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Preliminary communication

Identification of fused 16β,17β-oxazinone-estradiol derivatives as a new family of non-estrogenic 17β-hydroxysteroid dehydrogenase type 1 inhibitors

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ABSTRACT

A new family of cyclic carbamate-estradiol derivatives has been designed to remove the intrinsic estrogenic activity of a parent acyclic compound reported as a potent inhibitor of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1). The synthesis of two series of fused 16β,17β-oxazinone-estradiol derivatives, saturated compounds **7a**–**d** and unsaturated compounds **10a**–**d**, led to the identification of **10b**, a 17β-HSD1 inhibitor (IC_{50} = 1.4 μM) without estrogenic activity in estrogen-sensitive T-47D cells. Interestingly, this compound was found selective over 17β-HSD2 and 17β-HSD12. A computational analysis of inhibitors into 17β-HSD1 by molecular docking also revealed interesting structure–activity relationships that could be helpful in the design of new generation of 16β,17β-oxazinone-estradiol analogs.

1. Introduction

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) is involved in the conversion of estrone (E1, Fig. 1) into estradiol (E2), the most potent estrogen in women. This enzyme is also involved in the conversion of dehydroepiandrosterone (DHEA) into 5-androstene-3β,17β-diol (5-diol), a less potent estrogen largely present in post-menopausal women [1–4]. 17β-HSD1 is thus of prime importance for women and has been suspected to play a pivotal role in estrogen-dependent diseases such as breast cancer [5–12], endometrial cancer [13], and endometriosis [14,15]. The development of steroidal inhibitors of 17β-HSD1 was initiated in the eighties and different approaches led to the discovery of several families of inhibitors [16–22]. However, residual estrogenic activity associated with these molecules has often limited their development as therapeutic agents.

In our continuing efforts to obtain non-estrogenic and potent steroidal inhibitors of 17β-HSD1, the 16β-*m*-carbamoylbenzyl-E2 (CC-156, Fig. 1) was identified as a promising inhibitor. Unfortunately, this steroidal derivative showed residual estrogenic activity on estrogen-sensitive cells [23,24]. Consequently, different modifications of A ring of the E2 core of CC-156 have been explored in order to decrease the affinity of the molecule for the estrogen receptor, and hopefully to remove this undesirable estrogenic activity. From these efforts, the first steroidal non-estrogenic and irreversible 17β-HSD1 inhibitor has recently emerged [25–27].

Using a new strategy, we were interested in designing novel mimics of CC-156 toward non-estrogenic reversible inhibitors of 17β-HSD1. Our idea was to rigidify the 16β-side chain of CC-156 around D ring by synthesizing fused oxazinone derivatives at position C15 and C16 of E2 using a diversity-oriented synthesis

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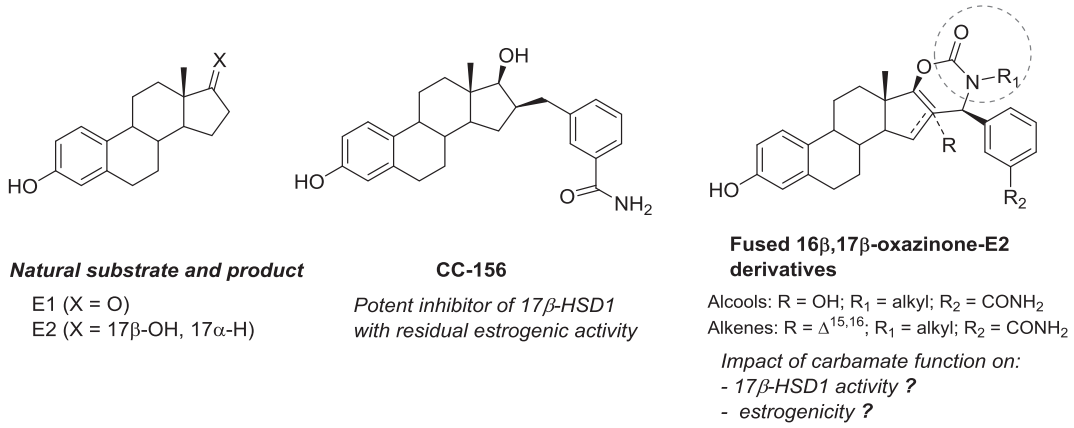


Fig. 1. Structures of 17β-HSD1 natural substrate (E1) and product (E2), potent reversible inhibitor (CC-156) and proposed CC-156 mimics as 16β,17β-oxazinone-E2 derivatives.

methodology [28]. Indeed, the proposed steroidal oxazinones possess a rigid frame that is composed of five fused rings (A, B, C, D and E) with a benzylamide side chain at position C16β (Fig. 1). We were intrigued by the impact on 17β-HSD1 inhibition and estrogenic activity of the introduction of a carbamate functionality between the 17β-OH and the 16β-methylene (CH_2) group of CC-156, as well as the addition of an N-alkyl chain.

2. Results and discussion

2.1. Design

We were initially interested in observing the superimposition potential of the 16β,17β-oxazinone-E2 derivatives over CC-156 lead compound before engaging the synthetic efforts. A flexible alignment of compounds **7a** and **10a** (Scheme 1) with CC-156 supports the potential of this series of E2 derivatives for inhibition of 17β-HSD1 (Fig. 2). Cyclisation through a carbamate formation also opens the door to a new substitution ($OCONR_1$) avenue to further interact with 17β-HSD1. Conformational similarity of these new analogs, which is represented in Fig. 2, was rendered by PyMOL [29]. The result of the superimposition study, through highlighting the conformational similarity of these new analogs, comforts us toward the potential of this series of E2 derivatives as inhibitors of 17β-HSD1.

2.2. Chemical synthesis

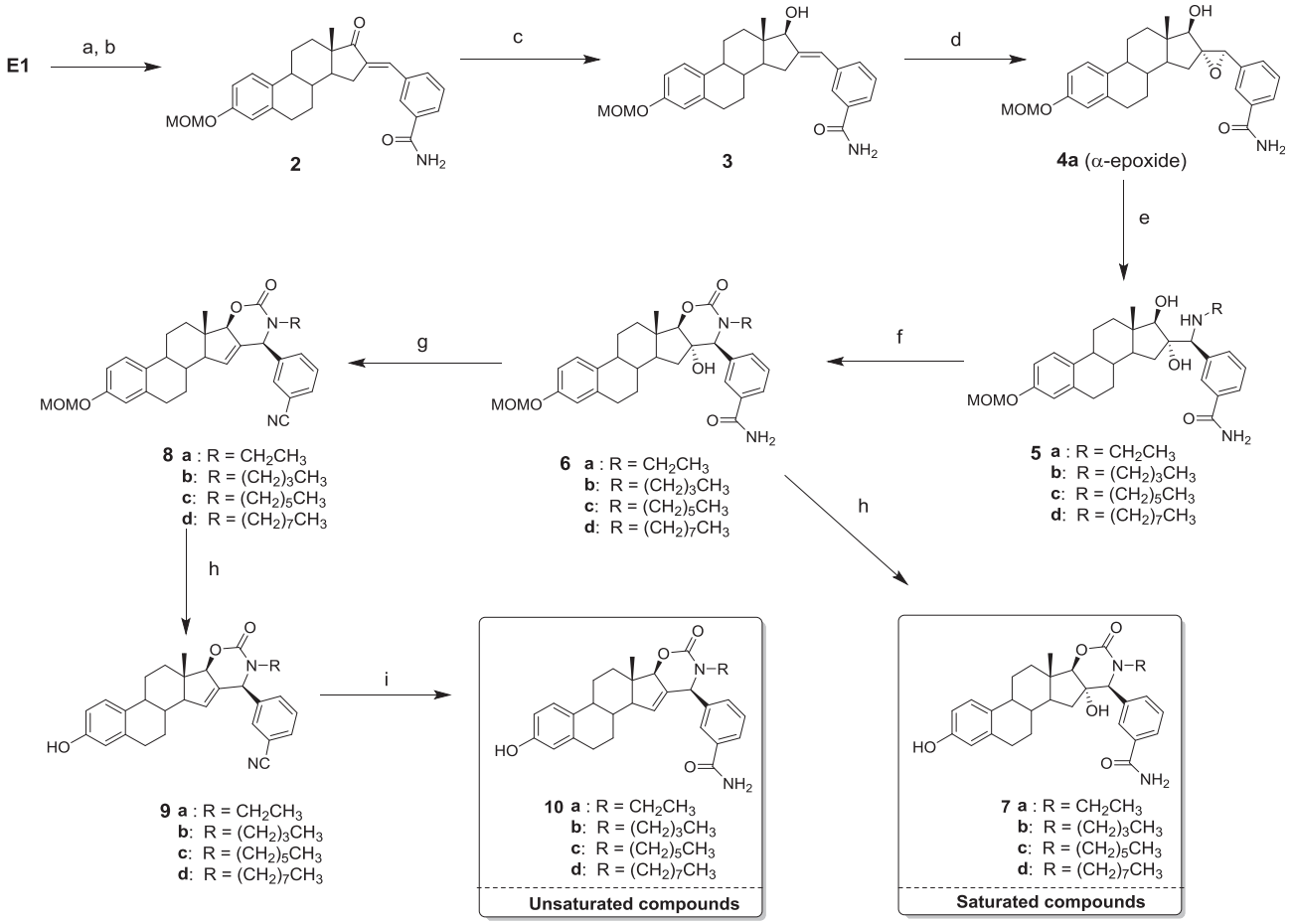
Based on the preliminary results of superimposition, we engaged the synthesis of a series of steroidal oxazinone derivatives, in both saturated (**7a**–**d**) and unsaturated (**10a**–**d**) forms at positions C15 and C16 of E2 (Scheme 1). The chemical route used to obtain the series of proposed molecules was adapted from a diversity-oriented synthesis strategy recently reported by our research group for the synthesis of fused azacycles from molecular ketone templates, differing this time by the presence of a benzamide functionality on the phenyl ring of the azacycle [28]. Thus, we started the synthesis by adding the benzamide chain at position C16 using an aldol condensation reaction between 3-methoxymethyl-estrone and 3-formyl-benzonitrile. In these strong basic reaction conditions, the benzonitrile was readily hydrolyzed *in situ* to carboxamide, giving the steroid **2**. The C17-ketone was then stereoselectively reduced to 17β-OH, giving compound **3** in quantitative yield. The resulting C17–OH group was used in the next step to facilitate the subsequent epoxidation reaction. A mixture of α and β epoxides **4a** and **4b** (35:65) was obtained, easily separated by flash chromatography, and the stereochemistry of each epoxide was determined by NOESY experiments [28].

The aminolysis of α-epoxide **4a** with four appropriate amines under microwave heating conditions gave the β-aminodiols **5a**–**d** as the only isomer considering an axial attack on the epoxide. The treatment of **5a**–**d** with triphosgene then resulted in the formation of cyclic carbamates **6a**–**d**, which under acidic conditions gave the phenolic compounds **7a**–**d** (saturated compounds at positions C15 and C16). The dehydratation of C16-tertiary alcohols **6a**–**d** was performed by a treatment with phosphorous trichloride oxide giving alkenes **8a**–**d** as major elimination compounds at positions C15 and C16. However, under these conditions, the carboxamide group was transformed into a nitrile. The methoxymethylether of **8a**–**d** was hydrolyzed and the benzamide functionality of intermediate compounds **9a**–**d** was regenerated from the corresponding benzonitrile by a reversible hydration reaction [30] using catalytic palladium dichloride and acetamide in a mixture of THF/H_2O, to give the C15,C16-unsaturated derivatives **10a**–**d**.

2.3. 17β-HSD1 inhibition

The two series of oxazinone derivatives (compounds **7a**–**d** and **10a**–**d**) were tested at a concentration of 10 μM for their ability to inhibit the transformation of E1 to E2 by 17β-HSD1 in intact T-47D cells, a breast cancer cell line that expresses 17β-HSD1 [24–26]. The results show interesting tendencies regarding the N-alkyl substituent length (Fig. 3). For both series of compounds, a gradation of inhibitory activity was observed according to N-alkyl chain length with a peak of activity for the N-hexyl chain (compound **7c**) in the saturated series and for the N-butyl chain (compound **10b**) in the unsaturated series. The C15,C16-substitution pattern seems to be important, with a significantly different 17β-HSD1 inhibitory activity observed between the unsaturated and saturated series. Indeed, the N-alkyl derivatives **10a** (N-ethyl) and **10b** (N-butyl) were significantly better inhibitors than their corresponding 16β-OH analogs **7a** and **7b**. However, in the case of longest N-alkyl chain (octyl), this trend was reversed with a negative effect on inhibition observed for **7d** and **10d**.

For the purpose of comparison, the IC_{50} of the best compound of each series was then determined (Fig. 4). Compound **7c** gave IC_{50} values of 6.6 μM and compound **10b** an IC_{50} of 1.4 μM, thus confirming that unsaturated compounds have better inhibitory potential than saturated compounds. Although the IC_{50} value of **10b**



Scheme 1. Synthesis of two series of 16β,17β-oxazinone-E2 saturated derivatives **7a**–**d** and unsaturated derivatives **10a**–**d**. *Reagents and conditions*: (a) methoxymethyl-chloride (MOM-Cl), DIPEA, DCM, rt; (b) 3-CN-benzaldehyde, KOH, EtOH, 100 °C; (c) $NaBH_4$, MeOH, rt; (d) *1.* *m*-CPBA, DCM, rt; *2.* chromatography of epoxides **4a** (α) and **4b** (β); (e) appropiate alkylamine, EtOH, microwave, 170 °C; (f) triphosgene, DIPEA, DCM, rt; (g) $POCl_3$, pyridine, rt; (h) HCl 10% in MeOH, 50 °C; (i) $AcNH_2$, $PdCl_2$, THF/H_2O (3:1).

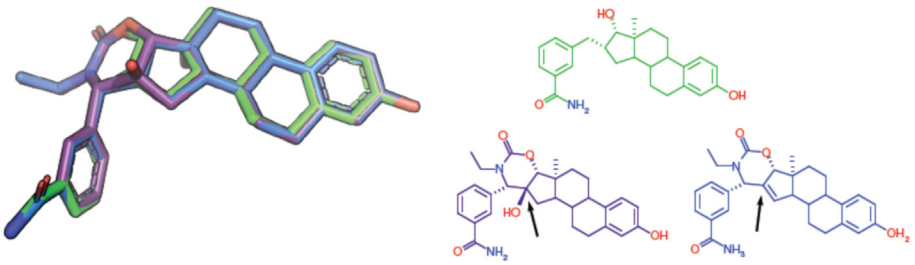


Fig. 2. Sticks (LEFT) and 2D (RIGHT) representation of the flexible alignment of CC-156 (green), compound **7a** (blue) and compound **10a** (purple). Black arrows highlight the difference between compounds **7a** and **10a**. Graphical representations were rendered with PyMOL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(1.4 μM) was considerably higher (about 50 times) than the reported IC_{50} value of CC-156 (0.027 μM) [26], compound **10b** represents a promising scaffold for iterative optimisation considering its potential for diversification at R_1 and R_2 (Fig. 1).

2.4. Estrogenic activity

The estrogenic activity of 17β-HSD1 inhibitors **7c**, **10b** and CC-156 was determined in estrogen-sensitive T-47D cells at two concentrations (Fig. 5). Under these conditions, the potent estrogen E2 stimulated the cell proliferation to 320 and 380% at 0.1 and 0.5 μM, respectively. We were pleased to see that **10b**, the most active compound from the saturated and unsaturated series of compounds, did not stimulate the cells. This result represents an important improvement over the lead compound CC-156, which similarly had a significant stimulatory effect on the proliferation of T-47D cells, as previously reported [23]. Compound **7c** was however found to be estrogenic at 0.5 μM, which potentially highlights the role of 16α-OH in estrogen receptor binding. With the best inhibitory activity on 17β-HSD1 without significant estrogenic activity, compound **10b** was thus retained as the most interesting molecule emerging from both series of saturated and unsaturated oxazinone derivatives.

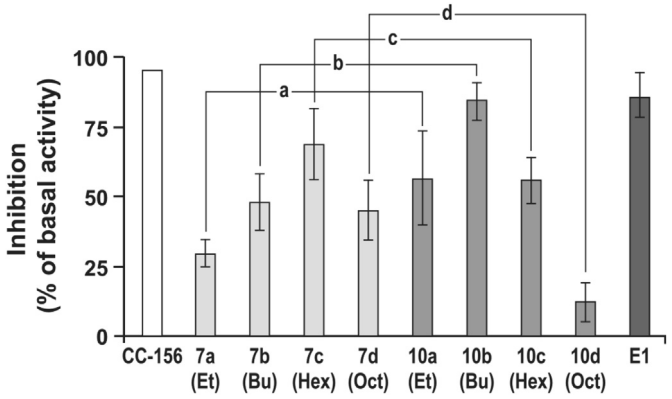


Fig. 3. Inhibition of 17β-HSD1 transformation of [^{14}C]-E1 to [^{14}C]-E2 tested for saturated compounds **7a**–**d** and unsaturated compounds **10a**–**d** compared to potent inhibitor CC-156 and unlabeled substrate (E1) at 10 μM in T-47D cells. Results (±SEM) with the same letter are significantly different: **7a** vs **10a** ($p < 0.01$); **7b** vs **10b** ($p < 0.01$); **7c** vs **10c** (not significant); **7d** vs **10d** ($p < 0.01$).

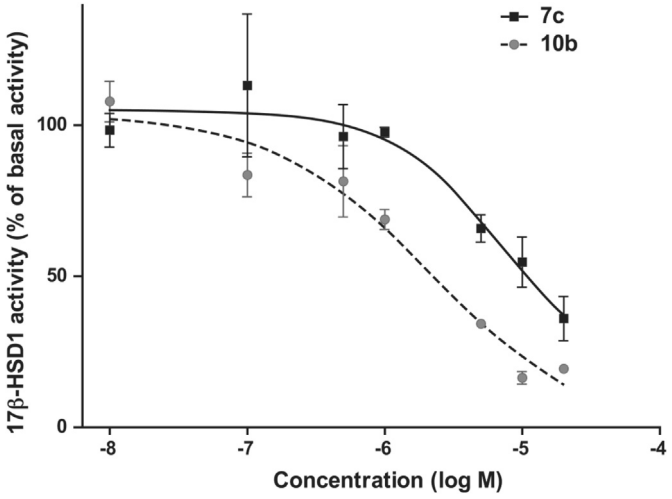


Fig. 4. Inhibition curves for the transformation of [^{14}C]-E1 to [^{14}C]-E2 by 17β-HSD1 in T-47D cells. Different concentrations of compounds **7c** and **10b** were used to determine the IC_{50} values (6.6 and 1.4 μM, respectively).

2.5. Selectivity of **10b** over 17β-HSD2 and 17β-HSD12

Compound **10b** was selected to address its selectivity for 17β-HSD1 inhibition over 17β-HSD2. In fact, to be used in breast cancer therapy, it is very important that an inhibitor of 17β-HSD1 not induce any estrogenic effect on ER^+ cells and not inhibit the oxidation of E2 into E1 by 17β-HSD2. The assay was performed with stably transfected 17β-HSD2 in intact HEK-293 cells using [^{14}C]-E2 as substrate [31]. The compound **10b** did show a very low inhibition of 17β-HSD2 (10 ± 5% and 15 ± 8% at 1 and of 10 μM, respectively) suggesting very good selectivity (data not shown). Furthermore, since we recently reported that saturated oxazinones, among which **7a** and **7b**, can activate the transformation of E1 into E2 by 17β-HSD12 [32], we were also interested in verifying the activity of the unsaturated oxazinone **10b** on this enzyme. As results, we observed that compound **10b** has no effect over 17β-HSD12 at 10 μM (data not shown). This result suggests a fine modulation of 17β-HSDs depending on the substitution nature at positions C15 and C16 of the E2-oxazinone scaffold.

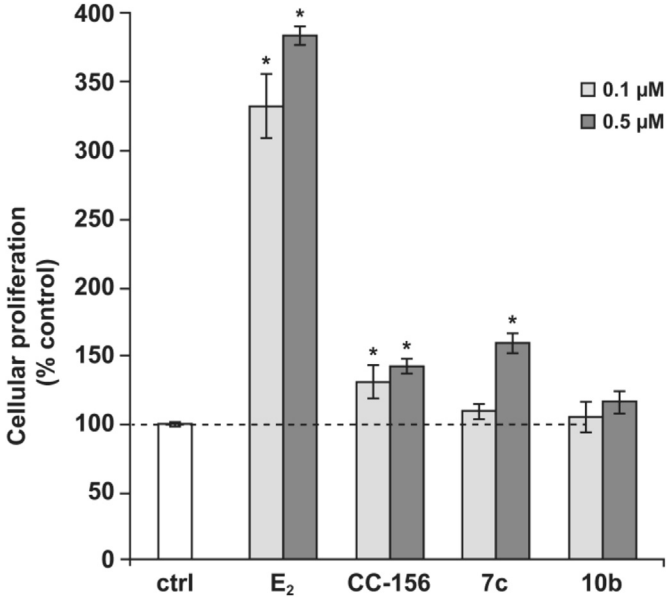


Fig. 5. Estrogenic activity of 17β-HSD1 inhibitors **7c**, **10b** and CC-156 in estrogen-sensitive T-47D cells. *$p < 0.01$ from control (ctrl).

2.6. Molecular modeling

Molecular docking and conformational sampling were achieved to further understand the structural differences between the potent inhibitor CC-156 and the two series of compounds (**7a**–**d** and **10a**–**d**, Scheme 1). The binding site of 17β-HSD1 is divided into two parts: 1) the estradiol (substrate) pocket, which is mainly hydrophobic, but has three H-bonding regions (Glu-282 and His-221; Ser-142; Leu 95) [33], and 2) the NADP (cofactor), which is mainly polar, but has a small non-polar region (Val-188 and Cys-185) that accommodates the nicotinamide moiety [34]. Molecular docking of compounds **7a**–**d** and **10a**–**d** indicated, for all compounds, that the R-alkyl chain is positioned at the same place as the nicotinamide moiety of NADP (Fig. 6, left). This result supports a competitive inhibition of compounds **7** and **10** with both E2 and NADP, and is likely to explain the potency difference with CC-156. Moreover, the short ethyl chain of compounds **7a** and **10a** did not fill the hydrophobic region as much as the medium-length chains (butyl and hexyl) of compounds **7b**–**c** and **10b**–**c**, which had the best activity. The longer octyl chain is more likely to displace water molecules located in the active site, such as in Fig. 6 (middle), where the docked conformation of compound **7d** would expel one or both molecule(s) of water bound to the backbone of Gly-15 and Ile-14. These results are also observed for compounds **7c**–**d** and **10c**–**d**.

The rigidity of the scaffold also impacts on the proper positioning of the alkyl side-chain (R group). A conformational search with simplified molecules (R = methyl and phenyl instead of a benzamide) leads to two scaffolds for series **7** and only one for series **10** (Fig. 7, left). This conformational disparity was increased for compounds **7a** and **10a**, with 17 and 7 conformations, respectively. A more flexible core would allow for a better accommodation of longer chains for the compounds of series **7** (**7d** compared to **10d**), but would also increase the loss of conformational entropy upon binding. The unsaturation generated between carbons C15 and C16, by removing the OH on carbon C16 (compounds **10a-d**) was favorable to the binding as it reduced the conformational space. In docking results, the OH at C16 was found in an hydrophobic environment formed by the side chains of Pro-187 and Phe-

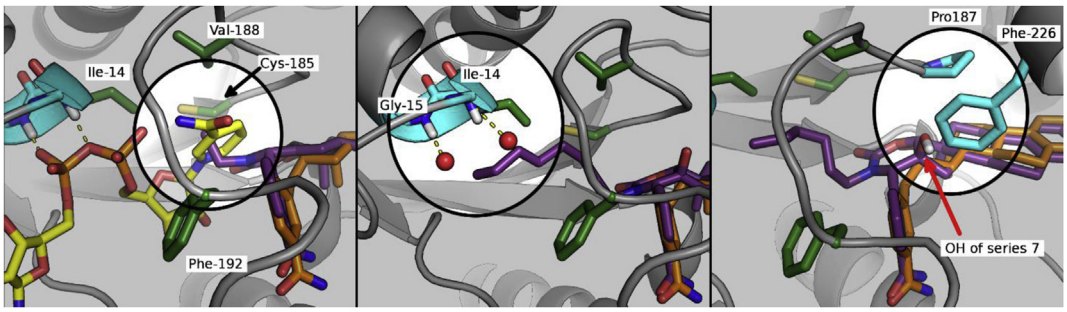


Fig. 6. Protein structure of 17β-HSD1 is represented in gray drawing, the cofactor NADP is represented by yellow sticks and CC-156, used as reference inhibitor, is represented in orange sticks. Docking results are represented in purple sticks. LEFT: Docking results for compound **10a** (purple sticks). Ethyl (R-chain) is positioned between the three amino acids Ile-14, Val-188 and Cys-185, on one side of the pocket, and Phe-192, on the other side. This R chain binds in the same region as the nicotinamide moiety of the NADP (from pdb: 3HB5). MIDDLE: In the absence of NADP, the alpha helix ending with residues Ile-14 and Gly-15 have their backbone N–H linked to water molecules (as in pdb: 3HB4). Docking results of compound **7d**, in purple sticks, show the octyl (R alkyl) chain in an extended conformation that overlaps with these water molecules. RIGHT: the OH group of the series **7** compounds, represented with the docking result of compound **7c** in purple sticks, is positioned far from any direct H-bonding groups and close to Pro-187 and Phe-226 side chains. Hydrogens are absent unless necessary, in which case they are represented by light gray sticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

226 (Fig. 7, right), and did not form any H-bond with the protein. Thus, the two differences between compound series **7** and **10** (the core flexibility and the presence of OH on carbon C16) make compounds **7a**–**d** less likely to bind with 17β-HSD1 than compounds **10a**–**d**. This conclusion is in agreement with the differences in inhibitory potency observed experimentally between compounds **7c** (IC_{50} = 6.6 μM) and **10b** (IC_{50} = 1.4 μM).

3. Conclusion

The development of a non-estrogenic steroidal inhibitor of 17β-HSD1 has been a real challenge for the past forty years, and has been addressed through different strategies. In our effort to reach this goal, we have identified a steroidal inhibitor (CC-156) with strong inhibition potency, but unfortunately with unwanted residual estrogenic activity. As a new strategy to remove this intrinsic estrogenicity, we have evaluated the impact of the rigidification of steroid D-ring and the benzylbenzamide moiety of CC-156 by using a diversity-oriented synthesis approach. We thus synthesized two series (saturated and unsaturated) of novel fused 16β,17β-oxazinone-estradiol derivatives (compounds **7a**–**d** and **10a**–**d**) with varied N-alkyl side chains. The compounds were tested on 17β-HSD1 and showed interesting inhibitions related to their level of saturation at positions C15 and C16 (unsaturated > saturated) as well as their N-alkyl substituent (hexyl = butyl > ethyl >> octyl). Furthermore, the two best candidates identified, compounds **7c** and **10b**, showed selectivity over 17β-HSD2 and most importantly, the best inhibitor identified (**10b**; IC_{50} = 1.4 μM) showed no significant estrogenic activity in estrogen-sensitive (ER^+) T-47D cells. Interestingly, the unsaturated (C15–C16) compound **10b** differs from saturated analogs (compounds **7a**–**d**) by showing no activation effect on 17β-HSD12. This result seems to point to a fine modulation of 17β-HSDs regarding to the saturation level at positions C15 and C16. Finally, the results of the molecular modeling study were in agreement with the inhibition results by showing that the presence of an OH group on carbon 16 makes the compounds **7a**–**d** of saturated series less likely to bind 17β-HSD1 than compounds **10a**–**d** of saturated series. Thus, a novel molecular template was identified as a new family of non-estrogenic selective inhibitors of 17β-HSD1 with a large potential for iterative optimization.

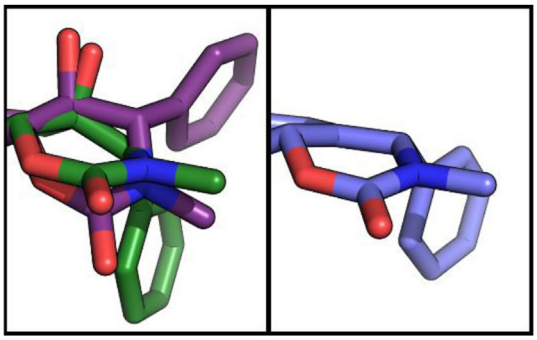


Fig. 7. Stick representation of the two accessible scaffold conformations for a simplified compound of series **7** (LEFT) and the only accessible scaffold conformation for a simplified compound of series **10** (RIGHT). For the simplified compounds of each series, the benzamide group was replaced by a phenyl and the side chain (R) is a methyl.

4. Experimental section

4.1. Chemical synthesis

4.1.1. General

The reagents for chemical synthesis were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). The usual solvents were obtained from Fisher Scientific (Montréal, QC, Canada) and were used as received. Anhydrous tetrahydrofuran (THF) and anhydrous dichloromethane (DCM) were from Sigma–Aldrich. Chemical steps using microwave heating were performed with the Initiator microwave synthesizer from Biotage (Charlotte, NC, USA). Phase separator syringes were purchased from Biotage (Isolute phase separator, 6 mL). Thin-layer chromatography (TLC) and flash-column chromatography were performed on 0.20-mm silica gel 60 F254 plates and with Silicycle R10030B 230-400-mesh silica gel (Québec, QC, Canada). Infrared spectra (IR) were recorded on a ABB model MB3000 FT-IR spectrophotometer (Québec, QC, Canada), and the significant bands were reported in cm^{-1}. Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz for ^1H and 100.6 MHz for ^{13}C with a Bruker Avance 400 digital spectrometer (Billerica, MA, USA). The chemical shifts (δ) are expressed in ppm and referenced to acetone (2.06 ppm, ^1H, and 29.8 ppm, ^{13}C), methanol (3.31 ppm, ^1H, and 49.0 ppm, ^{13}C) or chloroform (7.26 ppm, ^1H, 77.0 ppm, ^{13}C). In addition to the classic ^1H NMR and ^{13}C NMR data reported in experimental procedures, representative compounds were also analyzed by HSQC, HMBC, COSY and NOESY experiments to support the assignment of protons and carbons. The

numbering used for assignment of NMR signals was reported in Fig. 8. The HPLC purity of the final compounds was determined with a Shimadzu apparatus using a Shimadzu SPD-M20A Photodiode array detector, an Altima HPC18 reversed-phase column (250 mm × 4.6 mm, 5 μm) and as solvent gradient of MeOH:H_2O. The wavelength of the UV detector was selected between 190 and 205 nm. Low-resolution mass spectra (LRMS) were recorded on a Perkin–Elmer Sciex API-150ex apparatus (Foster City, CA, USA) equipped with a turbo ion-spray source and expressed in *m/z*. The names of new compounds were obtained using ACD/Labs (Chemist's version) software (Toronto, ON, Canada).

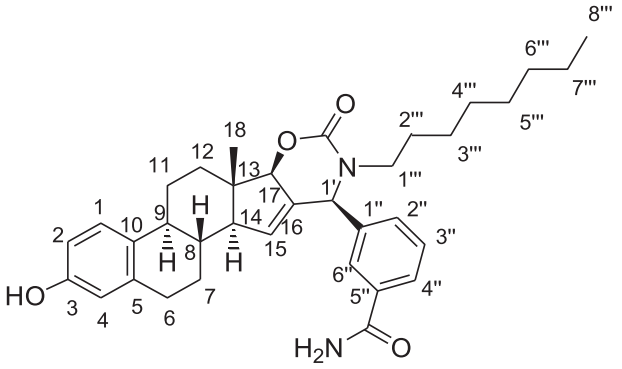


Fig. 8. Carbon numbering used for the assignment of representative ^{1}H NMR and ^{13}C NMR signals.

*4.1.2. General procedure for the synthesis of compounds **5a–d***

To a solution of α-epoxide **4a** [32] (500 mg, 1.08 mmol) in absolute ethanol (5 mL) was added the appropriate primary amine (10.0 mmol). The solution was placed in a microwave reactor and heated at 170 °C for 12 h. The reaction mixture was then evaporated under reduced pressure to dryness and purified by flash chromatography (DCM/MeOH, 95:5 to 90:10) to give compounds **5a–d**. Characterization of compounds **5a** and **5b** was previously reported [32].

*4.1.2.1. 3-[(S)-[(16α,17β)-16,17-dihydroxy-3-(methoxymethoxy) estra-1(10),2,4-trien-16-yl](hexylamino)methyl]benzamide (**5c**).* White amorphous solid (50 mg, 41%). IR (KBr) υ: 3402 (NH_2, NH and OH), 1666 ($CONH_2$); ^{1}H NMR (Acetone-d_6) δ: 0.86 (t, J = 6.8 Hz, 6‴-CH_3), 0.89 (s, 18-CH_3), 1.20–1.62 (m, 7α-CH, 8β-CH, 11β-CH, 12α-CH, 14α-CH, 15α-CH_3, 3‴-CH_2, 4‴-CH_2 and 5‴-CH_2), 1.80 (m, 7β-CH), 1.95 (m, 15β-CH and 12β-CH), 2.21 (m, 9α-CH), 2.32 (m, 11α-CH and 1H of 1‴-CH_2), 2.48 (m, 1H of 1‴-CH_2), 2.78 (m, 6-CH_2), 3.41 (s, CH_3O), 3.74 (s, 17α-CH), 3.94 (s, 1′-CH), 5.13 (s, OCH_2O), 6.60 (broad s, 1H of $CONH_2$), 6.71 (d, J = 2.5 Hz, 4-CH), 6.79 (dd, J_1 = 8.5 Hz, J_2 = 2.6 Hz, 2-CH), 7.19 (d, J = 8.5 Hz, 1-CH), 7.41 (t, J = 7.7 Hz, 3″-CH), 7.48 (broad s, 1H of $CONH_2$), 7.65 (d, J = 7.6 Hz, 2″-CH), 7.83 (d, J = 7.7 Hz, 4″-CH), 8.05 (s, 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 12.3 (C18), 14.3 (C6‴), 23.2 (C5‴), 27.4 (C11), 27.7 (C4‴), 28.5 (C7 and C3‴), 30.0 (C6), 32.4 (C2‴), 35.5 (C15), 38.7 (C8), 39.6 (C12), 45.0 (C9), 45.9 (C13), 47.0 (C14), 47.9 (C1‴), 55.8 (CH_3O), 68.5 (C1′), 82.7 (C16), 94.0 (C17), 95.0 (OCH_2O), 114.6 (C2), 117.0 (C4), 126.9 (C1 and C4″), 128.4 (C3″), 129.1 (C6″), 133.1 (C2″), 134.0 (C5″), 134.6 (C10), 138.5 (C5), 141.7 (C1″), 156.2 (C3), 167.2 ($CONH_2$); LRMS for $C_{34}H_{49}N_2O_5$ $[M+H]^+$: 565.4.

*4.1.2.2. 3-[(S)-[(16α,17β)-16,17-dihydroxy-3-(methoxymethoxy) estra-1(10),2,4-trien-16-yl](octylamino)methyl]benzamide (**5d**).* White amorphous solid (40 mg, 31%). IR (KBr) υ: 3348 (NH_2, NH and OH), 1666 ($CONH_2$); ^{1}H NMR (Acetone-d_6) δ: 0.86 (t, J = 6.8 Hz, 8‴-CH_3), 0.89 (s, 18-CH_3), 1.20–1.62 (m, 7α-CH, 8β-CH, 11β-CH, 12α-CH, 14α-CH, 15α-CH_3, 4‴-CH_2, 5‴-CH_2, 6‴-CH_2 and 7‴-CH_2), 1.80 (m, 7β-CH), 1.95 (m, 15β-CH and 12β-CH), 2.21 (m, 9α-CH), 2.32 (m, 11α-CH and 1H of 1‴-CH_2), 2.48 (m, 1H of 1‴-CH_2), 2.78 (m, 6-CH_2), 3.41 (s, CH_3O), 3.74 (s, 17α-CH), 3.94 (s, 1′-CH), 5.13 (s, OCH_2O), 6.59 (br s, 1H of $CONH_2$), 6.71 (d, J = 2.5 Hz, 4-CH), 6.79 (dd, J_1 = 8.5 Hz, J_2 = 2.6 Hz, 2-CH), 7.19 (d, J = 8.5 Hz, 1-CH), 7.41 (t, J = 7.7 Hz, 3″-CH), 7.42 (br s, 1H of $CONH_2$), 7.65 (d, J = 7.6 Hz, 2″-CH); 7.83 (d, J = 7.7 Hz, 4″-CH), 8.05 (s, 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 12.3 (C18), 14.3 (C8‴), 23.3 (C7‴), 27.4 (C11), 28.0 (C3‴), 28.5 (C7), 30.0 (C6), 30.1 (C4‴), 32.5 (C5‴), 34.6 (C2‴ and C6‴), 35.5 (C15), 38.7 (C8), 39.5 (C12), 45.0 (C9), 45.9 (C13), 47.0 (C14), 47.9 (C1‴), 55.8 (CH_3O), 68.5 (C1′), 84.3 (C16), 94.0 (C17), 95.0 (OCH_2O), 114.6 (C2), 117.0 (C4), 127.0 (C1 and C4″), 128.4 (C3″), 129.1 (C6″), 133.1 (C2″), 134.0 (C5″), 134.6 (C10), 138.5 (C5), 142.3 (C1″), 156.1 (C3), 167.2 ($CONH_2$); LRMS for $C_{36}H_{53}N_2O_5$ $[M+H]^+$: 593.4.

*4.1.3. General procedure for the synthesis of compounds **6a–d***

To a solution of aminodiol **5a**, **5b**, **5c** or **5d** (0.1 mmol) in anhydrous DCM (5 mL) was added triphosgene (0.1 mmol) and diisopropylethylamine (DIPEA) (0.3 mmol) at room temperature under an argon atmosphere. The solution was stirred for 30 min at room temperature. The solution was diluted with DCM (25 mL), washed with water, filtered over a phase-separator (Biotage), and evaporated under reduced pressure. The crude compound was purified by flash chromatography (DCM/MeOH, 97:3) to give compound **6a**, **6b**, **6c** or **6d**. Characterization of compounds **6a** and **6b** was previously reported [32].

*4.1.3.1. 3-[(4bS,6aS,6bR,10S,10aS,11aS,11bR)-9-hexyl-10a-hydroxy-2-(methoxymethoxy)-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,10a,11,11a,11b,12,13-tetradecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzamide (**6c**).* White amorphous solid (18 mg, 39%). IR (KBr) υ: 3402 (NH_2 and OH), 1666 (OCON and $CONH_2$); ^{1}H NMR (Acetone-d_6) δ: 0.40 (s, 18-CH_3), 0.85 (t, J = 7.0 Hz, 6‴-CH_3), 1.34 (m, 7α-CH, 8β-CH, 11β-CH, 15β-CH, 3‴-CH_2, 4‴-CH_2 and 5‴-CH_2), 1.54 (m, 12α-CH and 2‴-CH_2), 1.81 (m, 14α-CH, 7β-CH and 15α-CH), 1.99 (m, 12β-CH), 2.26 (m, 9α-CH and 11α-CH), 2.74 (m, 6-CH_2), 2.89 and 3.67 (2m, 1‴-CH_2), 3.39 (s, CH_3O), 4.12 (s, 17α-CH), 4.77 (s, 1′-CH), 5.11 (s, OCH_2O), 6.68 (d, J = 2.5 Hz, 4-CH), 6.71 (broad s, 1H of $CONH_2$), 6.76 (dd, J_1 = 8.6 Hz, J_2 = 2.6 Hz, 2-CH), 7.15 (d, J = 8.6 Hz, 1-CH), 7.39 (broad m, 2″-CH), 7.49 (t, J = 7.9 Hz, 3″-CH and 1H of $CONH_2$), 7.88 (d, J = 7.2 Hz, 4″-CH), 7.89 (s, 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 12.5 (C18), 14.3 (C6‴), 23.2 (C5‴), 27.0 (C11 and C4‴), 28.4 (C7), 28.6 (C3‴), 30.1 (C6), 32.5 (C2‴), 38.0 (C8), 38.6 (C12 and C15), 44.6 (C9), 45.1 (C13), 48.4 (C14), 49.5 (C1‴), 55.8 (CH_3O), 72.0 (C1′), 82.7 (C16), 95.0 (OCH_2O), 97.8 (C17), 114.6 (C2), 117.0 (C4), 127.0 (C1), 127.4 (C4″ and C6″), 129.1 (C2″ and C3″), 134.0 (C10), 135.3 (C5″), 138.4 (C5), 139.5 (C1″), 156.2 (C3), 156.3 (OCON), 168.5 ($CONH_2$); LRMS calculated for $C_{35}H_{47}N_2O_6$ $[M+H]^+$: 591.4.

*4.1.3.2. 3-[(4bS,6aS,6bR,10S,10aS,11aS,11bR)-9-octyl-10a-hydroxy-2-(methoxymethoxy)-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,10a,11,11a,11b,12,13-tetradecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzamide (**6d**).* White amorphous solid (10 mg, 32%). IR (film) υ: 3356 (NH_2 and OH), 1674 (OCON and $CONH_2$); ^{1}H NMR (Acetone-d_6) δ: 0.40 (s, 18-CH_3), 0.86 (t, J = 6.8 Hz, 8‴-CH_3), 1.28 (m, 7α-CH, 8β-CH, 11β-CH, 15β-CH, 3‴-CH_2, 4‴-CH_2, 5‴-CH_2, 6‴-CH_2 and 7‴-CH_2), 1.53 (m, 12α-CH and 2‴-CH_2), 1.80 (m, 14α-CH, 7β-CH and 15α-CH), 1.99 (m, 12β-CH), 2.26 (m, 9α-CH and 11α-CH), 2.74 (m, 6-CH_2), 2.93 and 3.68 (2m, 1‴-CH_2), 3.39 (s, CH_3O), 4.12 (s, 17α-CH), 4.77 (s, 1′-CH), 5.11 (s, OCH_2O), 6.68 (d, J = 2.5 Hz, 4-CH), 6.70 (broad s, 1H of $CONH_2$), 6.76

(dd, J_1 = 8.6 Hz, J_2 = 2.6 Hz, 2-CH), 7.15 (d, J = 8.6 Hz, 1-CH), 7.39 (broad m, 2″-CH), 7.49 (t, J = 7.9 Hz, 3″-CH and 1H of $CONH_2$), 7.77 (d, J = 7.0 Hz, 4″-CH), 7.88 (s, 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 12.5 (C18), 14.3 (C8‴), 23.3 (C7‴), 27.0 (C11), 27.1 (C6‴), 27.4 (C5‴), 28.4 (C7), 28.6 (C4‴), 29.9 (C3‴) 30.1 (C6, 32.5 (C2‴), 38.0 (C8), 38.6 (C12 and C15), 44.6 (C9), 45.1 (C13), 48.4 (C14), 49.5 (C1‴), 55.8 (CH_3O), 72.0 (C1′), 82.7 (C16), 95.0 (OCH_2O), 97.8 (C17), 114.6 (C2), 117.0 (C4), 127.0 (C1), 127.5 (C4″ and C6″), 129.1 (C2″ and C3″), 134.0 (C10), 135.3 (C5″), 138.4 (C5), 139.5 (C1″), 156.2 (C3), 156.3 (OCON), 168.5 ($CONH_2$); LRMS for $C_{37}H_{51}N_2O_6$ $[M+H]^+$: 619.5.

*4.1.4. General procedure for the synthesis of compounds **7a**–**d***

To a solution of compound **6a**, **6b**, **6c** or **6d** in MeOH (3.0 mL) was added an aqueous solution of 10% HCl. The reaction mixture was stirred overnight at 60 °C and then poured into a saturated solution of sodium bicarbonate. The solution was extracted with EtOAc, washed with brine, dried over sodium sulfate, filtered, and evaporated under reduced pressure. The crude compound was purified by flash chromatography (DCM/MeOH, 9:1) to give compound **7a**, **7b**, **7c** or **7d**. Characterization of compounds **7a** and **7b** was previously reported [32].

*4.1.4.1. 3-[(4bS,6aS,6bR,10S,10aS,11aS,11bR)-9-hexyl-2,10a-dihydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,10a,11,11a,11b,12,13-tetradecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzamide (**7c**).* White solid (8 mg, 55%). IR (KBr) υ: 3379 (NH_2 and OH), 1674 (OCON and $CONH_2$); ^{1}H NMR (Acetone-d_6) δ: 0.39 (s, 18-CH_3), 0.84 (t, J = 7.0 Hz, 6‴CH_3), 1.29 (m, 7α-CH, 8β-CH, 11β-CH, 15β-CH and 3‴-CH_2, 4‴-CH_2 and 5‴-CH_2), 1.52 (m, 12α-CH and 2‴-CH_2), 1.79 (m, 14α-CH, 7β-CH and 15α-CH), 1.98 (m, 12β-CH), 2.17 (m, 9α-CH), 2.28 (m, 11α-CH), 2.68 (m, 6-CH_2), 2.9 (under H_2O solvent peak) and 3.66 (2m, 1‴-CH_2), 4.11 (s, 17α-CH), 4.77 (d, J = 3.8 Hz, 1′-CH), 6.48 (d, J = 2.5 Hz, 4-CH), 6.56 (dd, J_1 = 8.5 Hz, J_2 = 2.5 Hz, 2-CH), 6.70 (broad s, 1H of $CONH_2$), 7.04 (d, J = 8.5 Hz, 1-CH), 7.39 (broad d, 2″-CH), 7.48 (t, J = 8.0 Hz, 3″-CH), 7.50 (broad s, 1H of $CONH_2$), 7.88 (d, J = 7.3 Hz, 4″-CH), 8.02 (s, 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 12.5 (C18), 14.1 (C6‴), 23.2 (C5‴), 27.0 (C4‴), 27.1 (C11), 28.5 (C7 and C3‴), 30.2 (C6), 32.3 (C2‴), 38.2 (C8), 38.6 (C12 and C15), 44.6 (C9), 45.1 (C13), 48.4 (C14), 49.5 (C1‴), 72.0 (C1′), 82.8 (C16), 97.9 (C17), 113.6 (C2), 115.9 (C4), 126.9 (C1), 127.5 (C4″ and C6″), 129.1 (C2″ and C3″), 131.5 (C10), 134.0 and 135.2 (C5″), 138.2 (C5), 139.5 (C1″), 156.0 (C3), 156.0 (OCON), 168.7 ($CONH_2$); LRMS for $C_{33}H_{43}N_2O_5$ $[M+H]^+$: 547.4; HPLC purity: 97.5%.

*4.1.4.2. 3-[(4bS,6aS,6bR,10S,10aS,11aS,11bR)-9-octyl-2,10a-dihydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,10a,11,11a,11b,12,13-tetradecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzamide (**7d**).* White solid (7 mg, 70%). ^{1}H NMR (Acetone-d_6) δ: 0.39 (s, 18-CH_3), 0.86 (t, J = 6.9 Hz, 8‴-CH_3), 1.33 (m, 7α-CH, 8β-CH, 11β-CH, 15β-CH, 3‴-CH_2, 4‴-CH_2, 5‴-CH_2, 6‴-CH_2 and 7‴-CH_2), 1.52 (m, 12α-CH and 2‴-CH_2), 1.78 (m, 14α-CH, 7β-CH and 15α-CH), 1.98 (m, 12β-CH), 2.17 (m, 9α-CH), 2.28 (m, 11α-CH), 2.70 (m, 6-CH_2), 2.93 and 3.67 (2m, 1‴-CH_2), 4.12 (s, 17α-CH), 4.71 (s, OH), 4.76 (d, J = 3.8 Hz, 1′-CH), 6.48 (d, J = 2.5 Hz, 4-CH), 6.57 (dd, J_1 = 8.4 Hz, J_2 = 2.6 Hz, 2-CH), 6.70 (broad s, 1H of $CONH_2$), 7.05 (d, J = 8.6 Hz, 1-CH), 7.40 (broad d, 2″-CH), 7.48 (t, J = 8.0 Hz, 3″-CH and 1H of $CONH_2$), 7.87 (d, J = 6.2 Hz, 4″-CH), 7.93 (s, 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 12.5 (C18), 14.2 (C8‴), 23.3 (C7‴), 27.1 (C11 and C6‴), 28.5 (C7 and C5‴), 28.6 (C4‴), 29.9 (C3‴), 30.1 (C6), 32.5 (C2‴), 38.2 (C8), 38.6 (C12 and C15), 44.6 (C9), 45.1 (C13), 48.4 (C14), 49.5 (C1‴), 72.0 (C1′), 82.8 (C16), 97.9 (C17), 113.6 (C2), 115.9 (C4), 127.0 (C1), 127.5 (C4″ and C6″), 129.1 (C2″ and C3″), 131.6 (C10), 134.0 and 135.2 (C5″), 138.2 (C5), 139.5 (C1″), 156.0 (C3), 156.3 (OCON), 168.5 ($CONH_2$); LRMS for $C_{35}H_{47}N_2O_5$ $[M+H]^+$: 575.4; HPLC purity: 93.7%.

*4.1.5. General procedure for the synthesis of compounds **8a**–**d***

To a solution of compound **6a**, **6b**, **6c** or **6d** (0.2 mmol) in dry pyridine was added $POCl_3$ (10 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature overnight and then poured into water. The solution was extracted with EtOAc, washed with brine, dried over sodium sulfate, filtered, and evaporated under reduced pressure. The crude compound was purified by flash chromatography (EtOAc/hexanes, 2:8) to give compound **8a**, **8b**, **8c** or **8d** as major compound.

*4.1.5.1. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-ethyl-2-(methoxymethoxy)-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzonitrile (**8a**).* White solid (46 mg, 42%). IR (film) υ: 2230 (CN), 1697 (OCON); ^{1}H NMR (Acetone-d_6) δ: 0.92 (s, 18-CH_3), 1.03 (t, J = 7.1 Hz, 2‴-CH_3), 1.36 (m, 7α-CH), 1.53 (8β-CH and 11β-CH), 1.84 (m, 12α-CH), 1.97 (m, 7β-CH), 2.06 (m, 12β-CH), 2.26 (m, 9α-CH and 14α-CH), 2.39 (m, 11α-CH), 2.75 (m, 6-CH_2) 2.75 and 3.59 (2m, 1‴-CH_2), 3.39 (CH_3O), 4.92 (s, 17α-CH), 5.12 (OCH_2O), 5.46 (m, 1′-CH), 5.63 (d, J = 2.1 Hz, 15-CH), 6.70 (d, J = 2.6 Hz, 4-CH), 6.79 (dd, J_1 = 8.6 Hz, J_2 = 2.6 Hz, 2-CH), 7.18 (d, J = 8.6 Hz, 1-CH), 7.65 (d, J = 7.9 Hz, 2″-CH), 7.69 (t, J = 7.6 Hz, 3″-CH), 7.80 (m, 4″-CH and 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 12.9 (C18), 13.3 (C2‴), 26.7 (C11), 28.4 (C7), 30.0 (C6), 35.0 (C12), 36.6 (C8), 42.8 (C1‴), 45.0 (C9), 52.0 (C13), 55.8 (CH_3O), 56.6 (C14), 60.3 (C1′), 89.0 (C17), 95.0 (OCH_2O), 113.7 (C5″), 114.6 (C2), 117.0 (C4), 119.1 (CN), 126.9 (C1), 129.3 (C15), 131.2 (C3″), 131.9 (C6″), 132.6 (C2″), 132.7 (C4″), 134.0 (C10), 138.3 (C5), 138.8 (C1″), 144.0 (C16), 154.2 (C3), 156.2 (OCON); LRMS for $C_{31}H_{35}N_2O_4$ $[M+H]^+$: 499.4.

*4.1.5.2. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-butyl-2-(methoxymethoxy)-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzonitrile (**8b**).* White solid (40 mg, 29%). IR (film) υ: 2230 (CN), 1697 (OCON); ^{1}H NMR (Acetone-d_6) δ: 0.85 (t, J = 7.3 Hz, 4‴CH_3), 0.92 (s, 18-CH_3), 1.20–1.60 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2 and 3‴-CH_2), 1.85 (m, 12α-CH), 1.98 (m, 7β-CH), 2.06 (m, 12β-CH), 2.26 (m, 9α-CH and 14α-CH), 2.39 (m, 11α-CH), 2.60 and 3.64 (2m, 1‴-CH_2), 2.80 (m, 6-CH_2), 3.40 (CH_3O), 4.94 (s, 17α-CH), 5.12 (OCH_2O), 5.46 (t, J = 2.1 Hz, 1′-CH), 5.67 (d, J = 1.9 Hz, 15-CH), 6.70 (d, J = 2.6 Hz, 4-CH), 6.79 (dd, J_1 = 8.6 Hz, J_2 = 2.6 Hz, 2-CH), 7.18 (d, J = 8.6 Hz, 1-CH), 7.63 (d, J = 7.9 Hz, 2″-CH), 7.70 (t, J = 7.6 Hz, 3″-CH), 7.80 (m, 4″-CH and 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 13.3 (C18), 14.0 (C4‴), 20.5 (C3‴), 26.7 (C11), 28.3 (C7), 30.0 (C6 and C2‴), 35.0 (C12), 36.6 (C8), 45.0 (C9), 47.4 (C1‴), 52.0 (C13), 55.8 (CH_3O), 56.6 (C14), 60.5 (C1′), 89.0 (C17), 95.0 (OCH_2O), 113.7 (C5″), 114.7 (C2), 117.1 (C4), 119.1 (CN), 126.9 (C1), 129.2 (C15), 131.2 (C3″), 131.8 (C6″), 132.5 (C2″), 132.7 (C4″), 134.0 (C10), 138.3 (C5), 138.8 (C1″), 144.0 (C16), 154.6 (C3), 156.2 (OCON); LRMS for $C_{33}H_{39}N_2O_4$ $[M+H]^+$: 527.4.

*4.1.5.3. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-hexyl-2-(methoxymethoxy)-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzonitrile (**8c**).* White solid (35 mg, 22%). IR (KBr) υ: 2230 (CN), 1690 (OCON); ^{1}H NMR (Acetone-d_6) δ: 0.85 (t, J = 7.0 Hz, 6‴-CH_3), 0.91 (s, 18-CH_3), 1.20–1.65 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2, 3‴-CH_2, 5‴-CH_2 and 4‴-CH_2), 1.85 (m, 12α-CH), 1.98 (m, 7β-CH), 2.06 (m, 12β-CH), 2.23 (m, 9α-CH and 14α-CH), 2.38 (m, 11α-CH), 2.63 and 3.61 (2m, 1‴-CH_2), 2.77 (m, 6-CH_2), 3.40 (CH_3O), 4.93 (s, 17α-CH), 5.12 (OCH_2O), 5.46 (t, J = 2.1 Hz, 1′-CH), 5.67 (d, J = 1.8 Hz, 15-CH), 6.70 (d, J = 2.5 Hz, 4-CH), 6.79 (dd, J_1 = 8.6 Hz, J_2 = 2.6 Hz, 2-CH), 7.17 (d, J = 8.6 Hz, 1-CH), 7.64 (d, J = 6.4 Hz, 2″-CH), 7.69 (t, J = 7.6 Hz, 3″-CH), 7.80 (m, 4″-CH and 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 13.2 (C18), 14.3 (C6‴), 23.2 (C5‴), 26.7 (C11), 27.7 (C4‴) 28.3 (C7), 30.0 (C6),

30.2 (C3‴), 34.6 (C2‴), 35.0 (C12), 36.6 (C8), 45.0 (C9), 47.8 (C1‴), 52.0 (C13), 55.8 (CH_3O), 56.6 (C14), 60.6 (C1′), 89.0 (C17), 95.0 (OCH_2O), 113.7 (C5″), 114.6 (C2), 117.0 (C4), 119.1 (CN), 126.8 (C1), 129.2 (C15), 131.2 (C3″), 131.8 (C6″), 132.5 (C2″), 132.7 (C4″), 134.0 (C10), 138.3 (C5), 138.8 (C1″), 144.0 (C16), 154.5 (C3), 156.2 (OCON); LRMS for $C_{35}H_{43}N_2O_4$ $[M+H]^+$: 555.4.

*4.1.5.4. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-octyl-2-(methoxymethoxy)-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzonitrile (**8d**).* White solid (40 mg, 19%). IR (film) υ: 2230 (CN), 1697 (OCON); ^{1}H NMR (Acetone-d_6) δ: 0.87 (t, J = 6.9 Hz, 8‴-CH_3), 0.92 (s, 18-CH_3), 1.20–1.65 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2, 3‴-CH_2, 4‴-CH_2, 5‴-CH_2, 6‴-CH_2 and 7‴-CH_2), 1.85 (m, 12α-CH), 1.98 (m, 7β-CH), 2.06 (m, under solvent peak, 12β-CH), 2.28 (m, 9α-CH and 14α-CH), 2.37 (m, 11α-CH), 2.63 and 3.61 (2m, 1‴-CH_2), 2.78 (m, under solvent peak, 6-CH_2), 3.40 (CH_3O), 4.93 (s, 17α-CH), 5.13 (OCH_2O), 5.46 (t, J = 2.1 Hz, 1′-CH), 5.67 (d, J = 1.8 Hz, 15-CH), 6.71 (d, J = 2.5 Hz, 4-CH), 6.79 (dd, J_1 = 8.5 Hz, J_2 = 2.5 Hz, 2-CH), 7.18 (d, J = 8.6 Hz, 1-CH), 7.64 (d, J = 6.4 Hz, 2″-CH), 7.70 (t, J = 7.6 Hz, 3″-CH), 7.80 (m, 4″-CH and 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 13.3 (C18), 14.3 (C8‴), 23.3 (C7‴), 26.6 (C11), 27.3 (C6‴), 27.7 (C5‴) 28.3 (C7), 29.9 (C4‴), 30.0 (C6 and C3‴), 32.5 (C2‴), 35.0 (C12), 36.6 (C8), 45.0 (C9), 47.8 (C1‴), 52.0 (C13), 55.8 (CH_3O), 56.6 (C14), 60.6 (C1′), 89.0 (C17), 95.0 (OCH_2O), 113.7 (C5″), 114.6 (C2), 117.0 (C4), 119.1 (CN), 126.8 (C1), 129.2 (C15), 131.2 (C3″), 131.8 (C6″), 132.5 (C2″), 132.7 (C4″), 133.9 (C10), 138.3 (C5), 138.8 (C1″), 144.0 (C16), 154.5 (C3), 156.2 (OCON); LRMS for $C_{37}H_{47}N_2O_4$ $[M+H]^+$: 583.4.

*4.1.6. General procedure for the synthesis of compounds **9a–d***

To a solution of compound **8a**, **8b**, **8c** or **8d** in MeOH (3.0 mL) was added an aqueous solution of 10% HCl. The solution was stirred overnight at 60 °C and then poured into a saturated solution of sodium bicarbonate. The solution was extracted with EtOAc, washed with brine, dried over sodium sulfate, filtered, and evaporated under reduced pressure. The crude compound was purified by flash chromatography (DCM/MeOH, 9:1) to give compounds **9a, 9b**, **9c** or **9d**.

*4.1.6.1. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-ethyl-2-hydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzonitrile (**9a**).* White solid (39 mg, 95%). IR (KBr) υ: 3387 (OH), 2230 (CN), 1666 (OCON); ^{1}H NMR ($CDCl_3$) δ: 0.91 (s, 18-CH_3), 1.07 (t, J = 7.1 Hz, 2‴-CH_3), 1.36 (m, 7α-CH), 1.55 (8β-CH and 11β-CH), 1.78 (m, 12α-CH), 1.88 (m, 7β-CH), 2.11 (m, 12β-CH and 14α-CH), 2.26 (m, 9α-CH), 2.32 (m, 11α-CH), 2.72 and 3.70 (m, 1‴-CH_2), 2.75 (m, 6-CH_2), 4.88 (s, 17α-CH), 5.15 (m, 1′-CH), 5.44 (d, J = 1.6 Hz, 15-CH), 5.70 (broad s, OH), 6.56 (d, J = 2.5 Hz, 4-CH), 6.65 (dd, J_1 = 8.5 Hz, J_2 = 2.5 Hz, 2-CH), 7.09 (d, J = 8.5 Hz, 1-CH), 7.48 (d, J = 8.0 Hz, 2″-CH), 7.55 (t, J = 7.7 Hz, 3″-CH and 6″-CH), 7.67 (d, J = 7.6 Hz, 4″-CH); ^{13}C NMR ($CDCl_3$) δ: 12.5 (C18), 12.8 (C2‴), 25.9 (C11), 27.6 (C7), 29.1 (C6), 34.4 (C12), 35.6 (C8), 42.2 (C1‴), 44.2 (C9), 51.6 (C13), 55.9 (C14), 60.0 (C1′), 88.5 (C17), 112.9 (C2), 113.2 (C5″), 115.3 (C4), 118.2 (CN), 126.2 (C1), 129.7 (C15), 130.2 (C3″), 130.8 (C6″), 131.5 (C2″), 131.6 (C10), 132.2 (C4″), 136.6 (C5), 137.7 (C1″), 142.0 (C16), 153.7 (OCON), 154.2 (C3); LRMS for $C_{29}H_{31}N_2O_3$ $[M+H]^+$: 455.3.

*4.1.6.2. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-butyl-2-hydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzonitrile (**9b**).* White solid (6 mg, 95%). IR (film) υ: 3340 (OH), 2230 (CN), 1674 (OCON); ^{1}H NMR (Acetone-d_6) δ: 0.85 (t, J = 7.3 Hz, 4‴-CH_3), 0.91 (s, 18-CH_3), 1.20–1.60 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2 and 3‴-CH_2), 1.86 (m, 12α-CH), 1.96 (m, 7β-CH), 2.06 (m, under solvent peak, 12β-CH), 2.24 (m, 9α-CH and 14α-CH), 2.36 (m, 11α-CH), 2.61 and 3.62 (2m, 1‴-CH_2), 2.73 (m, 6-CH_2), 4.94 (q, J = 1.9 Hz, 17α-CH), 5.46 (t, J = 2.1 Hz, 1′-CH), 5.67 (d, J = 1.9 Hz, 15-CH), 6.50 (d, J = 2.5 Hz, 4-CH), 6.59 (dd, J_1 = 8.4 Hz, J_2 = 2.6 Hz, 2-CH), 7.08 (d, J = 8.5 Hz, 1-CH), 7.63 (d, J = 7.9 Hz, 2″-CH), 7.70 (t, J = 7.6 Hz, 3″-CH), 7.78 (m, 4″-CH and 6″-CH); ^{13}C NMR ($CDCl_3$) δ: 12.8 (C18), 13.8 (C4‴), 19.9 (C3‴) 25.9 (C11), 27.6 (C7), 29.1 (C6 and C2‴), 34.3 (C12), 35.6 (C8), 44.2 (C9), 46.8 (C1‴), 51.6 (C13), 55.9 (C14), 60.0 (C1′), 88.4 (C17), 112.9 (C2), 113.2 (C5″), 115.3 (C4), 118.3 (CN), 126.3 (C1), 129.5 (C15), 130.2 (C3″), 130.7 (C6″), 131.3 (C2″), 131.8 (C10), 132.2 (C4″), 136.7 (C5), 137.7 (C1″), 142.1 (C16), 153.5 (OCON), 154.4 (C3); LRMS for $C_{31}H_{35}N_2O_3$ $[M+H]^+$: 483.3.

*4.1.6.3. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-hexyl-2-hydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzonitrile (**9c**).* White solid (30 mg, 90%). IR (film) υ: 3340 (OH), 2230 (CN), 1674 (OCON); ^{1}H NMR (Acetone-d_6) δ: 0.85 (t, J = 6.9 Hz, 6‴CH_3), 0.91 (s, 18-CH_3), 1.15–1.65 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2, 3‴-CH_2, 4‴-CH_2 and 5‴-CH_2), 1.84 (m, 12β-CH), 1.97 (m, 7β-CH), 2.06 (m, under solvent peak, 12α-CH), 2.23 (m, 9α-CH and 14α-CH), 2.37 (m, 11α-CH), 2.63 and 3.61 (2m, 1‴-CH_2), 2.73 (m, 6-CH_2), 4.94 (s, 17α-CH), 5.46 (t, J = 2.1 Hz, 1′-CH), 5.67 (d, J = 1.9 Hz, 15-CH), 6.50 (d, J = 2.5 Hz, 4-CH), 6.59 (dd, J_1 = 8.4 Hz, J_2 = 2.6 Hz, 2-CH), 7.07 (d, J = 8.5 Hz, 1-CH), 7.63 (d, J = 7.9 Hz, 2″-CH), 7.69 (t, J = 7.6 Hz, 3″-CH), 7.79 (m, 4″-CH and 6″-CH), 7.99 (s, OH); ^{13}C NMR (Acetone-d_6) δ: 13.3 (C18), 14.3 (C6‴), 23.2 (C5‴), 26.7 (C11), 27.0 (C4‴), 27.7 (C3‴) 28.4 (C7), 30.1 (C6), 32.2 (C2‴), 35.0 (C12), 36.8 (C8), 45.0 (C9), 47.8 (C1‴), 52.0 (C13), 56.6 (C14), 60.6 (C1′), 89.0 (C17), 113.6 (C2), ~114 (C5″), 116.0 (C4), 119.1 (CN), 126.8 (C1), 129.3 (C15), 131.2 (C3″), 131.4 (C10), 131.8 (C6″), 132.5 (C2″), 132.7 (C4″), 138.2 (C5), 138.7 (C1″), 144.0 (C16), 154.6 (OCON), 156.1 (C3); LRMS for $C_{33}H_{39}N_2O_3$ $[M+H]^+$: 511.4.

*4.1.6.4. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-octyl-2-hydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzonitrile (**9d**).* White solid (27 mg, 95%). IR (KBr) υ: 3348 (OH), 2230 (CN), 1666 (OCON); ^{1}H NMR (Acetone-d_6) δ: 0.87 (t, J = 6.9 Hz, 8‴CH_3), 0.91 (s, 18-CH_3), 1.15–1.65 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2, 3‴-CH_2, 4‴-CH_2, 5‴-CH_2, 6‴-CH_2 and 7‴-CH_2), 1.84 (m, 12α-CH), 1.97 (m, 7β-CH), 2.06 (m, under solvent peak, 12β-CH), 2.22 (m, 9α-CH and 14α-CH), 2.36 (m, 11α-CH), 2.63 and 3.62 (2m, 1‴-CH_2), 2.74 (m, 6-CH_2), 4.93 (s, 17α-CH), 5.46 (t, J = 2.1 Hz, 1′-CH), 5.66 (d, J = 1.9 Hz, 15-CH), 6.51 (d, J = 2.5 Hz, 4-CH), 6.59 (dd, J_1 = 8.4 Hz, J_2 = 2.5 Hz, 2-CH), 7.07 (d, J = 8.5 Hz, 1-CH), 7.63 (d, J = 7.9 Hz, 2″-CH), 7.69 (t, J = 7.7 Hz, 3″-CH), 7.79 (m, 4″-CH and 6″-CH), 7.99 (s, OH); ^{13}C NMR (Acetone-d_6) δ: 13.3 (C18), 14.4 (C8‴), 23.3 (C7‴), 26.8 (C11), 27.4 (C6‴), 27.8 (C5‴) 28.4 (C7), 29.9 (C4‴), 30.0 (C6), 30.1 (C3‴), 32.5 (C2‴), 35.1 (C12), 36.8 (C8), 45.1 (C9), 47.8 (C1‴), 52.1 (C13), 56.6 (C14), 60.6 (C1′), 89.0 (C17), 113.6 (C2), 113.7 (C5″), 116.0 (C4), 119.1 (CN), 126.8 (C1), 129.3 (C15), 131.2 (C3″), 131.4 (C10), 131.8 (C6″), 132.5 (C2″), 132.7 (C4″), 138.2 (C5), 138.7 (C1″), 144.0 (C16), 154.6 (OCON), 156.1 (C3); LRMS for $C_{35}H_{43}N_2O_3$ $[M+H]^+$: 539.5.

*4.1.7. General procedure for the synthesis of compounds **10a–d***

To a solution of compound **9a**, **9b**, **9c** or **9d** (0.09 mmol) in THF (3 mL) was added water (1 mL), acetamide (0.9 mmol) and $PdCl_2$ (0.14 mmol). The solution was stirred at 50 °C for 3 h under argon atmosphere. The resulting mixture was poured in water, extracted with EtOAc, washed with brine, dried over sodium sulfate, filtered, and evaporated under reduced pressure. The crude compound was purified by flash chromatography (DCM/MeOH, 95:5 to 90:10) to give compound **10a**, **10b**, **10c** or **10d**.

*4.1.7.1. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-ethyl-2-hydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzamide (**10a**).* White solid (15 mg, 38%). IR (KBr) υ: 3356 (OH and NH_2), 1666 (OCON and $CONH_2$); ^{1}H NMR (Acetone-d_6) δ: 0.93 (s, 18-CH_3), 1.02 (t, J = 7.1 Hz, 2‴-CH_3), 1.32 (m, 7α-CH), 1.53 (8β-CH and 11β-CH), 1.82 (m, 12α-CH), 1.90 (m, 7β-CH), 2.06 (m, under solvent peak, 12β-CH), 2.18 (m, 9α-CH and 14α-CH), 2.35 (m, 11α-CH), 2.71 (m, 6-CH_2), 2.74 and 3.57 (m, 1‴-CH_2), 4.90 (t, J = 1.6 Hz, 17α-CH), 5.39 (t, J = 2.2 Hz, 1′-CH), 5.52 (d, J = 1.8 Hz, 15-CH), 6.50 (d, J_1 = 2.5 Hz, 4-CH), 6.59 (dd, J_1 = 8.5 Hz, J_2 = 2.6 Hz, 2-CH), 6.75 (broad s, 1H of $CONH_2$), 7.07 (d, J = 8.5 Hz, 1-CH), 7.46 (d, J = 7.7 Hz, 2″-CH), 7.54 (t, J = 8.3 Hz, 3″-CH), 7.62 (broad s, 1H of $CONH_2$), 7.95 (m, 4″-CH and 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 12.8 (C2‴), 13.4 (C18), 26.8 (C11), 28.4 (C7), 29.9 (C6), 35.1 (C12), 36.8 (C8), 42.6 (C1‴), 45.1 (C9), 52.0 (C13), 56.6 (C14), 60.8 (C1′), 89.0 (C17), 113.6 (C2), 116.0 (C4), 126.8 (C1), 127.7 (C4″), 128.0 (C6″), 128.8 (C15), 129.9 (C3″), 130.9 (C2″), 131.4 (C10), 136.0 (C1″), 138.2 (C5), 139.2 (C5″), 142.5 (C16), 154.3 (OCON), 156.1 (C3), 168.6 ($CONH_2$); LRMS for $C_{29}H_{33}N_2O_4$ $[M+H]^+$: 473.3; HPLC purity: 97.5%.

*4.1.7.2. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-butyl-2-hydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzamide (**10b**).* White solid (12 mg, 55%). IR (KBr) υ: 3356 (OH and NH_2), 1666 (OCON and $CONH_2$); ^{1}H NMR (CD_3OD) δ: 0.85 (t, J = 7.3 Hz, 4‴-CH_3), 0.94 (s, 18-CH_3), 1.20–1.60 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2 and 3‴-CH_2), 1.86 (m, 12α-CH and 7β-CH), 2.08 (m, 12β-CH and 14α-CH), 2.18 (m, 9α-CH), 2.34 (m, 11α-CH), 2.68 and 3.56 (2m, 1‴-CH_2), 2.72 (m, 6-CH_2), 4.94 (s, 17α-CH), 5.34 (t, J = 2.1 Hz, 1′-CH), 5.49 (d, J = 1.7 Hz, 15-CH), 6.44 (d, J_1 = 2.5 Hz, 4-CH), 6.53 (dd, J_1 = 8.5 Hz, J_2 = 2.6 Hz, 2-CH), 7.05 (d, J = 8.5 Hz, 1-CH), 7.42 (d, J = 7.8 Hz, 2″-CH), 7.53 (t, J = 7.7 Hz, 3″-CH), 7.82 (d, J = 1.6 Hz, 4″-CH) 7.87 (dd, J_1 = 6.4 Hz, J_2 = 1.4 Hz, 6″-CH); ^{13}C NMR (CD_3OD) δ: 13.5 (C18), 14.0 (C4‴), 20.9 (C3‴), 27.1 (C11), 28.7 (C7), 30.1 (C6), 30.3 (C2‴), 35.4 (C12), 37.3 (C8), 45.4 (C9), 48.0 (C1‴), 52.5 (C13), 56.9 (C14), 61.6 (C1′), 90.0 (C17), 113.8 (C2), 116.1 (C4), 127.0 (C1), 128.2 (C4″), 128.6 (C6″), 130.3 (C15), 130.4 (C3″), 131.8 (C2″), 132.0 (C10), 135.8 (C1″), 138.5 (C5), 138.6 (C5″), 142.2 (C16), 156.0 (OCON), 157.0 (C3), 167.7 ($CONH_2$); LRMS for $C_{31}H_{37}N_2O_4$ $[M+H]^+$: 501.4; HPLC purity: 97.8%.

*4.1.7.3. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-hexyl-2-hydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzamide (**10c**).* White solid (15 mg, 56%). IR (KBr) υ: 3364 (OH and NH_2), 1666 (OCON and $CONH_2$); ^{1}H NMR (Acetone-d_6) δ: 0.85 (t, J = 6.9 Hz, 6‴-CH_3), 0.93 (s, 18-CH_3), 1.18–1.64 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2, 3‴-CH_2, 4‴-CH_2 and 5‴-CH_2), 1.84 (m, 12α-CH), 1.93 (m, 7β-CH), 2.06 (m, under solvent peak, 12β-CH), 2.20 (m, 9α-CH and 14α-CH), 2.34 (m, 11α-CH), 2.64 and 3.60 (2m, 1‴-CH_2), 2.72 (m, 6-CH_2), 4.90 (s, 17α-CH), 5.38 (t, J = 2.2 Hz, 1′-CH), 5.55 (d, J = 1.8 Hz, 15-CH), 6.50 (d, J_1 = 2.4 Hz, 4-CH), 6.59 (dd, J_1 = 8.4 Hz, J_2 = 2.5 Hz, 2-CH), 6.71 (broad s, 1H of $CONH_2$), 7.07 (d, J = 8.5 Hz, 1-CH), 7.45 (d, J = 7.7 Hz, 2″-CH), 7.54 (t, J = 8.0 Hz, 3″-CH), 7.57 (br s, 1H of $CONH_2$), 7.95 (m, 4″-CH and 6″-CH); ^{13}C NMR (Acetone-d_6) δ: 13.4 (C18), 14.3 (C6‴), 23.2 (C5‴), 26.8 (C11), 27.1 (C4‴), 27.7 (C3‴) 28.4 (C7), 29.9 (C6), 32.2 (C2‴), 35.1 (C12), 36.8 (C8), 45.1 (C9), 47.6 (C1‴), 52.0 (C13), 56.5 (C14), 61.2 (C1′), 89.0 (C17), 113.6 (C2), 116.0 (C4), 126.8 (C1), 127.6 (C4″), 127.9 (C6″), 128.7 (C15), 129.9 (C3″), 130.8 (C2″), 131.4 (C10), 136.1 (C1″), 138.2 (C5), 139.2 (C5″), 142.6 (C16), 154.6 (OCON), 156.1 (C3), 168.5 ($CONH_2$); LRMS for $C_{29}H_{33}N_2O_4$ $[M+H]^+$: 529.2; HPLC purity: 95.6%.

*4.1.7.4. 3-[(4bS,6aS,6bR,10S,11aR,11bR)-9-octyl-2-hydroxy-6a-methyl-8-oxo-4b,5,6,6a,6b,8,9,10,11a,11b,12,13-dodecahydronaphtho[2′,1′:4,5]indeno[2,1-e][1,3]oxazin-10-yl]benzamide (**10d**).* White solid (15 mg, 61%). IR (KBr) υ: 3356 (OH and NH_2), 1666 (OCON and $CONH_2$); ^{1}H NMR (Acetone-d_6) δ: 0.86 (t, J = 6.9 Hz, 8‴-CH_3), 0.92 (s, 18-CH_3), 1.15–1.63 (m, 7α-CH, 8β-CH, 11β-CH, 2‴-CH_2, 3‴-CH_2, 4‴-CH_2, 5‴-CH_2, 6‴-CH_2 and 7‴-CH_2), 1.84 (m, 12α-CH), 1.94 (m, 7β-CH), 2.06 (m, under solvent peak, 12β-CH), 2.20 (m, 9α-CH and 14α-CH), 2.34 (m, 11α-CH), 2.63 and 3.60 (m, 1‴-CH_2), 2.72 (m, 6-CH_2), 4.91 (s, 17α-CH), 5.38 (t, J = 2.0 Hz, 1′-CH), 5.55 (d, J = 1.7 Hz, 15-CH), 6.50 (d, J_1 = 2.4 Hz, 4-CH), 6.59 (dd, J_1 = 8.5 Hz, J_2 = 2.5 Hz, 2-CH), 6.72 (broad s, 1H of $CONH_2$), 7.07 (d, J = 8.5 Hz, 1-CH), 7.45 (d, J = 7.7 Hz, 2″-CH), 7.54 (t, J = 7.7 Hz, 3″-CH), 7.59 (broad s, 1H of $CONH_2$), 7.93 (m, 4″-CH and 6″-CH), 8.00 (s, OH); ^{13}C NMR (Acetone-d_6) δ: 13.4 (C18), 14.3 (C8‴), 23.3 (C7‴), 26.8 (C11), 27.4 (C6‴), 27.7 (C5‴) 28.4 (C7), 29.9 (C6), 30.0 (C4‴), 30.1 (C3‴), 32.5 (C2‴), 35.1 (C12), 36.8 (C8), 45.1 (C9), 47.6 (C1‴), 52.0 (C13), 56.5 (C14), 61.2 (C1′), 89.0 (C17), 113.6 (C2), 116.0 (C4), 126.8 (C1), 127.6 (C4″), 127.9 (C6″), 128.7 (C15), 129.9 (C3″), 130.8 (C2″), 131.4 (C10), 136.1 (C1″), 138.2 (C5), 139.2 (C5″), 142.5 (C16), 154.6 (OCON), 156.1 (C3), 168.5 ($CONH_2$); LRMS for $C_{35}H_{45}N_2O_4$ $[M+H]^+$: 557.2; HPLC purity: 93.4%.

4.2. Biological evaluation

4.2.1. Inhibition assays for 17β-HSD1

T-47D cells obtained from the American Type Culture Collection (ATCC) were used as a source of 17β-HSD1. They were maintained in RPMI medium supplemented 10% foetal bovine serum (FBS), 2 nM glutamax, 100 IU/mL peniciline, 100 mg/mL streptomycin and 1 nM estradiol (E2) in T-75 culture flasks. For assays, 5000 cells were seeded in each 24 wells of culture plates in steroid-deprived medium containing 50 ng/mL insulin and 5% FBS charcoal-stripped instead of 10% complete FBS, and without E2 supplement contrary to standard culture medium. Cells were treated 24 h later by adding a DMSO solution of the compound (**7a**–**d**, **10a**–**d**, CC-156 or E2) to obtain a final concentration of 10 μM (DMSO concentration <0.5%) and by adding the radioactive substrate (60 nM of [^{14}C]-estrone; American Radiolabeled Chemicals, St. Louis, MO, USA). Cell medium were collected after overnight incubation for quantification of radiolabeled steroids ([^{14}C]-E1 and [^{14}C]-E2). Steroids were extracted with diethyl ether, separated by thin layer chromatography (TLC) and quantified using a Storm 860 system (Molecular Dynamics, Sunnyvale, CA, USA).

The percentage of transformation and the percentage of inhibition were calculated as follow: % transformation = 100 × [^{14}C]-E2 /([^{14}C]-E1 + [^{14}C]-E2) and % of inhibition = 100 × (% transformation without inhibitor - % transformation with inhibitor) / % transformation without inhibitor.

4.2.2. IC_{50} determination for compounds **7c** and **10b**

17β-HSD1 activity was measured as above except that the two selected compounds were tested at 0, 0.01, 0.1, 0.5, 1, 5, 10 and 20 μM. IC_{50} values were calculated from the percentage of transformation using GraphPad Prism version 5.00 for Windows (GraphPad Software, www.graphpad.com) and "log(inhibitor) vs. response – Variable slope" algorithm.

4.2.3. Inhibition assay for 17β-HSD2

Selectivity of compounds **7c** and **10b** were assessed as previously described [26] in stably transfected HEK-293 cells that were kindly provided by Dr. Van Luu-The (CHU de Quebec-Research Center). Cells were seeded in 24-well plates in protocol medium (MEM medium supplemented with 5% dextran-coated charcoal stripped, G418 (700 μg/mL), penicillin (100 IU/mL), streptomycin (100 μg/mL), insulin (50 ng/mL), glutamine (2 mM), nonessential amino acids (0.1 mM), and pyruvate (1 mM)). After 48 h of

incubation, treatment with DMSO solution (<0.5% final) of compound **7c** or **10b** (at concentrations of 1 μM and 10 μM) in protocol medium was conducted. Radiolabeled substrate ([^{14}C]-E2), obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA), was added to obtain a final concentration of 60 nM. Once a substrate transformation of 25% was reached, radiolabeled steroids ([^{14}C]-E1 and [^{14}C]-E2) were extracted with diethyl ether, separated by TLC and quantified as described in the section on 17β-HSD1 inhibition assays.

*4.2.4. Estrogenic activity of compounds **7c** and **10b***

T-47D cells were suspended in RPMI supplemented with insulin (50 ng/mL), instead of estradiol, and 5% charcoal-stripped FBS to deprive the media of estrogens. The cells were plated in 96-well plates at a density of 3000 cells/well and allowed to attach for 48 h. After this pre-incubation period, the inhibitors and the reference compounds dissolved in DMSO were diluted in fresh culture media, added to the wells (final DMSO concentration <0.5%) and replaced every 2 days for a total of 7 days of treatment.

CellTitter 96® Aqueous One Solution Cell Proliferation Assay was used as an indirect colorimetric measurement of cell proliferation according to the manufacturer's instructions. Briefly, after the treatments, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution (MTS) were added to each well and the mixture incubated at 37 °C for 4 h. The absorbance at 490 nm was then measured with a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA, USA). The control (culture media + DMSO) is set to 100% of cell proliferation.

4.2.5. Molecular modeling

Molecular modeling was performed using MOE 2013.08 [35]. The crystal structure coordinates of 17B-HSD1, including the inhibitor CC-156 and the NADP, were taken from PDB ID 3HB5 [33]. Hydrogen atoms were added using the Protonate 3D tool included in MOE. The protein was prepared using the LigX tool with the OPLSAA force field included in MOE to adjust the position of the hydrogens, the side chain rotamers, and to minimize the energy of the system, as previously described [36]. From the resulting structure, the water molecules, the ligand and the cofactor were removed prior to docking. Docking simulations were performed using the induced fit protocol with the side chains of the receptor tethered with a weight derived from the b-factor, with all other parameters set to default. Validation of this docking protocol was carried out by a self-docking of CC-156, leading to a heavy atoms Root Mean Square Deviation (RMSD) of 0.40 Å between the docked and CC-156 conformation from the crystallographic structure. No further optimization of the docking protocol was considered.

Compounds **7a**–**d** and **10a**–**d** and simplified scaffolds were built using MOE. Hydrogens were adjusted and molecules were energy-minimized using the OPLSAA force field. The best two conformations of each docked compound were used for analysis. The conformations were selected according to the lowest RMSDs between the heavy atoms of the docked conformations and their equivalent from the CC-156 crystal structure. The comparisons between selected docked conformations and NADP were realized by superposition of the selected docking results and crystal structure. Protein structure preparation was also performed with PDB ID 3HB4 which contains active-site water molecules instead of the NADP as shown in Fig. 6 (MIDDLE).

The Conformational Search tool in MOE was used with the LowModeMD protocol to sample the accessible conformations of each compound. The flexible alignments were realized for molecules **7a**, **10a** and CC-156 using the flexible alignment tool in MOE with default parameters. Graphical representations were rendered with PyMOL [29].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.01.059.

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