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Parallel Solid-Phase Synthesis of 3β-Peptido-3α-hydroxy-5α-androstan-17-one Derivatives for Inhibition of Type 3 17β-Hydroxysteroid Dehydrogenase

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Abstract—Type 3 17β-hydroxysteroid dehydrogenase (17β-HSD), a key steroidogenic enzyme, transforms 4-androstene-3,17-dione (Δ^4-dione) into testosterone. In order to produce potential inhibitors, we performed solid-phase synthesis of model libraries of 3β-peptido-3α-hydroxy-5α-androstan-17-ones with 1, 2, or 3 levels of molecular diversity, obtaining good overall yields (23–58%) and a high average purity (86%, without any purification steps) using the Leznoff's acetal linker. The libraries were rapidly synthesized in a parallel format and the generated compounds were tested as inhibitors of type 3 17β-HSD. Potent inhibitors were identified from these model libraries, especially six members of the level 3 library having at least one phenyl group. One of them, the 3β-(*N*-heptanoyl-L-phenylalanine-L-leucine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (**42**) inhibited the enzyme with an IC_{50} value of 227 nM, which is twice as potent as the natural substrate Δ^4-dione when used itself as an inhibitor. Using the proliferation of androgen-sensitive (AR^+) Shionogi cells as model of androgenicity, the compound **42** induced only a slight proliferation at 1 μM (less than previously reported type 3 17β-HSD inhibitors) and, interestingly, no proliferation at 0.1 μM.

Introduction

Type 3 17β-hydroxysteroid dehydrogenase (17β-HSD)[1–4] is a key steroidogenic enzyme transforming the inactive androgen 4-androstene-3,17-dione (Δ^4-dione) into testosterone (T), the second most active androgen in men after dihydrotestosterone (DHT) (Fig. 1).[5] This enzyme constitutes an interesting target for blocking the biosynthesis of testosterone and thus its effects in androgen-sensitive diseases,[6,7] such as prostate cancer.[8–10] Furthermore, considering that type 3 17β-HSD is found exclusively in the testis,[11] the development of potent inhibitors could be of interest for blocking spermatogenesis and as an anti-fertility agent for males.[12,13] The first studies on the inhibition of type 3 17β-HSD were reported in 1983 by Pittaway[14] and revealed that a steroid scaffold with a carbonyl at position 17 and a non aromatic A-ring are important contributing factors for inhibition. More recently, we screened 80 steroids from different classes as inhibitors of type 3 17β-HSD,[15] and identified the C-19 steroid androsterone (ADT) to be a suitable lead compound for further development (Fig. 2). The subsequent synthesis of ADT derivatives bearing various hydrophobic 3β substituents led to inhibitors more potent than ADT. In fact, with an IC_{50} value of 57 nM, 3β-phenylmethyl-ADT (**1**) is one of the most potent type 3 17β-HSD inhibitors known to date.[16] Based on these results, we were interested in developing strategies for the rapid generation of a large number of diversified ADT 3β-substituted derivatives. Our plan was to use the combinatorial chemistry approach to generate libraries of steroid derivatives either in liquid phase or solid-phase.[17–20] Recently, we first published a method for the liquid-phase combinatorial synthesis of 3β-amido-3α-hydroxy-5α-androstan-17-ones of general formula **2**.[21] This time, taking advantage of the solid-phase synthesis for the generation of peptido-steroids,[18,22] we now report the parallel synthesis of 3β-peptido-3α-hydroxy-5α-androstan-17-ones of general formula **3** and their evaluation as inhibitors of type 3 17β-HSD.

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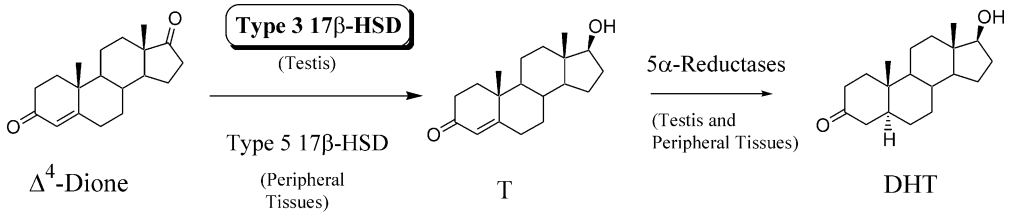


Figure 1. The role of type 3 17β-HSD in the transformation of the inactive androgen 4-androstene-3,17-dione (Δ^4-dione) into active androgens testosterone (T) and dihydrotestosterone (DHT).

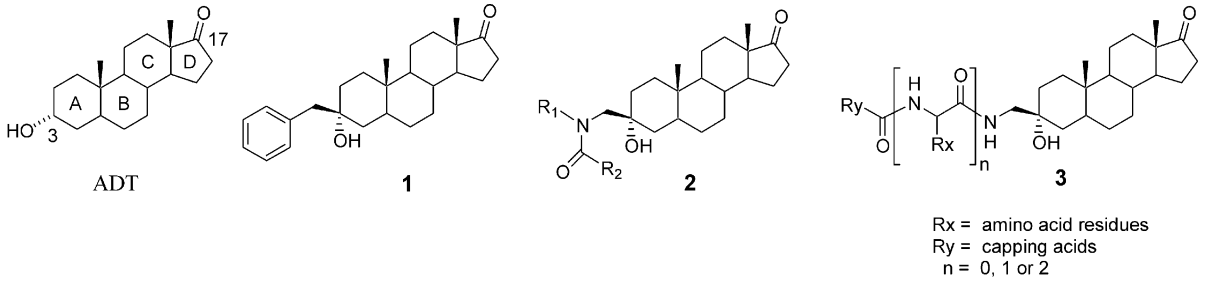


Figure 2. Steroidal inhibitors of type 3 17β-HSD derived from androsterone (ADT) and previously reported by us (compound **1** and general formula **2**) or proposed (general formula **3**).

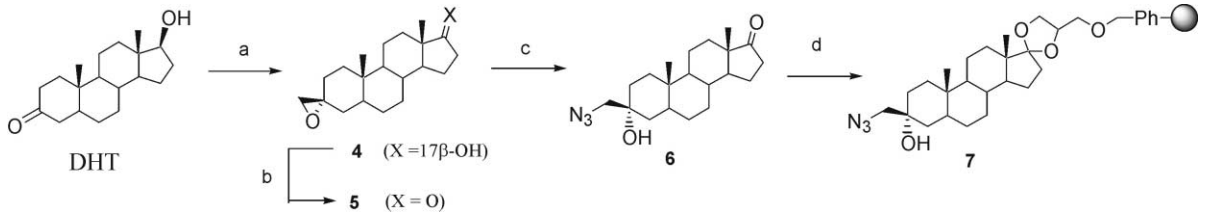
Results and Discussion

Chemical synthesis

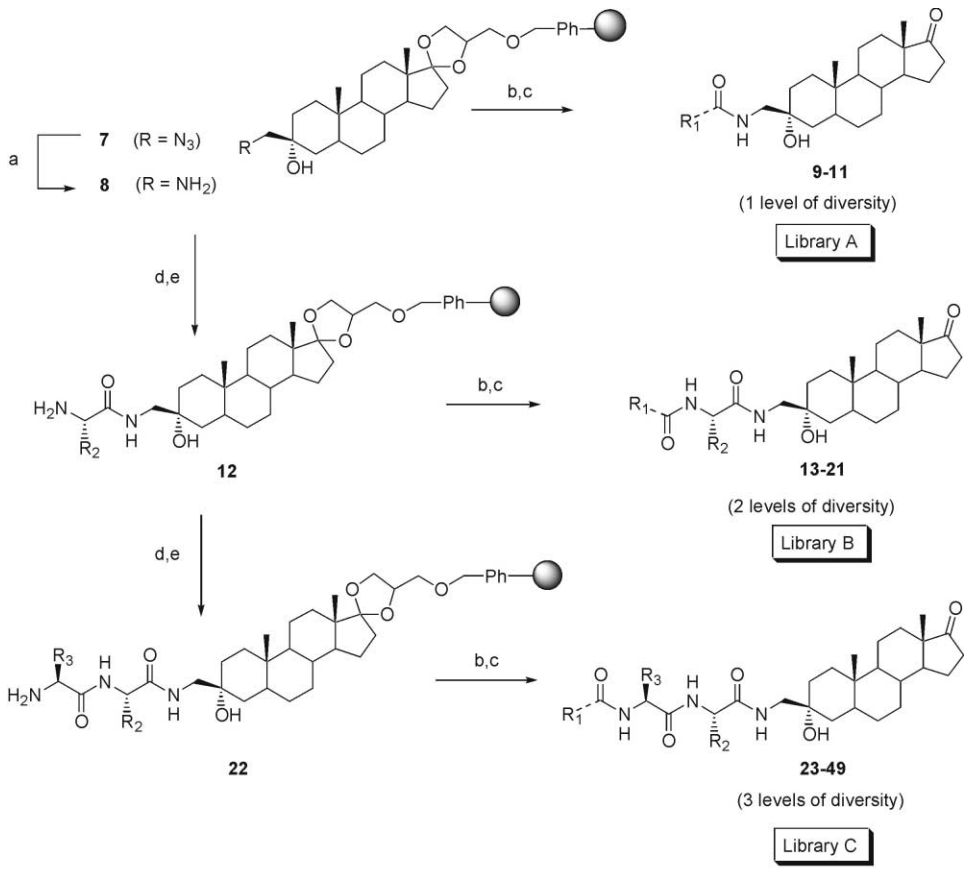
The use of amino acids at position 3β of a C19-steroid nucleus was very attractive in the perspective of promoting complementary interactions with amino acids of type 3 17β-HSD and of potentially increasing the affinity of such derivatives for this enzyme. Accordingly, based on our previously reported biological results,[15,16] we planned the synthesis of different ADT derivatives having a 3β-side chain ranging from 0 to 2 amino-acid units (1, 2 or 3 levels of diversity) including a capping with a carboxylic acid at the end of the sequence of reactions (general formula **3**). Based on preliminary work on the efficient coupling of steroidal ketone using polymer-bound glycerol,[23] we chose the Leznoff's acetal linker as the most appropriate and compatible one to link our library precursor **6** on solid support (Scheme 1) and to carry out the proposed solid-phase sequence producing the libraries A, B, and C (Scheme 2).

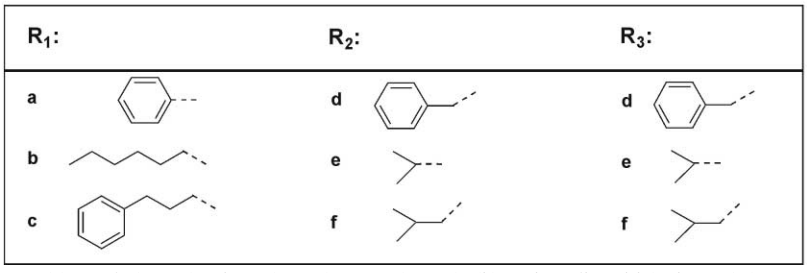
The library precursor **6** was easily obtained with good yield following a three-step synthesis. Starting with DHT, oxirane **4** was first stereoselectively synthesized using in situ generated dimethylsulfoxoniummethylide.[17,24] The subsequent oxidation of the 17β-OH group was performed using tetrapropylammonium perruthenate (TPAP) in dichloromethane to give the ketone **5** in a nearly quantitative yield. The final opening of the oxirane group of steroid **5** was accomplished by heating sodium azide and boric acid in dimethylformamide (DMF) to give the desired 3β-azidomethyl-3α-hydroxy-5α-androstan-17-one (**6**). Keto-steroid **6** was then coupled onto polymer-bound glycerol using the optimized acetal exchange conditions [trimethylorthoformate and $Sc(OTf)_3$ in toluene] previously reported by us.[23] The presence of the acetal linkage (119.4 ppm) and of the azide function (2098 cm^{-1}) was confirmed by ^{13}C gel phase NMR[25,26] (Fig. 3) and IR spectrum of resin **7**.

We selected three hydrophobic amino acids [L-phenylalanine (L-Phe), L-valine (L-Val) and L-leucine (L-Leu)] and three hydrophobic carboxylic acids (benzoic acid, heptanoic acid and phenylbutyric acid) as building blocks to generate the convenient model libraries A–C with 1, 2 or 3 levels of diversity (Scheme 2). The solid-phase synthesis of libraries started by reducing the azide function of **7** to obtain free amine **8** using the conditions of Bartra et al.[27] The second intermediate, amine **12**, was then synthesized from **8** by a treatment with a Fmoc protected amino acid using PyBOP, HOBt, and DIPEA in dry DMF under argon for 1 h followed by the cleavage of the Fmoc protective group with a solution of piperidine (20%) in dichloromethane. The third intermediate amine **22** was obtained from **12** using the same sequence of reactions (amino acid coupling and



Scheme 1. Reagents and conditions: (a) NaH, Me_3SOI, DMSO; (b) TPAP, NMO, molecular sieves, CH_2Cl_2; (c) NaN_3, H_3BO_3, DMF, 100 °C; (d) polymer-bound glycerol (1.0 mmol/g), TMOF, $Sc(OTf)_3$, toluene, rt.





Scheme 2. Reagents and conditions: (a) $SnCl_2$ (0.2 M), PhSH (0.8 M), Et_3N (1.0 M); (b) carboxylic acid (a, b, or c) (0.75 mmol), PyBOP (0.75 mmol), HOBt (0.75 mmol), DIPEA (1.5 mmol), DMF, rt; (c) HCl 2.0 N in dioxane (containing 1% of H_2O), rt; (d) L-amino acid (d, e, or f) (0.5 mmol), PyBOP (0.5 mmol), HOBt (0.5 mmol), DIPEA (1.0 mmol), DMF, rt; (e) piperidine/CH_2Cl_2 (2:8), rt.

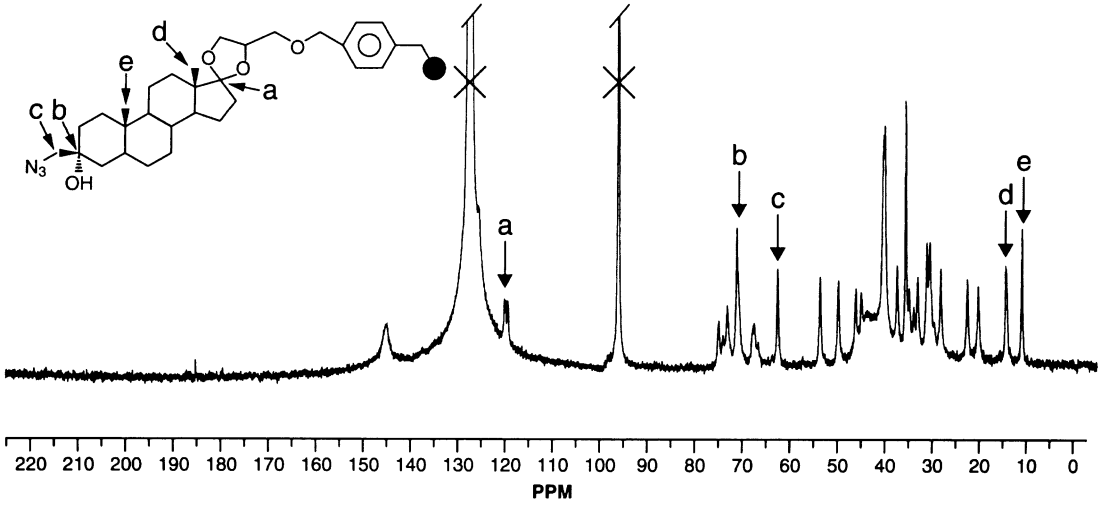


Figure 3. ^{13}C NMR spectrum of resin **7**, showing the characteristic signals a–e. The spectrum was recorded at 75.5 MHz using 100 mg resin **7** swollen in 0.6 mL of CCl_4/C_6D_6 (4:1) within a total experiment time of 1 h with the following conditions: $d_1 = 1.0$ s, $p_1 = 30°$ (3.0 μs), aq = 430 ms, RG 800, SI = 32 K.

Fmoc removal) reported above. Finally, the libraries with one (**9**–**11**), two (**13**–**21**), and three (**23**–**49**) levels of diversity were obtained from amines **8**, **12**, and **22** after a two-step sequence of reactions: (i) treatment with the mixed anhydride resulting from a carboxylic acid, PyBOP, HOBt, and DIPEA in dry DMF and (ii) final release of the 3β-ADT derivatives from the solid support using a solution of 2.0 N HCl in dioxane at room temperature.

The three model libraries A–C were synthesized in the parallel format giving individual compounds with overall yields varying from 23 to 58%. In a typical experiment, 60 mg of resin **7** yielded between 8 and 20 mg of the desired final compounds. The three members of library A, the nine members of library B, and a sampling of nine members of library C were characterized by IR, ^{1}H NMR and LR-MS. Their purity was determined by HPLC analysis (Table 1). The average crude purity (HPLC) of the library A (88%), library B (84%), and library C (88%) was found to be high enough for the compounds to be tested without further purification.

Biological activity

After we developed our parallel solid-phase methodology for the preparation of various 3β-peptido-steroids, we were interested in testing the ability of the members of the libraries A–C to inhibit type 3 17β-HSD. The assays were performed at a concentration of 0.3 μM of inhibitor and the results were expressed as the inhibition (%) of the enzymatic reduction of Δ^4-dione to testosterone (Fig. 4). In library A, the compounds **9**, **10**, and **11** show very similar inhibitory activities (47–60%), with a slightly slower inhibition value for the compounds bearing no aromatic ring. In library B, the compounds **13**, **16**, and **19**, bearing a benzoyl building block at position R_1, showed the best inhibition (57–65%) irrespective of the amino acid at position R_2. Finally in library C, a benzoyl group at position R_1 was clearly not appropriate to inhibit the enzyme ($<53\%$) and the best inhibitory activities ($>65\%$) were observed only in compounds having a heptanoyl or a phenylbutyryl as capping group. From these library C members originate the strongest inhibitors of our study (Table 2). As a general tendency through the three libraries, the compounds

Table 1. Characterization of selected library members

Compound[a]	Library	R_1	R_2	R_3	Theorical mass	Observed mass[b]	Overall yield[c] (%)	Crude purity[d] (%)
9	A	a	—	—	423.3	424.4	47	91
10	A	b	—	—	431.3	432.9	58	80
11	A	c	—	—	465.3	466.6	47	94
13	B	a	d	—	570.4	571.3	44	85
14	B	b	d	—	578.4	579.5	40	88
15	B	c	d	—	612.4	613.4	43	40
16	B	a	e	—	522.4	523.7	53	93
17	B	b	e	—	530.4	531.5	55	83
18	B	c	e	—	564.4	565.5	43	92
19	B	a	f	—	536.4	535.7	45	85
20	B	b	f	—	544.4	545.4	43	92
21	B	c	f	—	578.4	579.5	43	94
23	C	a	d	d	717.9	718.5	37	90
24	C	b	d	d	—	—	34	—
25	C	c	d	d	—	—	42	—
26	C	a	d	e	—	—	40	—
27	C	b	d	e	—	—	39	—
28	C	c	d	e	712.0	712.5	35	92
29	C	a	d	f	683.9	684.4	41	96
30	C	b	d	f	692.0	692.6	41	86
31	C	c	d	f	—	—	40	—
32	C	a	e	d	—	—	48	—
33	C	b	e	d	—	—	34	—
34	C	c	e	d	—	—	37	—
35	C	a	e	e		—	43	—
36	C	b	e	e	629.5	630.8	27	82
37	C	c	e	e	663.5	664.4	23	84
38	C	a	e	f	635.9	636.6	42	90
39	C	b	e	f	643.5	644.5	41	81
40	C	c	e	f	—	—	44	—
41	C	a	f	d	—	—	46	—
42	C	b	f	d	—	—	39	—
43	C	c	f	d	725.5	726.6	43	90
44	C	a	f	e	—	—	39	—
45	C	b	f	e	—	—	39	—
46	C	c	f	e	—	—	47	—
47	C	a	f	f	—	—	39	—
48	C	b	f	f	—	—	41	—
49	C	c	f	f	—	—	38	—

[a]See Scheme 2 for the chemical structures of **9**–**11**, **13**–**21**, and **23**–**49** and the signification of R_1, R_2 and R_3.
[b]Low resolution mass spectra (LR-MS) in positive mode $(M+H)^+$, except compound **19** in negative mode $(M-H)^-$.
[c]Calculated for the sequence of reaction starting from resin **7** and using the theorical loading of polymer-bound glycerol (1.0 mmol/g).
[d]High-performance liquid chromatography (HPLC) purity of steroid derivatives released from resin (without purification steps).

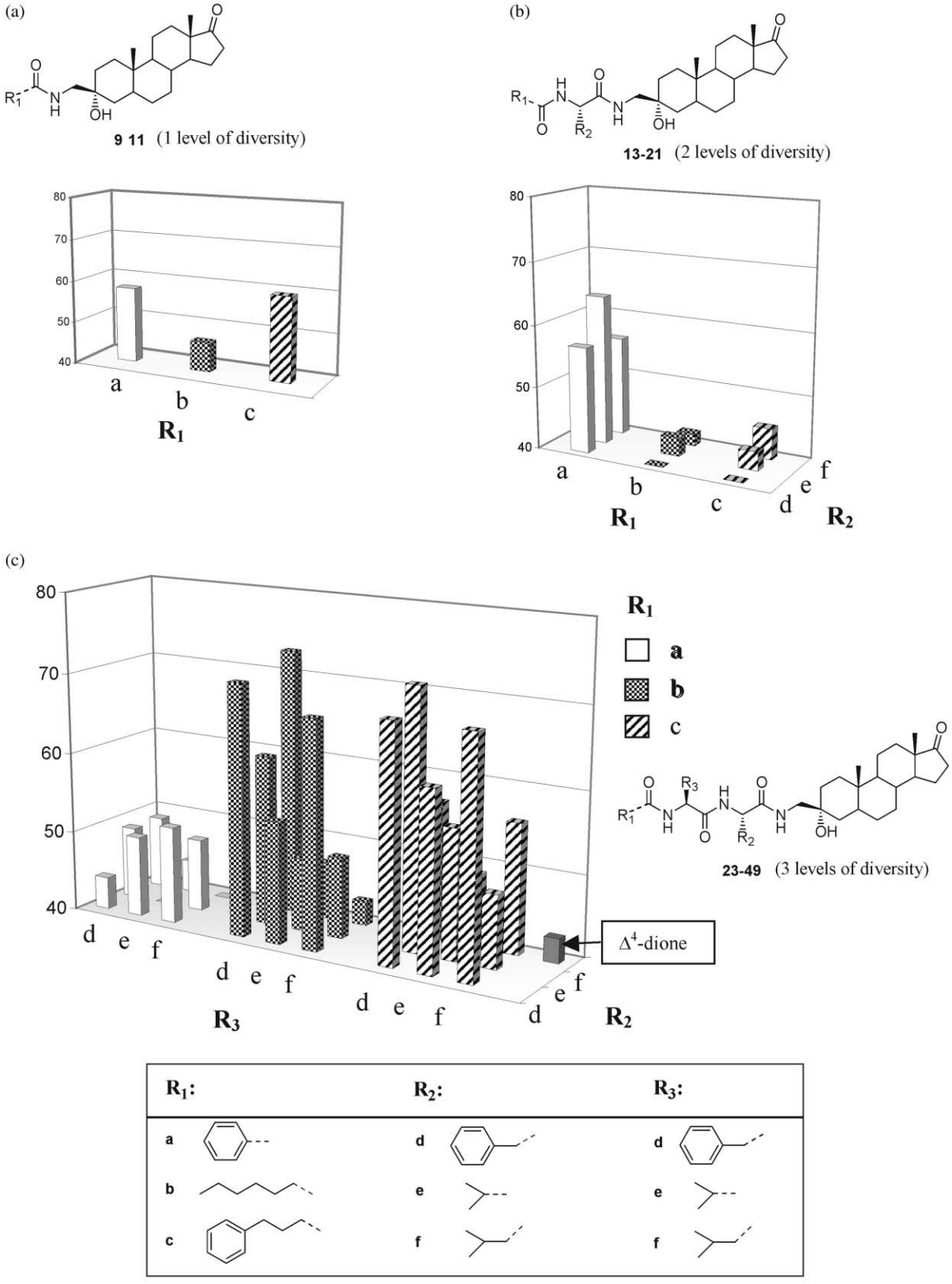
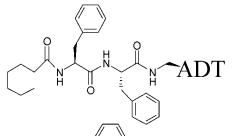
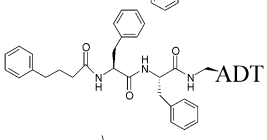
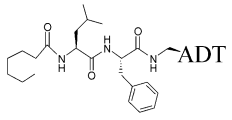
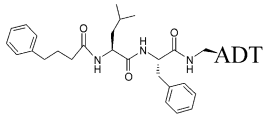
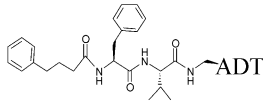
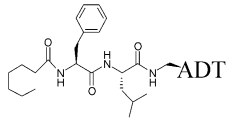


Figure 4. Inhibition (%) of type 3 17β-HSD by 0.3 μM of compounds **9**–**11** (Library A), **13**–**21** (library B), and **23**–**49** (library C). The reaction tested was the reductive transformation of [^{14}C] Δ^4-dione (0.05 μM) to [^{14}C] testosterone catalysed by type 3 17β-HSD transfected in HEK-293 cells (homogenized).

Table 2. The most potent inhibitors from our study: chemical structures, inhibitory activity on type 3 17β-HSD, and proliferative activity in Shionogi (AR^+) cells

Compd	Structure	Type 3 17β HSD inhibition[a]			Proliferative activity[b]	
		% at 0.3 μM	% at 0.03 μM	IC_{50} (nM)	% at 1 μM	% at 0.1 μM
24		71	26	N	41	1
25		69	26	N	18	0
30		68	29	N	30	8
31		69	23	N	3	2
34		72	26	N	46	8
42		73	29	227±37	30	5
—	ADT	N	N	182±46	2	14
—	Δ^4-dione (natural substrate)	43	16	489±112	N	N
1	3β-phenylmethyl-ADT	91	24	98±9	100	27

N, not determined.
[a]Inhibition of type 3 17β-HSD activity transforming [^{14}C] Δ^4-dione (0.05 μM) to [^{14}C] T.
[b]Proliferative activity (%) in androgen-sensitive (AR^+) Shionogi cells compared to the stimulation induced by 0.3 nM of androgen DHT (100%).

having a phenyl ring in their 3β-side chain generally achieved a good inhibition of type 3 17β-HSD. Thus, the six best inhibitors of these libraries have at least one phenyl group, suggesting its importance for inhibition.

For comparison purposes, we determined the IC_{50} value of compound **42**, the most potent inhibitor of libraries A–C, of ADT, the steroidal nucleus precursor, and of Δ^4-dione the natural substrate (used as inhibitor) (Table 2). Despite a bulky peptido chain at position 3β of ADT, compound **42** ($IC_{50} = 227 \pm 37$ nM) was found to be as equally potent as ADT ($IC_{50} = 182 \pm 46$ nM). This observation highlights the fact that the enzyme probably possesses a relatively large hydrophobic pocket that could tolerate a long and bulky substituent at position 3β of ADT. This interesting finding opens the door to additional optimization steps. Indeed, a compound with 3 levels (R_1, R_2 and R_3) of diversity, such as library C members, will favor more beneficial interactions with the enzyme than compounds having just one (R_1) or two (R_1 and R_2) levels of diversity. Compound **42** is even two times more potent than Δ^4-dione ($IC_{50} = 489 \pm 112$ nM), the natural substrate of the enzyme, but two times less potent than our first reported inhibitor (compound **1**; $IC_{50} = 98 \pm 9$ nM).

In the context of potentially using these type 3 17β-HSD inhibitors in the treatment of androgen-sensitive diseases, we verified their androgenic profile using the androgen-sensitive (AR^+) Shionogi cells as a model of androgenicity. Interestingly, compound **42** caused only a very slight proliferative activity at 1 μM (only 30% of the stimulation induced by 0.3 nM of DHT) and no significant proliferative activity at 0.1 μM. At these two concentrations (0.1 and 1 μM), the known inhibitor **1** (Fig. 2) strongly induced (27 and 100%, respectively) the proliferation of androgen-sensitive cells.

Conclusion

Steroidal ketone **6** was easily synthesized in solution and linked with good yield to a solid support (polymer-bound glycerol) by an efficient transacetalization reaction. Using Fmoc chemistry, the coupling of different amino acids on the aminomethyl group of resin **8** led to model libraries of 1, 2, and 3 levels of molecular diversity (libraries A–C). Six 3β-peptido-3α-hydroxy-5α-androstane-17-ones of the library C showed potent inhibition of the targeted enzyme, type 3 17β-HSD. One

of them, compound **42**, inhibited the enzyme with an $IC_{50} = 227$ nM and induce only a slight proliferation of androgen-sensitive (AR^+) Shionogi cells at high concentration (1 μM), and no proliferation at 0.1 μM. These preliminary results are very encouraging considering the potency of the parallel solid-phase methodology developed to generate a huge number of ADT derivatives and, consequently, to facilitate the optimization of such type 3 17β-HSD inhibitors. Furthermore, the chance to find a perfect fitting between the 3β-side chain of ADT derivatives and amino acids of the enzyme hydrophobic pocket will be increased by the possible combinations allowed by compounds of three levels of diversity. Thus, the next step will be to prepare new libraries with three levels of diversity, containing more diversified amino acids and carboxylic acids. These second generation will be synthesized in the parallel format (individual compounds) or in the split and pool format[28,29] (mixture of compounds) using the methodology that we reported above.

Experimental

General methods

Dihydrotestosterone (DHT) was purchased from Steraloids (Wilton, NH, USA). Polymer-bound glycerol with a loading of 1.0 mmol/g and chemical reagents 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 dichloromethane (CH_2Cl_2), acetonitrile (CH_3CN), dimethyl sulfoxide (DMSO), and dimethyformamide (DMF) were obtained from Sigma-Aldrich. Anhydrous tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl and kept under argon. The libraries of steroid derivatives were realized with ACT LabTech manual synthesizer (Advanced ChemTech; Louisville, KY, USA) using either a 40 or 96 solid-phase reaction block. Thin-layer chromatography (TLC) and flash-column chromatography were performed on 0.20 mm silica gel 60 F254 plates and with 230–400 mesh ASTM silica gel 60, respectively (E. Merck, Darmstadt, Germany). The purity of every library A and B members and a sampling of library C members was determined by HPLC (Waters Associates, Milford, MA, USA) using a NovaPak C18 reversed-phase column (150×3.9 mm id) and an ultraviolet detector (205 or 225 nM). Infra-red spectra (IR) were recorded on a Perkin–Elmer series 1600 FT-IR spectrometer (Norwalk, CT, USA) and the significant bands reported in cm^{-1}. Nuclear magnetic resonance spectra (NMR) were recorded at 300 MHz for ^1H and 75.5 MHz for ^{13}C on a Brucker AC/F300 spectrometer (Billerica, MA, USA) and reported in ppm. Low-resolution mass spectra (LR-MS) were recorded on a PE Sciex API-150ex apparatus (Foster City, CA, USA) equipped with a turbo ionspray source.

Synthesis of spiro-3(*R*)-oxirane-5α-androstan-17-one (5). To a solution of hydroxy-steroid **4**[17] (7.3 g, 24.0 mmol) in dry CH_2Cl_2 (60 mL) were added molecular sieves 4 Å (14.0 g) and 4-methylmorpholine-*N*-oxide (NMO) (4.9 g, 41.8 mmol) and tetrapropylammonium perruthenate (TPAP) (490 mg, 1.4 mmol). The reaction mixture was stirred for 3 h and then directly purified by flash chromatography (EtOAc/hexanes, 3:7) to give the desired keto-steroid **5** (7.0 g, 95% yield). IR (KBr): 1740 (C=O). ^1H NMR ($CDCl_3$) δ 0.86 (s, 6H, CH_3-18 and CH_3-19), 0.80–2.20 (21H), 2.43 (dd, $J_1 = 8.6$ Hz and $J_2 = 18.9$ Hz, 1H of CH_2-16), 2.62 (s, 2H, OCH_2 of oxirane); ^{13}C NMR ($CDCl_3$) δ 11.3, 13.8, 20.3, 21.8, 23.3, 29.1, 30.7, 31.5, 35.0, 35.6, 35.8, 35.9 (2×), 43.7, 47.8, 51.4, 53.5, 54.1, 58.4, 221.3. LR-MS for $C_{20}H_{30}O_2 + NH_4$ [M^+]: m/z 320.3.

Synthesis of 3β-azidomethyl-3α-hydroxy-5α-androstan-17-one (6). To a solution of keto-steroid **5** (4.0 g, 13.2 mmol) in DMF (60 mL) under an atmosphere of argon was added sodium azide (1.07 g, 16.5 mmol) and boric acid (3.40 g, 55.0 mmol). The solution was stirred at 50 °C for 5 h. The reaction mixture was poured in cold water and extracted with EtOAc (2×250 mL). The organic layer was washed with water (500 mL), brine (250 mL) and dried with $MgSO_4$. After filtration and evaporation of solvent, the resulting crude product was purified by flash chromatography using EtOAc/hexanes (2:8) as eluent to give compound **6** (3.4 g, 75% yield). IR (KBr) 3462 (OH), 2100 (N_3), 1723 (C=O, ketone). ^1H NMR ($CDCl_3$) δ 0.75 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.90–2.20 (22H), 2.41 (dd, $J_1 = 8.5$ Hz, $J_2 = 19.0$ Hz, 1H of CH_2-16), 3.19 (s, 2H, CH_2N_3); ^{13}C NMR ($CDCl_3$) δ 11.1, 13.7, 20.2, 21.7, 28.0, 30.7 (2×), 31.5, 33.1, 34.9, 35.8, 35.9, 37.4, 40.3, 47.7, 51.3, 54.1, 62.7, 71.7, 221.4. LR-MS for $C_{20}H_{31}N_3O_2 + NH_4$ [M^+]: m/z 363.2.

Coupling of keto-steroid 6 to polymer-bound glycerol (synthesis of 7). To polymer-bound glycerol (5.0 g, 5.0 mmol) swollen in dry toluene (20 mL) were added a solution of keto-steroid **6** (4.98 g, 14.4 mmol) in dry toluene (30 mL), trimethylorthoformate (TMOF) (1.53 g, 15.0 mmol), and scandium triflate (150 mg, 0.30 mmol). The suspension was stirred for 16 h at room temperature under an atmosphere of argon. The resulting resin was filtered and washed with toluene (60 mL), CH_2Cl_2 (2×60 mL) and MeOH (60 mL). The excess of keto-steroid **6** was then recovered after evaporation of the filtrate, while the resin **7** was dried overnight under reduced pressure to give 6.64 g. IR (KBr) 3462 (OH) and 2098 (N_3). ^{13}C NMR (CCl_4/C_6D_6, 80:20) δ 11.2, 14.7, 20.5, 22.8, 28.3, 30.6, 31.3, 33.2, 35.0, 35.7 (2×), 37.5, 40.1, 45.1, 46.2, 49.8, 53.7, 62.6, 67.5, 71.2, 73.2, 74.1, 74.9, 96.1 (CCl_4), 119.4, 120.2 (CH–aromatic), 122–125 (C_6D_6 and CH aromatic), 145.0 (CH aromatic) (Fig. 3).

Reduction of azide 7 to amine 8. To resin **7** (6.49 g) were added a solution of tin (II) chloride (2.38 g, 0.2 M), thiophenol (4.92 mL, 0.8 M) and triethylamine (8.36 mL, 1.0 M) in dry THF (65 mL). The resulting mixture was stirred under an atmosphere of argon for 8 h at room temperature. The resin was then filtered and washed with THF (2×70 mL), DMF (2×70 mL), CH_2Cl_2 (2×70 mL), and MeOH (2×70 mL) to give, after 2 days of drying under reduced pressure, the

corresponding amine **8**. IR (KBr): 3405 (OH and NH_2), and no azide (N_3) band observed at 2098.

General procedure for synthesis of compounds 9–11 (library A)

The compounds possessing a single level of diversity (R_1) were obtained by treatment of resin **8** (60 mg) (assuming a loading of 70% or 0.7 mmol/g) with a solution of carboxylic acid (2 equiv of benzoic acid, heptanoic acid or 4-phenylbutyric acid), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (2 equiv), *N*-hydroxybenzotriazole (HOBt) (2 equiv) and diisopropylethylamine (DIPEA) (4 equiv) in dry DMF and under an atmosphere of argon for 2 h. Each resin was then washed with DMF (1×), CH_2Cl_2 (2×) and MeOH (1×), and dried under vacuum for 1 h. The decoupling of the final compound from the solid support was achieved by stirring the resin for 2 h with a solution of 1,4-dioxane/HCl 2.0 N containing 1% of water. The resulting mixture was filtered, diluted with EtOAc, and transferred in a test tube to be washed (1×) with a cold aqueous solution of 10% $NaHCO_3$. The organic layer was finally evaporated using a speedvac apparatus (Jouan RC1010, Winchester, VA, USA) and dried under reduced pressure for 3 days at 50 °C to give compounds **9**–**11** in good average yield (51%) and high average HPLC purity (88%) without any purification step (Table 1).

3β-(*N*-Benzoyl-aminomethyl)-3α-hydroxy-5α-androstan-17-one (9). IR (KBr) 3385 (NH and OH), 1740 (C=O, ketone), 1638 (C=O, amide). ^{1}H NMR (acetone-d_6) δ 0.83 and 0.84 (2s, 6H, 18-CH_3 and 19-CH_3), 0.80–2.10 (22 H), 2.37 (dd, $J_1 = 8.9$ Hz, $J_2 = 18.0$ Hz, 1H of CH_2-16), 3.40 (d, $J = 6.2$ Hz, 2H, CH_2NHCO), 7.49 (m, 3H, CH aromatics), 7.73 (broad, NH), 7.92 (d, $J = 7.3$ Hz, 2H, CH aromatics). LR-MS for $C_{27}H_{38}NO_3$ $[M+H]^+$ m/z 424.4. HPLC ($CH_3CN/H_2O/CH_3OH$, 30:45:25) 91% of purity.

3β-(*N*-Heptanoyl-aminomethyl)-3α-hydroxy-5α-androstan-17-one (10). IR (KBr) 3385 (NH and OH), 1740 (C=O, ketone), 1651 (C=O, amide). ^{1}H NMR (acetone-d_6) δ 0.81 (s, 3H, 19-CH_3), 0.84 (s, 3H, 18-CH_3), 0.88 (t, $J = 7.2$ Hz, 3H, CH_3CH_2), 0.80–2.10 (30 H), 2.21 (t, $J = 7.4$ Hz, 2H, CH_2CO), 2.37 (dd, $J_1 = 8.8$ Hz, $J_2 = 18.0$ Hz, 1H of CH_2-16), 3.16 (d, $J = 4.5$ Hz, 2H, CH_2NHCO), 7.12 (broad, NH). LR-MS for $C_{27}H_{46}NO_3$ $[M+H]^+$ m/z 432.9. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:40:25) 80% of purity.

3β-(*N*-Phenylbutyryl-aminomethyl)-3α-hydroxy-5α-androstan-17-one (11). IR (KBr) 3378 (NH and OH), 1740 (C=O, ketone), 1654 (C=O, amide). ^{1}H NMR (acetone-d_6) δ 0.81 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.80–2.10 (25H), 2.25 (t, $J = 7.3$ Hz, 2H, CH_2CO), 2.37 (dd, $J_1 = 9.0$ Hz, $J_2 = 18.0$ Hz, 1H of CH_2-16), 2.64 (t, $J = 7.7$ Hz, 2H, CH_2Ph), 3.17 (d, $J = 5.7$ Hz, 2H, CH_2NHCO), 7.22 (m, 5H, CH aromatics). LR-MS for $C_{30}H_{44}NO_3$ $[M+H]^+$ m/z 466.6. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:40:25): 94% of purity.

General procedure for synthesis of compounds 13–21 (library B)

The compounds possessing two levels of diversity (R_1 and R_2) were obtained by the reaction of resin **8** with the three different *N*-protected amino acid (Fmoc-L-Phe-OH, Fmoc-L-Val-OH, Fmoc-L-Leu-OH) using a solution of PyBOP (2 equiv), HOBt (2 equiv) and DIPEA (4 equiv) in dry DMF and under an atmosphere of argon for 1 h. The resulting resins were stirred with a solution of 20% piperidine in CH_2Cl_2 for 1 h to remove the Fmoc protecting group. The resins were then washed with CH_2Cl_2 (3×) and dried under vacuum for 1 h to give three resins of general structure **12**. Each of the three resins was then split into three portions (60 mg) and submitted to acylation using three carboxylic acids (benzoic acid, heptanoic acid and 4-phenylbutyric acid) and the same conditions as reported above for library A. The acid cleavage of the resulting resins with 1,4 dioxane/HCl 2.0 N containing 1% of water (as above), led to compounds **13**–**21** in good average yield (45%) and high average HPLC purity (84%) without any purification step (Table 1).

3β-(*N*-Benzoyl-L-phenylalanin-aminomethyl)-3α-hydroxy-5α-androstane-17-one (13). IR (KBr) 3306 (NH and OH), 1737 (C=O, ketone), 1662 (C=O, amide), 1646 (C=O, amide). ^{1}H NMR (acetone-d_6) δ 0.78 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.80–2.10 (24H), 2.37 (m, 1H of CH_2-16), 3.20 (m, 4H, CH_2NHCO and CH_2Ph), 4.90 (m, 1H, NH-*CH*-CO), 7.35 (m, 8H, CH aromatics), 7.84 (d, $J = 7.3$ Hz, 2H, CH aromatics. LR-MS for $C_{36}H_{47}N_2O_4$ $[M+H]^+$: m/z 571.3. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:35:30) 85% of purity.

3β-(*N*-Heptanoyl-L-phenylalanine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (14). IR (KBr) 3283 (NH and OH), 1738 (C=O, ketone), 1657 (C=O, amide), 1646 (C=O, amide). ^{1}H NMR (acetone-d_6) δ 0.80 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.86 (t, $J = 7.0$ Hz, 3H, CH_3CH_2), 0.80–2.40 (30H), 2.14 (t, $J = 7.4$ Hz, 2H, CH_2CO), 2.37 (m, 1H of CH_2-16), 2.91 (dd, $J_1 = 9.0$ Hz, $J_2 = 13.8$ Hz, 1H of CH_2Ph), 3.12 (m, 3H, CH_2NHCO and 1H of CH_2Ph), 4.70 (q, $J_1 = 5.7$ Hz, $J_2 = 8.5$ Hz, 1H, NH-*CH*-CO), 7.28 (m, 5H, CH aromatics), 7.44 (m, 2H, NH). LR-MS for $C_{36}H_{55}N_2O_4$ $[M+H]^+$: m/z 579.5. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:30:35) 88% of purity.

3β-(*N*-Phenylbutyryl-L-phenylalanine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (15). IR (KBr) 3294 (NH and OH), 1738 (C=O, ketone), 1670 (C=O, amide), 1640 (C=O, amide). ^{1}H NMR (acetone-d_6) δ 0.79 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.80–2.10 (25H), 2.18 (t, $J = 7.5$ Hz, 2H, CH_2CO), 2.37 (dd, $J_1 = 8.8$ Hz, $J_2 = 17.9$ Hz, 1H of CH_2-16), 2.52 (t, 2H, $J = 7.8$ Hz, CH_2CH_2Ph), 3.15 (m, 4H, CH_2NHCO and CH_2Ph), 4.74 (m, 1H, NH–*CH*–CO), 7.20 (m, 10H, CH aromatics), 7.38 (broad, NH). LR-MS for $C_{39}H_{53}N_2O_4$ $[M+H]^+$ m/z 613.4. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:30:35): 40% of purity. In addition to compound **15** (40%) another unknow compound (55%) was also observed but not identified.

3β-(*N*-Benzoyl-L-valine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (16). IR (KBr) 3299 (NH and OH), 1742 (C=O, ketone), 1636 (C=O, amide). ^{1}H NMR (acetone-d_6) 0.79 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 1.02 (d, 6H, J = 6.7 Hz, $(CH_3)_2$-CH), 0.80–2.40 (23H), 3.21 (d, J = 5.9 Hz, 2H, CH_2NHCO), 4.50 (t, J = 7.9 Hz, 1H, NH-*CH*-CO), 7.48 (m, 4H, NH and 3×CH aromatics), 7.52 (d, J = 7.1 Hz, NH), 7.67 (d, J = 8.3 Hz, NH), 7.93 (d, J = 7.4 Hz, 2H, CH aromatics). LR-MS for $C_{32}H_{47}N_2O_4$ $[M+H]^+$ m/z 523.7. HPLC (CH_3CN/H_2O/CH_3OH, 30:40:30): 93% of purity.

3β-(*N*-Heptanoyl-L-valine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (17). IR (KBr) 3467, 3339 and 3288 (NH and OH), 1741 (C=O, ketone), 1637 (C=O, amides). ^{1}H NMR (acetone-d_6) δ 0.81 (s, 3H, 19-CH_3), 0.84 (s, 3H, 18-CH_3), 0.91 (t, J = 7.2 Hz, 3H, CH_3CH_2), 0.94 (d, J = 5.8 Hz, 6H, $(CH_3)_2$CH), 0.80–2.10 (21 H), 2.24 (m, 2H, CH_2CO), 2.37 (dd, J_1 = 9.0 Hz, J_2 = 17.8 Hz, 1H of CH_2-16), 3.18 (d, J = 5.8 Hz, 2H, CH_2NHCO), 4.28 (t, J = 8.1 Hz, 1H, NH-*CH*-CO), 7.20 (d, J = 8.6 Hz, NH), 7.33 (broad, NH). LR-MS for $C_{32}H_{55}N_2O_4$ $[M+H]^+$ m/z 531.5. HPLC (CH_3CN/H_2O/CH_3OH, 35:30:35) 83% of purity.

3β-(*N*-Phenylbutyryl-L-valine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (18). IR (KBr) 3462, 3334 and 3283 (NH and OH), 1740 (C=O, ketone), 1637 (C=O, amides). ^{1}H NMR (acetone-d_6) 0.79 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.94 (t_{app}, J = 5.8 Hz, 6H, $(CH_3)_2$CH), 0.80–2.20 (31H), 2.35 (m, 3H, CH_2CO and 1H of CH_2-16), 2.63 (t, J = 7.7 Hz, 2H, CH_2Ph), 3.18 (t, J = 5.2 Hz, 2H, CH_2NHCO), 4.30 (t, J = 7.8 Hz, 1H, NH-*CH*-CO), 7.23 (m, 6H, NH and CH aromatics), 7.36 (broad, NH). LRMS for $C_{35}H_{53}N_2O_4$ $[M+H]^+$ m/z 565.5. HPLC (CH_3CN/H_2O/CH_3OH, 35:30:35) 92% of purity.

3β-(*N*-Benzoyl-L-leucine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (19). IR (KBr) 3376 (NH and OH), 1741 (C=O, ketone), 1640 (C=O, amides). ^{1}H NMR (acetone-d_6) 0.78 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.95 (t_{app}, J = 5.5 Hz, 6H, $(CH_3)_2$CH), 0.80–2.10 (25H), 2.36 (dd, J_1 = 8.8 Hz, J_2 = 17.8 Hz, 1H of CH_2-16), 3.17 (s, 2H, CH_2NHCO), 4.70 (m, 1H, NH–*CH*–CO), 7.48 (m, 4H, NH and 3×CH aromatics), 7.93 (m, 3H, NH and 2×CH aromatics). LR-MS for $C_{33}H_{47}N_2O_4$ $[M-H]^-$ 535.7 m/z. HPLC (CH_3CN/H_2O/CH_3OH, 30:40:30) 85% of purity.

3β-(*N*-Heptanoyl-L-leucine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (20). IR (KBr) 3359 (NH and OH), 1740 (C=O, ketone), 1654 (C=O, amides). ^{1}H NMR (acetone-d_6) δ 0.81 (s, 3H, 19-CH_3), 0.84 (s, 3H, 18-CH_3), 0.90 (t_{app}, J = 7.6 Hz, 9H, $(CH_3)_2$CH and CH_3CH_2), 0.80–2.10 (35H), 2.22 (t, J = 7.4 Hz, 2H, CH_2CO), 2.38 (m, 1H of CH_2-16), 3.15 (t, J = 5.5 Hz, 2H, CH_2NHCO), 4.46 (m, 1H, NH–*CH*–CO), 7.33 (broad, 2×NH). LR-MS for $C_{33}H_{57}N_2O_4$ $[M+H]^+$ m/z 545.4. HPLC (CH_3CN/H_2O/CH_3OH, 35:35:30) 92% of purity.

3β-(*N*-Phenylbutyryl-L-leucine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (21). IR (KBr) 3300 (NH and OH), 1741 (C=O, ketone), 1654 (C=O, amides). ^{1}H NMR (acetone-d_6) δ 0.79 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.91 (t_{app}, J = 6.5 Hz, 6H, $(CH_3)_2$CH), 0.80–2.10 (27H), 2.27 (t, J = 7.3 Hz, 2H, CH_2CO), 2.37 (dd, J_1 = 8.8 Hz, J_2 = 18.0 Hz, 1H of CH_2-16), 2.63 (t, J = 7.7 Hz, 2H, CH_2Ph), 3.15 (m, 2H, CH_2NHCO), 4.48 (m, 1H, NH–*CH*–CO), 7.22 (m, 6H, NH and CH aromatics), 7.42 (broad, NH). LR-MS for $C_{36}H_{55}N_2O_4$ $[M+H]^+$ m/z 579.5. HPLC (CH_3CN/H_2O/CH_3OH, 30:40:30) 94% of purity.

General procedure for synthesis of compounds 23–49 (library C)

The three different resins of general structure **12** were split into three equal portions (nine reactors, 190 mg each) and the second level of diversity (R_3) was introduced by coupling the three different *N*-protected amino acids (Fmoc-L-Phe-OH, Fmoc-L-Val-OH, Fmoc-Leu-OH) using a solution of PyBOP (2 equiv), HOBt (2 equiv) and (DIPEA) (4 equiv) in dry DMF and under an atmosphere of argon for 1 h. The resulting resins were stirred with a solution of 20% piperidine in CH_2Cl_2 for 1 h to remove the Fmoc protecting group. The resins were then washed with CH_2Cl_2 (3×) and dried under vacuum for 1 h to give resins of general structure **22**. The nine resins **22** were then split into three equal portions (27 reactors, 60 mg each) and the third level of diversity (R_1) was obtained by performing the acylation with carboxylic acids (benzoic acid, heptanoic acid and 4-phenylbutyric acid) as reported above for library A. The acid cleavage of the resulting resins with 1,4 dioxane/HCl 2.0 N containing 1% of water (as above) led to compounds **23**–**49** in good average yield (39%) and high average HPLC purity (88%) for a random sampling of nine members (**23**, **28**, **29**, **30**, **36**, **37**, **38**, **39**, and **43**) without any purification step (Table 1).

3β-(*N*-Benzoyl-L-phenylalanine-L-phenylalanine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (23). IR (film) 3283 (NH and OH), 1738 (C=O, ketone), 1635 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.73 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.80–2.20 (23H), 2.46 (dd, J_1 = 10.5 Hz, J_2 = 19.2 Hz, 1H of CH_2-16), 2.80–3.30 (m, 6H, 2×CH_2Ph and CH_2NHCO), 4.69 (m, 2H, 2×NH–*CH*–CO), 6.23 (d, J = 8.2 Hz, NH), 6.52 (broad, NH), 6.90–7.60 (m, 15H, CH aromatics); LR-MS for $C_{45}H_{56}N_3O_5$ $[M+H]^+$ m/z 718.5. HPLC (CH_3CN/H_2O/CH_3OH, 35:30:35) 90% of purity.

3β-(*N*-Phenybutyryl-L-valine-L-phenylalanine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (28). IR (film) 3283 (NH and OH), 1738 (C=O, ketone), 1636 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.71 (s, 3H, 19-CH_3), 0.84 (s, 3H, 18-CH_3), 0.87 (2d, J = 6.6 Hz, 6H, $(CH_3)_2$CH), 0.80–2.30 (25H), 2.43 (dd, J_1 = 8.6 Hz, J_2 = 19.0 Hz, 1H of CH_2-16), 2.60 (t, J = 7.6 Hz, 2H, CH_2CH_2Ph), 3.10 (m, 4H, CH_2Ph and CH_2NHCO), 4.33 (t, J = 7.1 Hz, 1H, NH–*CH*–CO), 4.74 (q, broad, 1H, NH–*CH*–CO), 6.57 (broad, NH), 6.81 (broad, NH), 7.20 (m, 11H, CH aromatics and NH). LR-MS for $C_{43}H_{60}N_3O_5$ $[M+H]^+$ m/z 712.5. HPLC (CH_3CN/H_2O/CH_3OH, 35:30:35) 92% of purity.

3β-(*N*-Benzoyl-L-leucine-L-phenylalanine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (29). IR (film) 3289 (NH and OH), 1738 (C=O, ketone), 1636 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.73 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.94 (2d, $J=5.8$ Hz, 6H, (CH_3)$_2$CH), 0.80–2.20 (25H), 2.43 (dd, $J_1=8.5$ Hz, $J_2=19.0$ Hz, 1H of CH_2-16), 3.14 (m, 4H, CH_2Ph and CH_2NHCO), 4.45 (q, broad, 1H, NH–*CH*–CO), 4.67 (q, $J_1=7.3$ Hz, $J_2=6.6$ Hz, 1H, NH–*CH*–CO), 6.48 (d, $J=5.9$ Hz, NH), 6.55 (d, $J=7.6$ Hz, 2×NH), 7.13 (d, broad, 5H, CH aromatics), 7.45 (t_{app}, $J=7.4$ Hz, 2H, CH aromatics), 7.57 (t_{app}, $J=7.3$ Hz, 1H, CH aromatics), 7.66 (d, $J=7.4$ Hz, 2H, CH aromatics). LR-MS for $C_{42}H_{58}N_3O_5$ $[M+H]^+$ *m/z* 684.4. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:30:35) 96% of purity.

3β-(*N*-Heptanoyl-L-leucine-L-phenylalanine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (30). IR (film) 3284 (NH and OH), 1739 (C=O, ketone), 1636 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.74 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.89 (m, 9H, (CH_3)$_2$CH and CH_3CH_2), 0.80–2.20 (36H), 3.15 (m, 4H, CH_2Ph and CH_2NHCO), 4.27 (m, 1H, NH–*CH*–CO), 4.68 (q, 1H, $J=7.5$ Hz, NH–*CH*–CO), 6.03 (d, $J=5.9$ Hz, NH), 6.65 (broad, 2H, 2×NH), 7.22 (m, 5H, CH aromatics). LR-MS for $C_{42}H_{66}N_3O_5$ $[M+H]^+$ *m/z* 692.6. HPLC ($CH_3CN/H_2O/CH_3OH$, 40:25:35) 86% of purity.

3β-(*N*-Heptanoyl-L-valine-L-valine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (36). IR (KBr) 3448 and 3296 (NH and OH), 1742 (C=O, ketone), 1637 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.76 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.90 (m, 15H, 2×(CH_3)$_2$-CH and CH_3CH_2), 0.80–2.30 (35 H), 3.24 (d, $J=5.4$ Hz, 2H, CH_2NHCO), 4.24 (t, $J=8.4$ Hz, 1H, NH–*CH*–CO), 4.41 (t, 1H, $J=8.2$ Hz, NH–*CH*–CO), 6.85 (d, $J=7.4$ Hz, NH), 7.07 (broad, NH), 7.42 (broad, NH). LR-MS for $C_{37}H_{64}N_3O_5$ $[M+H]^+$ *m/z* 630.8. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:30:35) 82% of purity.

3β-(*N*-Phenylbutyryl-L-valine-L-valine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (37). IR (KBr) 3426 and 3293 (NH and OH), 1736 (C=O, ketone), 1637 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.75 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.91 and 0.93 (2d, $J=7.5$ Hz, 12H, 2×(CH_3)$_2$CH), 0.80–2.35 (28 H), 2.43 (dd, $J_1=8.7$ Hz, $J_2=19.1$ Hz, 1H of CH_2-16), 2.62 (t, $J=7.6$ Hz, 2H, CH_2CH_2Ph), 3.23 (d, 2H, $J=4.5$ Hz, CH_2NHCO), 4.24 (t, $J=8.2$ Hz, 1H, NH–*CH*–CO), 4.39 (t, $J=8.2$ Hz, 1H, NH–*CH*–CO), 6.65 (broad, NH), 6.89 (broad, NH), 7.19 (m, 6H, CH aromatics and NH). LR-MS for $C_{40}H_{62}N_3O_5$ $[M+H]^+$ *m/z* 664.4. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:35:30) 84% of purity.

3β-(*N*-Benzoyl-L-leucine-L-valine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (38). IR (film) 3295 (NH and OH), 1739 (C=O, ketone), 1636 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.75 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.92 (m, 12H, 2×(CH_3)$_2$CH), 0.80–2.40 (26 H), 2.43 (dd, $J_1=8.5$ Hz, $J_2=18.9$ Hz, 1H of CH_2-16), 3.27 (m, 2H, CH_2NHCO), 4.27 (t, $J=7.8$ Hz, 1H, NH-*CH*-CO), 4.72 (q, broad, 1H, NH-*CH*-CO), 6.85 (broad, NH), 6.97 (d, $J=7.5$ Hz, NH), 7.10 (d, $J=8.5$ Hz, NH), 7.42 (t_{app}, $J=7.8$ Hz, 2H, CH aromatics), 7.51 (t_{app}, $J=7.3$ Hz, 1H, CH aromatics), 7.79 (d, $J=7.4$ Hz, 2H, CH aromatics). LR-MS for $C_{38}H_{58}N_3O_5$ $[M+H]^+$ *m/z* 636.6. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:33:32) 90% of purity.

3β-(*N*-Heptanoyl-L-leucine-L-valine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (39). IR (KBr) 3286 (NH and OH), 1743 (C=O, ketone), 1637 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.75 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.90 (m, 15H, 2×(CH_3)$_2$CH and CH_3CH_2), 0.80–2.30 (36H), 2.43 (dd, $J_1=8.5$ Hz, $J_2=18.8$ Hz, 1H of CH_2-16), 3.23 (m, 2H, CH_2NHCO), 4.24 (t, $J=8.3$ Hz, 1H, NH–*CH*–CO), 4.59 (m, 1H, NH–*CH*–CO), 6.68 (d, $J=7.9$ Hz, NH), 7.07 (broad, NH), 7.37 (d, $J=8.6$ Hz, NH). LRMS for $C_{38}H_{66}N_3O_5$ $[M+H]^+$ *m/z* 644.5. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:25:40) 55% of purity.

3β-(*N*-Phenylbutyryl-L-phenylalanine-L-leucine-aminomethyl)-3α-hydroxy-5α-androstan-17-one (43). IR (KBr) 3473 and 3274 (NH and OH), 1736 (C=O, ketone), 1634 (C=O, amides). ^{1}H NMR ($CDCl_3$) δ 0.75 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.87 (d, $J=6.2$ Hz, 6H, (CH_3)$_2$CH), 0.80–2.15 (29H), 2.17 (t, $J=7.4$ Hz, 2H, CH_2CO), 2.43 (dd, $J_1=8.6$ Hz, $J_2=19.1$ Hz, 1H of CH_2-16), 2.54 (t, $J=7.5$ Hz, 2H, CH_2CH_2Ph), 3.11 (m, 4H, CH_2Ph and CH_2NHCO), 4.43 (m, 1H, NH–*CH*–CO), 4.68 (q, broad, 1H, NH–*CH*–CO), 6.28 (d, $J=6.8$ Hz, NH), 6.70 (d, $J=7.8$ Hz, NH), 6.76 (broad, NH), 7.18 (m, 10H, CH aromatics). LR-MS for $C_{45}H_{64}N_3O_5$ $[M+H]^+$ *m/z* 726.6. HPLC ($CH_3CN/H_2O/CH_3OH$, 35:30:35) 90% of purity.

Inhibition of type 3 17β-HSD

Preparation of the enzymatic source. The expression vectors encoding for type 3 17β-HSD were transfected into human embryonic kidney (HEK)-293 cells using the calcium phosphate procedure.[4] Cells were then sonicated in 50 mM sodium phosphate buffer (pH 7.4), containing 20% glycerol and 1 mM of ethylenediaminetetraacetic acid (EDTA), and centrifugated at 10,000*g* for 1 h to remove the mitochondria, plasma membranes, and cells fragments. The supernatant was further centrifugated at 100,000*g* to separate the microsomal fraction which was used as source of type 3 17β-HSD activities for the enzymatic assay.

Enzymatic assay. The inhibition test was carried out at 37 °C in 1 mL of 50 mM sodium phosphate buffer (pH 7.4), containing 20% glycerol and 1 mM of EDTA, 5 mM of cofactor (NADPH), 0.05 μM [4-^{14}C] 4-androstene-3-17-dione (New England Nuclear, Boston, MA, USA) and the indicated concentration of compounds to be tested. The reaction was stopped after 2 h by adding 2 mL of diethyl ether containing 10 μM of unlabeled 4-androstene-3-17-dione (Δ^4-dione) and testosterone (T). The metabolites were extracted twice with 2 mL of diethyl ether, evaporated, and then dissolved in CH_2Cl_2 before being applied on silica gel 60 TLCs. TLC plates were developed in a mixture of toluene and acetone (4:1). Substrate [^{14}C] Δ^4-dione and metabolite [^{14}C] T were identified by comparison with reference steroids

and revealed by autoradiography, then quantified using the PhosphorImager (Molecular Dynamics, SunnyVale, CA, USA). The percentage of transformation and the percentage of inhibition were calculated from equations 1 and 2, respectively:

$$\%\ \text{transformation} = ([^{14}C]T/[^{14}C]\ T + [^{14}C]\ \Delta^4\text{-dione}) \times 100 \quad (1)$$

$$\%\ \text{inhibition} = [(\%\ \text{transf. without inhibitor} - \%\ \text{transf. with inhibitor})\ /\%\ \text{transf. without inhibitor}] \times 100 \quad (2)$$

The IC_{50} value, the concentration of inhibitor that provokes 50% of enzymatic inhibition, was calculated by computer (DE_{50} program, CHUL Research Center, Québec, Canada).

Proliferative activity in Shionogi (AR^+) mammary cells. Assays on the proliferation of androgen-sensitive (AR^+) Shionogi mammary carcinoma cells were carried out according to the procedure previously described by Sam et al.[30] Two concentrations of tested compound (0.1 and 1 μM) were used in the assay. The results were reported as the percentage (%) of cell proliferation compared to the stimulation induced by 0.3 nM of androgen dihydrotestosterone (100%).

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