds showed no inhibitory activity (Table **2**). The most potent inhibitors within the range of compounds were **11b** and **12b** which were both found to possess ~35% inhibition against 17β-HSD3 at [I]=100μM. Under similar conditions, the parent non-brominated derivatives (compounds **3a** to **11a**, Table **1**) were found to be potent inhibitors of 17β-HSD3 [12] - **10a** was found to inhibit this enzyme by ~84% under similar conditions (and was found to possess an IC_{50} value of 7.84μM). In comparison, the two standard compounds were also found to possess weak inhibitory activity against 17β-HSD3 in comparison to compounds **3a** to **11a** with **1** and **2** possessing ~39% and ~54% inhibition (at [I]=100μM).

As previously mentioned, the acidity of the phenolic OH moiety was proposed to be an important factor in the inhibitory activity observed in compounds **1a** to **11a**. In particular, we proposed that the hydroxy moiety undergoes hydrogen bonding interaction with an amino acid moiety hypothesised to exist within the active site of 17β-HSD3 corresponding to the C(15) and C(16) area of the steroid backbone. More specifically, we postulated that if the ability of the OH moiety to undergo the hydrogen bonding interaction was affected in any way, then this would result in an overall decrease in the inhibitory activity observed within these compounds. The consideration of the determination of the pK_a values for a small range of the dibrominated and the non-brominated derivatives (Table **3**) shows that the dibromination of the phenyl ring moiety results in a significant increase in the acidity of the OH moiety, as a result, the dibrominated derivatives are found to possess significantly reduced pK_a values than the non-brominated derivatives. For example, consideration of the inhibitory activity of **10a** and **10b** shows that the two compounds possess ~84% and ~23% inhibitory activity respectively (under similar conditions) whilst possessing pK_a values of 8.40 and 6.82 respectively, i.e. a dramatic increase in acidity is observed in the dibrominated derivative. Similar trends are observed in the other compounds (Table **3**), for example, compounds **3a** and **3b** are found to possess ~37% and 0% inhibitory activity respectively (under similar conditions) whilst possessing pK_a values of 8.00 and 6.47 respectively. The consideration of the pK_a data for the dibrominated

Table 2. 3,5-Dibromo Derivatives of 3a to 17a Evaluated Against 17β-HSD3



Compound	R	Initial Screening Against 17β-HSD3 at ([I]=100μM)
1	-	38.8 ± 1.4
2	-	53.9 ± 1.1
3b	CH_3	0
4b	C_2H_5	0
5b	C_3H_7	0
6b	C_4H_9	0
7b	C_5H_{11}	9.91±0.2
8b	C_6H_{13}	8.94±0.03
9b	C_7H_{15}	11.11±0.18
10b	C_8H_{17}	22.51±0.10
11b	C_9H_{19}	35.1±2.2
12b	$C_{11}H_{23}$	35.1±0.8
13b	cycloC_4H_7	17.09±5.18
14b	cycloC_5H_9	18.78±0.07
15b	cycloC_6H_{11}	30.23±2.40
16b	H	21.27±3.59
17b	C_6H_5	16.75±0.08

Table 3. Table of pK_a Values for a Small Range of Brominated and Non-Brominated Derivatives of 4-Hydroxypenyl Ketone-Based Compounds

Compound	Experimentally Determined pK_a Value
3a	8.00
4a	8.08
6a	8.12
8a	8.55
10a	8.40
17a	8.01
Average	8.12
3b	6.47
4b	6.84
6b	7.33
8b	6.80
10b	6.82
17b	6.38
Average	6.63

derivatives would therefore appear to support our initial hypothesis with regard to the importance of the OH moiety in undergoing hydrogen bonding with the active site, i.e. an increase in acidity within the 4-hydroxyphenyl ketone-based inhibitors results in an overall decrease in inhibitory activity against 17β-HSD3.

It could be argued that the dibromination of the phenyl moiety could result in the OH moiety being sterically hindered resulting in a decrease in the level of hydrogen bonding interaction between the inhibitor and the active site. As such, the observed reduction in inhibitory activity could be postulated to be due to the steric effect of the two bromine atoms as opposed to the increase in acidity. In an effort to evaluate the role of the steric effect in comparison to the pK_a, we modelled the brominated and non-brominated derivatives onto the previously reported TS for the reaction catalysed by 17β-HSD3, in particular, we minimised the inhibitors within the representation of the active site so as to obtain the binding conformer. The molecular modelling study shows that the inhibitors are indeed able to fit within the active site and undergo hydrogen bonding with the active site, as well as undergo interaction with the NADPH functionality. For example, the modelling of compound **10b** (Fig. **4**) shows that the 4-hydroxy moiety is able to interact with the group at the active site [which is postulated to exist close to the C(15) and C(16) position of the steroid backbone] whilst still undergoing the interaction between the NADPH and the C=O moiety, resulting in a hydrogen bond distance of 2.2Å. The modelling study therefore suggests that the decrease in inhibitory activity was due to the increase in acidity of the OH moiety as opposed to the bromine atoms causing a steric effect and therefore resulting in a decrease in the extended hydrogen bonding (leading to a decrease in inhibitory activity).

In an effort to rationalise the increased acidity within the dibrominated derivatives of the parent compounds (**1**a to **11**a), we undertook a modelling study so as to determine the electron distribution within the dibrominated and the parent compounds. The results of our additional molecular modelling study show that the bromine atoms result in an overall decrease in the electron density on the oxygen atom of the OH moiety. For example, consideration of the compounds **4a** and **4b** shows that within the former compound, the partial charge on the oxygen atom of the hydroxy moiety was found to be -0.221, whereas the oxygen atom within compound **4b** possessed a partial charge of -0.187. That is, the dibromination of **4a** resulted in an overall decrease in electron density within the OH moiety, the resulting decrease in electron density on the oxygen atom of the OH functionality results in the resulting O^- to be stabilised within the dibrominated derivatives as opposed to the non-brominated compounds. This therefore adds further support that the brominated compounds are acidic in nature compared to the non-brominated derivatives (hence resulting in a decrease in pK_a), as such, the potential for the OH moiety to 'donate' the H to an appropriate amino acid within the active site is decreased, thereby resulting in an overall decrease in hydrogen bonding interaction between the inhibitor and the active site (as well as a decrease in the stability of the inhibitor enzyme complex), leading to an overall decrease in inhibitory activity.

CONCLUSION

In conclusion, the biochemical evaluation of a series of dibrominated and non-brominated derivatives of 4-hydroxyphenyl ketone-based compounds adds further support to our initial hypothesis that the hydroxy moiety within the non-brominated range of compounds undergoes hydrogen bonding interaction with the active site of 17β-HSD3. Within the compounds considered in the current study, we have also shown that the dibromination of the parent compound (**1a** to **11a**) resulted in an increase in the acidity of the OH moiety, resulting in a disruption of the

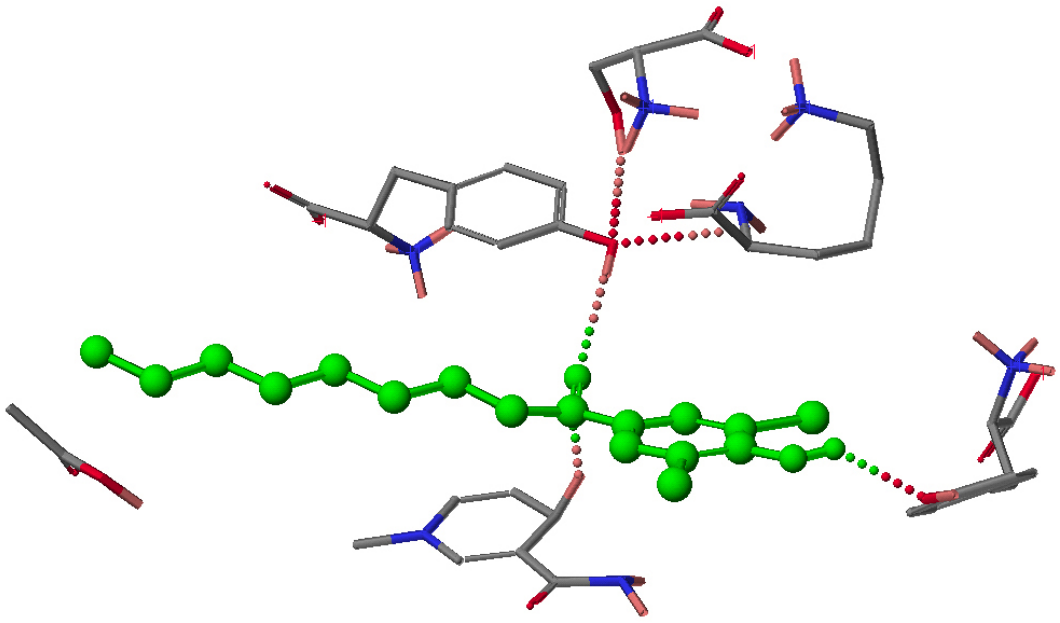


Fig. (4). To show the modelling of **10b** within the representation of the 17β-HSD3 active site.

hydrogen bonding, leading to an overall reduction of the inhibitory activity. The results of the current study would therefore appear to support our hypothesis regarding the importance of hydrogen bonding interaction in determining the overall inhibitory activity of potential inhibitors of 17β-HSD3 and therefore supports our previous observations [14].

DISCLOSURE

The overall conclusions in the two manuscripts "Synthesis, biochemical evaluation and rationalisation of a series of 3,5-dibromo derivatives of 4-hydroxyphenyl ketone-based compounds as probes of the active site of type 3 of 17β-hydroxysteroid dehydrogenase (17β-HSD3) family of enzymes – an insight into the importance of hydrogen bonding interaction in the inhibition of 17β-HSD3" and "Synthesis and Biochemical Evaluation of a Series of Methanesulfonate Derivatives of 4-Hydroxyphenyl Ketone as Probes of the Active Site of Type 3 OF 17β-Hydroxysteroid Dehydrogenase Family of Enzymes" are almost similar.

CONFLICT OF INTEREST

Declared none.

ACKNOWLEDGEMENTS

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