

Article

Structure Confirmation and Evaluation of a Nonsteroidal Inhibitor of 17 β -Hydroxysteroid Dehydrogenase Type 10

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Abstract: 17 β -Hydroxysteroid dehydrogenase type 10 (17 β -HSD10) is a steroidogenesis enzyme known for its potential role in Alzheimer's disease. For comparison purposes between steroidal and nonsteroidal 17 β -HSD10 inhibitors **1** and **2**, respectively, we attempted the chemical synthesis of benzothiazole phosphonate derivative **2**. Instead of a one-pot synthesis, we report a two-step synthesis with characterization of both imine intermediate **5** and final compound **2**. Furthermore, complete assignment of ¹H and ¹³C nuclear magnetic resonance (NMR) signals of **2** is provided, as we observed a divergence of NMR data with those published previously. Finally, biological assays showed that **1** and **2** inhibited the oxidation of estradiol (E2) into estrone (E1) by the 17 β -HSD10 recombinant protein. However, in human embryonic kidney (HEK)-293 intact cells transfected with 17 β -HSD10, only the steroidal inhibitor **1** induced a dose-dependent inhibition of E2 to E1 transformation.

Keywords: NMR; chemical synthesis; enzyme inhibitor; 17 β -HSD10; Alzheimer's

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative pathology characterized by the loss of important brain functions, notably memory [1]. It was reported that mitochondrial 17 β -hydroxysteroid dehydrogenase type 10 (17 β -HSD10), also known as amyloid- β (A β) binding alcohol dehydrogenase (ABAD), can form a harmful complex with the A β -42 peptide and, consequently, could play a role in AD [2–6]. This complex may lead to the accumulation of amyloid plaques, a pathological marker of AD. 17 β -HSD10 also metabolizes estradiol (E2), a steroidal hormone reported to demonstrate neuroprotective action [2,7]. In fact, E2 exerts neuroprotective effects by its regulation of A β protein precursor trafficking and metabolism [7]. It was also reported that an E2 treatment reduced the formation of A β in both in vivo and in vitro experiments [8]. Interestingly, the expression of 17 β -HSD10 was greatly up-regulated in the activated astrocytes of AD patients [9,10]. Taken together, these observations suggest that 17 β -HSD10 may exacerbate the progress of AD and the inhibition of 17 β -HSD10 enzymatic activity could provide a new approach for the treatment of AD.

In our efforts to develop inhibitors of the 17 β -HSD family, particularly inhibitors of types 1, 2, 3, 5, and 7 for the treatment of estrogen- or androgen-dependent diseases [11–15], we fortuitously identified a first family of steroidal inhibitors of 17 β -HSD10 represented by compound **1** [16] (Figure 1). Nonsteroidal inhibitors of this enzyme, although few, have already been reported [17–20]. Among these inhibitors, we focused on the family of benzothiazole phosphonate derivatives because of their very promising biological properties [21]. To have at our disposal a reference inhibitor for comparison

with our steroid inhibitors, we therefore undertook the chemical synthesis of compound **2** (Figure 1), one of the most active benzothiazole phosphonate derivatives [18,21]. The difficulties we encountered, as well as the divergence of nuclear magnetic resonance (NMR) data, forced us to further investigate the synthesis, the characterization, and the biological activity of inhibitor **2**.

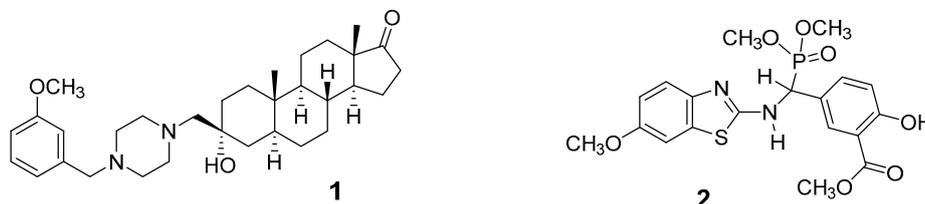


Figure 1. Steroidal and nonsteroidal compounds **1** and **2**, respectively, representative of two families of 17β -hydroxysteroid dehydrogenase type 10 (17β -HSD10) inhibitors.

2. Results and Discussion

2.1. Chemical Synthesis of Nonsteroidal Inhibitor **2**

Our first attempts to synthesize **2** were performed using the one-pot chemical procedures provided in the patent [21] and published article [18]. Because there is some ambiguity regarding the quantity of each component of the reaction (number of millimoles and equivalent), we used the same mass in grams for our assay as used in the patent. Even after allowing the reaction to continue for 20 h instead of 6 h, ^1H NMR spectra did not show the characteristic N-CH-P signal reported in literature [18,21], but through NMR analysis, we observed the formation of the intermediate imine. The modification of the ratio of reagents, the reaction time, and the temperature did not produce the desired compound **2**.

Because we did not obtain **2** under the one-pot synthesis conditions, we tested a two-step procedure: (1) formation of the imine **5**, and (2) addition of dimethylphosphite to generate **2** (Figure 2). For the first step, we used magnesium perchlorate because it was reported to be an efficient catalyst for the synthesis of imines [22]. Methyl-5-formyl-2-hydroxybenzoate (**3**) was first stirred with magnesium perchlorate (1 equiv) to allow its activation by the salt acting as a Lewis acid, and then 6-methoxy-benzo[*d*]thiazole-2-amine (**4**) in excess (5 equiv) was added and the mixture refluxed. The reaction was monitored by ^1H NMR by taking an aliquot of the supernatant at 22 and 42 h. The aliquot was taken after allowing the reaction mixture to return to room temperature (rt), as benzothiazole **4** is slightly soluble in toluene at rt. Therefore, only the benzaldehyde **3** and the imine **5** were obtained after evaporation of the aliquot. After 22 and 42 h, the proportions of remaining benzaldehyde **3** were 72% and 43%, respectively, while the proportions of imine **5** were 28% and 57%, respectively. After work-up (66 h), the proportions of benzaldehyde **3** and imine **5** were calculated as 15% and 85%, respectively, and after purification, imine **5** was obtained as a yellow solid in a 57% yield. Thus, the aromatic imine **5** was stable under the work-up and chromatographic conditions, but its formation requires more time and more magnesium perchlorate because of the low reactivity of an aromatic amine such as **4**. In the second step, imine **5** was reacted with dimethylphosphite in excess (5 equiv) for 22 h in refluxing toluene. After purification, compound **2** was obtained in a 33% yield. Thus, the global yield for the two steps was low (19%), but it was not optimized, and it allowed us to obtain a sufficient amount of compound **2** for characterization and biological testing.

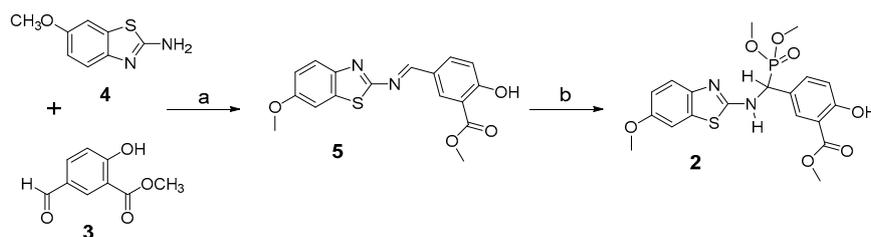


Figure 2. Chemical synthesis of nonsteroidal 17β -hydroxysteroid dehydrogenase type 10 (17β -HSD10) inhibitor **2**. Reagents and conditions: (a) $\text{Mg}(\text{ClO}_4)_2$, toluene, reflux, 66 h; (b) $\text{HPO}(\text{OCH}_3)_2$, toluene, reflux, 22 h.

2.2. Preliminary Characterization of Compound 2

In the mass spectrum, the compound obtained by the two-step procedure showed the appropriate $M + H$ molecular peak at $453.2 m/z$, as well as another signal at $343.2 m/z$ corresponding to the loss of a $\text{PO}(\text{OCH}_3)_2$ fragment. As expected, the infrared spectrum showed characteristic bands at 3232 cm^{-1} (OH/NH), 1682 cm^{-1} (conjugated ester), and 1543 cm^{-1} (double bonds), and the retention time ($R_f = 0.35$) also agreed with literature data ($R_f = 0.26$) [18]. However, ^1H and ^{13}C NMR results did not fully agree with data reported in the literature obtained in the same solvent (dimethylsulfoxide (DMSO)- d_6). In the ^1H NMR spectrum (Figure 3), the four OCH_3 signals roughly showed the same chemical shifts, but the characteristic CH-1 appeared as a doublet at 5.62 ppm contrary to a doublet at 4.32 ppm [21] or 5.35 ppm [18], whereas some aromatic CH signals showed different chemical shifts. In the ^{13}C NMR spectrum, some aromatic C and CH signals as well as the characteristic CH-1 signals (52.1/53.7 vs. 65.4/66.7 ppm) showed different chemical shifts. However, comparison between the experimental and literature data was not easy, because two sets of different data were published by the same research group [18,21] (Supplementary Materials). These results were very intriguing, as the authors obtained a crystalline structure demonstrating the desired benzothiazole phosphonate derivative **2**.

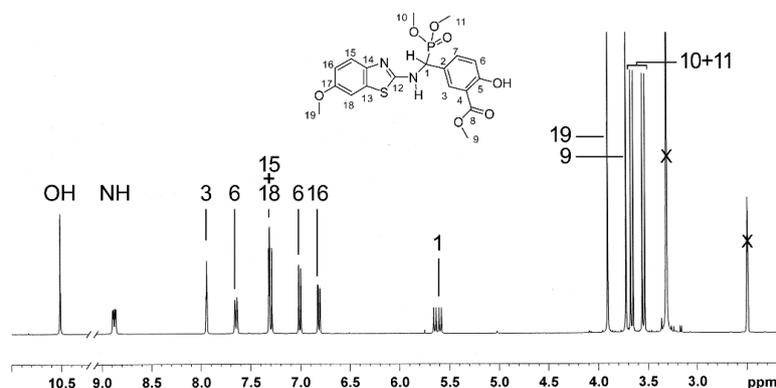


Figure 3. ^1H nuclear magnetic resonance (NMR) spectrum of compound **2** in dimethylsulfoxide (DMSO)- d_6 and full signal assignment.

To further characterize the product that we obtained, we crystallized compound **2** in methanol and performed an X-ray analysis that identified a monoclinic crystal in space group $P2_1/c$. In fact, the crystal mesh contained four molecules of compound **2** (two *R* and two *S*) and two molecules of disorganized methanol at a 2:1 ratio. Our crystal data thus confirmed the expected structure as illustrated with the *R* isomer in Figure 4. Because the presence of methanol could not explain the divergence observed between the literature data and our preliminary NMR analysis, we next fully characterized and assigned all ^1H and ^{13}C NMR signals.

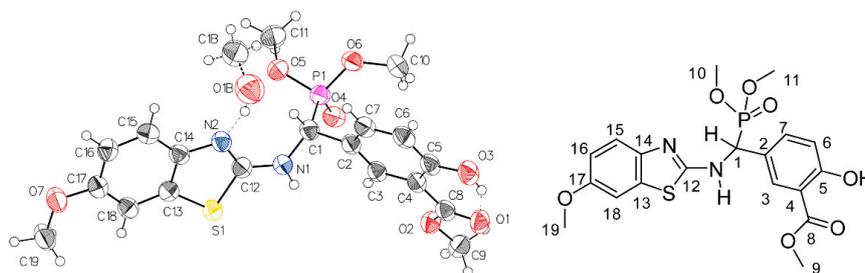


Figure 4. ORTEP diagram resulting from X-ray analysis of compound **2**. Methanol (CH₃OH) was present in the crystal in a 1:2 proportion with compound **2** (represented as the *R* isomer).

2.3. NMR-Analyses of Compound **2**

Divergent NMR data and the fact that the assignments of ¹H and ¹³C signals were not provided in literature for compound **2** prompted us to perform additional NMR experiments. Three experiments, heteronuclear single quantum correlation (HSQC), heteronuclear multiple-bond correlation (HMBC), and homonuclear correlation spectroscopy (COSY) allowed full assignment of all ¹H and ¹³C NMR signals (Table 1 and Supplementary Materials).

Table 1. Assignment of proton and carbon nuclear magnetic resonance (NMR) signals related to compounds **2**.

No. ¹	¹ H NMR ²	¹³ C NMR ²	¹ H NMR ³ (Estimated)	Difference ⁴ (ppm) (%)	¹³ C NMR ³ (Estimated)	Difference ⁴ (ppm) (%)
CH-1	5.62 (dd, 9.5; 21.3)	52.9 (154.8) ⁵	3.9	−1.72 (30.6)	69.0	16.1 (30.4)
C-2	—	126.8	—	—	128.6	1.8
CH-3	7.94 (t, 2.2)	129.3 (d, 6.0)	7.78	−0.16 (2.0)	129.4	0.1
C-4	—	113.0	—	—	111.7	−1.3
C-5	—	159.5	—	—	159.8	0.3
CH-6	7.01 (d, 8.6)	117.5	6.95	−0.06 (0.9)	116.7	−0.8
CH-7	7.65 (dt, 1.8; 8.6)	135.3 (d, 5.3)	7.32	−0.33 (4.0)	132.6	−2.7 (2.0)
C-8	—	168.8	—	—	169.7	0.9
CH ₃ -9	3.91 (s)	52.5	3.95	0.04 (1.0)	51.5	−1.0
CH ₃ -10/11	3.55/3.67 (2d, 10.6; 10.6)	53.2/53.5 (2d, 6.7/7.1)	3.66	0.11/−0.01 (3.0/0.3)	53.4	−0.1/0.2
C-12	—	163.6 (d, 10.5)	—	—	174.5	10.9 (6.7)
C-13	—	131.9	—	—	131.9	0
C-14	—	145.5	—	—	145.5	0
CH-15	7.30 (d, 9.1)	118.7	7.53	0.23 (3.2)	118.2	−0.5
CH-16	6.82 (dd, 2.7; 8.8)	113.1	7.00	0.18 (2.6)	114.6	1.5
C-17	—	154.6	—	—	156.7	2.1
CH-18	7.31 (d, 3.4)	105.6	7.53	0.22 (3.0)	104.9	−0.7
CH ₃ -19	3.73 (s)	55.5	3.80	0.07 (1.8)	55.8	0.3
NH	8.88 (dd, 3.5; 9.5)	—	6.79	−2.09 (23.5)	—	—
OH	10.51 (s)	—	15.2	4.69 (44.6)	—	—

¹ See Figure 4 for all carbon numbering. ² Chemical shift (δ) and coupling constant (J) expressed in ppm and in hertz, respectively. Dimethylsulfoxide (DMSO)-d₆ was used as solvent. ³ Data estimated by ChemDraw 17. ⁴ Estimated δ values and experimental δ values expressed in ppm or %. ⁵ Two signals at 52.1 and 53.7 ppm.

In the HSQC spectra, there was no correlation between the two signals at 10.51 (s) and 8.88 (dd) ppm, which were assigned to the OH (having any nearby proton for coupling) and NH protons, respectively. Using the HMBC results between the OH and carbons nearby, C-4, C-5, and CH-6 could be assigned to 159.5, 117.5, or 113.0 ppm. Because two of these carbons, C-4 and C-5, are not linked to any protons, a ¹³C NMR attached proton test (APT) and HSQC were used to identify CH-6 (117.5 and

7.01 ppm). C-4 and C-5 could not be differentiated between at this point. Using COSY and HSQC experiments, CH-7 was assigned to the signals at 7.65 and 135.3 ppm, having a correlation with CH-6. Because CH-7 correlated with only one other aromatic C-H in the HMBC spectra, then CH-3 could be assigned (7.94 and 129.3 ppm). Knowing that there was no possible correlation between C-4 and CH-7 in HMBC, C-5 (159.5 ppm, correlating with CH-7) and C-4 (113.0 ppm) could thereafter be deduced. Making a correlation with CH-6 in HMBC, the last carbon signal to be assigned at 126.8 ppm was in fact C-2. From the correlation with CH-3 in HMBC, the carbonyl (C-8) was assigned at 168.8 ppm.

The four methoxy signals of compound **2** were assigned with a combination of one dimensional (1D)- and two dimensional (2D)-NMR experiments. Because phosphorus has a spin of 1/2, the two methoxy groups on this atom are coupled. Therefore, the two doublets at 3.55 and 3.67 ppm ($^3J_{HP}$) were assigned to either CH₃-10 or CH₃-11. As determined with the HSQC experiment (Figure 5), C-10 and C-11 corresponded to the two doublets at 53.2 and 53.5 ppm. In the HMBC spectra, a correlation with the carbonyl C-8 allowed us to confirm CH₃-9 by signals at 3.91 (1H) and 52.5 (^{13}C) ppm. Thus, the remaining methoxy (CH₃-19) was assigned to the signals at 3.73 (1H) and 55.5 (^{13}C) ppm.

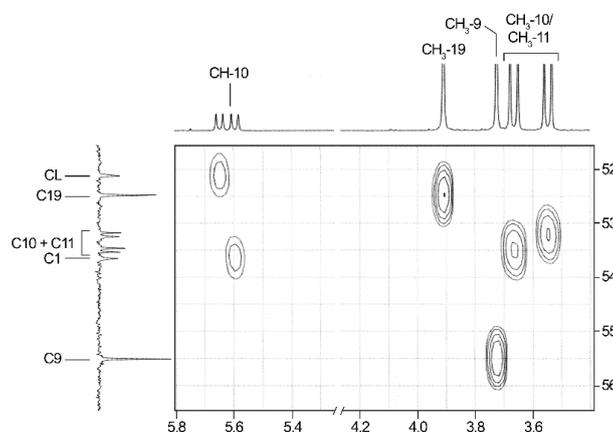


Figure 5. Partial spectra of heteronuclear single quantum correlation (HSQC) experiment showing key HC-correlations for both CH-1 and CH₃O signals.

The characteristic CH-1 signal (dd at 5.62 ppm) was deduced from correlations in HMBC with C-2, C-3, and C-7 and in COSY with N-H. Using HSQC, C-1 was assigned to the two signals at 52.1 and 53.7 ppm (52.9 ppm, d, $^1J_{CP} = 154.8$ Hz) (Figure 5). The phosphorus atom neighbouring CH-1 was responsible for a $^2J_{HP}$ coupling (21.3 Hz) as well as a $^1J_{CP}$ coupling (154.8 Hz). From the CH-1 signal and knowing that three of the four correlations previously observed in the HMBC experiment were with C2, C3, and C7 (Figure 6), it was possible to identify the last unassigned signal at 163.6 ppm as C-12. The C-17 signal was deduced from its correlation (in HMBC) with the known CH₃-19, and it was the starting point for the identification of the benzothiazole proton and carbon signals. Thus, in HMBC, C-17 could make correlations with CH-15, CH-16, and CH-18, and the corresponding signals were at 7.30 ppm (d, $J = 9.0$ Hz), 6.82 ppm (dd, $J = 8.8, 2.7$ Hz), and 7.31 ppm (d, $J = 3.4$ Hz). In 1H NMR, CH-16 was coupled with CH-15 and CH-18, thus producing the doublet of doublets at 6.82 ppm, and it was positioned at 113.1 ppm in ^{13}C NMR, as deduced from HSQC. The doublet of CH-15, being in ortho of CH-16, had the highest coupling constant ($J = 8.8$ Hz) in comparison to CH-18 ($J = 3.4$ Hz) and could be assigned to the signals at 7.30 and 118.7 ppm. Therefore, CH-18 was positioned at 7.31 ppm (d) in 1H NMR and at 105.6 ppm in ^{13}C NMR. Knowing that CH-16 would produce a correlation with C-14 in HMBC but not with C-13, C-14 was consequently assigned to the signal at 145.5 ppm, and, by deduction, C-13 was assigned to the last signal at 131.9 ppm.

After assigning all the protons and carbons of compound **2** through 2D NMR analysis, we compared these experimental values with those predicted by the ChemDraw software (Table 1).

In ^1H NMR, the predicted chemical shift values were very close to the experimental values (with a difference of less than 4%), except for three signals. The proton found between the phosphorus and nitrogen atoms (CH-1) and two labile protons (NH and OH) showed differences of 30.6%, 23.5%, and 44.6%, respectively. In ^{13}C NMR, the predicted values were also relatively good (<2.0%), except for C-1 and C-12. The worst prediction with a difference of 30.4% was observed for CH-1 (69.0 vs. 52.9 ppm) and to a lesser extent (6.7%) for C-12 (174.5 vs. 163.6 ppm). These two carbons are close to the phosphorus atom.

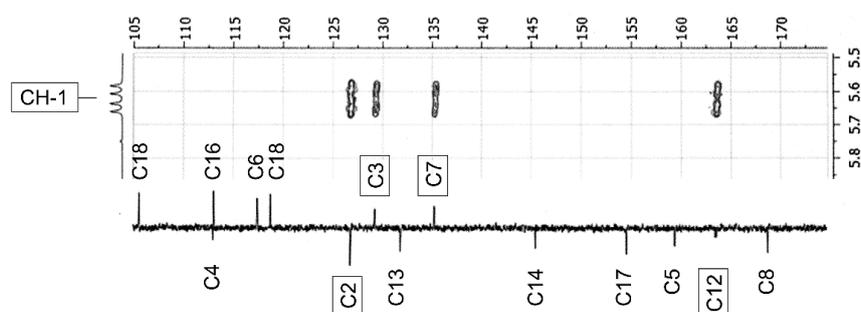


Figure 6. Partial spectra of heteronuclear multiple-bond correlation (HMBC) experiment showing four key correlations between CH-1 and C-2, C-3, C-7, and C-12.

2.4. Enzyme Inhibition

The inhibitory potency of steroidal compound **1** and nonsteroidal compound **2** for the transformation of E2 into estrone (E1) was assessed using two different sources of 17β -HSD10: the recombinant purified protein and transfected HEK-293[17β -HSD10] cells. For both enzymatic assays, we used the same substrate concentration (1.0 μM , E2/ ^{14}C -E2 in 9:1 proportion) and incubation time (40 h). The unreacted substrate (E2) and metabolite (E1) were extracted, separated by thin-layer chromatography (TLC) and quantified to obtain the percentage of transformation and then the percentage of inhibition. In the first assay with purified protein (Figure 7A), nonsteroidal inhibitor **2** was a more potent inhibitor than the steroidal inhibitor **1**. At a concentration of 100 μM , **1** and **2** inhibited 30.3% \pm 8.2% and 53.1% \pm 8.1% of E2 to E1 transformation by 17β -HSD10, respectively. In the second assay, using HEK-293[17β -HSD10] intact cells (Figure 7B), **2** did not significantly inhibit the E2 into E1 transformation in the range of concentrations tested (0.001–25 μM). However, steroidal inhibitor **1** inhibited E2 into E1 transformation with a dose-dependent relationship. A significant inhibition (30%) was first observed at 0.3 μM and increased until 85% at the higher concentration of 25 μM . From these data, the concentration inhibiting 50% of the transformation (IC_{50}) was estimated at 1.7 μM .

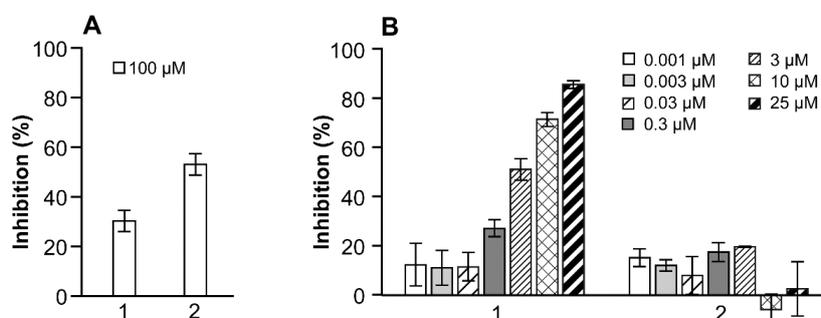


Figure 7. Inhibition by compounds **1** and **2** of the transformation of estradiol (E2) into estrone (E1) by recombinant 17β -hydroxysteroid dehydrogenase type 10 (17β -HSD10) (A) and HEK-293[17β -HSD10] intact cells (B). Results of a triplicate expressed in % \pm SEM.

The results obtained in the two biological assays showed a discrepancy, as steroidal compound **1** was more active as an inhibitor when tested in intact cells than with the purified protein. Although the number of mRNA copies for 17 β -HSD10 is very high in HEK-293[17 β -HSD10] compared to the mRNA of other endogenous enzymes, a possible explanation would be that an enzyme in very small quantities but that is much more efficient contributes to the oxidation of E2 into E1, a transformation that would be blocked by compound **1**. Additional work will, however, be needed to clarify this point as well as the reason for the failure of compound **2** to inhibit 17 β -HSD10 in intact cells.

These results report for the first time the inhibitory potency of nonsteroidal inhibitor **2** using E2 as a substrate. Previously, the inhibitory potency of **2** on the 17 β -HSD10 protein was assessed using the reduction of S-acetoacetyl-CoA (SAAC), another substrate of 17 β -HSD10, by measuring the decrease of the cofactor nicotinamide adenine dinucleotide reduced (NADH) (absorbance at 340 nm) and thus the oxidation of the cofactor [21]. 17 β -HSD10 is an enzyme known to transform a large range of substrates [23] and to have both oxidative and reductive activities depending on the cofactor used. To keep the level of neuroprotective E2 as high as possible, and to avoid its transformation to E1, it would be more appropriate to develop inhibitors of the oxidative reaction catalyzed by 17 β -HSD10.

3. Materials and Methods

3.1. Chemical Synthesis of **2**

3.1.1. General

Reagents and solvents were purchased from commercial suppliers and were used as received. TLC and flash-column chromatography were performed on 0.20 mm silica gel 60 F254 plates (E. Merck; Darmstadt, Germany) and with 230–400 mesh ASTM silica gel 60 (Silicycle, Québec, QC, Canada), respectively. X-ray analysis was performed by Thierry Marris at the Laboratoire de Diffraction des Rayons X (University of Montréal, Montréal, QC, Canada). High-resolution mass spectra (HRMS) were provided by Pierre Audet from the Department of Chemistry at Université Laval (Québec, QC, Canada). Fourier transform infrared (FTIR) spectra were recorded with a Horizon MB 3000 ABB FTIR spectrometer (Québec, QC, Canada). NMR spectra were recorded at rt in CDCl₃ with a 5 mm NMR tube on a Bruker AVANCE 400 spectrometer (Billerica, MA, USA). ¹H and ¹³C NMR chemical shifts were referenced to the residual peaks of DMSO-d₆ (2.50 and 39.5 ppm, respectively). For characterization, we used the following experiments: APT, COSY, HSQC, and HMBC. These NMR experiments were performed according to the manufacturers' instructions.

3.1.2. Synthesis of Imine **5**

To a solution of methyl-5-formyl-2-hydroxybenzoate (**3**) (100 mg, 0.56 mmol) in anhydrous toluene (20 mL) was added Mg(ClO₄)₂ (120 mg, 0.54 mmol), and the mixture was stirred for 15 min at rt. Then, 6-methoxy-benzo[*d*]thiazole-2-amine (**4**) (500 mg, 2.77 mmol) was added, and the mixture was stirred at reflux for 66 h. The solvent was removed under reduced pressure, water was added, and the aqueous phase was extracted with EtOAc. The organic phases were combined, washed with water and brine, and dried over Na₂SO₄. After filtration and evaporation under reduced pressure, the crude orange solid was purified by flash chromatography using a gradient of CH₂Cl₂/MeOH/NEt₃ (99:0:1) to CH₂Cl₂/MeOH/Et₃N (97:2:1) to afford the imine intermediate **5** as an amorphous yellow solid (108 mg, 57%), but starting benzaldehyde and amine were not recovered. Methyl 2-hydroxy-5-(((6-methoxybenzo[*d*]thiazol-2-yl)imino)methyl)benzoate (**5**): IR (ν , cm⁻¹): 3379 (O–H), 1674 (C=O), 1574 (C=N), 1227 (C–O), 1142 (C–O); ¹H NMR (DMSO-d₆) δ : 3.84 (s, 3H, CH₃-19), 3.93 (s, 3H, CH₃-9), 7.10 (dd, ³J_{HH} = 8.9 Hz, ⁴J_{HH} = 2.6 Hz, 1H, CH-16), 7.17 (d, ³J_{HH} = 8.7 Hz, 1H, CH-6), 7.64 (d, ⁴J_{HH} = 2.6 Hz, 1H, CH-18), 7.80 (d, ³J_{HH} = 8.9 Hz, 1H, CH-15), 8.19 (dd, ³J_{HH} = 8.7 Hz, ⁴J_{HH} = 2.2 Hz, 1H, CH-7), 8.49 (d, *J* = 2.1 Hz, 1H, CH-3), 9.07 (s, 1H, CH-1), 11.10 (s, 1H, OH); ¹³C NMR (DMSO-d₆) δ : 52.6 (CH₃-9), 55.7 (CH₃-19), 105.1 (CH-18), 114.7 (C-4), 115.6 (CH-16), 118.5 (CH-6),

123.1 (C-15), 126.2 (C-2), 133.1 (CH-3), 135.3 (C-13), 135.9 (CH-7), 145.6 (C-14), 157.2 (C-17), 163.3 (C-5), 164.8 (CH-1), 167.9 (C-8), 168.8 (C-12); HRMS: $[M + H]^+$ calculated for $C_{17}H_{15}N_2O_4S$, 343.0747; found, 343.0747.

3.1.3. Synthesis of Inhibitor 2

To a solution of imine **5** (40 mg, 0.12 mmol) in anhydrous toluene (15 mL) was added dimethyl phosphite (54 μ L, 0.58 mmol), and the mixture was stirred at reflux for 22 h. The solvent was then removed under reduced pressure, water was added, and the aqueous phase was extracted with EtOAc. The organic phases were combined, washed with water, dried over Na_2SO_4 , filtered, and evaporated under reduced pressure. The resulting crude brown solid was purified by flash chromatography using a gradient of hexanes/EtOAc (1:1 to 3:7) to afford **2** as a light brown amorphous solid (18 mg, 33%). Methyl 5-((dimethoxyphosphoryl)((6-methoxy-benzo[d]thiazol-2-yl) amino)methyl)-2-hydroxy-benzoate (**2**): IR (ν , cm^{-1}): 3232 (O–H/N–H), 1682 (C=O), 1543 (C=C), 1219 (P=O), 1049 (C–O), 1026 (P–O–C); 1H NMR (DMSO- d_6) δ : 3.55 and 3.67 (2d, $^3J_{HP}$ = 10.6 Hz, 6H, CH_3 -10 and CH_3 -11), 3.73 (s, 3H, CH_3 -19), 3.91 (s, 3H, CH_3 -9), 5.62 (dd, $^2J_{HP}$ = 21.3 Hz, $^3J_{HH}$ = 9.5 Hz, 1H, CH-1), 6.82 (dd, $^3J_{HH}$ = 8.8 Hz, $^4J_{HH}$ = 2.7 Hz, 1H, CH-16), 7.01 (d, $^3J_{HH}$ = 8.6 Hz, 1H, CH-6), 7.30 (d, $^3J_{HH}$ = 9.6 Hz, 1H, CH-15), 7.31 (d, $^4J_{HH}$ = 2.4 Hz, 1H, CH-18), 7.65 (dt, $^3J_{HH}$ = 8.6 Hz, $^4J_{HH}$ = 1.8 Hz, 1H, CH-7), 7.94 (t, $^4J_{HH}$ = 2.2 Hz, 1H, CH-3), 8.88 (dd, $^3J_{HP}$ = 9.5 Hz, $^3J_{HH}$ = 3.5 Hz, 1H, NH), 10.51 (s, 1H, OH); ^{13}C NMR (DMSO- d_6) δ : 52.9 (d, $^1J_{CP}$ = 154.8 Hz, CH-1), 53.2 and 53.5 (2d, $^3J_{CP}$ = 6.7 and 7.1 Hz, 2C, CH_3 -10 and CH_3 -11), 52.5 (CH_3 -9), 55.5 (CH_3 -19), 105.6 (CH-18), 113.0 (s, C-4), 113.1 (CH-16), 117.5 (CH-6), 118.7 (CH-15), 126.8 (C-2), 129.3 (d, $^3J_{CP}$ = 6.0 Hz, CH-3), 131.9 (C-13), 135.3 (d, $^3J_{CP}$ = 5.3 Hz, CH-7), 145.5 (C-14), 154.6 (C-17), 159.5 (C-5), 163.6 (d, $^3J_{CP}$ = 10.5 Hz, C-12), 168.8 (C-8); HRMS: $[M + H]^+$ calculated for $C_{19}H_{22}N_2O_7PS$, 453.0880; found 453.0890.

3.2. Biological Evaluation of **1** and **2**

3.2.1. Inhibition of E2 to E1 Transformation by 17β -HSD10 Protein

The inhibitory potency of **1** and **2** was evaluated on the 17β -HSD10 recombinant protein using E2 as a substrate. Briefly, to each well was added 5 μ L of inhibitor stock solution (15 mM in DMSO) and 50 μ L of nicotinamide adenine dinucleotide oxidized (NAD^+) (5 mM in Tris-BSA buffer) to obtain final concentrations of 100 μ M and 1 mM, respectively. The final concentration of DMSO in each well was adjusted to 2%. Additionally, 10 μ L of a solution containing [^{14}C]-E2 (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) and E2 (Sigma-Aldrich) (1:9) was added to obtain a final concentration of 1 μ M. Tris-BSA buffer was then added to obtain a final volume of 200 μ L. A stock solution of 17β -HSD10 protein (MyBioSource, Inc., San Diego, CA, USA) was prepared in Tris-BSA buffer (2 ng/ μ L). The enzymatic reaction in triplicate was started by the addition of 50 μ L of 17β -HSD10 protein solution to obtain a final concentration of 400 ng/mL. The assay tubes were incubated for 40 h at 37 $^\circ$ C. Then, the steroids (substrate E2 and metabolite E1) were extracted from the buffer medium with diethyl ether, and the organic phase was evaporated to dryness under nitrogen. The residue was dissolved in dichloromethane and dropped on silica gel TLC plates (EMD Chemicals Inc., Gibbstown, NJ, USA) and eluted with a mixture of toluene/acetone (4:1). Substrate [^{14}C]-E2 and metabolite [^{14}C]-E1 were identified by comparing them with reference steroids (E2 and E1) and quantified using the Storm 860 system (Molecular Dynamics, Sunnyvale, CA, USA). Percentages of transformation and inhibition were calculated as follows: % of transformation = $100 \times [^{14}C]\text{-E1}/([^{14}C]\text{-E2} + [^{14}C]\text{-E1})$; % of inhibition = $100 \times (\% \text{ transformation without inhibitor} - \% \text{ transformation with inhibitor})/\% \text{ transformation without inhibitor}$.

3.2.2. Inhibition of E2 to E1 Transformation by HEK-293 Cells Overexpressing 17β -HSD10

HEK-293 cells stably transfected with 17β -HSD10 [16] were seeded at 2.5×10^5 cells in a 24-well plate at 37 $^\circ$ C under a 95% air/5% CO_2 humidified atmosphere in 990 μ L of culture medium. Inhibitor

stock solutions were prepared in DMSO (10 mM) and diluted with culture medium. After 24 h, 5 μ L of these solutions was added to the cells to obtain final inhibitor concentrations ranging from 0.001 to 25 μ M. The final concentration of DMSO in each well was adjusted to 0.5%. Additionally, 5 μ L of a solution containing [14 C]-17 β -E2 and E2 (1:9) was added to obtain a final concentration of 1 μ M, and cells were incubated for 40 h. Each inhibitor was assessed in triplicate. After incubation, the culture medium was removed, and labeled steroids (E1 and E2) were extracted with diethyl ether and quantified as reported above.

4. Conclusions

As part of our experiments to obtain a reference inhibitor of 17 β -HSD10 for comparison with our steroidal inhibitors, we tried to synthesize the benzothiazole phosphonate derivative **2**. After our attempts did not provide the desired compound using the one-pot synthesis procedure published previously, we performed a two-step synthesis and obtained the nonsteroidal inhibitor **2**. Furthermore, we obtained complete 1 H and 13 C NMR assignments of **2** and the intermediate imine **5** using 2D-NMR (COSY, HSQC, and HMBC) experiments. X-ray analysis of the crystallized compound also confirmed the desired product.

Nonsteroidal inhibitor **2** was also, for the first time, tested against the transformation of E2 as a substrate into E1 by 17 β -HSD10. The assays with the recombinant 17 β -HSD10 protein and E2 as a substrate proved that **1** and **2** are both inhibitors. However, the use of intact cells showed that only the steroidal inhibitor **1** induces a significant inhibition of E2 into E1 transformation. These surprising results raise questions about the validity of the models used to test inhibitors, and additional studies are needed for their validation.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2312-7481/4/3/32/s1>: (1) Listing of 1 H and 13 C NMR data reported in literature for compound **2**; (2) Crystal data and structure refinement for CHUL08 (**2**); (3) NMR spectra for compound **2** (1 H NMR, 13 C NMR (APT), COSY, HSQC, and HMBC).

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