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Studies on the Synthesis and Anti-Osteoporosis of Estrogen-GHRPs Linkers

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Abstract—The linkers of estrogen-GHRPs were prepared by the combination of estradiol, estrone, TyrGlyGlyPheLeuOH, and TyrGlyGlyPheLeuOH. Their anti-osteoporosis effect was evaluated by analyzing the data, for instance the weight of the body, femur, femur ash, the content of calcium and phosphor in the femur, the content of calcium and ALP activity in the serum, obtained from the corresponding bioassay in vivo. The results indicated that the anti-osteoporosis potency for estradiol, estrone, TyrGlyGlyPheLeuOH and TyrGlyGlyPheLeuNH_2 may be totally enhanced each other via the corresponding linkers.

It is commonly accepted that the decrease of skeletal muscle and bone mass depends on the lower level of estrogen and growth hormone (GH).[1,2] Thus supplement of growth hormone releasing peptides (GHRPs) and estrogen may prevent osteoporosis of elderly women.[3,4] The interaction between estrogen and growth hormone-insulin like growth factor (GH-IGF) axis observed so far, for instance estrogen effected obviously on the secretion of human growth hormone (hGH), encourage us studying the synthesis and analgesic effects of kyotorphin-steroid linkers.[5] The phenomenon that the effects of the peptides were enhanced by steroids indicated that this kind of combination provided an useful way to find the lead compounds. In the present paper TyrGlyGlyPheLeuOH, and TyrGlyGlyPheLeuNH_2[6] were coupled with estradiol and estrone to simulate their enhanced anti-osteoporosis effect via the corresponding estrogen-GHRPs.

Chemistry

The protective tetrapeptide (**1**) was prepared by use of stepwise synthesis (from C terminal to N terminal) via solution method in which dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) were used as the coupling agents in the peptide formation, hydrogen chloride in ethyl acetate was used as the deprotective agent in the removal of Boc, the yields for all of the reactions were in 77–90%. At 0 °C **1** was treated with 2 mol/L of NaOH and hydrochloric acid successively to offer the corresponding tetrapeptide acid (**2**) in 96% yield. Under the normal conditions **2** was coupled with L-LeuOMe and L-LeuNH_2 to give **3** and **6** in 73 and 76% yield, respectively. Removal of Boc with 4 mol/L of hydrogen chloride in ethyl acetate **3** and **6** were converted into **4** and **7** in 96% yield. Saponification and acidification of **3** gave **5** in 88% yield. In the presence of Pd/C (10%), H_2, and 4.4% of formic acid in methanol **4** and **7** were converted into **8** and **9** in 94 and 91% yield, respectively (Scheme 1).

The *O*-alkylation of estrone with ethyl bromoacetate and sodium ethoxide provided ethyl estrone-3-oxymethylenecarboxylated (**10**) in 83% yield. Treating **10** with 2 mol/L of NaOH and 2 mol/L of hydrochloric acid **11** was obtained in 94% yield, which was coupled with **4** and **7** in the presence of DCC and HOBt to offer 3-protective pentapeptide-estrone (**12a**,**b**) in 64 and 85% yield, respectively. In the presence of Pd/C (10%), H_2, and 4.4% of formic acid in methanol **12a**,**b** were converted into the C-terminal protective 3-pentapeptide-estrone (**13a**,**b**) in 92 and 90%, yield, respectively Saponification and acidification of **13a** gave the goal linker **14a** in 51% yield. Reducing **12b** and **14a** with sodium

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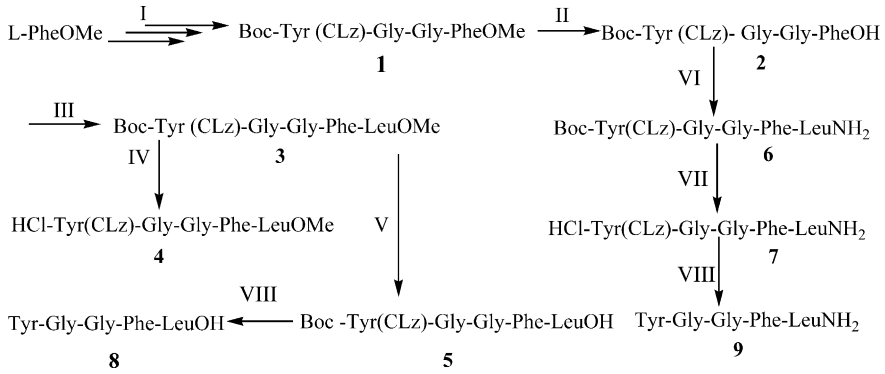
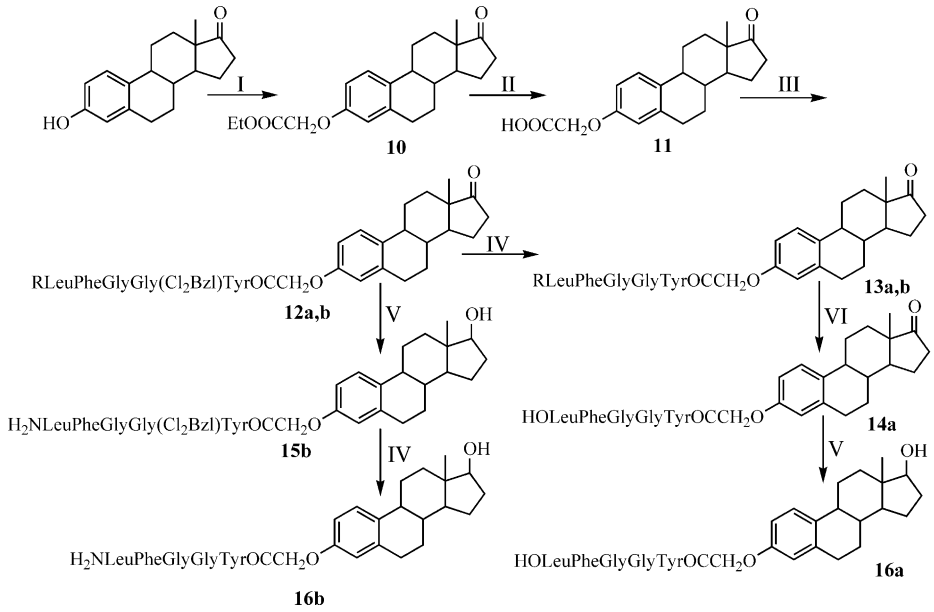


Scheme 1. (I) With DCC, HOBt as the coupling agent; $HCl/CH_3CO_2C_2H_5$ as the deprotection agent of Boc; (II) 2 mol/L of NaOH, and 4 mol/L of HCl, 0 °C; (III) DCC, HOBt $LeuOCH_3$; (IV) 4 mol/L of hydrochloride in ethyl acetate; (V) 2 mol/L of NaOH, 0 °C; (VI) DCC, HOBt $LeuNH_2$; (VII) 4 mol/L of hydrochloride in ethyl acetate; (VIII) Pd/C (10%), H_2 and 4.4% of HCOOH in CH_3OH.

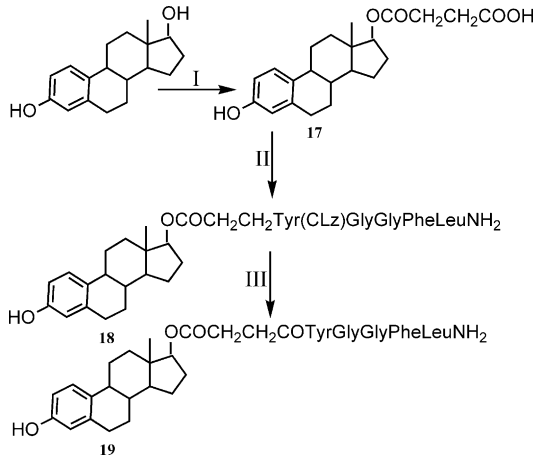


Scheme 2. (I) NaOEt, C_2H_5OH, $BrCH_2CO_2C_2H_5$; (II) 2 mol/L of NaOH; (III) DCC, HOBt, NMM, **4**, **7**; (IV) Pd/C (10%), H_2 and 4% of HCOOH in CH_3OH; (V); $NaBH_4$; (VI) 2 mol/L of NaOH; in **12a** and **13a** R = OCH_3 in **12b** and **13b** R = NH_2.

borohydride gave 3-protective pentapeptide-estradiol (**15b** and **16a**) in 92 and 94% yield, respectively. Using the same reaction conditions as that for **12a**,**b** **15b** was converted into the goal linker **16b** in 91% yield (Scheme 2).

Acylation of the 17-hydroxyl in estradiol with succinic anhydride and DMAP estradiol-17-oxycarbonylpropionic acid (**17**) was obtained in 66% yield. Treating **17** with **7** and DCC estradiol-17-oxycarbonylpropionyl-protective pentapeptide amide (**18**) was provided in 70% yield, which was deprotected in the presence of Pd/C (10%), H_2, and 4.4% of formic acid in methanol to give estradiol-17-oxycarbonylpropionylpentapeptide amide (**19**) in 90% yield (Scheme 3).

In the presence of DCC and DMAP estradiol was acylated by **5** and the corresponding protective pentapeptidyl-17-*O*-estradiol (**20**) was obtained in 67% yield. Treating **20** with Pd/C (10%), H_2, and 4.4% of formic acid in methanol, and 4 mol/L of hydrogen chloride in

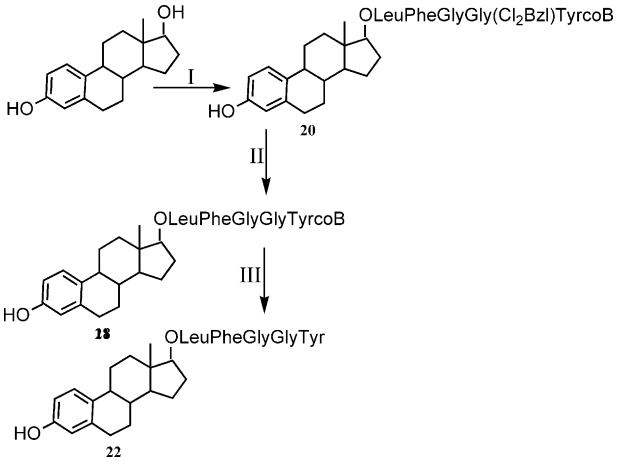


Scheme 3. (I) Succinic anhydride, DMAP; (II) DCC, HOBt, NMM and **7**; (III) Pd/C (10%), H_2 and 4.4% of HCOOH in CH_3OH.

ethyl acetate successively **22** was obtained in 88% yield (Scheme 4).

Bioassay in vivo[7]

Male ICR rats, weighting 20±2 g, housed in a 12/12 light/dark cycle at 21±2 °C for 24 h before being used. Except the normal saline (NS) group the animal was administrated special food (containing 0.1% of calcium and 0.4% of phosphor) and distilled water, and injected 2 μL of the solution of prednisone (6.3 mg/kg) in NS intramuscularly twice a week and 2 μL of NS once a day for the prednisone control group, and injected 2 μL of the solution of prednisone (6.3 mg/kg) in NS intramuscularly twice a week and 2 μL of the solution of estradiol, or estrone, or GHRPs or estrogen-GHRPs linkers (18 μg/kg) in NS once a day for the drug receiving groups. For NS group the animal was administrated normal food (containing 1.76% of calcium and 1.01% of phosphor) and running water, and injected 2 μL of NS once a day. All of the animals were treated according to the corresponding procedure for 4 weeks after which their weight of body, femur, and femur ash, the content of calcium and phosphor in the femur, the level of calcium and the activity of alkaline phosphatase (ALP) in the serum were tested. The results are listed in Tables 1–3. The statistical analysis of the data were carried out by use of ANOVA test; $p<0.05$ was considered significant.



Scheme 4. (I) DCC, HOBt, NMMand **7**; (II) Pd/C (10%), H_2 and 4.4% of HCOOH in CH_3OH; (III) 4 mol/L of hydrochloride in ethyl

Discussion

In the preparation of TyrGlyGlyPheLeuOH, TyrGlyGlyPheLeuNH_2 and the corresponding estrogen-GHRPs the Cl_2Bzl (2,6-dichlorobenzyl) group in Tyr

Table 1. Weight of the body, femur and femur ash

Group	Body (g)	Femur (mg)	Femur ash (mg)
NS	39.40±2.27	44.75±3.96	27.21±2.71
Prednisone	31.89±2.68[c]	33.39±3.01[c]	21.26±1.67[c]
Estradiol	35.39±3.40[a,e]	35.09±3.97[c]	22.82±3.84[b]
Estrone	33.62±2.90[c]	33.93±3.81[c]	21.69±1.79[c]
8	33.29±2.29[c]	39.49±3.27[b,f,h,k]	21.74±2.89[c]
9	34.16±2.14[c,d]	40.27±3.37[b,f,h,k]	22.80±2.73[b]
14a	34.27±2.98[c,d]	40.60±4.41[a,f,j]	23.75±2.19[b,d,j]
13b	35.83±3.16[b,e]	41.68±3.04[a,f,k]	24.90±2.46[a,f,k]
16a	36.46±2.18[b,f,n]	42.64±3.62[f,m,l]	25.36±1.70[f,h,m]
16b	38.00±2.01[f,g,q]	43.68±3.22[f,m,o]	26.39±3.31[f,I,n]
19	37.24±3.93[f,o]	42.56±4.31[f,m,n]	25.46±2.85[f,g,n]
22	36.87±3.16[f,n]	41.13±4.23[a,f,h]	24.84±2.79[a,f,l]

N = 12; NS = vehicle; (a) compare to NS, $p<0.05$; (b) compare to NS, $p<0.01$; (c) compare to NS, $p<0.001$; (d) compare to prednisone, $p<0.05$; (e) compare to prednisone, $p<0.01$; (f) compare to prednisone, $p<0.001$;(g) compare to estradiol, $p<0.05$; (h) compare to estradiol, $p<0.01$; (i) compare to estradiol, $p<0.001$; (j) compare to estrone, $p<0.05$; (k) compare to estrone, $p<0.001$; (l) compare to **8**, $p<0.05$; (m) compare to **8**, $p<0.01$; (n) compare to **9**, $p<0.05$; (o) compare to **9**, $p<0.01$.

Table 2. Content of calcium and phosphor in femur

Group	Calcium (%)	Phosphor (%)
NS	47.85±5.28	22.24±2.16
Prednisone	39.54±6.07[b]	20.87±2.36
Estradiol	42.11±5.75[a]	21.85±2.43
Estrone	40.83±4.75[b]	22.02±2.28
8	43.75±6.42	22.27±2.71
9	43.97±6.10	22.35±3.50
14a	45.30±9.79	23.12±2.39[c]
13b	46.94±8.50	23.48±3.04[c]
16a	47.73±6.80	24.33±.07[d,e,g]
16b	48.94±6.15	25.10±3.08[a,e]
19	48.63±7.80	25.24±2.92[a,f,h]
22	46.03±6.13	23.40±2.44[c]

N = 12; NS = vehicle; (a) compare to NS, $p<0.05$; (b) compare to NS, $p<0.01$; (c) compare to prednisone, $p<0.05$; (d) compare to prednisone, $p<0.001$; (e) compare to estradiol, $p<0.05$; (f) compare to estradiol, $p<0.01$; (g) compare to **8**, $p<0.05$; (h) compare to **9**, $p<0.05$.

Table 3. Content of calcium and ALP activity in serum

Group	C alcium (mmol/L)	ALP
NS	1.91±0.25	43.50±7.91
Prednisone	2.42±0.32[c]	48.70±7.35
Estradiol	2.13±0.29[d]	44.10±8.43
Estrone	2.28±0.30[b]	45.84±8.03
8	2.18±0.21[a,d]	37.70±9.52[e]
9	2.01±0.20[e]	34.22±8.50[a,f,g]
14a	2.00±0.13[f,j,l]	32.01±7.02[b,f,k]
13b	1.99±0.27[f,i]	30.89±9.04[b,f,k]
16a	1.95±0.24[f,l]	29.92±8.07[c,f,h,j]
16b	1.90±0.15[f,g]	28.10±8.08[c,f,h]
19	1.92±0.21[f]	28.24±7.92[c,f,h]
22	1.94±0.13[f,m]	29.40±8.44[c,f,h,l]

N = 12; NS = vehicle; (a) compare to NS, $p<0.05$; (b) compare to NS, $p<0.01$; (c) compare to NS, $p<0.001$; (d) compare to prednisone, $p<0.05$; (e) compare to prednisone, $p<0.01$; (f) compare to prednisone, $p<0.001$; (g) compare to estradiol, $p<0.05$; (h) compare to estradiol, $p<0.001$; (i) compare to estrone, $p<0.05$; (j) compare to estrone, $p<0.01$; (k) compare to estrone, $p<0.001$; (l) compare to **8**, $p<0.05$; (m) compare to **8**, $p<0.001$.

residue of all the intermediates, some of which are unstable to HF, can be removed in satisfactory yield in the presence of Pd/C (10%), H_2, and 4.4% of formic acid in methanol. The data in Table 1 indicated that the prednisone induced weight decrease of the body, the femur and the femur ash of the rats was obviously improved by **13b**, **14a**, **16a**, **16b**, **19** and **22** treating. The differences of the weights between estradiol, **8**, **16a** and **22** groups; between estradiol, **9**, **16b** and **19** groups; between estrone, **8** and **14a** groups; and between estrone, **9** and **13b** groups suggested that the anti-osteoporosis potency of estrogen and GHRPs was enhanced each other. The data in Table 2 indicated that the content of calcium in the femur of the rats treated with NS or **8**, **9**, **13b**, **14a**, **16a**, **16b**, **19**, **22** gave no significant difference. The phosphor level in the femur of the rats treated with **13b**, **14a**, **16a**, **16b**, **19** and **22** was obviously higher than the others. The data in Table 3 indicated that the content of calcium in serum of the rats treated with **13b**, **14a**, **16a**, **16b**, **19** and **22** was reduced to the level of NS treating rats and their ALP activity was significantly lower than that of normal rats. All of the results obtained here showed that the combination of estrogen and GHRPs may be an useful way to find the corresponding lead compounds.

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