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# The homodimer approach to the design of a new long-acting depot prodrug of abiraterone

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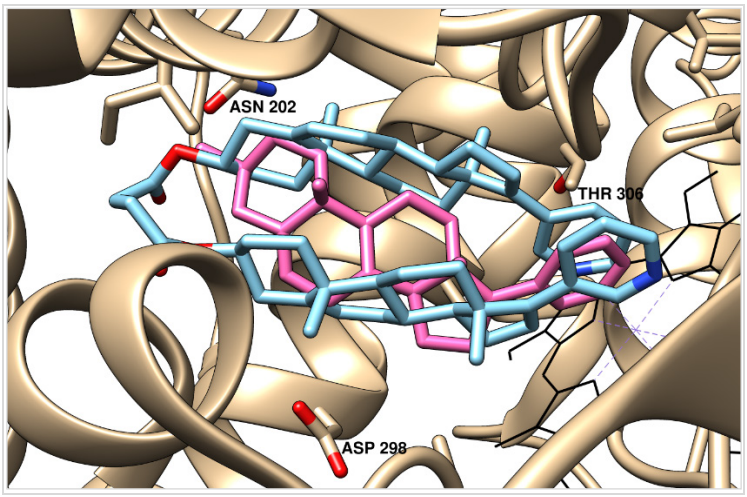
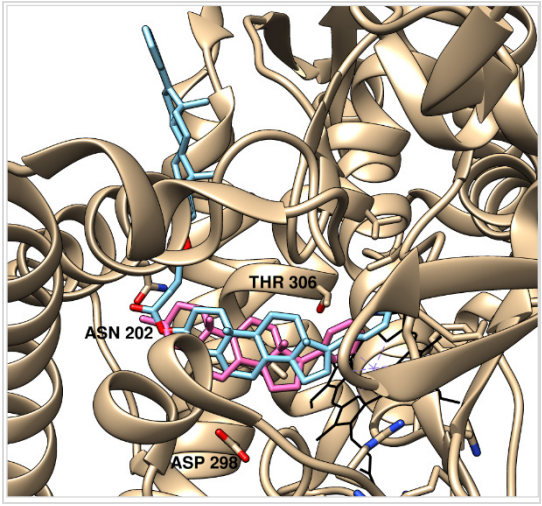
## 1. Molecular modeling

Visual analysis of the abiraterone binding site and adjacent region in CYP17A1 (PDB ID: 3RUK) shows that the volume of free cavities is not sufficient to pack the second steroid residue. Molecular docking of conjugate **6** into the active site of CYP17A1 X-ray structure was performed using AutoDock Vina 1.1.2 software[S1] as described in.[S2,S3] Before docking procedure all water molecules and abiraterone were removed. Due to the large size of the compound **6** a large docking box was used: 15.0 Å×28.5 Å×18.0 Å, grid center size $x$ = 26.358 Å, $y$ = –0.048 Å, $z$ = 33.783 Å, energy range=4, exhaustiveness=30. The resulting complexes (visualized using UCSF Chimera 1.15 software[S4]) belong to two clusters of solutions (see the figures below).




***Docking pose 1***




***Docking pose 2***

**Figure S1** In *docking pose 1* the steroid residues of the conjugate are 'sandwiched' in the abiraterone binding site, while in the *docking pose 2* the additional steroid moiety is located in the cavity between the protein helices. In both docking poses the scoring function is positive and not a single cluster of solutions with a negative value of the scoring function was found.

## 2. Chemistry

All starting materials and reagents were purchased as high-grade commercial products and used without further purification; the solvents were technical grade and distilled from standard drying agents. *N*,*N′*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) and succinic acid anhydride were used as commercial products. Malic and citric acid anhydrides were synthesized by literature methods,[S5,S6] physico-chemical characteristics of **5** were described earlier.[S7] Liquid column chromatography was performed using silica gel 'Macherey–Nagel' (0.063–0.2 mm). Thin-layer chromatography (TLC) was performed on ALUGRAM Xtra G/UV254 silica gel sheets. $^{1}$H and $^{13}$C NMR spectra were recorded on Agilent 400-MR spectrometer (400.0 MHz for $^{1}$H; 100.6 MHz for $^{13}$C) at 28°C. Chemical shifts (δ) are reported in ppm referenced to residual solvent peak ($CDCl_3$, $\delta_H$=7.24 ppm, $\delta_C$=77.0 ppm; methanol-$d_4$, $\delta_H$=4.87 ppm, $\delta_C$=49.15 ppm); spin-spin coupling constants (*J*) are reported in Hz. Liquid chromatography (LC) and ElectroSpray ionization mass spectrometry (ESI-MS) data were obtained on an Agilent 1100 LC/MSD with an Agilent 1100 SL quadrupole mass spectrometer, eluting with 0.05 % TFA in $H_2O$ and 0.05 % TFA in $CH_3CN$ (positive-ion monitoring mode). CHN elemental analysis was performed using a Carlo-Erba ER-20 analyzer. IR-spectra were registered on FT-IR Termo Nicolet IR200 Spectrometer with 4 $cm^{-1}$ resolution, absorption bands are given in $cm^{-1}$. Melting points were determined using a capillary melting point apparatus and were uncorrected.

**(3*S*)-3-Hydroxy-4-oxo-4-{[(3b)-17-pyridin-3-ylandosta-5,16-dien-3-yl]oxy}butanoic acid (3).** Anhydride (*S*)-2-(trifluoroacetyloxy)succinic anhydride[S5] (0.25 g, 1.18 mmol) in a mixture with abiraterone (**1a**) (2.06 g, 5.9 mmol) suspension in dichloromethane (100 ml) and catalytic amount of DMAP (0.005 g, 0.05 mmol) was stirred at room temperature for 4 days. According to LC-MS analysis the reaction mixture contained nonreacted abiraterone (**1a**) ([M+1] = 350), the product of mono-esterification **3** ([M+1] = 466), the product of double acetylation of malic acid hydroxyl group ([M+1] = 582) and trifluoroacetic ester of abiraterone ([M+1] = 446). The reaction mass was concentrated under reduced pressure, the residue was chromatographed on silica gel (eluent: ethyl acetate – petroleum ether 40–70°C, gradient 1:5 – 1:2) to give **3** as white solid (0.044 g, yield 8%).

M.p. 117-119°C.

$^{1}$H NMR (δ, methanol-$d_4$): 0.99 (s, 3 H, $CH_3$); 1.00 (s, 3 H, $CH_3$); 0.95–1.08 (m, 2 H); 1.36–1.43 (m, 1 H); 1.48–1.64 (m, 5 H); 1.69 (td, 1 H, *J* = 4.3, *J* = 10.7); 1.76–1.82 (m, 2 H); 1.92–2.05 (m, 3 H); 2.19 (dd, 1 H, *J* = 3.1, *J* = 6.0); 2.22–2.28 (m, 2 H); 2.59 (dd, 1 H, $CH_2CO_2$, *J* = 7.0, *J* = 16.0); 2.67 (dd, 1 H, $CH_2CO_2$, *J* = 4.8, *J* = 16.0); 4.36 (m, 1 H, C<u>H</u>OH, *J* = 1.6, *J* = 6.0); 4.46–4.54 (m, 1 H, H3); 5.32–5.34

(m, 1 H, H6); 6.12–6.13 (m, 1 H, H16); 7.51 (dd, 1 H, H5Py, $J$ = 5.4, $J$ = 8.0); 8.01 (d, 1 H, H4Py, $J$ = 8.0); 8.38–8.42 (m, 1 H, H6Py); 8.55 (s, 1 H, H2Py).

$^{13}$C NMR (δ, methanol-$d_4$): 17.1 ($CH_3$), 19.5, 19.9 ($CH_3$), 22.1, 28.8, 31.8, 32.0, 32.7, 33.0, 36.4, 38.1, 38.3, 39.1, 40.1, 48.6, 51.9, 59.1, 68.9 (CHOH), 76.4 (C3), 123.5, 123.6, 126.2, 132.9, 138.9, 141.4, 145.6, 145.8, 151.8, 174.1 (C=O), 174.3 (C=O).

MS (ESI): 466.3 [M+H]. Calculated for $C_{28}H_{36}NO_5$: 466.6

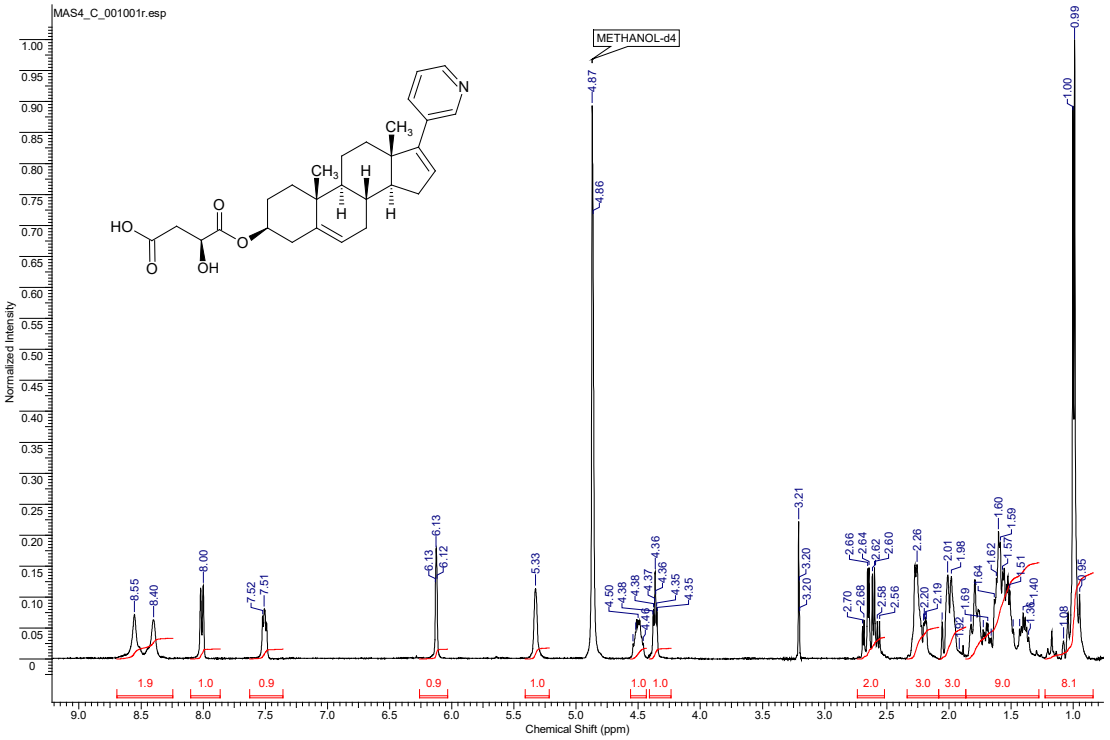



**Figure S2** $^{1}$H NMR spectrum of compound **3**.

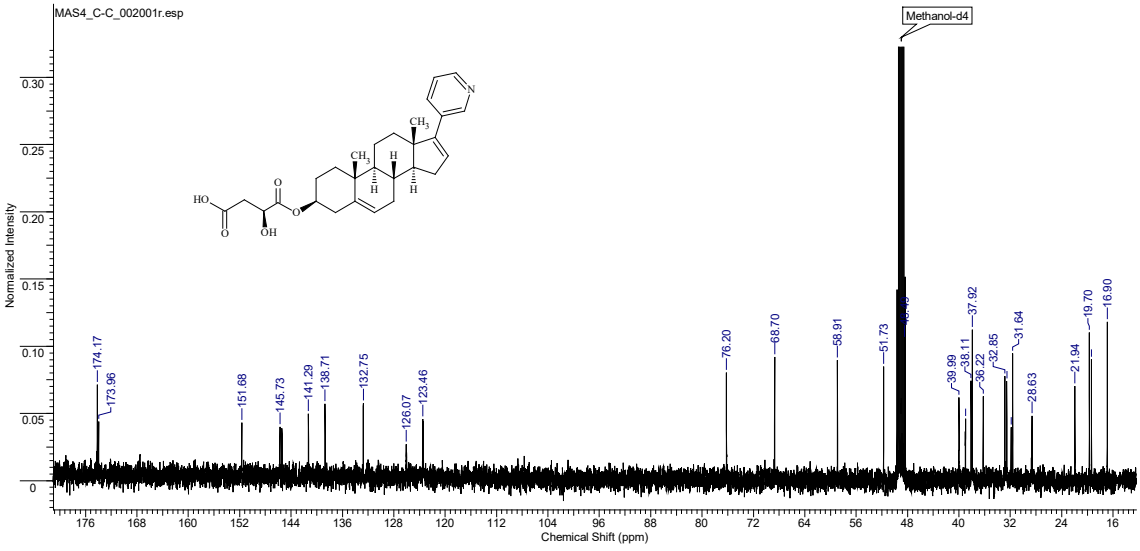



**Figure S3** $^{13}$C NMR spectrum of compound **3**.

**2-(2-(((3b)-17-Pyridin-3-ylandrosta-5,16-dien-3-yl)oxy)-2-oxoethyl)-2-hydroxysuccinic acid (4).** Anhydride of citric acid[S6] (0.25 g, 1.44 mmol) in suspension of abiraterone **1a** (2.51 g, 7.2 mmol) in dichloromethane (80 ml) and catalytic amount of DMAP (0.005 g, 0.05 mmol) was stirred at room temperature for 4 days. The reaction mass was concentrated under reduced pressure, the residue was chromatographed on silica gel (eluent: ethyl acetate – petroleum ether 40–70°C, gradient 1:5 – 1:1) to give **4** as white solid (0.037 g, yield 5%) with purity more than 83% by HPLC data (254 nm) and more than 80% by $^1$H NMR data.

$^1$H NMR (δ, methanol-d$_4$): 1.02 (s, 3 H, $CH_3$); 1.04 (s, 3 H, $CH_3$); 0.98–1.09 (m, 2 H); 1.25–1.28 (m, 1 H); 1.40–1.94 (m, 9 H); 1.98–2.12 (m, 3 H); 2.25–2.31 (m, 3 H); 2.66 (d, 1 H, $CH_2CO_2$, $J$ = 15.6); 2.69 (d, 1 H, $CH_2CO_2$, $J$ = 15.6); 2.79 (d, 1 H, $CH_2CO_2$, $J$ = 15.0); 2.80 (d, 1 H, $CH_2CO_2$, $J$ = 15.0); 4.43–4.54 (m, 1 H, H3); 5.32–5.33 (m, 1 H, H6, $J$ = 4.3); 6.37 (m, 1 H, H16, $J$ = 1.3, $J$ = 3.2); 7.90 (dd, 1 H, H5Py, $J$ = 5.1, $J$ = 8.0); 8.50 (d, 1 H, H4Py, $J$ = 8.0); 8.52 (d, 1 H, H6Py, $J$ = 3.5); 8.75 (s, 1 H, H2Py).

$^{13}$C NMR (δ, methanol-d$_4$): 17.0 ($CH_3$), 20.0 ($CH_3$), 20.74, 22.0, 26.25, 28.5, 31.7, 32.7, 36.4, 37.9, 38.1, 38.9, 39.0, 48.5, 51.8, 59.0, 72.5 (C3), 76.5 (COH), 123.3, 125.0, 130.0, 133.0, 136.0, 141.5, 147.9, 150.6, 152.3, 170.0 (C=O), 173.7 (C=O), 175.5 (C=O).

MS (ESI): 524.3 [M+H]. Calculated for $C_{30}H_{38}NO_7$: 524.6

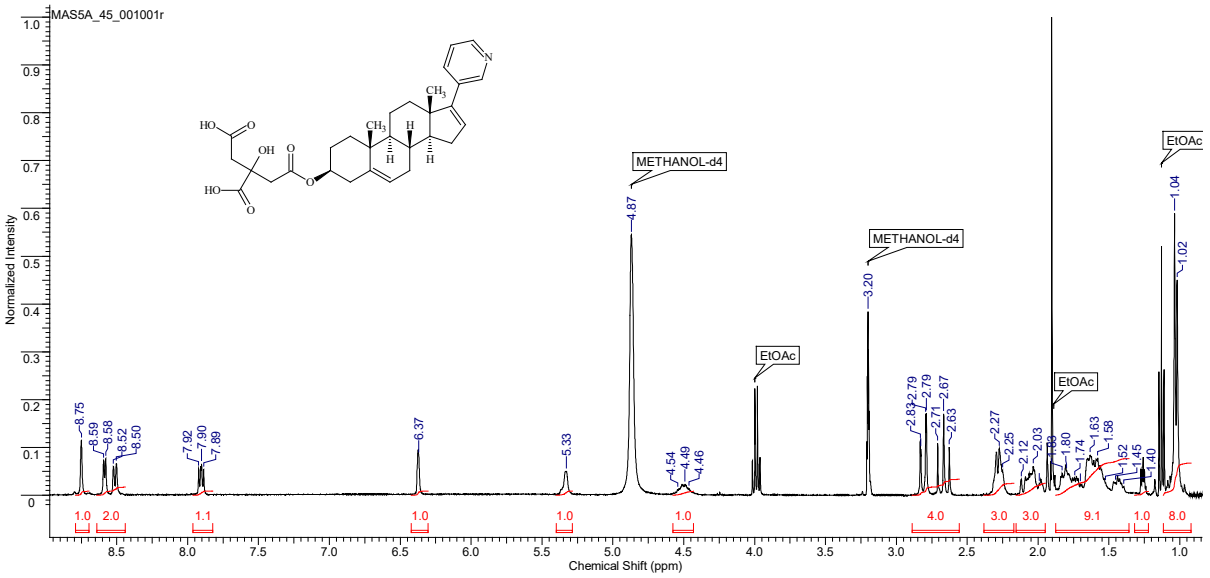



**Figure S4** $^1$H NMR spectrum of compound **4**.

**4-Oxo-4-{[(3b)-17-pyridin-3-ylandrosta-5,16-dien-3-yl]oxy}butanoic acid (succinic acid monoabirateron-3-yl ester) (5)**. A solution of abiraterone **1a** (0.150 g, 0.43 mmol) and succinic anhydride (0.100 g, 1.0 mmol) in anhydrous THF (20 ml) was stirred at reflux in the presence of catalytic amount of DMAP (0.005 g, 0.05 mmol) for 12 h. The mixture was cooled and concentrated under reduced pressure, the residue was chromatographed on silica gel (eluent: ethyl acetate – petroleum ether

40–70°C, gradient 1:3 – 1:1) to give **5** as white solid (0.175 g, yield 91%). M.p. 240-242°C (Lit. M.p. 240-242°C[S7]).

$^1$H NMR (δ, $CDCl_3$): 0.99 (s, 3 H, $CH_3$); 1.02 (s, 3 H, $CH_3$); 0.95–1.13 (m, 2 H); 1.40 (td, 1 H, *J* = 4.9, *J* = 12.2); 1.50–1.73 (m, 7 H); 1.80–1.84 (m, 2 H); 1.94–2.05 (m, 3 H); 2.22 (m, 1 H, *J* = 2.9, *J* = 15.7); 2.28–2.30 (m, 3 H); 2.50–2.55 (m, 2 H); 4.53–4.60 (m, 1 H, H3); 5.35 (d, 1 H, H6, *J* = 3.4); 5.96 (d, 1 H, H16, *J* = 2.9); 7.22 (dd, 1 H, H5Py, *J* = 4.9, *J* = 7.8); 7.65 (d, 1 H, H4Py, *J* = 7.8); 8.34 (d, 1 H, H6Py, *J* = 4.9); 8.50 (s, 1 H, H2Py).

MS (ESI): 450.4 [M+H]. Calculated for $C_{28}H_{36}NO_4$: 450.6

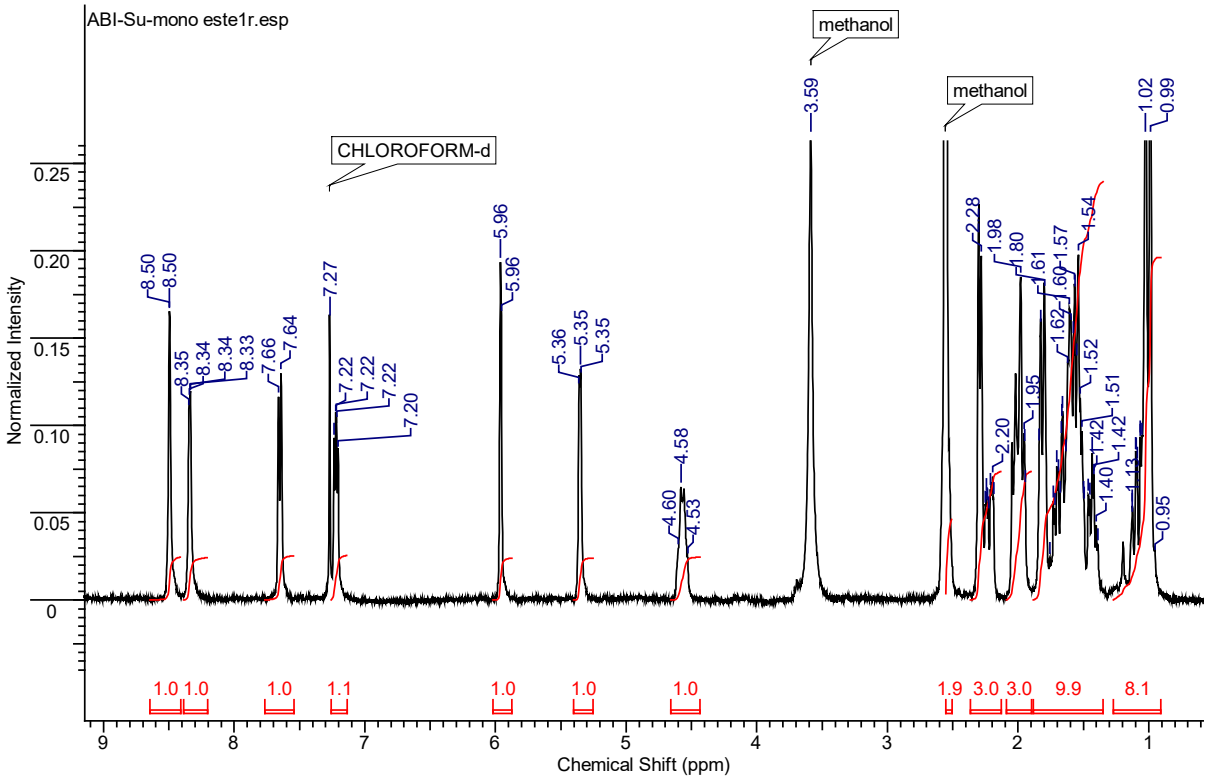



**Figure S5** $^1$H NMR spectrum of compound **5**.

**Bis[(3b)-17-pyridin-3-ylandrosta-5,16-dien-3-yl] butanedioate (6).** A solution of succinic acid monoabirateron ester **5** (0.110 g, 0.24 mmol) and abiraterone **1a** (0.100 g, 0.29 mmol) in anhydrous dichloromethane (20 ml) was stirred with EDCI (0.080 g, 0.42 mmol) and catalytic amount of DMAP (0.005 g, 0.05 mmol) at room temperature for 12 h. The reaction mixture was washed with brine (2*10 ml), dried over $Na_2SO_4$ and concentrated under reduced pressure, the residue was chromatographed on silica gel (eluent: methylene dichloride – methanol, gradient 0 – 1%) to give **6** as a white solid (0.105 g, yield 56%). M.p. 254-256°C.

$^1$H NMR (δ, $CDCl_3$): 1.04 (s, 6 H, $CH_3$); 1.08 (s, 6 H, $CH_3$); 1.04–1.16 (m, 2 H); 1.45–1.78 (m, 16 H); 2.03–2.09 (m, 6 H); 2.27 (m, 2 H, *J* = 2.7, *J* = 15.7); 2.35–2.38 (m, 4 H); 2.61 (m, 4 H); 4.60–4.68 (m,

2 H, H3); 5.41 (d, 2 H, H6, $J$ = 3.9); 6.00 (m, 2 H, H16, $J$ = 1.6); 7.23 (dd, 2 H, H5Py, $J$ = 4.7, $J$ = 7.8); 7.66 (d, 2 H, H4Py, $J$ = 7.8); 8.45 (d, 2 H, H6Py, $J$ = 4.7); 8.62 (s, 2 H, H2Py).

$^{13}$C NMR (δ, $CDCl_3$): 16.5 ($CH_3$), 19.2 ($CH_3$), 20.7, 20.8, 25.3, 27.7, 29.5, 31.5, 31.8, 35.1, 36.7, 36.8, 38.0, 47.3, 50.2, 57.4, 74.2 (C3), 122.3, 123.1, 129.4, 130.1, 133.9, 139.9, 147.6, 147.7, 151.5, 171.7 (C=O).

MS (ESI): 783.0 [M+2H], 392.0. Calculated for $C_{52}H_{66}N_2O_4$: 783.1

Anal. Calcd for $C_{52}H_{64}N_2O_4$: C, 79.96; H, 8.26; N, 3.59; O, 8.19. Found: C, 79.99, H, 8.28, N, 3.62.

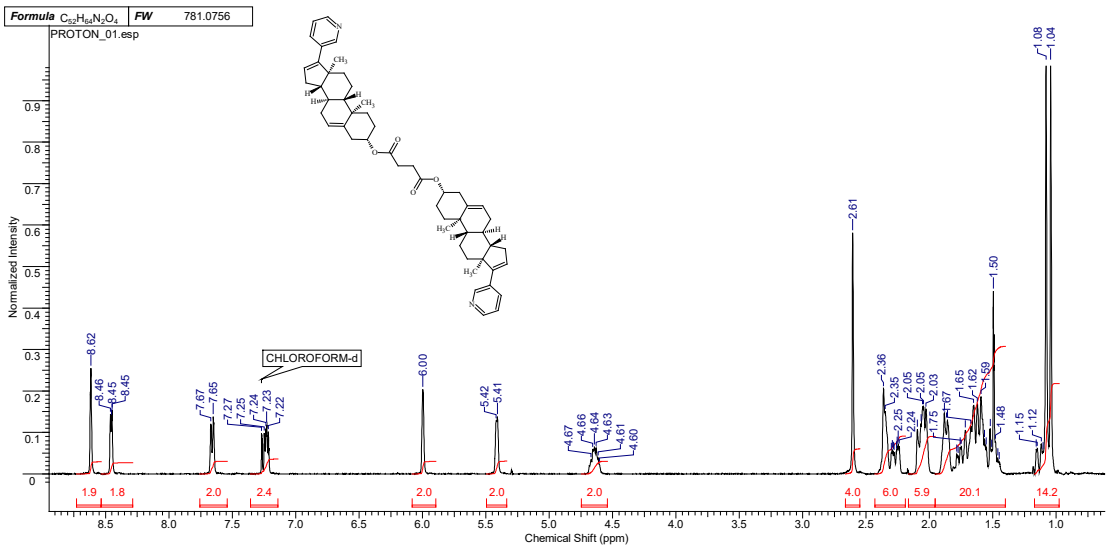



**Figure S6** $^1$H NMR spectrum of compound **6**.

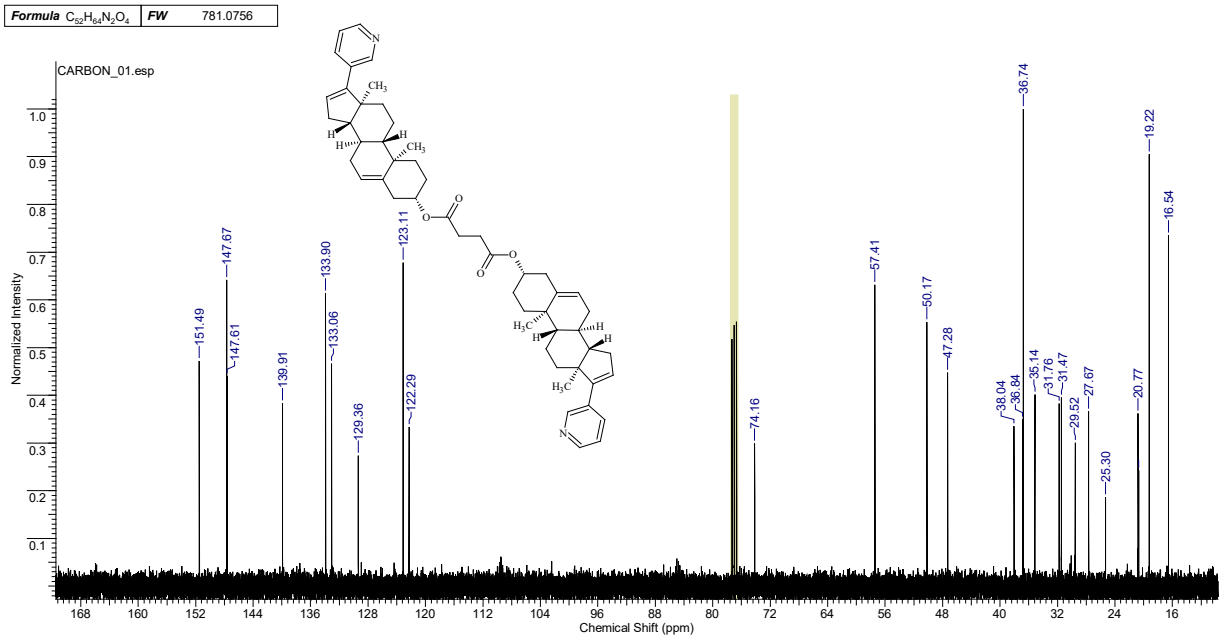



**Figure S7** $^{13}$C NMR spectrum of compound **6**.

## 3. Biochemical and Biological Assays

**Recombinant protein expression and purification.** Human CYP17A1 Δ2-19 variant with C-terminal four-residue histidine tag,[S8] was expressed in *E. coli* JM109 cells. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when $OD_{600}$ have reached 0.8. δ-aminolevulinic acid (δ-ALA) was also added to the culture medium at a final concentration of 1 mM to enhance heme production in cells. The expression was continued for 48 hours at 26 °C with continuous shaking at 130 rpm. At the end of the expression, the cells were harvested by centrifugation at 2500 *g* for 20 minutes and then resuspended in three volumes of 0.1 M potassium phosphate buffer (pH 7.4) that contained 0.5 M NaCl, 20% glycerol and 0.6 mM phenylmethylsulfonyl fluoride (PMSF). After resuspension in the buffer, the cells were disrupted using high-pressure homogenizer Avestin Emulsifex C3 (Avestin, Canada). The protein was solubilized by adding CHAPS detergent to a final concentration of 1.1%. The resulting suspension was centrifuged at 60000 *g* and 4 °C for 1 hour. The obtained supernatant was applied to a Ni-NTA column equilibrated with 0.1 M potassium phosphate buffer (pH 7.4) with 0.2% CHAPS, 0.5 M NaCl, 20% glycerol and 5 mM imidazole (buffer A). After protein immobilization, the column was washed with 30 column volumes of buffer A, containing 25 mM imidazole (buffer B). CYP17A1 was eluted with buffer B using linear gradient of imidazole (25-500 mM). After elution, CYP17A1 was analyzed by SDS-PAGE and MALDI-TOF mass-spectrometry to confirm purity and expected molecular mass (55.6 kDa), respectively. Finally, CHAPS detergent and imidazole were removed by gel-filtration with Sephadex G-15, equilibrated with 0.1 M potassium phosphate buffer (pH 7.4) with 0.5 M NaCl, 20% glycerol and 0.1 M glycine. Protein was concentrated to 150 μM using 30 kDa molecular weight cut-off Amicon Ultra-15 Centrifugal filter units. All purification steps were conducted at 4 °C.

***In vitro* ligand binding assays**. The interaction of compound **6** and abiraterone (as a control) with recombinant human CYP17A1 was measured using differential spectrophotometric titration method. Difference spectra were recorded at 25 °C using dual-beam UV-Vis-NIR spectrophotometer Cary 5000 (Agilent Technologies, USA) equipped with temperature-controlled cuvette holders. All measurements were performed in 0.1 M potassium phosphate buffer (pH 7.4) with 0.15 M NaCl, 20% glycerol at 25 °C in the wavelength range of 350–500 nm. The protein concentration and optical path length were 1 μM and 1 cm, respectively.

For the titration curves fitting, the tight binding equation (Morison equation) (S1) or the simple two site binding equation (S2) was used:

$$\Delta A = \Delta A_{max}\left\{([P_T] + [L_T] + K_D) - \left[([P_T] + [L_T] + K_D)^2 - 4[P_T][L_T]\right]^{\frac{1}{2}}\right\}/2[P_T] \quad (S1)$$

$$\Delta A = \frac{\Delta A_{max1}[L_T]}{K_{D_1} + [L_T]} + \frac{\Delta A_{max2}[L_T]}{K_{D_2} + [L_T]} \quad \text{(S2)}$$

where ΔA is the observed absorbance change, $\Delta A_{max}$ is the maximum absorbance change, $K_D$ is the apparent equilibrium dissociation constant, $[P_T]$ and $[L_T]$ are the total concentrations of CYP17A1 and inhibitor, respectively.

Abiraterone and compound **6** were dissolved in ethanol and in DMF, respectively. An equivalent amount of the appropriate solvent was added to the reference cuvette to eliminate solvent dependent difference spectrum perturbation. The total amount of solvent in the titration experiment did not exceed 2% of the sample volume.

During CYP17A1 titration with compound **6**, a slight increase in the absorbance is observed in the region of 419–425 nm and a decrease at 392 nm, which reflects the interaction of heme iron with the lone electron pair of the nitrogen atom of the pyridine ring in a non-standard fasion. The absence of a clear (sharp) maximum at 425 nm in the difference spectrum theoretically might be associated with the π-interaction of two pyridine rings in the conjugate, leading to the bathochromic shift. Theoretically, parallel interaction of compound **6** in both extended and folded forms is possible. The titration curve for abiraterone is well fitted by the tight binding equation, while a simple two-site binding equation is appropriate for compound **6**. The apparent equilibrium dissociation constant ($K_D$) for the tighter interaction state of compound **6** with CYP17A1 ($K_{D1}$) is 131.5 ± 26 nM ($K_{D2}$ ~ 132 μM), but $\Delta A_{max1}$ is 7 times lower than $\Delta A_{max}$ for abiraterone (0.034). Low value of $\Delta A_{max1}$ is not primarily an indication of low CYP17A1 saturation by an inhibitor, because the presence of a different mode of interaction with the enzyme active site can lead to a mixed averaged difference spectrum. In order to exclude such situation from the consideration, we extra added abiraterone to a final concentration of 1 μM to the solution of CYP17A1 saturated by the compound **6** (Figure 3A, main text). Such abiraterone addition results in almost full saturation of CYP17A1 (type II interaction), indicating that compound **6** specifically interacts only with a small fraction of protein from the conformational ensemble. The affinity of the compound **6** to truncated CYP17A1 is approximately 44 times lower than that of the abiraterone ($K_D$ – 3 ± 1.1 nM). In general, it can be argued that compound **6** is a poor inhibitor of CYP17A1 compared to abiraterone.

**The hydrolysis of conjugate 6 in PBS** was analyzed by means of HPLC system with Shimadzu LC-8A on the chromatographic column Reprosil-Pur C-18-AQ, 10 µm (250×20 mm) equipped with PE SCIEX API 150EX mass detector, and Shimadzu spectrophotometric detector (254 nm) at a flow rate of 25 mL/min in gradient mode with mobile phase MeCN/water +0.05% TFA.

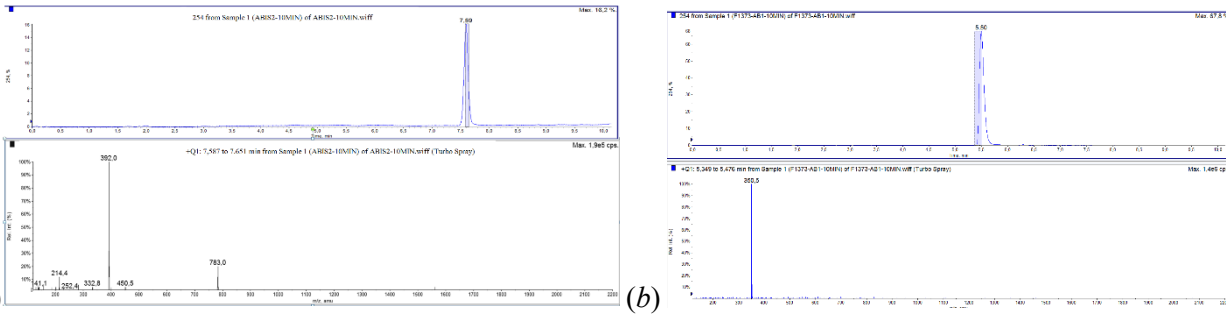



**Figure S8** (*a*) HPLC-MS spectrum for stock solution of compound **6** in PBS (pH 7.4) incubated at 37°C for 72 h, single chromatographic peak of compound **6** (RT = 7.59 min) with corresponding molecular ion [M+2H] = 783.0 is observed, no hydrolysis products are detected; (*b*) reference HPLC-MS spectrum of abiraterone (RT = 5.50 min).

**Quantification of metabolic release** of compound **6 in rat plasma** was carried out using liquid chromatography tandem mass spectrometry method (HPLC-MS/MS). The procedure is based on the incubation of the test or reference compound in rat plasma at 37°C, followed by determination of the content of the remaining compound at points 0, 3 and 22 h. For this determination, at the end of incubation, the reaction was stopped by deproteinization of blood plasma (precipitation of proteins) with ice-cold acetonitrile and a quantitative analysis of the compound was carried out using HPLC-MS/MS. The experiment was carried out in duplicate. Based on the data obtained, the half-life of the test compound is calculated.[S9,S10]

**Reagents** were purchased from the companies indicated in the parentheses: $Na_2HPO_4$ and NaCl (Fluka, USA), $NaH_2PO_4$ (DiaM, Russia), DMSO (Panreac, Spain), HPLC grade acetonitrile (Biosolve, Spain), formic acid (Acros Organics, Germany). Deionized water (18 MΩ*$cm^{-1}$) was obtained using Millipore system (Bedford, MA, USA) and was used in all aqueous solutions. Propranolol hydrochloride (Sigma, USA) served as reference compound.

**Blood plasma** was obtained from adult (10–12-weeks old) male white not purebred rats *Rattus norvegicus* (180-200 g) from laboratory animal nursery branch "Stolbovaya" FSBIS "Scientific Center for Biomedical Technologies FMBA» (Russia, Moscow region). The procedure was carried out in compliance with Ministry of Health of Russia Order No. 199n of Apr. 1, 2016, On Approval of Good Laboratory Practice Rules and was approved by a resolution of the Bioethics Committee of Federal Research Center for Original and Prospective Biomedical and Pharmaceutical Technologies. The

animals were kept in stationary conditions under natural light conditions and a standard diet (complete food, water). Blood was collected after decapitation into vials containing 5% $K_2$EDTA (100 μL per mL of blood). The plasma samples were separated by centrifugation (3000 *rpm*, $4^0$C, 15 min) and stored at -20ºC until used.

**Stock solutions** of the tested compounds were individually prepared in DMSO: 5 mM for compound **6** and 10 mM for propranolol. The standard solutions of tested and reference compounds (10 μM for compound **6** and 20 μM for propranolol) were prepared by diluting the appropriate amounts from individual stock solutions in phosphate-buffered saline (PBS, pH7.4). All stock and working solutions were stored at 4ºC until used.

**Equipment and chromatographic conditions.** The chromatographic separation was achieved using HPLC system Ultimate 3000 (Thermo Scientific, USA). LC conditions are summarized in Table S1.

**Table S1** LC conditions.

<table>
<tr><td>Mobile phase (eluent)</td><td colspan="4">Eluent A – 0.1% formic acid in $H_2O$.<br>Eluent B – 0.1% formic acid in AcCN.</td></tr>
<tr><td rowspan="13">Pump operating mode – Gradient</td><td>Time, min</td><td colspan="2">Flow rate, μL/min</td><td>B%</td></tr>
<tr><td colspan="4">Analyte 6</td></tr>
<tr><td>0.0</td><td colspan="2">1000</td><td>55</td></tr>
<tr><td>1.0</td><td colspan="2">1000</td><td>100</td></tr>
<tr><td>2.2</td><td colspan="2">1000</td><td>100</td></tr>
<tr><td>2.3</td><td colspan="2">1000</td><td>55</td></tr>
<tr><td>3.5</td><td colspan="2">1000</td><td>55</td></tr>
<tr><td colspan="4">Analyte 5, Analyte 1a, propranolol</td></tr>
<tr><td>0.0</td><td colspan="2">1000</td><td>5</td></tr>
<tr><td>1.0</td><td colspan="2">1000</td><td>95</td></tr>
<tr><td>2.2</td><td colspan="2">1000</td><td>95</td></tr>
<tr><td>2.3</td><td colspan="2">1000</td><td>5</td></tr>
<tr><td>3.5</td><td colspan="2">1000</td><td>5</td></tr>
<tr><td>Stationary phase (column)</td><td colspan="4">Luna C18(2), 3 μm, 20×4.0 mm<br>(Phenomenex, USA, part#00M-4251-D0)</td></tr>
<tr><td>Column oven</td><td colspan="4">40ºC</td></tr>
<tr><td>Autosampler temperature</td><td colspan="4">5ºC</td></tr>
<tr><td rowspan="2">Retention time, min</td><td>6</td><td>5</td><td>1a</td><td>Propranolol</td></tr>
<tr><td>2.77±0.01</td><td>1.18±0.01</td><td>1.09±0.01</td><td>0.84±0.01</td></tr>
<tr><td>Injection volume</td><td colspan="4">5 μL</td></tr>
<tr><td>Total analysis time</td><td colspan="4">3.5 min</td></tr>
</table>

Eluents were prepared by mixing of 1 mL of concentrated formic acid either with 1000 mL of deionized water (eluent A) or with 1000 mL of acetonitrile (eluent B). Stored at 4ºC.

**Mass spectrometric detection** was performed on triple quadrupole mass spectrometer TSQ Altis (Thermo Scientific, USA) equipped with a heated electrospray ionization source (HESI). HESI was operated in positive mode. The ion source parameters for all analytes were as follows: ion spray voltage 3500 V; vaporizer temperature 350ºC; sheath gas, auxiliary gas and sweep gas pressure: 60, 15 and 2 arbitrary units, respectively; ion transfer tube temperature 380ºC. Argon pressure in the collision cell (CID Gas) was 1.5 mTorr. Dwell 399.27 (ms). Analytes were detected using SRM (Selected Reaction Monitoring) mode to monitor precursor → product ion transitions (see Table S2).

**Table S2**

| Resolution | Q1=0.7<br>Q3=1.2 | | |
|---|---|---|---|
| SRM transition (Q1/ Q3)<br>(Compound parameters) | Q1<br>Precursor ion, m/z | Q3<br>Product ion, m/z | Collision Energy, eV |
| **6** | $[M+2H]^{2+}$=391.4 | 332.3 | 30 |
| **5** | $[M+H]^{+}$=450.4 | 332.2 | 27 |
| **1a** | $[M+H]^{+}$=350.3 | 156.1 | 55 |
| Propranolol | $[M+H]^{+}$=260.3 | 155.2 | 30 |

Data acquisition and processing were accomplished using Xcalibur v.4.2.28.14 software (Thermo Scientific, USA).

**Sample preparation.** An aliquot of 760 µl of rat plasma and 40 µl of the tested (compound **6**) or reference compound (propranolol) in PBS were pipetted out into the polypropylene tubes Eppendorf (the final concentration of **6** was 0.5 µM, of propranolol – 1 µM). To prepare samples that did not contain compound **6** or propranolol (bl/Blank probes) 40 µl of PBS buffer (pH 7.4) was added to 760 µl of plasma. All samples were prepared in duplicate. The tubes with the reaction mixture were placed in a thermostatic shaker (37ºC).

Sample preparation at time points 0, 3 and 22 h: at a certain time interval, 100 µl of the reaction mixture was transferred to each new tube containing 400 µl of 100% ice-cold acetonitrile and immediately vortexed for 10 sec, then shaken for 15 min on a high-speed shaker and afterwards centrifuged at 13000 *rpm* for 15 min at +4ºC. After centrifugation 100 µl of the supernatant was transferred into chromatographic amber glass vials with screw caps (Agilent, USA) and aliquot of 5 µl was injected into the HPLC-MS/MS system (see results in Table S3).

**Table S3**.

| **Time, h** | **6 (Analyte Area, cps*)** | | | **1a (Analyte Area, cps)** | | | **1a / 6 (%)** | |
|---|---|---|---|---|---|---|---|---|
| | **Mean** | **SD** | **CV, %** | **Mean** | **SD** | **CV, %** | **Mean** | **SD** |
| *22* | 10072.0 | 274.4 | 2.7 | 1176.0 | 86.3 | 7.3 | 11.7% | 0.5% |
| *3* | 17295.0 | 237.6 | 1.4 | 923.0 | 49.5 | 5.4 | 5.3% | 0.4% |
| *0* | 31521.5 | 2393.6 | 7.6 | 345.5 | 54.4 | 15.8 | 1.1% | 0.1% |
| **Blank** | 0 | - | - | 0 | - | - | 0 | - |

*cps – counts per second

**Quantitative determination of half-life ($t_{1/2}$) of compound 6** (the time required for plasma concentration of **6** to decrease by 50%). The value of the peak area of the analyte determines the content of the remaining substance as a percentage of the initial amount, taken as 100% at the zero point ($t$ = 0 min). The first-order reaction rate constant (k) is determined by the tangent of the linear slope of the linear dependence of the logarithm of the % content of the substance on the logarithm of the incubation time: $k$ = [ln (100) - ln (% at t =22 h) ]/ (t = 22 h). Accordingly, the half-life ($t_{1/2}$) is determined by the formula: $t_{1/2}$ (min) = – ln 2/$k$ (see results in Table S4).

**Table S4**.

| **Time, h** | **Remain,%** | | | |
|---|---|---|---|---|
| | **6** | | **Propranolol** | |
| | *Mean* | *CV, %* | *Mean* | *CV, %* |
| *22* | 32.0 | 2.7 | 64.7 | 5.3 |
| *3* | 54.9 | 1.4 | 74.4 | 3.6 |
| *0* | 100.0 | - | 100.0 | - |
| **$t_{1/2}$, h** | **15.9** | | **45.2** | |

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