

## Supporting Information

# Novel nano-materials, RGD-tetrapeptides modified 17 $\beta$ -amino-11 $\alpha$ -hydroxyandrost-1,4-diene-3-one: Synthesis, self-assembly based nano-images and *in vivo* anti-osteoporosis evaluation †

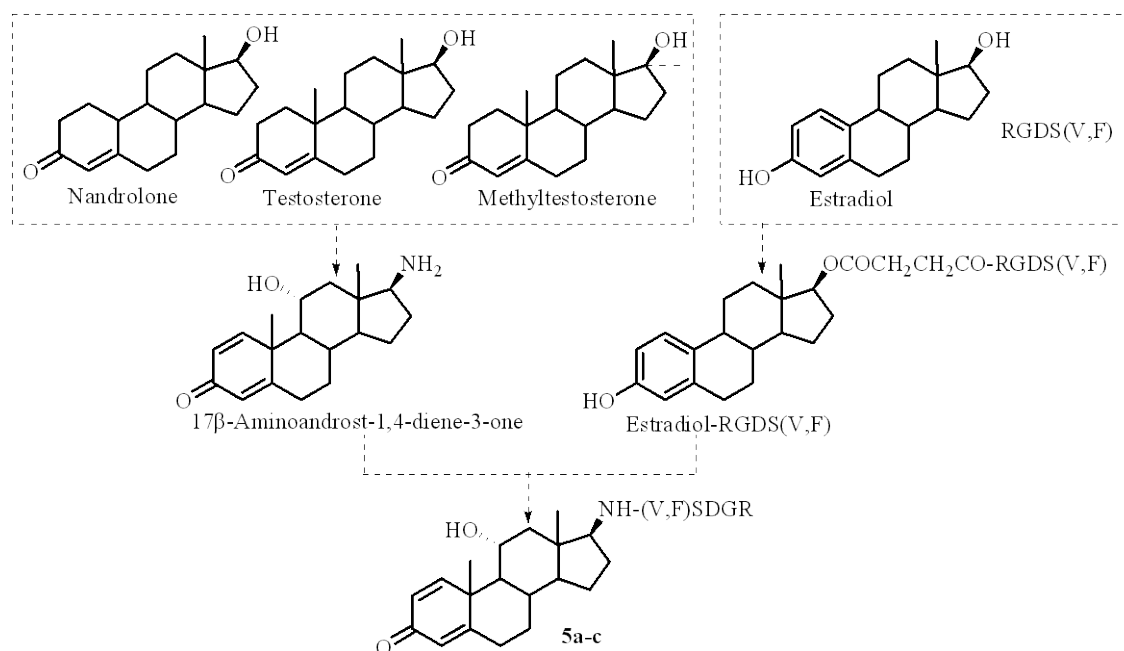
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### 1. Design of novel anti-osteoporosis agents, 5a-c

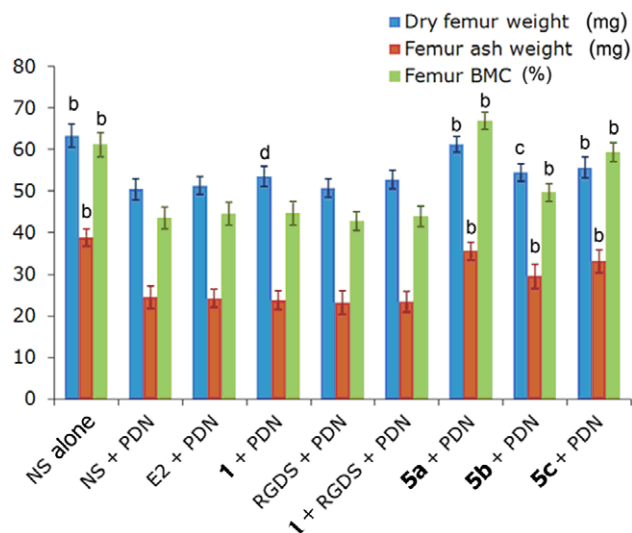
In the design of the anti-osteoporosis agents the structures and the *in vivo* activities of the estrogens, estrogen-RGDS, estrogen-RGDV, estrogen-RGDF and three novel amphiphiles, androst-1,4-diene-3-one-RGD-AA (AA = S, V and F) in Figure 1 were compared. The comparison predicted that androst-1,4-diene-3-one-RGD-AA should have high anti-osteoporosis efficacy and few side effects.<sup>1-3</sup>



**Figure 1** From androgenic compounds and estradiol-RGD-peptides with anti-osteoporosis efficacy the present novel 17 $\beta$ -(Arg-Gly-Asp-Ser/Val/Phe-amido)-androst-1,4-diene-3-one. (**5a-c**) could be designed.

## 2. Dry femur weights, femur ash weights and femur BMC of **5a-c** treated mice

In the *in vivo* anti-osteoporosis assay NS only was used as the background control, NS plus prednisone was used as the blank control, estradiol plus prednisone was used as the positive control and the *in vivo* anti-osteoporosis activities of **5a-c** were evaluated, represented with dry femur weights, femur ash weights and femur BMC of the treated mice and are shown in Figure 2. The data indicate that the *in vivo* anti-osteoporosis activities of **5a-c** are significantly higher than that of estradiol.



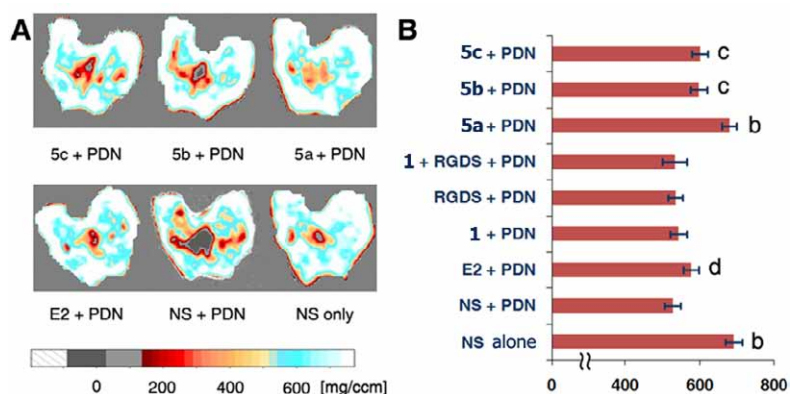
**Figure 2** Dry femur weights, femur ash weights and femur BMC of **5a-c** treated mice. a) Weights of dry femur and femur ash are represented with mean  $\pm$  SD mg, BMC (femur mineral content) is represented with mean  $\pm$  SD %,  $n = 12$ , PDN = prednisone, E2 = estradiol, **1** =  $17\beta$ -amino-11 $\alpha$ -hydroxyandrost-1,4-diene-3-one; Dose of E2, RGDS, **1** and **5a-c** = 110 nmol/kg. **For dry femur weight:** b) Compared to NS + PDN, E2 + PDN, **1** + PDN, RGDS + PDN and **1** + RGDS + PDN  $p < 0.05$ ; c) Compared to NS + PDN, E2 + PDN, RGDS + PDN and **1** + RGDS + PDN  $p < 0.05$ ; d) Compared to NS + PDN, E2 + PDN and RGDS + PDN  $p < 0.05$ . **For femur ash weight:** b) Compared to NS + PDN, E2 + PDN, **1** + PDN, RGDS + PDN and **1** + RGDS + PDN  $p < 0.01$ . **For femur BMC:** b) Compared to NS + PDN, E2 + PDN, **1** + PDN, RGDS + PDN and **1** + RGDS + PDN  $p < 0.01$ .

### 3. Trabecula bone densities of **5a-c** treated mice

In the *in vivo* anti-osteoporosis assay NS only was used as the background control, NS plus prednisone was used as the blank control, estradiol plus prednisone was used as the positive control and the *in vivo* anti-osteoporosis activities of **5a-c** were evaluated, represented with trabecula bone densities of 0.2 mm to 0.4 mm under the caput articularis of knee joint of the femurs of the treated mice and are shown in Figure 3.

The data indicate that the *in vivo* anti-osteoporosis activities of **5a-c** are significantly

higher than that of estradiol.

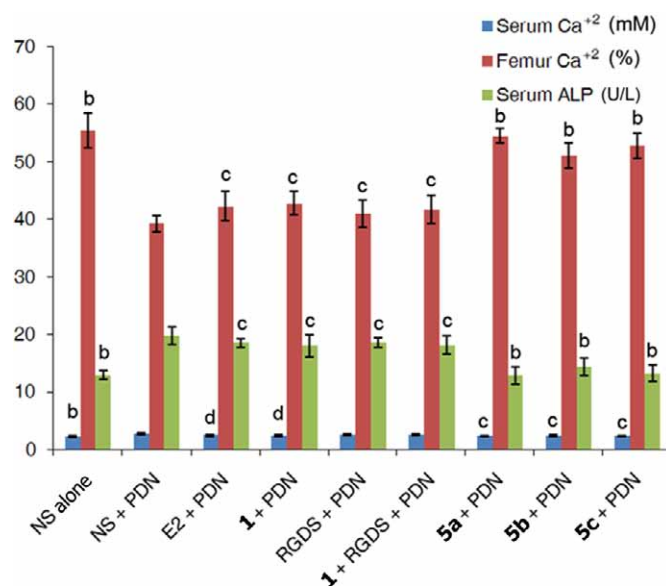


**Figure 3** (A) Images of the 0.2 mm to 0.4 mm under the caput articularis of knee joint of the femurs of **5a-c** treated mice, and (B) Trabecular bone density (TBD) of the 0.2 mm to 0.4 mm under the caput articularis of knee joint of the femurs of **5a-c** treated mice.

a) TBD (trabecular bone density) is represented with mean  $\pm$  SD mg/cm<sup>3</sup>, n = 12, PDN = prednisone, E2 = estradiol, **1** = 17 $\beta$ -amino-11 $\alpha$ -hydroxyandrost-1,4-diene-3-one; Dose of E2, RGDS, **1** and **5a-c**: 110 nmol/kg. b) Compared to NS + PDN, E2 + PDN, **1** + PDN, RGDS + PDN and **1** + RGDS + PDN p < 0.01; c) Compared to NS + PDN, E2 + PDN, RGDS + PDN and **1** + RGDS + PDN p < 0.01, to **1** + PDN p < 0.05; d) Compared to NS + PDN p < 0.05.

#### 4. Serum Ca<sup>+2</sup>, femur Ca<sup>+2</sup> and serum ALP of **5a-c** treated mice

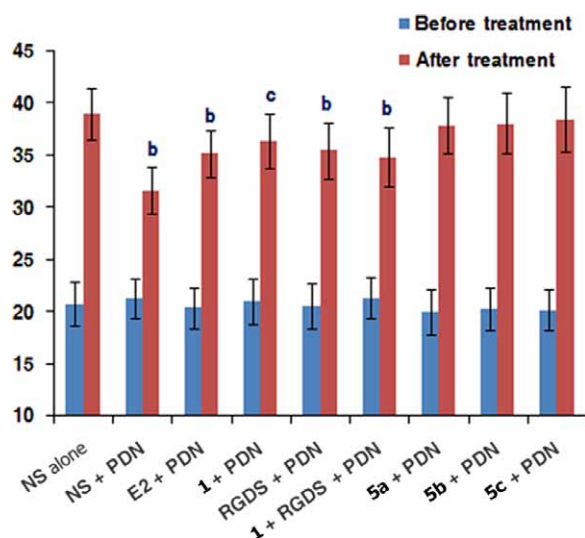
In the *in vivo* anti-osteoporosis assay NS only was used as the background control, NS plus prednisone was used as the blank control, estradiol plus prednisone was used as the positive control the *in vivo* anti-osteoporosis activities of **5a-c** were evaluated, represented with serum Ca<sup>+2</sup>, femur Ca<sup>+2</sup> and serum ALP of the treated mice and are shown with Figure 4. The data indicate that the *in vivo* anti-osteoporosis activities of **5a-c** are significantly higher than that of estradiol.



**Figure 4** Serum Ca<sup>2+</sup>, femur Ca<sup>2+</sup> and serum ALP of **5a-c** treated mice. a) ALP = alkaline phosphatase is represented by mean ± SD U/L, serum Ca<sup>2+</sup> is represented by mean ± SD mM, femur Ca<sup>2+</sup> is represented by mean ± SD %, n = 12, PDN = prednisone, E2 = estradiol, **1** = 17β-amino-11α-hydroxyandrost-1,4-diene-3-one; Dose of E2, RGDS, **1** and **5a-c**: 110 nmol/kg. **For serum Ca<sup>2+</sup>**: b) Compared to NS + PDN, E2 + PDN, **1** + PDN, RGDS + PDN and **1** + RGDS + PDN p < 0.01; c) Compared to NS + PDN p < 0.01, to E2 + PDN, RGDS + PDN and **1** + RGDS + PDN p < 0.05; d) Compared to NS + PDN p < 0.05. **For femur Ca<sup>2+</sup>**: b) Compared to NS + PDN, E2 + PDN, **1** + PDN, RGDS + PDN and **1** + RGDS + PDN p < 0.01; c) Compared to NS + PDN p < 0.05. **For serum ALP**: b) Compared to NS + PDN, E2 + PDN, **1** + PDN, RGDS + PDN and **1** + RGDS + PDN p < 0.01; c) Compared to NS + PDN p < 0.05.

## 5. Body weights of **5a-c** treated mice

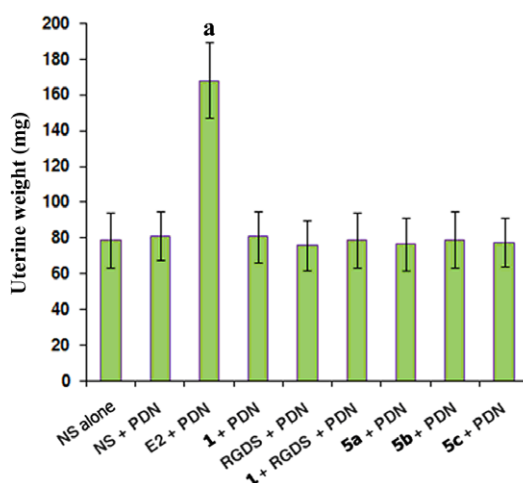
The effect of **5a-c** on the health of the treated mice was estimated by comparing the body weights before the treatment with the body weights after the treatment and is shown in Figure 5. The data indicate that **5a-c** do not influence the health of the treated mice.



**Figure 5** Body weights of **5a-c** treated mice. a) Body weight is represented by mean  $\pm$  SD g,  $n = 12$ , PDN = prednisone, E2 = estradiol, **1** =  $17\beta$ -amino- $11\alpha$ -hydroxyandrost-1,4-diene-3-one; Dose of E2, RGDS, **1** and **5a-c**: 110 nmol/kg. b) Compared to NS only  $p < 0.01$ ; c) Compared to NS only  $p < 0.05$ .

## 6. Uteri weights of **5a-c** treated mice

The effect of **5a-c** on the endometrial cell hyperplasia of the treated mice was estimated by comparing the uterine weights of **5a-c** treated mice with that of NS and estradiol treated mice, and are shown in Figure 6. The data indicate that **5a-c** do not induce the treated mice to develop endometrial hyperplasia.



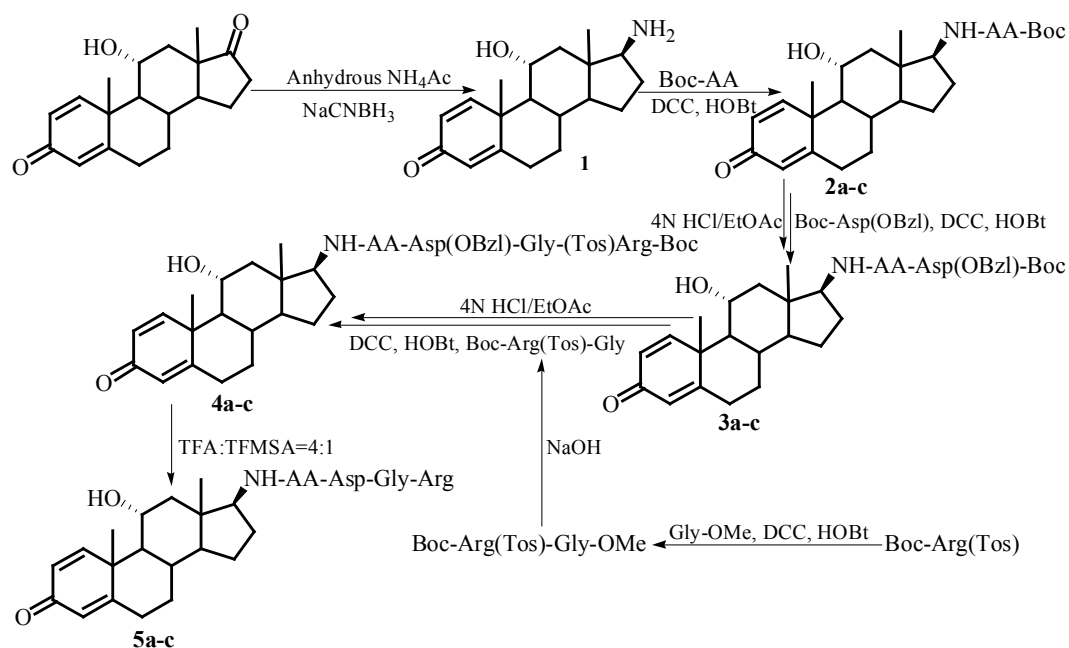
**Figure 6** Uterine weights of **5a-c** treated mice. Uterine weight is represented by mean  $\pm$  SD mg,  $n = 12$ , PDN = prednisone, E2 = estradiol, **1** =  $17\beta$ -amino- $11\alpha$ -hydroxyandrost-1,4-diene-3-one; Dose of E2, RGDS, **1** and **5a-c**: 110 nmol/kg. a) Compared to NS only  $p < 0.01$ .

## 7. Tail bleeding time of 5a-c treated mice <sup>4</sup>

After the 4-week treatment of estradiol or **5a-c** the mice in anti-osteoporosis assays were weighed to record body weights. Thirty and ninety min after the last administration, the mice were given *in vivo* tail bleeding time assay by use of a standard procedure. Briefly, the mouse was placed in a tube holder with its tail protruding, and a 2 mm cut was made on the tail. Flowing blood until it stopped was gently wiped away with a filter paper every 30 s, yielding the bleeding time. The tail bleeding time of the mice after 30 min and 90 min of the last oral administration for estradiol plus prednisone ( $214 \pm 20$  and  $225 \pm 19$  seconds, respectively) is significantly shorter than that for NS plus prednisone ( $268 \pm 18$  and  $272 \pm 19$  seconds, respectively). The tail bleeding time after 30 min and 90 min of the last oral administration for **5a-c** plus prednisone (ranging from  $270 \pm 19$  to  $276 \pm 21$  seconds and from  $279 \pm 20$  to  $280 \pm 22$  seconds, respectively) is substantially equal to that of NS plus prednisone. These observations suggest that when the osteoporosis mice are given oral administration for 4 successive weeks, **5a-c** have no blood coagulation risk.

## 8. Synthesis of the intermediates of 5a-c

Via a five-step-procedure **5a-c** were synthesized. The preparations and the data of intermediates **1**, **2a-c**, **3a-c** and **4a-c** were given in the Figure 7.



**Figure 7** Synthetic route of 17 $\beta$ -(Arg-Gly-Asp-Ser/Val/Phe-amido)-11 $\alpha$ -hydroxyandrost-1,4-diene-3-ones. In **2a-4a**: AA = Ser(Bzl); in **5a**, AA = Ser; in **2b-5b**: AA = Val; in **2c-5c**: AA = Phe.

### 8.1 17 $\beta$ -amino-11 $\alpha$ -hydroxyandrost-1,4-diene-3-one (**1**)

To a solution of 5.0 g (16.6 mmol) of 11 $\alpha$ -hydroxyandrost-1,4-dien-3,17-dione in 50 mL of anhydrous methanol 12.8 g (16.6 mmol) of anhydrous ammonium acetate was added. After the salt dissolved completely, 1.2 g (18.0 mmol) of NaBH<sub>3</sub>CN was added partly. The mixture was stirred at room temperature for 24 h and TLC (ethyl acetate: petrol ether, 2:1) indicated the disappearance of 11 $\alpha$ -hydroxyandrost-1,4-dien-3,17-dione. After removal of methanol under reduced pressure, the residue was added into 20 mL of water. Anhydrous K<sub>2</sub>CO<sub>3</sub> was added to adjust pH to 10 and the mixture was extracted with 100 mL of chloroform. The organic phase was dried over anhydrous sodium sulphate. After filtration and evaporation under reduced pressure the residue was recrystallization with methanol to yield the title compound 4.51g (90 %) as a



yellow powder. Mp 154-158 °C. ESI-MS (m/e): 302 [M + H]<sup>+</sup>. [ $\alpha$ ]<sub>D</sub><sup>20</sup> = 28.2 (c = 0.01, CH<sub>3</sub>OH). IR (KBr): 1658, 3372, 3289, 3380 cm<sup>-1</sup>. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz)  $\delta$  = 7.16 (d, *J* = 10.2 Hz, 1H), 6.25 (dd, *J* = 10.2 Hz, *J* = 1.8 Hz, 1H), 6.05 (s, 1H), 3.72 (w, 1H), 2.75 (m, 1H), 2.76 (t, *J* = 3.0 Hz), 2.53 (w, 2H), 2.00 - 2.20 (m, 9H), 1.56 (m, 1H), 1.40 (s, 3H), 1.33 (m, 2H), 0.70 (s, 3H). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 125 MHz)  $\delta$  = 186.2, 166.1, 154.2, 132.4, 124.0, 119.9, 66.7, 60.0 (C<sub>11</sub>), 58.1, 47.8, 43.0, 40.0, 38.8, 33.9, 33.3, 31.1, 30.4, 26.6, 13.8. Anal. Calcd. for C<sub>19</sub>H<sub>27</sub>NO<sub>2</sub>: C, 75.71; H, 9.03, N, 4.65. Found C, 75.55; H, 8.91; N, 4.48.

## 8.2 17 $\beta$ -[Boc-Ser(Bzl)-amido]-11 $\alpha$ -hydroxyandrost-1,4-diene-3-one (2a)

At 0 °C to a solution of 590 mg (2.0 mmol) of Boc-Ser(Bzl) in 20 mL of anhydrous tetrahydrofuran (THF) was mixed with 270 mg (2.0 mmol) of N-hydroxybenzotriazole (HOBT) and 444 mg (2.2 mmol) of dicyclohexylcarbodiimide (DCC). This was solution A and at 0 °C stirred for 30 min. At 0 °C to a solution of 560 mg (2.0 mmol) of **1** in 10 mL of anhydrous THF 0.1 mL of N-methylmorpholine was added. This was solution B and at 0 °C stirred for 10 min. At 0 °C the solution A and solution B were combined, the formed mixture was stirred at room temperature for 8 h, and TLC (ethyl acetate: petroleum ether, 1:1) indicated complete disappearance of **1**. The reaction mixture was evaporation at reduced pressure, the residue was extracted with ethyl acetate (3 × 50 mL) and the ethyl acetate phase was successively washed with aqueous KHSO<sub>4</sub> (5 %, 3 × 50 mL) and saturated aqueous NaCl (3 × 50 mL), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated at reduced pressure and the residue was purified on silica gel column (chloroform/methanol, 30/1) to give 970 mg (84 %)

of the title compound as colorless powder. Mp 118 - 119 °C,  $[\alpha]_D^{20} = 24.0$  (c = 0.11, MeOH), ESI-MS (m/e) 579 [M + H]<sup>+</sup>.

### 8.3 17β-(Boc-Val-amido)-11α-hydroxyandrost-1,4-diene-3-one (2b)

Using the procedure of preparing **2a** from 440 mg (2.0 mmol) of Boc-Val and 560 mg (2.0 mmol) of **1** 840 mg (84 %) of the title compound was obtained as colorless powder. Mp 121 - 122 °C,  $[\alpha]_D^{20} = 25.0$  (c = 0.12, MeOH), ESI-MS (m/e) 501 [M + H]<sup>+</sup>.

### 8.4 17β-(Boc-Phe-amido)-11α-hydroxyandrost-1,4-diene-3-one (2c)

Using the procedure of preparing **2a** from 530 mg (2.0 mmol) of Boc-Phe and 560 mg (2.0 mmol) of **1** 905 mg (82 %) of the title compound was obtained as colorless powder. Mp 131 - 132 °C,  $[\alpha]_D^{20} = 29.0$  (c = 0.11, MeOH), ESI-MS (m/e) 549 [M+H]<sup>+</sup>.

### 8.5 17β-[Boc-Asp(OBzl)-Ser(Bzl)-amido]-11α-hydroxyandrost-1,4-diene-3-one (3a)

At 0 °C a solution of 230 mg (0.71 mmol) of Boc-Asp(OBzl) in 10 mL of anhydrous THF was mixed with 270 mg (2.0 mmol) of HOBT and 444 mg (2.2 mmol) of DCC. This was solution A and was at 0 °C stirred for 30 min. At room temperature 400 mg (0.71 mmol) of **2a** was treated with 3 mL of solution of hydrogen chloride in anhydrous ethyl acetate for 4 h. After completely removing the free hydrogen chloride and ethyl acetate, the colorless powder of 17β-[Ser(Bzl)-amido]-11α-hydroxyandrost-1,4-diene-3-one was obtained, which was dissolved in 10 mL of anhydrous THF, at 0 °C mixed with 0.1 mL of N-methylmorpholine, and stirred for 10 min. This was

solution B. At 0 °C the solution A and solution B were combined, the formed mixture was stirred at room temperature for 8 h, and TLC (ethyl acetate: petroleum ether, 1:1) indicated complete disappearance of **2a**. The reaction mixture was evaporation at reduced pressure, the residue was extracted with ethyl acetate (3 × 50 mL) and the ethyl acetate phase was successively washed with aqueous KHSO<sub>4</sub> (5 %, 3 × 50 mL) and saturated aqueous NaCl (3 × 50 mL), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated at reduced pressure and the residue was purified on silica gel column (chloroform/methanol, 30/1) to give 490 mg (88 %) of the title compound as colorless powder. Mp 114 - 115 °C,  $[\alpha]_D^{20} = 13.0$  (c = 0.12, MeOH), ESI-MS (m/e) 784 [M + H]<sup>+</sup>.

#### **8.6 17β-[Boc-Asp(OBzl)-Val- amido]-11α-hydroxyandrost-1,4-diene-3-one (3b)**

Using the procedure of preparing **3a** from 500 mg (1.0 mmol) of **2b** and 330 mg (1.0 mmol) of Boc-Asp(OBzl) 660 mg (94 %) of the title compound was obtained as light yellow powder. Mp 114 - 115 °C,  $[\alpha]_D^{20} = 19.0$  (c = 0.12, MeOH), ESI-MS (m/e) 706 [M + H]<sup>+</sup>.

#### **8.7 17β-[Boc-Asp(OBzl)-Phe- amido]-11α-hydroxyandrost-1,4-diene-3-one (3c)**

Using the procedure of preparing **3a** from 500 mg (0.9 mmol) of **2c** and 300 mg (0.9 mmol) of Boc-Asp(OBzl) 600 mg (88 %) of the title compound was obtained as colorless powder. Mp 124 - 125 °C,  $[\alpha]_D^{20} = 21.0$  (c = 0.12, MeOH), ESI-MS (m/e) 754 [M + H]<sup>+</sup>.

#### **8.8 17β-[Boc-Arg(Tos)-Gly-Asp(OBzl)-Ser(Bzl)-amido]-11α-hydroxyandrost-1,4-diene-3-one (4a)**

At 0 °C a solution of 240 mg (0.5 mmol) of Boc-Arg(Tos)-Gly in 10 mL of anhydrous THF was mixed with 68 mg (0.5 mmol) of HOBt and 111 mg (0.5 mmol) of DCC. This was solution A and was at 0 °C stirred for 30 min. At room temperature 392 mg (0.5 mmol) of **3a** was treated with 3 mL of solution of hydrogen chloride in anhydrous ethyl acetate for 4 h. After completely removing the free hydrogen chloride and ethyl acetate, the colorless powder of 17 $\beta$ -[Asp(OBzl)-Ser(Bzl)-amido]-11 $\alpha$ -hydroxyandrost-1,4-diene-3-one was obtained, which was dissolved in 10 mL of anhydrous THF, was at 0 °C mixed with 0.1 mL of N-methylmorpholine, and then stirred for 10 min. This was solution B. At 0 °C the solution A and solution B were combined, the formed mixture was stirred at room temperature for 8 h, and TLC (ethyl acetate: petroleum ether, 1:1) indicated complete disappearance of **3a**. The reaction mixture was evaporation at reduced pressure, the residue was extracted with ethyl acetate (3  $\times$  50 mL) and the ethyl acetate phase was successively washed with aqueous KHSO<sub>4</sub> (5%, 3  $\times$  50 mL) and saturated aqueous NaCl (3  $\times$  50 mL), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated at reduced pressure and the residue was purified on silica gel column (chloroform/methanol, 30/1) to give 521 mg (89 %) of the title compound as light yellow powder. Mp 103 - 104 °C,  $[\alpha]_D^{20} = 3.7$  (c = 0.11, MeOH), ESI-MS (m/e) 1151 [M + H]<sup>+</sup>.

#### **8.9 17 $\beta$ -[Boc-Arg(Tos)-Gly-Asp(OBzl)-Val-amido]-11 $\alpha$ -hydroxyandrost-1,4-diene-3-one (4b)**

Using the procedure of preparing **4a** from 402 mg (0.57 mmol) of **3b** and 265 mg (0.57 mmol) of Boc-Arg(Tos)-Gly 530 mg (87 %) of the title compound was obtained

as colorless powder. Mp 99 - 100 °C,  $[\alpha]_D^{20} = 4.0$  (c = 0.12, MeOH), ESI-MS (m/e) 1073 [M + H]<sup>+</sup>.

**8.10 17β-[Boc-Arg(Tos)-Gly-Asp(OBzl)-Phe-amido]-11α-hydroxyandrost-1,4-diene-3-one (4c)**

Using the procedure of preparing **4a** from 400 mg (0.53 mmol) of **3c** and 245 mg (0.53 mmol) of Boc-Arg(Tos)-Gly 504 mg (91 %) of the title compound was obtained as colorless powder. Mp 104 - 105 °C,  $[\alpha]_D^{20} = 9.0$  (c = 0.11, MeOH), ESI-MS (m/e) 1121 [M + H]<sup>+</sup>.

**References**

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