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The synthesis and immunosuppressive activities of steroid–urotoxin linkers

Chao Wang,[b] Ming Zhao,[b] Xuecai Qiu[b] and Shiqi Peng[a,*]

[a]*College of Pharmaceutical Sciences, Capital University of Medical Sciences, Beijing 100054, China*
[b]*College of Pharmaceutical Sciences, Peking University, Beijing 100083, China*

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Abstract—The urotoxins (Glu-Asp-Gly-OH, His-Gly-Glu-OH, His-Gly-Lys-OH, and His-Gly-Lys-$NHNH_2$) were introduced into the convenient sites of hydrocortisone and prednisolone via the amidation or condensation reactions to form the corresponding linkers **7a**–**d**, **8a**–**d**, **9a**,**b**, and **10a**,**b** in acceptable yields. The bioassays such as prolongation of heterotopic transplanted cardiac tissue survival in vivo, inhibitory effects on phagocytosis of mouse peritoneal macrophages and concanavalin (ConA) or lipopolysaccharide (LPS) induced proliferation of mouse spleen lymphocytes in vitro show that at the comparable concentrations the immunosuppressive activities of the steroid–urotoxin linkers **7a**–**d**, **8a**–**d**, **9a**,**b**, and **10a**,**b** were higher than that of hydrocortisone, prednisolone, and the urotoxins alone, as well as significantly higher than that of the mixture of hydrocortisone and urotoxins or prednisolone and urotoxins. The so-called 'permissive action' may be responsible for the enhancement of the mentioned bioactivities of the steroid–urotoxin linkers **7a**–**d**, **8a**–**d**, **9a**,**b**, and **10a**,**b**.

1. Introduction

In the 1950s it was reported that when a peptide was mixed with a steroid the bioactivity of the peptide was significantly enhanced by the steroid through increasing the peptide's receptor numbers. Since then this kind of phenomenon was known as 'permissive action'.[1,2] Afterwards it was observed that glucocorticoids exhibited rapid in vivo effects on the integrity of rat lymphocyte genomic deoxyribonucleic acid.[3] This means that on the cell membrane both steroid and peptide hormones have their receptors. All of those observations encouraged us to synthesize the linkers consisting of steroid and kyotophin or GHRPs, which exhibited analgesic or prevented osteoporosis effects, to simulate the 'permissive action' via the chemical combination of steroids and peptides.[4,5] The linkers of steroids and peptides indeed exhibited interesting biological phenomenon and provided a number of possible modifications either for peptides or for steroids, which obviously provides the useful ways to find the lead compounds. The importance of the immunosuppressive lead compounds, the immunosuppressive activity of hydrocortisone, prednisolone, and urotoxins isolated from uremic fluid, the available synthetic route of hydrocortisone–urotoxin,[6,7] and the preliminary immunosuppressive activity of four hydrocortisone–urotoxins,[8] drives us to prepare a series of hydrocortisone–urotoxins and prednisolone–urotoxins, to evaluate their in vitro and in vivo immunosuppressive activities, and to analyze the related chemical, structural, and biological knowledge.

2. Results

2.1. Solution method and stepwise synthesis giving urotoxins in 78–89% total yields

According to Scheme 1 the protective intermediate Boc-Glu(OBzl)-Asp(OBzl)-Gly-OEt was prepared via the solution method and stepwise synthesis (from C-terminal to N-terminal) with Gly-OEt as the starting material in 76% total yield. After removal of ethyl ester group the resulted Boc-Glu(OBzl)-Asp(OBzl)-Gly-OH was deprotected using the standard procedure in the presence of TFMSA/TFA to provide Glu-Asp-Gly-OH (**1**) in 89% yield. With the substantially similar stepwise synthesis (from C to N terminal in 78–81% total yield) depicted in

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* Corresponding author. Tel.: +86-10-8280-2482; fax: +86-10-8280-2311; e-mail: sqpeng@mail.bjmu.edu.cn

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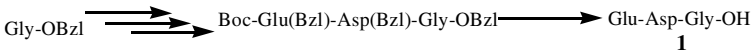
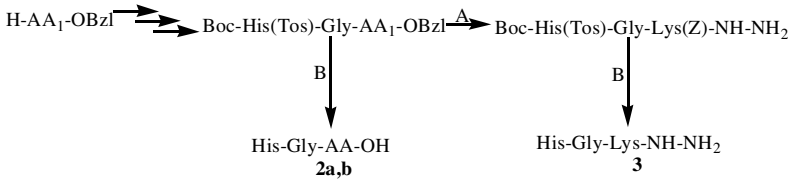


Scheme 1. Preparation of Glu-Asp-Gly–OH. (1) General procedure for coupling of C-terminal and N-terminal components: The solution of 1.5 mmol of the N-terminal component, 1.5 mmol of HOBt and 1.9 mmol of DCC in 15 mL of anhydrous THF was stirred at 0 °C for 24 h. After the addition of 1.5 mmol of C-terminal component and 1.9 mmol of *N*-methylmorpholine the solution was stirred at 0 °C for another 24 h. (2). General procedure for removal of Boc of the C-terminal component: 1.5 mmol of Boc protected compound were treated with 8 mL of hydrogen chloride in ethyl acetate (4 mol/L) at room temperature for 3 h. (3) General procedure for removal of all the semi-permanent groups: The solution of 1.0 mmol of protected peptide were treated with 6 mL of dimethyl sulfide, 6 mL of phenyl methyl ether, and 10 mL of CF_3COOH–CF_3SO_3H (4:1) at 0 °C for 2 h.

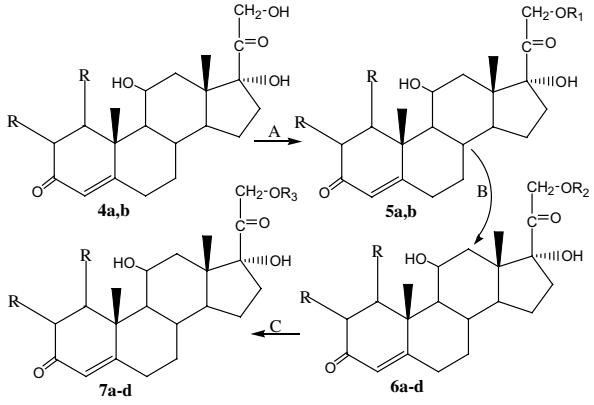


Scheme 2. Preparation of His-Gly-Glu-OH, His-Gly-Lys-OH, and His-Gly-Lys-$NHNH_2$. The general procedure for coupling of C-terminal and N-terminal components, general procedure for removal of Boc of the C-terminal component, and general procedure for removal of all the semi-permanent groups were the same as those described in Scheme 1; (A) 1 N NaOH/MeOH; NH_2NH_2/MeOH; (B) TFMSA/TFA. Wherein AA_1 = Glu(OBzl) and Lys(Z); in **2a,** AA = Glu; in **2b** AA = Lys.

Scheme 2 His-Gly-Glu-OH (**2a**), His-Gly-Lys-OH (**2b**), and His-Gly-Lys-$NHNH_2$ (**3**) were obtained.

2.2. Introducing urotoxin into 21-*O* of the steroid via carbonylpropionyl in 75-80% yield

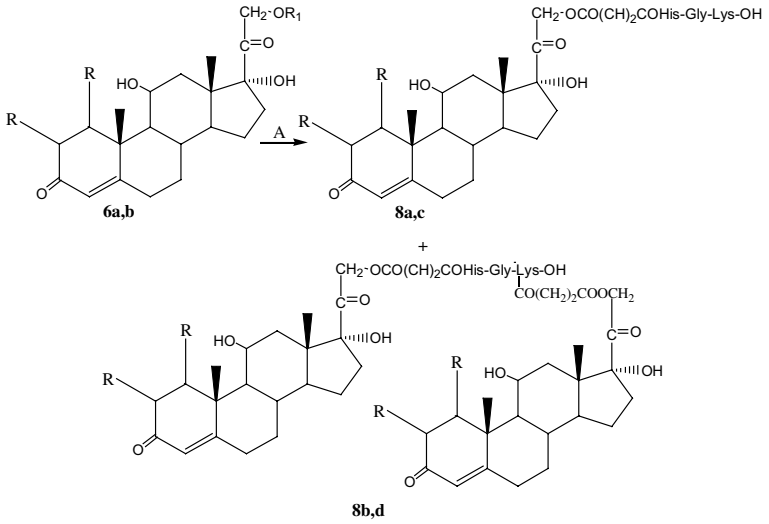
As indicated in Scheme 3 in the presence of pyridine hydrocortisone and prednisolone were treated with succinic anhydride to give hydrocortisone-21-*O*-β-carbonylpropionic acid (**5a**) and prednisolone-21-*O*-β-carbonylpropionic acid (**5b**) in 98% and 92% yield, respectively. The reaction of *p*-nitrophenol and **5a**,**b** resulted in the corresponding hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitrophenol ester (**6a**), and prednisolone-21-*O*-β-carbonylpropionic acid *p*-nitrophenol ester (**6b**) in 95% and 90% yield, respectively. The amidation of **6a**,**b** with urotoxin **1** provided the corresponding hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH (**7a**) and prednisolone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH (**7b**) in 50% and 52% yield, respectively. The amidation of **6a**,**b** with urotoxin **2a** provided the corresponding hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Glu-OH (**7c**) and prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Glu-OH (**7d**) in 48% and 46% yield, respectively.



Scheme 3. Preparation of hydrocortisone–urotoxin tripeptide and prednisolone–urotoxin tripeptide. The general procedure for coupling of C-terminal and N-terminal components was the same as that described in Scheme 1; (A) succinic anhydride/pyridine; (B) DCC/HOSu/HONp/THF; (C) Glu-Asp-Gly-OH (**1**) or His-Gly-Glu-OH (**2a**) in DMF/H_2O; in **4**–**6a**,**c**, and **7a**,**c** both of R = H and the steroid moiety = hydrocortisone; in **4**–**6b**,**d**, and **7b**,**d** both of R together constitute an olefinic bond and the steroid moiety = prednisolone; in **5a**,**b** $R_1 = COCH_2CH_2COOH$; in **6a**,**b** $R_2 = COCH_2CH_2COO(C_6H_4\text{-}p\text{-}NO_2)$; in **6c**,**d** $R_2 = COCH_2CH_2COOSu$; in **7a**,**c** $R_3 = COCH_2CH_2CO$-Glu-Asp-Gly-OH; in **7b**,**d** $R_3 = COCH_2CH_2CO$-His-Gly-Glu-OH.

The amidation of **6a** and **2b** gave the mono-steroid substituted peptide hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH (**8a** in 45% yield) and the bis-steroid substituted peptide hydrocortisone-His-Gly-Lys(hydrocortisone)-OH (**8b** 21% yield) simultaneously. The amidation of **6b** and **2b** gave also the mono-steroid substituted peptide prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH (**8c** in 42% yield) and the bis-steroid substituted peptide prednisolone-His-Gly-Lys(prednisolone)-OH (**8d** in 23% yield) simultaneously (Scheme 4).

In the presence of dicyclohexylcabodiimide (DCC) and *N*-hydroxysuccinimide (HOSu) **5a**,**b** were converted into hydrocortisone-21-*O*-β-carbonylpropionic acid HOSu ester (**6c**) and prednisolone-21-*O*-β-carbonylpropionic acid HOSu ester (**6d**), which were treated with urotoxin **1** and **2a** in situ to provide the corresponding hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH (**7a**), prednisolone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH (**7b**), hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Glu-OH (**7c**), and prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Glu-OH (**7d**) in 79%, 75%, 80%, and 77% yield, respectively. The amidation of hydrocortisone-21-*O*-β-carbonylpropionic acid HOSu ester (**6c**) and **2b** gave the mono-steroid substituted

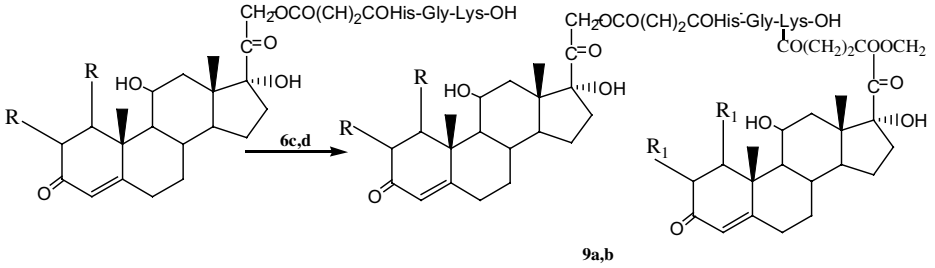


Scheme 4. Preparation of hydrocortisone–His-Gly-Lys-OH, prednisolone–His-Gly-Lys-OH, hydrocortisone–His-Gly-Lys(hydrocortisone)-OH, and prednisolone–His-Gly-Lys(prednisolone)-OH. The general procedure for coupling of C-terminal and N-terminal components was the same as that described in Scheme 1; (A) His-Gly-Lys-OH (**2b**) in DMF/H_2O. In **8a**,**b** both of R = H and the steroid moiety = hydrocortisone; in **8c**,**d** both of R together constitute an olefinic bond and the steroid moiety = prednisolone.

peptide hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH (**8a** in 50% yield) and bis-steroid substituted peptide hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys(hydrocortisone)-OH (**8b** in 20% yield) simultaneously. The amidation of prednisolone-21-*O*-β-carbonylpropionic acid HOSu ester (**6d**) and **2b** gave also the mono-steroid substituted peptide prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH (**8c** in 52% yield) and bis-steroid substituted peptide prednisolone-His-Gly-Lys(prednisolone)-OH (**8d** in 21% yield) simultaneously. The amidation of hydrocortisone-21-*O*-β-carbonylpropionic acid HOSu ester (**6c**) and prednisolone-21-*O*-β-carbonylpropionic acid HOSu ester (**6d**) with mono-steroid substituted peptide hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH (**8a)** and prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH (**8b)** gave the corresponding mixed bis-steroid substituted peptide hydrocortisone-His-Gly-Lys(prednisolone)-OH (**9a**) and prednisolone-His-Gly-Lys(hydrocortisone)-OH (**9b**) in 78% and 74% yield, respectively (Scheme 5).

2.3. Introducing urotoxin into 3-position of the steroid via hydrazine in 70–80% yield

In the presence of glacial acetic acid with methanol as the reaction solvent the selective condensation of the hydrazine group in Boc-His(Tos)-Gly-Lys(Z)-$NHNH_2$ and His-Gly-Lys-$NHNH_2$ (**3**) with the 3-carbonyl group in hydrocortisone and prednisolone provided the 3-[Boc-His(Tos)-Gly-Lys(Z)-NHN]-substituted hydrocortisone (**11a**) and 3-[Boc-His(Tos)-Gly-Lys(Z)-NHN]-substituted prednisolone (**11b**), 3-(His-Gly-Lys-NHN)-substituted hydrocortisone (**10a**), and 3-(His-Gly-Lys-NHN)-substituted prednisolone (**10b**) in 80%, 77%, 75%, and 70% yield, respectively. When inorganic acid, for instance HCl or H_2SO_4, was used instead of glacial acetic acid as the catalyst the yield of **10a**,**b**, and **11a**,**b** was lowed to 15–40%. This dependence may mean that the protonation of hydrazine group in **3** or Boc-His-(Tos)-Gly-Lys(Z)-$NHNH_2$ has a negative effect on the condensation reaction. Using the standard deprotected procedure in the presence of trifluoromethylsulfonic



Scheme 5. Preparation of hydrocortisone–His-Gly-Lys(prednisolone)-OH, and prednisolone–His-Gly-Lys(hydrocortisone)-OH. The general procedure for coupling of C-terminal and N-terminal components was the same as that described in Scheme 1; In **9a** both of R = H and the steroid moiety = hydrocortisone, and both of R_1 together constituted an olefinic bond and the steroid moiety = prednisolone; in **9b** both of R together constituted an olefinic bond and the steroid moiety = prednisolone, and both of R = H and the steroid moiety = hydrocortisone.

acid/trifluoroacetic acid (TFMSA/TFA) **11a**,**b** may be converted into **10a**,**b** in moderate yields (Scheme 6).

2.4. The steroid–urotoxin significantly inhibiting ConA and LPS induced lymphocyte proliferation

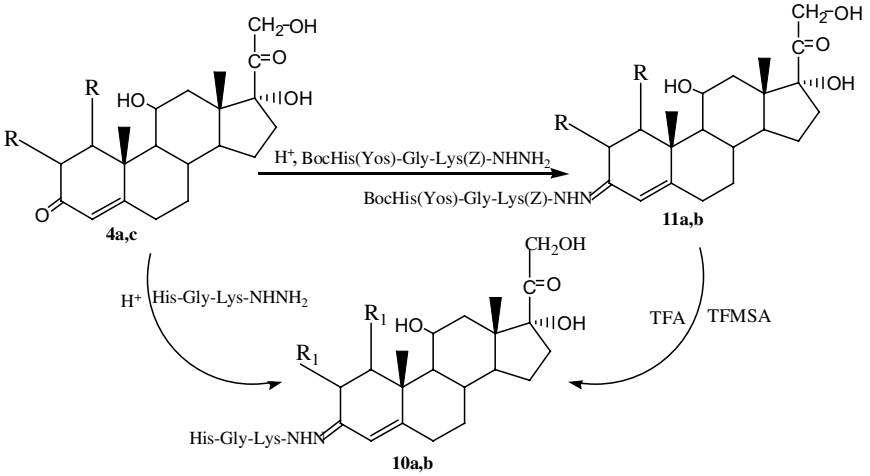
The spleen cells of BALB/C mice were collected according to the common method and incubated in a 96-well microplate with the controls and the synthetic steroid–urotoxin using the standard procedure.[9,10] The inhibitory effect of the synthetic steroid–urotoxins on ConA or LPS induced proliferation of BALB/C mouse spleen lymphocytes was evaluated. Based on the tested optical density (OD) value and the equation, 'Inhibitory rate = $(OD_{drug} - OD_{ConA\ or\ LPS})/(OD_{ConA}$ or $OD_{LPS} - OD_{NS})$' [wherein OD_{drug} represents the OD value derived from the lymphocytes treated with peptide, steroid, or steroid–peptide; OD_{ConA} or OD_{LPS} represents the OD value derived from the lymphocytes treated with concanavalin A (ConA) or lipopolysaccharide (LPS); OD_{NS} represents the OD value derived from the lymphocytes treated with normal saline (NS)], the inhibitory rate of the synthetic steroid–urotoxin was calculated. The data are listed in Tables 1–4 and the statistical analysis of the data is carried out by use of ANOVA test, $p < 0.05$ is considered significant. The results indicated that in the presence of the synthetic steroid–urotoxins the ConA and LPS induced proliferations of BALB/C mouse spleen lymphocytes were significantly inhibited (comparing to hydrocortisone, prednisolone, urotoxin, or the mixture of hydrocortisone or prednisolone and urotoxin, $P < 0.05$, $P < 0.01$, or $P < 0.001$).

2.5. The steroid–urotoxin significantly inhibiting phagocytosis of mouse peritoneal macrophages

The peritoneal macrophages were harvested according to the general method and incubated in a 96-well microplate with the controls and the synthetic steroid–urotoxin using the standard procedure.[11–13] After 24 h of incubation and the peritoneal macrophages being activated to the residue in the cultured well a solution of neutral red-stained zymosan was added and the neutral red-stained zymosan phagocytosis of mouse peritoneal macrophages was reflected by the OD value. The OD value of the incubation solution was read at 570 nm with Biorad. The inhibitory effect of the synthetic steroid–urotoxins on the phagocytosis of mouse peritoneal macrophages was then represented by the tested OD value. The data are listed in Tables 5 and 6, and the statistical analysis of the data are carried out by use of ANOVA test, $p < 0.05$ is considered significant. The results indicated that in the presence of the synthetic steroid–urotoxins the phagocytosis of mouse peritoneal macrophages were significantly inhibited (comparing to hydrocortisone, prednisolone, urotoxin, or the mixture of hydrocortisone or prednisolone and urotoxin, $P < 0.05$ or $P < 0.001$).

2.6. The steroid–urotoxin significantly lengthening the survival time of the split heart

The split hearts (3×3 mm in size) were obtained from C_{57}bl/6 mice (ranging in age from near full term fetuses up to 48 h old) according to the general method and transplanted into the base of the 'pocket' near the distal edge of the ear of BALB/C mice (male, ranged in age from 12–14 weeks at the time of transplantation).[14,15] One day after transplantation the mice were given an injection (ip) of 0.1 mL of DMSO/H_2O (1:4, v/v), of corresponding dosage of hydrocortisone in 0.1 mL of DMSO/H_2O (1:4, v/v), and of corresponding dosage of hydrocortisone–urotoxin in 0.1 mL of DMSO/H_2O (1:4, v/v) per day until the electrocardiograms of the grafts disappeared. Six days after transplantation the animals were anesthetized with sodium pentobarbital at a dosage of 75 μg/g body weight and the electrocardiograms of the grafts were recorded



Scheme 6. Preparation of His-Gly-Lys-NHN=hydrocortisone and His-Gly-Lys-NH-N=prednisolone. In **10a** and **11a** both of R = H and the steroid moiety = hydrocortisone; in **10b** and **11b** both of R together constituteed an olefinic bond and the steroid moiety = prednisolone.

Table 1. Effect of hydrocortisone–urotoxin on ConA induced spleen lymphocyte proliferation ($\overline{X}$±SD%)

Compound	10^{-8} mol/L	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L	10^{-4} mol/L
4a	6.3 ± 5.1	36.0 ± 5.2	56.4 ± 4.2	70.6 ± 4.6	72.9 ± 5.5
1	14.7 ± 4.3	21.7 ± 4.1	32.8 ± 4.9	55.1 ± 6.1	64.0 ± 6.8
2a	5.0 ± 5.3	22.4 ± 5.3	35.9 ± 6.3	55.7 ± 4.2	65.2 ± 7.2
2b	8.8 ± 4.2	34.5 ± 3.5	37.4 ± 5.6	62.4 ± 4.1	64.3 ± 8.2
3	4.9 ± 4.3	20.6 ± 4.2	33.7 ± 5.4	54.9 ± 5.0	63.8 ± 6.9
4a+1	16.9 ± 4.8	30.0 ± 6.2	46.4 ± 7.2	60.6 ± 6.6	64.9 ± 4.5
4a+2a	7.3 ± 6.1	28.4 ± 6.1	40.9 ± 5.8	60.1 ± 7.3	67.0 ± 6.9
4a+2b	10.8 ± 5.4	32.9 ± 5.3	42.1 ± 6.9	65.5 ± 7.1	66.4 ± 7.9
4a+3	5.8 ± 5.3	24.5 ± 5.6	38.7 ± 6.2	62.9 ± 7.0	65.6 ± 7.3
7a	36.2 ± 4.0[c,d]	49.2 ± 3.5[c,d]	67.9 ± 6.0[b,d]	78.5 ± 6.0[a,d]	88.8 ± 5.4[c,d]
7b	37.8 ± 3.6[c,f]	51.2 ± 3.1[c,f]	77.9 ± 5.7[c,f]	77.7 ± 5.5[a,f]	77.0 ± 5.7[e]
8a	29.3 ± 5.9[c,g]	31.4 ± 5.1	68.6 ± 5.0[b,g]	79.8 ± 5.6[a,g]	89.2 ± 6.1[c,g]
8b	29.9 ± 5.5[c,g]	49.0 ± 5.0[b,g]	67.1 ± 4.9[b,g]	78.9 ± 4.9[a,g]	90.1 ± 6.0[c,g]
9a	31.7 ± 4.8[c,i]	41.6 ± 6.2[a,i]	73.0 ± 6.9[b,i]	79.7 ± 5.8[a,i]	81.3 ± 6.5[h]
10a	30.7 ± 4.6[c,i]	40.0 ± 5.4[a,i]	71.8 ± 7.1[b,i]	78.3 ± 4.4[a,i]	79.2 ± 5.9[h]

$n = 6$.
[a] Compare to **4a**, $P < 0.05$.
[b] Compare to **4a**, $P < 0.01$.
[c] Compare to **4a**, $P < 0.001$.
[d] Compare to **1** and **4a+1,** $P < 0.001$.
[e] Compare to **2a** and **4a+2a**, $P < 0.01$.
[f] Compare to **2a** and **4a+2a**, $P < 0.001$.
[g] Compare to **2b** and **4a+2b**, $P < 0.001$.
[h] Compare to **3** and **4a+3**, $P < 0.01$.
[i] Compare to **3** and **4a+3**, $P < 0.001$.

Table 2. Effect of prednisolone–urotoxin on ConA induced spleen lymphocyte proliferation ($\overline{X}$±SD%)

Compound	$1/4 \times 10^{-8}$ mol/L	$1/4 \times 10^{-7}$ mol/L/4	$1/4 \times 10^{-6}$ mol/L	$1/4 \times 10^{-5}$ mol/L	$1/4 \times 10^{-4}$ mol/L
4b	6.0 ± 4.9	34.1 ± 6.1	52.3 ± 5.2	68.9 ± 5.7	75.0 ± 6.4
1	14.7 ± 4.3	21.7 ± 4.1	32.8 ± 4.9	55.1 ± 6.1	64.0 ± 6.8
2a	5.0 ± 5.3	22.45.3	35.9 ± 6.3	55.7 ± 4.2	65.2 ± 7.2
2b	8.8 ± 4.2	34.5 ± 3.5	37.4 ± 5.6	62.4 ± 4.1	64.3 ± 8.2
3	4.9 ± 4.3	20.6 ± 4.2	33.7 ± 5.4	54.9 ± 5.0	63.8 ± 6.9
4b+1	15.2 ± 4.4	28.9 ± 5.8	44.9 ± 6.6	59.4 ± 5.3	63.8 ± 4.3
4b+2a	7.1 ± 5.9	26.9 ± 5.8	39.6 ± 5.4	59.8 ± 6.8	66.8 ± 6.4
4b+2b	11.0 ± 5.6	33.2 ± 5.6	41.7 ± 5.6	64.7 ± 6.9	65.0 ± 6.7
4b+3	6.1 ± 5.5	23.8 ± 5.3	39.0 ± 5.7	63.2 ± 6.9	66.1 ± 6.9
7c	35.9 ± 3.8[c,d]	48.7 ± 3.6[c,d]	68.2 ± 5.9[b,d]	77.9 ± 6.2[a,d]	89.1 ± 5.6[c,d]
7d	38.2 ± 3.8[c,f]	50.9 ± 3.4[c,f]	78.4 ± 6.0[c,f]	78.3 ± 5.7[a,f]	76.8 ± 5.5[e]
8c	30.1 ± 6.2[c,g]	30.8 ± 4.8	69.1 ± 5.3[b,g]	80.0 ± 5.7[a,g]	88.8 ± 5.7[c,g]
8d	29.5 ± 5.4[c,g]	48.8 ± 4.7[b,g]	66.9 ± 4.7[b,g]	78.1 ± 4.8[a,g]	89.0 ± 5.8[c,g]
9b	32.2 ± 5.1[c,i]	42.0 ± 6.3[a,i]	72.7 ± 6.6[b,i]	80.3 ± 6.1[a,i]	80.9 ± 6.6[h]
10b	31.2 ± 4.8[c,i]	40.9 ± 5.6[a,i]	72.2 ± 6.8[b,i]	77.8 ± 4.6[a,i]	78.9 ± 6.0[h]

$n = 6$.
[a] Compare to **4b**, $P < 0.05$.
[b] Compare to **4b**, $P < 0.01$.
[c] Compare to **4b**, $P < 0.001$.
[d] Compare to **1** and **4b+1**, $P < 0.001$.
[e] Compare to **2a** and **4b+2a**, $P < 0.01$.
[f] Compare to **2a** and **4b+2a**, $P < 0.001$.
[g] Compare to **2b** and **4b+2b**, $P < 0.001$.
[h] Compare to **3** and **4b+3**, $P < 0.01$.
[i] Compare to **3** and **4b+3**, $P < 0.001$.

using the physiography. The interval between the tissue transplantation and the disappearance of pulsatile activity was as defined the survival time of the graft. The data are listed in Tables 7 and 8, and the statistical analysis of the data are carried out by use of ANOVA test, $p < 0.05$ is considered significant. The results indicated that after the administration of hydrocortisone–urotoxin and prednisolone-urotoxin the survival time of the grafts was significantly lengthened (comparing to hydrocortisone, prednisolone, urotoxin, or the mixture of hydrocortisone or prednisolone and urotoxin, $P < 0.01$, or $P < 0.001$).

Table 3. Effect of hydrocortisone–urotoxin on LPS induced spleen lymphocyte proliferation ($\overline{X}$±SD%)

Compound	10^{-8} mol/L	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L	10^{-4} mol/L
4a	10.3 ± 6.4	35.7 ± 6.6	51.6 ± 6.2	60.9 ± 5.3	66.8 ± 5.6
1	4.7 ± 4.3	21.7 ± 4.1	32.8 ± 4.9	45.1 ± 6.1	54.0 ± 6.8
2a	−7.5 ± 7.0	−5.0 ± 8.1	−15.9 ± 16.6	−12.1 ± 13.4	−15.8 ± 17.5
2b	−2.5 ± 4.2	−5.4 ± 3.0	15.6 ± 2.4	23.3 ± 7.0	33.4 ± 9.1
3	5.9 ± 4.3	19.3 ± 4.0	33.2 ± 5.3	44.8 ± 5.1	53.7 ± 6.8
4a+1	7.4 ± 5.5	27.1 ± 8.2	39.9 ± 8.8	54.3 ± 6.9	60.2 ± 8.6
4a+2a	6.9 ± 5.5	10.1 ± 7.8	5.9 ± 6.9	7.1 ± 6.0	5.8 ± 7.5
4a+2b	6.5 ± 4.8	6.8 ± 5.4	25.0 ± 9.8	43.3 ± 17.9	43.4 ± 19.6
4a+3	8.8 ± 7.5	26.8 ± 8.2	43.2 ± 15.1	54.0 ± 9.9	62.2 ± 9.9
7a	36.2 ± 4.0[c,d]	49.2 ± 3.5[b,d]	65.9 ± 6.0[b,d]	72.5 ± 6.0[b,d]	78.8 ± 5.4[b,d]
7b	33.9 ± 10.7[c,e]	43.5 ± 6.7[e]	64.2 ± 5.9[b,e]	70.9 ± 6.1[a,e]	64.5 ± 2.6[e]
8a	17.5 ± 5.1[a,f]	47.4 ± 2.4[b,f]	58.5 ± 3.8[a,f]	83.2 ± 12.5[b,f]	77.3 ± 6.6[a,f]
8b	29.9 ± 5.5[c,f]	44.0 ± 5.0[a,f]	65.1 ± 4.9[b,f]	72.9 ± 5.9[b,f]	80.1 ± 6.0[b,f]
9a	32.7 ± 9.2[c,g]	46.0 ± 4.6[a,g]	66.3 ± 4.9[b,g]	74.8 ± 5.6[b,g]	81.4 ± 5.7[b,g]
10a	31.3 ± 9.5[c,g]	44.7 ± 3.7[a,g]	64.9 ± 5.3[b,g]	73.2 ± 5.1[b,g]	79.2 ± 6.5[b,g]

$n = 6$.
[a] Compare to hydrocortisone, $P < 0.05$.
[b] Compare to hydrocortisone, $P < 0.01$.
[c] Compare to hydrocortisone, $P < 0.001$.
[d] Compare to **1** and hydrocortisone+**1**, $P < 0.001$.
[e] Compare to **2a** and hydrocortisone+**2a**, $P < 0.001$.
[f] Compare to **2b** and hydrocortisone+**2b**, $P < 0.001$.
[g] Compare to **3** and hydrocortisone+**3**, $P < 0.001$; minus quantity means enhanced action.

Table 4. Effect of prednisolone–urotoxin on LPS induced spleen lymphocyte proliferation ($\overline{X}$ ± SD%)

Compound	$1/4 \times 10^{-8}$ mol/L	$1/4 \times 10^{-7}$ mol/L	$1/4 \times 10^{-6}$ mol/L	$1/4 \times 10^{-5}$ mol/L	$1/4 \times 10^{-4}$ mol/L
4b	11.1 ± 6.2	36.2 ± 6.4	52.1 ± 6.6	61.4 ± 5.6	66.1 ± 5.4
1	5.1 ± 4.5	22.2 ± 4.4	33.3 ± 5.1	44.8 ± 5.8	53.7 ± 6.3
2a	−7.2 ± 6.7	−5.8 ± 7.4	−16.5 ± 16.0	−12.8 ± 13.1	−16.4 ± 16.9
2b	−4.2 ± 3.9	−6.2 ± 3.6	14.9 ± 2.6	22.7 ± 6.8	32.9 ± 8.7
3	6.3 ± 4.5	18.7 ± 4.3	34.5 ± 5.4	45.2 ± 5.3	54.5 ± 6.6
4b+1	7.2 ± 5.2	27.8 ± 7.6	40.2 ± 8.5	53.9 ± 6.6	59.9 ± 7.9
4b+2a	7.0 ± 5.4	10.6 ± 7.6	6.1 ± 6.0	7.7 ± 5.9	6.7 ± 7.1
4b+2b	6.9 ± 4.6	6.9 ± 5.0	24.8 ± 7.6	42.8 ± 16.8	42.6 ± 18.1
4b+3	9.2 ± 7.2	27.1 ± 7.9	42.8 ± 14.6	53.6 ± 9.3	63.0 ± 9.0
7c	35.7 ± 4.2[c,d]	48.8 ± 3.3[b,d]	65.1 ± 5.6[b,d]	73.0 ± 5.8[b,d]	78.0 ± 5.2[b,d]
7d	34.4 ± 9.5[c,e]	44.4 ± 6.5[e]	63.9 ± 6.0[b,e]	71.4 ± 5.8[a,e]	63.8 ± 3.1[e]
8c	18.1 ± 5.3[a,f]	46.9 ± 2.5[b,f]	59.3 ± 3.9[a,f]	82.7 ± 11.7[b,f]	78.0 ± 6.4[a,f]
8d	30.3 ± 5.4[c,f]	44.6 ± 5.2[a,f]	64.7 ± 4.6[b,f]	73.4 ± 5.7[b,f]	79.9 ± 5.9[b,f]
9b	33.2 ± 8.8[c,g]	45.8 ± 4.4[a,g]	67.0 ± 4.6[b,g]	75.4 ± 5.5[b,g]	81.0 ± 5.6[b,g]
10b	32.2 ± 9.3[c,g]	45.5 ± 3.6[a,g]	64.4 ± 5.0[b,g]	72.6 ± 5.0[b,g]	78.8 ± 6.2[b,g]

$n = 6$.
[a] Compare to **4b**, $P < 0.05$.
[b] Compare to **4b**, $P < 0.01$.
[c] Compare to **4b**, $P < 0.001$.
[d] Compare to **1** and **4b**+**1**, $P < 0.001$.
[e] Compare to **2a** and **4b**+**2a**, $P < 0.001$.
[f] Compare to **2b** and **4b**+**2b**, $P < 0.001$.
[g] Compare to **3** and **4b**+**3**, $P < 0.001$; minus quantity means enhanced action.

3. Discussion

3.1. The amidation of urotoxins preferring the HOSu ester to the *p*-nitrophenol ester

Using the solution method via stepwise synthesis (from C-terminal to N-terminal) the protective intermediates of urotoxins were smoothly obtained in relatively high yield. In the solution of TFMSA/TFA the protective intermediates were converted into urotoxins in satisfactory yield. The amidation of hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitrophenol ester and prednisolone-21-*O*-β-carbonylpropionic acid *p*-nitrophenol ester with urotoxins provided the desirable linkers in moderate yield. If hydrocortisone-21-*O*-β-carbonylpropionic acid HOSu ester and prednisolone-21-*O*-β-carbonylpropionic acid HOSu ester were used instead of hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitro-

Table 5. The phagocytosis of mouse peritoneal macrophages after treatment of hydrocortisone–urotoxin ($\overline{X} \pm SD$)

Compound	10^{-9} mol/L	10^{-8} mol/L	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
NS	0.78 ± 0.04				
4a	0.66 ± 0.03^{a}	0.56 ± 0.04^{c}	0.53 ± 0.05^{c}	0.50 ± 0.06^{c}	0.46 ± 0.03^{c}
1	0.80 ± 0.03	0.67 ± 0.02^{a}	0.63 ± 0.03^{a}	0.63 ± 0.04^{a}	0.60 ± 0.03^{b}
2a	0.74 ± 0.02	0.65 ± 0.05	0.63 ± 0.03^{a}	0.64 ± 0.04^{a}	0.59 ± 0.03^{c}
2b	0.81 ± 0.03	0.66 ± 0.02^{a}	0.62 ± 0.05^{a}	0.55 ± 0.04^{c}	0.54 ± 0.03^{c}
3	0.76 ± 0.03	0.66 ± 0.04	0.64 ± 0.03^{a}	0.64 ± 0.04^{a}	0.61 ± 0.03^{b}
4a+1	0.78 ± 0.04	0.66 ± 0.03^{a}	0.62 ± 0.04^{a}	0.62 ± 0.05^{a}	0.61 ± 0.04^{b}
4a+2a	0.73 ± 0.03	0.63 ± 0.06	0.64 ± 0.03^{a}	0.63 ± 0.05^{a}	0.58 ± 0.03^{c}
4a+2b	0.80 ± 0.04	0.65 ± 0.02^{a}	0.63 ± 0.03^{a}	0.54 ± 0.03^{c}	0.55 ± 0.02^{c}
4a+3	0.75 ± 0.04	0.65 ± 0.04	0.62 ± 0.04^{a}	0.63 ± 0.04^{a}	0.60 ± 0.03^{b}
7a	$0.58 \pm 0.03^{c,f,g}$	$0.53 \pm 0.04^{c,g}$	$0.44 \pm 0.02^{c,f,g}$	$0.48 \pm 0.03^{c,g}$	$0.43 \pm 0.03^{c,d,c}$
7b	$0.55 \pm 0.03^{c,f,h}$	$0.52 \pm 0.04^{c,d,h}$	$0.41 \pm 0.02^{c,f,h}$	$0.45 \pm 0.05^{c,d,h}$	$0.38 \pm 0.04^{c,f,h}$
8a	$0.68 \pm 0.04^{a,i}$	$0.52 \pm 0.02^{a,e,i}$	$0.45 \pm 0.03^{c,f,i}$	$0.40 \pm 0.05^{c,f,i}$	$0.41 \pm 0.03^{c,f,i}$
8b	$0.64 \pm 0.04^{c,i}$	$0.51 \pm 0.02^{c,f,i}$	$0.43 \pm 0.03^{c,f,i}$	$0.40 \pm 0.03^{c,f,i}$	$0.40 \pm 0.02^{c,f,i}$
9a	$0.60 \pm 0.04^{c,j}$	$0.51 \pm 0.03^{c,j}$	$0.42 \pm 0.04^{c,f,j}$	$0.42 \pm 0.04^{c,d,j}$	$0.40 \pm 0.03^{c,e,j}$
10a	$0.65 \pm 0.03^{c,j}$	$0.53 \pm 0.04^{c,j}$	$0.44 \pm 0.03^{c,f,j}$	$0.44 \pm 0.05^{c,d,j}$	$0.41 \pm 0.04^{c,e,j}$

$n = 12$; NS = vehicle.
[a] Compared to NS, $P < 0.05$.
[b] Compared to NS, $P < 0.01$.
[c] Compared to NS, $P < 0.001$.
[d] Compared to **4a**, $P < 0.05$.
[e] Compared to **4a**, $P < 0.01$.
[f] Compared to **4a**, $P < 0.001$.
[g] Compared to **1** and **4a+1**, $P < 0.001$.
[h] Compared to **2a** and **4a+2a**, $P < 0.001$.
[i] Compared to **2b** and **4a+2b**, $P < 0.001$.
[j] Compared to **3** and **4a+3**, $P < 0.001$.

Table 6. The phagocytosis of mouse peritoneal macrophages after treatment of prednisolone-urotoxin ($\overline{X} \pm SD$)

Compound	$1/4 \times 10^{-9}$ mol/L	$1/4 \times 10^{-8}$ mol/L	$1/4 \times 10^{-7}$ mol/L	$1/4 \times 10^{-6}$ mol/L	$1/4 \times 10^{-5}$ mol/L
NS	0.78 ± 0.04				
4b	0.64 ± 0.03^{a}	0.58 ± 0.05^{c}	0.56 ± 0.04^{c}	0.52 ± 0.05^{c}	0.48 ± 0.04^{c}
1	0.80 ± 0.03	0.67 ± 0.02^{a}	0.63 ± 0.03^{a}	0.63 ± 0.04^{a}	0.60 ± 0.03^{b}
2a	0.74 ± 0.02	0.65 ± 0.05	0.63 ± 0.03^{a}	0.64 ± 0.04^{a}	0.59 ± 0.03^{c}
2b	0.81 ± 0.03	0.66 ± 0.02^{a}	0.62 ± 0.05^{a}	0.55 ± 0.04^{c}	0.54 ± 0.03^{c}
3	0.76 ± 0.03	0.66 ± 0.04	0.64 ± 0.03^{a}	0.64 ± 0.04^{a}	0.61 ± 0.03^{b}
4b+1	0.80 ± 0.05	0.68 ± 0.04^{a}	0.66 ± 0.05^{a}	0.64 ± 0.05^{a}	0.63 ± 0.04^{b}
4b+2a	0.74 ± 0.04	0.62 ± 0.05	0.66 ± 0.04^{a}	0.65 ± 0.05^{a}	0.57 ± 0.04^{c}
4b+2b	0.79 ± 0.05	0.67 ± 0.03^{a}	0.65 ± 0.03^{a}	0.55 ± 0.03^{c}	0.57 ± 0.03^{c}
4b+3	0.77 ± 0.04	0.66 ± 0.04	0.64 ± 0.04^{a}	0.64 ± 0.04^{a}	0.62 ± 0.03^{b}
7c	$0.57 \pm 0.03^{c,f,g}$	$0.52 \pm 0.03^{c,g}$	$0.45 \pm 0.02^{c,f,g}$	$0.46 \pm 0.03^{c,g}$	$0.44 \pm 0.03^{c,d,g}$
7d	$0.56 \pm 0.03^{c,f,h}$	$0.51 \pm 0.04^{c,d,h}$	$0.42 \pm 0.02^{c,f,h}$	$0.44 \pm 0.04^{c,d,h}$	$0.37 \pm 0.04^{c,f,h}$
8c	$0.66 \pm 0.04^{a,i}$	$0.52 \pm 0.02^{c,e,i}$	$0.44 \pm 0.03^{c,f,i}$	$0.40 \pm 0.04^{c,f,i}$	$0.40 \pm 0.03^{c,f,i}$
8d	$0.65 \pm 0.04^{c,i}$	$0.50 \pm 0.02^{c,f,i}$	$0.44 \pm 0.03^{c,f,i}$	$0.41 \pm 0.03^{c,f,i}$	$0.41 \pm 0.02^{c,f,i}$
9b	$0.62 \pm 0.03^{c,j}$	$0.50 \pm 0.03^{c,j}$	$0.43 \pm 0.03^{c,f,j}$	$0.43 \pm 0.04^{c,d,j}$	$0.41 \pm 0.03^{c,e,j}$
10b	$0.63 \pm 0.03^{c,j}$	$0.51 \pm 0.03^{c,j}$	$0.43 \pm 0.03^{c,f,j}$	$0.43 \pm 0.05^{c,d,j}$	$0.42 \pm 0.03^{c,e,j}$

$n = 12$; NS = vehicle.
[a] Compared to NS, $P < 0.05$.
[b] Compared to NS, $P < 0.01$.
[c] Compared to NS, $P < 0.001$.
[d] Compared to **4b**, $P < 0.05$.
[e] Compared to **4b**, $P < 0.01$.
[f] Compared to **4b**, $P < 0.001$.
[g] Compared to **1** and **4b+1**, $P < 0.001$.
[h] Compared to **2a** and **4b+2a**, $P < 0.001$.
[i] Compared to **2b** and **4b+2b**, $P < 0.001$.
[j] Compared to **3** and **4b+3**, $P < 0.001$.

phenol ester and prednisolone-21-*O*-β-carbonylpropionic acid *p*-nitrophenol ester the yield of the amidation was increased by about 30%. These results indicated that for the amidation of urotoxins the HOSu ester is preferable to the *p*-nitrophenol ester.

3.2. The amidation of His-Gly-Lys-OH preferring the α-amino group of His to the ω-amino group of Lys

In principle the α-amino group at His residue and the ω-amino group at Lys residue of His-Gly-Lys-OH may be

Table 7. Survival time of the transplantation grafts after treatment of hydrocortisone–urotoxin ($\overline{X} \pm$ SD, h)

Compound	0.15 (mmol/20 g/d)	0.75 (mmol/20 g/d)	1.50 (mmol/20 g/d)
DMSO/H_2O (1:4)	210.9 ± 7.2		
4a	235.2 ± 7.7^{b}	250.6 ± 6.1^{b}	280.1 ± 7.2^{b}
1	220.9 ± 5.7^{a}	231.6 ± 6.4^{b}	250.6 ± 7.3^{b}
2a	222.0 ± 7.8^{a}	234.8 ± 7.3^{b}	247.2 ± 6.4^{b}
2b	225.2 ± 7.3^{b}	232.6 ± 6.4^{b}	252.1 ± 5.9^{b}
3	221.2 ± 6.7^{a}	230.6 ± 7.0^{b}	250.1 ± 6.2^{b}
4a+**1**	225.9 ± 6.7^{b}	232.6 ± 6.6^{b}	252.6 ± 7.5^{b}
4a+**2a**	226.0 ± 8.8^{b}	235.2 ± 7.1^{b}	250.1 ± 6.5^{b}
4a+**2b**	227.2 ± 7.5^{b}	234.6 ± 6.6^{b}	254.1 ± 6.4^{b}
4a+**3**	223.2 ± 7.1^{a}	232.0 ± 6.9^{b}	252.2 ± 6.0^{b}
7a	$245.9 \pm 6.7^{b,c,e}$	$270.8 \pm 7.4^{b,d,e}$	$299.4 \pm 5.2^{b,d,e}$
7b	$246.1 \pm 6.1^{b,c,f}$	$269.6 \pm 6.1^{b,d,f}$	$301.4 \pm 7.0^{b,d,f}$
8a	$250.1 \pm 6.5^{b,d,g}$	$268.0 \pm 5.8^{b,d,g}$	$302.7 \pm 5.4^{b,d,g}$
8b	$247.7 \pm 5.7^{b,d,g}$	$269.9 \pm 5.5^{b,d,g}$	$303.4 \pm 6.0^{b,d,g}$
9a	$250.5 \pm 6.7^{b,d,h}$	$273.2 \pm 5.9^{b,d,h}$	$305.1 \pm 6.3^{b,d,h}$
10a	$246.7 \pm 6.4^{b,d,h}$	$270.5 \pm 6.3^{b,d,h}$	$301.5 \pm 5.9^{b,d,h}$

$n = 12$; DMSO/H_2O (1:4) = vehicle.
[a] Compare to vehicle, $P < 0.01$.
[b] Compare to vehicle, $P < 0.001$.
[c] Compare to **4a**, $P < 0.01$.
[d] Compare to **4a**, $P < 0.001$.
[e] Compare to **1** and **4a**+**1**, $P < 0.001$.
[f] Compare to **2a** and **4a**+**2a**, $P < 0.001$.
[g] Compare to **2b** and **4a**+**2b**, $P < 0.001$.
[h] Compare to **3** and **4a**+**3**, $P < 0.001$.

Table 8. Survival time of the transplantation grafts after treatment of prednisolone–urotoxin ($\overline{X}\pm$SD, h)

Compound	0.04 (mmol/20 g/d)	0.19 (mmol/20 g/d)	0.38 (mmol/20 g/d)
DMSO/H_2O (1:4)	210.9 ± 7.2		
4b	232.4 ± 7.4^{b}	248.9 ± 5.9^{b}	278.8 ± 7.0^{b}
1	220.9 ± 5.7^{a}	231.6 ± 6.4^{b}	250.6 ± 7.3^{b}
2a	222.0 ± 7.8^{a}	234.8 ± 7.3^{b}	247.2 ± 6.4^{b}
2b	225.2 ± 7.3^{b}	232.6 ± 6.4^{b}	252.1 ± 5.9^{b}
3	221.2 ± 6.7^{a}	230.6 ± 7.0^{b}	250.1 ± 6.2^{b}
4b+**1**	223.6 ± 6.5^{b}	231.8 ± 6.4^{b}	251.6 ± 7.2^{b}
4b+**2a**	225.6 ± 8.6^{b}	234.8 ± 6.9^{b}	249.6 ± 6.3^{b}
4b+**2b**	226.7 ± 7.5^{b}	233.9 ± 6.4^{b}	253.5 ± 6.2^{b}
4b+**3**	222.7 ± 6.8^{a}	231.4 ± 6.7^{b}	251.7 ± 5.9^{b}
7c	$246.3 \pm 6.6^{b,c,e}$	$271.7 \pm 7.5^{b,d,e}$	$298.9 \pm 5.1^{b,d,e}$
7d	$246.7 \pm 6.1^{b,c,f}$	$268.9 \pm 6.0^{b,d,f}$	$302.0 \pm 7.1^{b,d,f}$
8c	$251.3 \pm 6.4^{b,d,g}$	$267.9 \pm 5.7^{b,d,g}$	$303.4 \pm 5.5^{b,d,g}$
8d	$248.4 \pm 5.8^{b,d,g}$	$269.2 \pm 5.4^{b,d,g}$	$304.3 \pm 6.1^{b,d,g}$
9b	$251.3 \pm 6.6^{b,d,h}$	$274.0 \pm 6.0^{b,d,h}$	$304.8 \pm 6.1^{b,d,h}$
10b	$247.2 \pm 6.3^{b,d,h}$	$271.2 \pm 6.1^{b,d,h}$	$302.2 \pm 5.8^{b,d,h}$

$n = 12$; DMSO/H_2O (1:4) = vehicle.
[a] Compare to vehicle, $P < 0.01$.
[b] Compare to vehicle, $P < 0.001$.
[c] Compare to **4b**, $P < 0.01$.
[d] Compare to **4b**, $P < 0.001$.
[e] Compare to **1** and **4b**+**1**, $P < 0.001$.
[f] Compare to **2a** and **4b**+**2a**, $P < 0.001$.
[g] Compare to **2b** and **4b**+**2b**, $P < 0.001$.
[h] Compare to **3** and **4b**+**3**, $P < 0.001$.

acylated either individually or simultaneously by the HOSu or *p*-nitrophenol ester of hydrocortisone- and prednisolone-21-*O*-β-carbonylpropionic acid and may result in the mono-steroid substituted peptides hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH, prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH, and bis-steroid substituted peptides hydrocortisone-His-Gly-Lys(hydrocortisone)-OH and prednisolon-His-Gly-Lys(prednisolone)-OH simultaneously. The ratio of the mono-steroid substituted peptide to bis-steroid substituted peptide depended on the ratio of **6** to **1** used in the amidation. When the ratio of raw material **6** to **1** was 1:1 the ratio of the mono-steroid substituted peptide to bis-steroid substituted peptide was about 2:1, when the ratio of raw material **6** to **1** was 1.5:1 the ratio of the mono-steroid substituted peptide to bis-steroid

substituted peptide was 1.5:1, when the ratio of raw material **6** to **1** was 2:1 the ratio of the mono-steroid substituted peptide to bis-steroid substituted peptide was 1:1. In all cases no mono-steroid substituted peptide with hydrocortisone- and prednisolone-21-*O*-β-carbonylpropionyl at the ω-amino group of the Lys residue was formed. The rather weak nucleophilicity of ω-amino group of the Lys residue may be responsible for the lack of the mono-steroid substituted peptide with hydrocortisone- and prednisolone-21-*O*-β-carbonylpropionyl at the ω-amino group of the Lys residue. When the amidations of urotoxins were carried out by use of hydrocortisone-21-*O*-β-carbonylpropionic acid HOSu ester as the amidation agent the yields of the linkers may be obviously increased.

3.3. The condensation of the hydrazine and the 3-carbonyl group depending on the catalyst

The yield of the condensation of the hydrazine group in His-Gly-Lys-$NHNH_2$ and Boc-His-(Tos)-Gly-Lys(Z)-$NHNH_2$ with the 3-carbonyl group in hydrocortisone and prednisolone was significantly dependent on the catalyst. With glacial acetic acid as the catalyst the yields of 3-(His-Gly-Lys-NHN)-substituted hydrocortisone, 3-(His-Gly-Lys-NHN)-substituted prednisolone, 3-[Boc-His(Tos)-Gly-Lys(Z)-NHN]-substituted hydrocortisone, and 3-[Boc-His-(Tos)-Gly-Lys(Z)-NHN]-substituted prednisolone were within 70–80%. When inorganic acid, for instance HCl or H_2SO_4, was used instead of glacial acetic acid as the catalyst their yields were lowed to 15–40%. This dependence perhaps means that the protonation of hydrazine group in His-Gly-Lys-$NHNH_2$ and Boc-His(Tos)-Gly-Lys(Z)-$NHNH_2$ has a negative effect on the condensation reaction.

3.4. Permissive action enhancing the immunosuppressive effect of both steroids and urotoxins

Both ConA or LPS induced proliferation of mouse spleen lymphocytes in vitro were used for evaluating the immunosuppressive effects of the synthetic steroid–urotoxins. The data in Tables 1 and 2 show that at the concentrations of 10^{-8}–10^{-4} mol/L the inhibition rates of **7a**–**d**, **8a**–**d**, **9a**,**b**, and **10a**,**b** for ConA or LPS induced proliferation of mouse spleen lymphocytes in vitro are significantly higher than that of hydrocortisone, prednisolone, urotoxins, or the mixture of hydrocortisone or prednisolone and urotoxins. On the other hand **2a** (at the concentrations of 10^{-8}–10^{-4} mol/L) and **2b** (at the concentrations of 10^{-8}–10^{-7} mol/L) exhibited (the minus inhibition rate) an enhanced action rather for LPS than for ConA induced proliferation of mouse spleen lymphocytes. This may mean that the immunosuppressive effects of **2a**,**b** on LPS and ConA induced proliferation of mouse spleen lymphocytes via distinct pathway. The phagocytosis of mouse peritoneal macrophages assay in vitro indicated that at the concentration of 10^{-6}–10^{-2} mol/L the inhibition effects of **7a**–**d**, **8a**–**d**, **9a**,**b**, and **10a**,**b** on the macrophage activity were also significantly higher than that of hydrocortisone, prednisolone, urotoxin, or the mixture of hydrocortisone or prednisolone and urotoxins. The rodent heterotopic ear–heart transplant assay in vivo suggested that the immunosuppressive effects, which was demonstrated by the survival times of grafts, of **7a**–**d**, **8a**–**d**, **9a**,**b**, and **10a**,**b** were again significantly higher than that of hydrocortisone, prednisolone, urotoxin, or the mixture of hydrocortisone or prednisolone and urotoxins. In conclusion, comparing to hydrocortisone, prednisolone, urotoxin, or the mixture of hydrocortisone or prednisolone and urotoxins, hydrocortisone–urotoxins, and prednisolone–urotoxins exhibited better immunosuppressive activities in vitro and in vivo, namely the immunosuppressive activities of both steroids and urotoxins were enhanced by the chemical combination of the steroids and urotoxins.

3.5. The possible chemical combination of steroids and peptides commonly enhancing bioactivity

In our previous studies it was found that the analgesic effects of hydrocortisone–KTP and estrone–KTP were significantly higher than that of KTP, hydrocortisone or estrone, and the mixture of KTP and hydrocortisone or estrone;[4] the anti-osteoporosis effects of estrogen–GHRPs were significantly higher than that of GHRPs, estrogen, and the mixture of estrogen and GHRPs.[5] According to the data from the in vitro and in vivo assays it is clear that the immunosuppressive activities of **7a**–**c**, **8a**–**c**, **10a**, and **10b** are at the same level and again significantly higher than that of hydrocortisone, prednisolone, and urotoxins. These observations indicated that not only the combination of steroid and peptide may result in an enhancement of the activity but also the substitution position of peptides at steroids gave no effect on the enhancing activity. The data from the in vitro and in vivo assays clearly point that the immunosuppressive activities of **9a** and **9b** are also at the same level as that of **7a**–**c**, **8a**–**c**, **10a**, and **10b** and again significantly higher than that of hydrocortisone, prednisolone, and urotoxins, meaning the increase of steroid gave no effect on the enhancing activity neither. Thus all the results suggested that the 'permissive action' should be generally simulated by the possibly chemical combination of steroids and peptides. Considering the immunosuppressive activities of **7a**–**c**, **8a**–**c**, **9a**,**b**, **10a**, and **10b** are significantly higher than that of the mixture of hydrocortisone or prednisolone and urotoxins, and the chemical stability of C–N bond in the 3-position of **10a** and **10b**, it is likely that the synthetic steroid–urotoxin in its entirety should be responsible to its immunosuppressive action. Thus the chemical combinations of steroids and peptides via simple ester bond are able to provide the useful way for the design of steroid and peptide related lead compounds enhancing the desirable bioactivity.

4. Experimental

4.1. Synthesis of peptides and steroid–urotoxins

4.1.1. General. The protected amino acids were of L-configuration. The purity of the intermediates and the

products was confirmed by TLC (Merck silica gel plates of type 60 F_{254}, 0.25 mm layer thickness) and HPLC (waters, C_{18} column 3.9 × 150 mm). Melting points were measured on a XT5 hot stage microscope (Beijing keyi electro-optic factory), and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 983 instrument. FAB-MS was determined by a VG-ZAB-MS and a HPES-5989 × instrument. ^{1}H NMR spectra were determined by a Varian INOVA-500 MHz spectrometer. Optical rotations were determined at 20 °C on a Schmidt+Haensch Polartronic D instrument. The amino acid analysis was performed by a Hitachi 835-50 instrument.

4.2. General procedure for coupling C-terminal and N-terminal components

To a solution of 1.5 mmol of the N-terminal component in 15 mL of anhydrous THF at 0 °C 1.5 mmol of HOBt and 1.9 mmol of DCC were added. The reaction mixture was stirred at 0 °C for 24 h and the precipitates of DCU were removed by filtration. The filtrate was evaporated under reduced pressure and the residue was triturated with petroleum ether to provide the corresponding active ester. To the solution of the active ester in 30 mL of anhydrous THF 1.5 mmol of C-terminal component and 1.9 mmol of N-methylmorpholine were added. The reaction mixture was stirred at room temperature for 24 h. After evaporation the residue was dissolved in 50 mL of ethyl acetate. The solution was washed successively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride and the organic phase was dried over anhydrous sodium sulfate. After filtration and evaporation under reduced pressure the residue was purified by chromatography ($CHCl_3$/CH_3OH, 30:1) to provide the protective intermediates.

4.3. Boc-Asp(OBzl)-Gly-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 450 mg (1.5 mmol) of Boc-Asp(OBzl)-OH and 248 mg (1.5 mmol) of Gly-OBzl 556 mg (80%) of the title compound were obtained as a colorless powder. Mp 93–95 °C, $[\alpha]_D^{20}$ +16.0 (*c* 1, $CHCl_3$), FAB-MS (*m*/*z*) 471 $[M+H]^+$. IR (KBr) 3344, 3336, 3090, 3075, 3029, 1735, 1691, 1600, 1500, 1460, 1450, 1395, 1365, 742, 693 cm^{-1}. ^{1}H NMR (DMSO-d_6): $\delta = 8.013$ (d, $J = 6.39$ Hz, 1H), 8.005 (t, $J = 6.54$ Hz, 1H), 7.335 (t, $J = 7.92$ Hz, 1H), 7.322 (t, $J = 7.96$ Hz, 1H), 7.310 (t, $J = 7.92$ Hz, 2H), 7.305 (t, $J = 7.96$ Hz, 2H), 7.257 (d, $J = 7.84$ Hz, 2H), 7.220 (d, $J = 7.80$ Hz, 2H), 5.181 (s, 2H), 5.172 (s, 2H), 4.904 (dt, $J = 4.56$ Hz, $J = 6.39$ Hz, 1H), 3.921 (d, $J = 6.54$ Hz, 2H), 2.901 (d, $J = 5.96$ Hz, 2H), 1.456 (s, 9H). Amino acid analysis: calcd, Asp/Gly = 1.0:1.0. Found, Asp/Gly = 1.00:0.99. Anal. Calcd for $C_{25}H_{30}N_2O_7$: C, 63.82; H, 6.43; N, 5.95. Found: C, 63.76; H, 6.50; N, 6.08.

4.4. Boc-Glu(OBzl)-Asp(OBzl)-Gly-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 507 mg (1.5 mmol) of Boc-Glu(OBzl)-OH and 599 mg (1.5 mmol) of HCl·Asp(OBzl)-Gly-OBzl 880 mg (87%) of the title compound were obtained as a colorless powder. Mp 78–80 °C, $[\alpha]_D^{20}$ +4.0 (*c* 1, $CHCl_3$), FAB-MS (*m*/*z*) 690 $[M+H]^+$. IR (KBr) 3351, 3346, 3328, 3088, 3072, 3031, 1741, 1688, 1605, 1504, 1459, 1448, 1396, 1366, 740, 691 cm^{-1}. ^{1}H NMR (DMSO-d_6): $\delta = 8.312$ (d, $J = 6.31$ Hz, 1H), 8.215 (d, $J = 6.52$ Hz, 1H), 8.069 (t, $J = 6.55$ Hz, 1H), 7.346 (t, $J = 7.90$ Hz, 1H), 7.340 (t, $J = 7.92$ Hz, 1H), 7.337 (t, $J = 7.88$ Hz, 1H), 7.304 (t, $J = 7.90$ Hz, 2H), 7.295 (t, $J = 7.92$ Hz, 2H), 7.254 (t, $J = 7.88$ Hz, 2H), 7.245 (d, $J = 7.87$ Hz, 2H), 7.191 (d, $J = 7.89$ Hz, 2H), 7.174 (d, $J = 7.86$ Hz, 2H), 5.162 (s, 2H), 5.142 (s, 2H), 5.135 (s, 2H), 4.921 (dt, $J = 6.31$ Hz, $J = 6.66$ Hz, 1H), 4.715 (d, $J = 6.55$ Hz, 2H), 4.705 (d, $J = 6.60$ Hz, 1H), 2.904 (t, $J = 6.60$ Hz, 2H), 2.721 (d, $J = 6.52$ Hz, 2H), 2.230 (m, $J = 6.60$ Hz, 2H), 1.455 (s, 9H). Amino acid analysis: calcd, Glu/Asp/Gly = 1.0:1.0:1.0. Found, Glu/Asp/Gly = 0.98:0.99:1.00. Anal. Calcd for $C_{37}H_{43}N_3O_{10}$: C, 64.43; H, 6.28; N, 6.09. Found: C, 64.22; H, 6.15; N, 6.14.

4.5. Boc-Gly-Glu(OBzl)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 261 mg (1.5 mmol) of Boc-Gly-OH and 489 mg (1.5 mmol) of Glu(OBzl)-OBzl 617 mg (85%) of the title compound were obtained as a colorless oil, $[\alpha]_D^{20}$ +8.0 (*c* 1, $CHCl_3$), FAB-MS (*m*/*z*): 485 $[M+H]^+$. IR (KBr) 3351, 3346, 3091, 3070, 3027, 1745, 1690, 1601, 1502, 1462, 1450, 1394, 1365, 741, 690 cm^{-1}. ^{1}H NMR (DMSO-d_6): $\delta = 8.202$ (t, $J = 6.32$ Hz, 1H), 8.101 (d, $J = 6.55$ Hz, 1H), 7.325 (t, $J = 7.78$ Hz, 1H), 7.315 (t, $J = 7.81$ Hz, 1H), 7.313 (t, $J = 7.58$ Hz, 2H), 7.306 (t, $J = 7.60$ Hz, 2H), 7.301 (d, $J = 7.52$ Hz, 2H), 7.234 (d, $J = 7.54$ Hz, 2H), 5.152 (s, 2H), 5.148 (s, 2H), 4.930 (dt, $J = 6.55$ Hz, $J = 6.78$ Hz, 1H), 4.906 (d, $J = 6.32$ Hz, 2H), 2.901 (t, $J = 6.54$ Hz, 2H), 2.202 (m, $J = 6.54$ Hz, 2H), 1.454 (s, 9H). Amino acid analysis: calcd, Gly/Glu = 1.0:1.0. Found, Gly/Glu = 1.00:0.98. Anal. Calcd for $C_{26}H_{32}N_2O_7$: C, 64.45; H, 6.66; N, 5.78. Found: C, 64.60; H, 6.80; N, 5.58.

4.6. Boc-His(Tos)-Gly-Glu(OBzl)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 588 mg (1.5 mmol) of Boc-His(Tos)-OH and 630 mg (1.5 mmol) of HCl·Gly-Glu(OBzl)-OBzl 907 mg (78%) of the title compound were obtained as a colorless powder. Mp 98–100 °C, $[\alpha]_D^{20}$ +2.5 (*c* 1, $CHCl_3$), FAB-MS (*m*/*z*) 776 $[M+H]^+$. IR (KBr) 3355, 3344, 3338, 3093, 3072, 3031, 1740, 1691, 1600, 1504, 1460, 1453, 1395, 1384, 1367, 740, 692 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 8.406$ (d, $J = 6.58$ Hz, 1H), 8.221 (t, $J = 6.52$ Hz, 1H), 8.032 (d, $J = 6.60$ Hz, 1H), 7.776 (t, $J = 7.83$ Hz, 1H), 7.423 (t, $J = 7.82$ Hz, 1H), 7.341 (s, 1H), 7.29 (d, $J = 7.78$ Hz, 2H), 7.320 (t, $J = 7.79$ Hz, 2H), 7.247 (t, $J = 7.76$ Hz, 2H), 7.244 (d, $J = 7.67$ Hz, 2H), 7.244 (d, $J = 7.78$ Hz, 2H), 7.244 (d, $J = 7.76$ Hz, 2H), 6.905 (s, 1H), 5.160 (s, 2H), 5.146 (s, 2H), 4.944 (dt, $J = 6.60$ Hz, $J = 6.54$ Hz, 1H), 4.909 (dt, $J = 6.58$ Hz, $J = 6.52$ Hz, 1H), 4.524 (d, $J = 6.52$ Hz,

2H), 3.130 (d, $J = 6.52$ Hz, 2H), 2.656 (t, $J = 6.54$ Hz, 2H), 2.371 (s, 3H), 1.812 (m, $J = 6.54$ Hz, 2H), 1.457 (s, 9H). Amino acid analysis: calcd, His/Gly/Glu = 1.0:1.0. Found, His/Gly/Glu = 0.97:1.00:0.98. Anal. Calcd for $C_{39}H_{45}N_5O_{10}S$: C, 60.37; H, 5.85; N, 9.03. Found: C, 60.19; H, 5.68; N, 9.22.

4.7. Boc-Gly-Lys(Z)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 261 mg (1.5 mmol) of Boc-Gly-OH and 555 mg (1.5 mmol) of Lys(Z)-OBzl 696 mg (88%) of the title compound were obtained as a colorless oil, $[\alpha]_D^{20}$ −2.6 (*c* 1, $CHCl_3$), FAB-MS (*m*/*z*): 528 $[M+H]^+$. IR (KBr) 3351, 3340, 3088, 3070, 3030, 1745, 1690, 1605, 1500, 1463, 1450, 1397, 1364, 744, 696 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 8.301$ (t, $J = 6.88$ Hz, 1H), 8.175 (d, $J = 6.79$ Hz, 1H), 8.100 (t, $J = 6.82$ Hz, 1H), 7.366 (t, $J = 7.82$ Hz, 1H), 7.336 (t, $J = 7.80$ Hz, 1H), 7.340 (t, $J = 7.77$ Hz, 2H), 7.318 (t, $J = 7.78$ Hz, 2H), 7.315 (d, $J = 7.77$ Hz, 2H), 7.312 (d, $J = 7.77$ Hz, 2H), 5.139 (s, 2H), 5.056 (s, 2H), 4.935 (dt, $J = 6.88$ Hz, $J = 6.60$ Hz, 1H), 4.515 (d, $J = 6.79$ Hz, 2H), 2.965 (dt, $J = 6.82$ Hz, $J = 5.02$ Hz, 2H), 1.674 (m, $J = 5.02$ Hz, 2H), 1.540 (m, $J = 4.96$ Hz, 2H), 1.521 (m, $J = 4.98$ Hz, 2H), 1.460 (s, 9H). Amino acid analysis: calcd, Gly/Lys = 1.0:1.0. Found, His/Gly/Lys = 1.00:0.98. Anal. Calcd for $C_{28}H_{37}N_3O_7$: C, 63.74; H, 7.07; N, 7.96, O, 21.23. Found: C, 63.52; H, 7.18; N, 7.96, O, 21.07.

4.8. Boc-His(Tos)-Gly-Lys(Z)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 612 mg (1.5 mmol) of Boc-His(Tos)-OH and 695 mg (1.5 mmol) of HCl·Gly-Lys(Z)-OBzl 1067 mg (87%) of the title compound were obtained as a colorless powder. Mp 70–72 °C, $[\alpha]_D^{20}$ −6.3 (*c* 1, $CHCl_3$), FAB-MS (*m*/*z*) 819 $[M+H]^+$. IR (KBr) 3363, 3347, 3329, 3092, 3068, 3029, 1751, 1693, 1600, 1512, 1458, 1451, 1395, 1381, 1365, 748, 690 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 8.412$ (d, $J = 6.59$ Hz, 1H), 8.241 (t, $J = 6.54$ Hz, 1H), 8.169 (d, $J = 6.60$ Hz, 1H), 8.112 (t, $J = 6.55$ Hz, 1H), 7.662 (d, $J = 7.80$ Hz, 2H), 7.422 (t, $J = 7.88$ Hz, 1H), 7.360 (t, $J = 7.85$ Hz, 1H), 7.352 (s, 1H), 7.338 (d, $J = 7.80$ Hz, 2H), 7.328 (t, $J = 7.86$ Hz, 2H), 7.310 (t, $J = 7.84$ Hz, 2H), 7.234 (d, $J = 7.90$ Hz, 2H), 7.230 (d, $J = 7.89$ Hz, 2H), 6.776 (s, 1H), 5.126 (s, 2H), 5.107 (s, 2H), 4.942 (dt, J= 6.78 Hz, $J = 6.59$ Hz, 1H), 4.752 (dt, $J = 6.60$ Hz, $J = 5.90$ Hz, 1H), 4.520 (d, $J = 6.54$ Hz, 2H), 3.162 (dt, $J = 6.55$ Hz, $J = 5.40$ Hz, 2H), 2.873 (m, $J = 5.90$ Hz, 2H), 2.373 (s, 3H), 2.015 (d, $J = 6.78$ Hz, 2H), 1.943 (m, $J = 5.41$ Hz, 2H), 1.524 (m, $J = 5.52$ Hz, 2H), 1.462 (s, 9H). Amino acid analysis: calcd, His/Gly/Lys = 1.0:1.0:1.0. Found, His/Gly/Lys = 0.99:1.00:0.98. Anal. Calcd for $C_{41}H_{50}N_6O_{10}S$: C, 60.13; H, 6.15; N, 10.26. Found: C, 60.25; H, 6.03; N, 10.11.

4.9. Boc-His(Tos)-Gly-Lys(Z)-$NHNH_2$

The solution of 818 mg (1.0 mmol) of Boc-His(Tos)-Gly-Lys(Z)-OBzl in 8 mL of methanol and 3 mL of hydrazine hydrate (50%) were mixed and stirred at room temperature for 60 h. After evaporation the residue was dissolved in 30 mL of ethyl acetate. The solution was washed successively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride and the organic phase was dried over anhydrous sodium sulfate. After filtration and evaporation under reduced pressure the residue was triturated with ether repeatedly to provide 579 mg (78%) of the title compound as a colorless oil, $[\alpha]_D^{20}$ −7.8, FAB-MS (*m*/*z*): 743 $[M+H]^+$. IR (KBr) 3431, 3410, 3365, 3342, 3334, 3090, 3072, 3031, 1750, 1690, 1609, 1507, 1450, 1438, 1395, 1380, 1365, 740, 692 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 8.422$ (d, $J = 6.58$ Hz, 1H), 8.223 (t, $J = 6.61$ Hz, 1H), 8.158 (d, $J = 6.68$ Hz, 1H), 8.112 (s, 1H), 8.012 (t, $J = 6.56$ Hz, 1H), 7.780 (d, $J = 7.84$ Hz, 2H), 7.431 (t, $J = 7.82$ Hz, 1H), 7.391 (t, $J = 7.75$ Hz, 2H), 7.315 (s, 1H), 7.308 (d, $J = 7.75$ Hz, 2H), 7.228 (d, $J = 7.82$ Hz, 2H), 6.760 (s, 1H), 5.112 (s, 2H), 4.935 (dt, $J = 6.58$ Hz, $J = 6.78$ Hz, 1H), 4.722 (d, $J = 6.61$ Hz, 2H), 4.526 (dt, $J = 6.68$ Hz, $J = 5.50$ Hz, 1H), 3.985 (d, $J = 6.78$ Hz, 2H), 3.084 (dt, $J = 6.56$ Hz, $J = 5.40$ Hz, 2H), 3.062 (m, $J = 5.50$ Hz, 2H), 2.369 (s, 3H), 1.884 (m, $J = 5.56$ Hz, 2H), 1.545 (m, $J = 5.40$ Hz, 2H), 1.326 (m, $J = 5.50$ Hz, 2H), 1.459 (s, 9H). Amino acid analysis: calcd, His/Gly/Lys = 1.0:1.0:1.0. Found, His/Gly/Lys = 0.98:1.00:0.98. Anal. Calcd for $C_{34}H_{46}N_8O_9S$: C, 54.97; H, 6.24; N, 15.08. Found: C, 54.79; H, 6.40; N, 15.22.

4.10. General procedure for removal of Boc of the C-terminal component

The solution of 1.5 mmol of Boc protected compound in 8 mL of hydrogen chloride in ethyl acetate (4 mol/L) was stirred at room temperature for 3 h. The reaction mixture was evaporated to remove the solvent. The residue was dissolved in 20 mL of ethyl acetate and the solution was evaporated to dry. The resulted solid was used for coupling reaction directly.

4.11. HCl·Asp(OBzl)-Gly-OBzl

Using the general procedure for removal of Boc of the C-terminal component from 693 mg (1.5 mmol) of Boc-Asp(OBzl)-Gly-OBzl 543 mg (91%) of the title compound were obtained as a colorless powder. Mp 72–75 °C, $[\alpha]_D^{20}$ +4.6 (*c* 1, $CHCl_3$), FAB-MS (*m*/*z*) 363 $[M+H]^+$.

4.12. HCl·Gly-Glu(OBzl)-OBzl

Using the general procedure for removal of Boc of the C-terminal component from 725 mg (1.5 mmol) of Boc-Gly-Glu(OBzl)-OBzl 566 mg (90%) of the title compound were obtained as a colorless powder. Mp 105–107 °C, $[\alpha]_D^{20}$ +4.0 (*c* 1, CH_3OH), FAB-MS (*m*/*z*) 384 $[M+H]^+$.

4.13. HCl·Gly-Lys(Z)-OBzl

Using the general procedure for removal of Boc of the C-terminal component from 791 mg (1.5 mmol) of

Boc-Gly-Lys(Z)-OBzl 577 mg (90%) of the title compound were obtained as a colorless powder. Mp 116–119 °C, $[\alpha]_D^{20}$ −10.0 (*c* 1, CH_3OH), FAB-MS (*m/z*) 428 $[M+H]^+$.

4.14. General procedure for removal of all the semi-permanent groups

The protected peptide (1.0 mmol) was mixed with 6 mL of dimethyl sulfide, 6 mL of phenyl methyl ether, and 10 mL of CF_3COOH–CF_3SO_3H (4:1). The mixture was stirred at 0 °C for 2 h. After removal of CF_3COOH–CF_3SO_3H the residue was triturated with ether and the residue was purified on Sephadex G_{10} and HPLC to provide the title compound as a colorless powder, among which **5b**–**11b**, **7d**, **8d**, **9a**, and **11a** are new compounds.

4.15. Glu-Asp-Gly-OH (1)

Using the general procedure for removal of all the semi-permanent groups from 673 mg (1.0 mmol) of Boc-Glu(OBzl)-Asp(OBzl)-Gly-OBzl 284 mg (89%) of the title compound were obtained as a colorless powder. Mp 143–145 °C (dec.), $[\alpha]_D^{20}$ +20.0 (*c* 0.5, H_2O), FAB-MS 320 $[M+H]^+$. IR (KBr) 3356, 3345, 3336, 3332, 3019, 2598, 2568, 2492, 1755, 1649, 1460 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 10.621$ (s, 1H), 10.605 (s, 1H), 10.401 (s, 1H), 8.668 (d, $J = 6.87$ Hz, 2H), 8.044 (d, $J = 6.68$ Hz, 1H), 7.986 (t, $J = 6.49$ Hz, 1H), 4.246 (m, $J = 6.55$ Hz, 1H), 4.035 (dt, $J = 6.68$ Hz, $J = 5.21$ Hz, 1H), 4.012 (d, $J = 6.49$ Hz, 2H), 2.670 (d, $J = 5.21$ Hz, 2H), 2.354 (t, $J = 4.96$ Hz, 2H), 2.165 (m, $J = 4.75$ Hz, 2H). Amino acid analysis: calcd, Glu/Asp/Gly = 1.0:1.0:1.0. Found, Glu/Asp/Gly = 0.98:0.98:1.00. Anal. Calcd for $C_{11}H_{17}N_3O_8$: C, 41.38; H, 5.37; N, 13.16. Found: C, 41.50; H, 5.19; N, 13.33.

4.16. His-Gly-Glu-OH (2a)

Using the general procedure for removal of all the semi-permanent groups from 775 mg (1.0 mmol) of Boc-His(Tos)-Gly-Glu(OBzl)-OBzl 266 mg (78%) of the title compound were obtained as a colorless powder. Mp 220 °C (dec.) $[\alpha]_D^{20}$ +8.0 (*c* 0.5, H_2O). FAB-MS 342 $[M+H]^+$. IR (KBr): 3352, 3341, 3335, 3325, 3010, 2605, 2489, 1652, 1463 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.787$ (s, 1H), 10.445 (s, 1H), 10.356 (s, 1H), 8.654 (d, $J = 6.78$ Hz, 2H), 8.230 (t, $J = 6.59$ Hz, 1H), 8.206 (d, $J = 6.70$ Hz, 1H), 7.554 (s, 1H), 6.785 (s, 1H), 4.460 (m, $J = 5.22$ Hz, 1H), 4.369 (t, $J = 6.59$ Hz, 2H), 3.846 (dt, $J = 6.70$ Hz, $J = 4.80$ Hz, 1H), 3.204 (t, $J = 4.89$ Hz, 2H), 2.678 (t, $J = 4.85$ Hz, m, $J = 4.90$ Hz, 2H), 1.976 (m, $J = 4.54$ Hz, 2H). Amino acid analysis: calcd, His/Gly/Glu = 1.0:1.0:1.0. Found, His/Gly/Glu = 0.98:1.00:0.99. Anal. Calcd for $C_{13}H_{19}N_5O_6$: C, 45.75; H, 5.61; N, 20.52. Found: C, 45.75; H, 5.61; N, 20.52.

4.17. His-Gly-Lys-OH (2b)

Using the general procedure for removal of all the semi-permanent groups from 818 mg (1.0 mmol) of Boc-His(Tos)-Gly-Lys(Z)-OBzl 286 mg (84%) of the title compound were obtained as a colorless powder. Mp 226 °C (dec.), $[\alpha]_D^{20}$ −16.0 (*c* 0.5, H_2O), FAB-MS 341 $[M+H]^+$. IR (KBr): 3350, 3344, 3339, 3331, 3021, 2489, 1648, 1460 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.755$ (s, 1H), 10.350 (s, 1H), 8.666 (d, $J = 6.80$ Hz, 2H), 8.617 (2H), 8.502 (t, $J = 6.57$ Hz, 1H), 7.895 (d, $J = 6.68$ Hz, 1H), 7.645 (s, 1H), 6.833 (s, 1H), 4.652 (dt, $J = 6.68$ Hz, $J = 4.55$ Hz, 1H), 4.474 (d, $J = 6.57$ Hz, 2H), 4.015 (dt, $J = 6.80$ Hz, $J = 4.86$ Hz, 1H), 3.103 (d, $J = 4.86$ Hz, 2H), 2.795 (t, $J = 4.59$ Hz, 2H), 1.804 (m, $J = 4.78$ Hz, 2H), 1.534 (m, $J = 4.66$ Hz, 2H), 1.267 (m, $J = 4.58$ Hz, 2H). Amino acid analysis: calcd, His/Gly/Lys = 1.0:1.0:1.0. Found, His/Gly/Lys = 0.98:1.00:0.98. Anal. Calcd for $C_{14}H_{24}N_6O_4$: C, 49.40; H, 7.11; N, 24.69, O, 18.80. Found: C, 49.40; H, 7.11; N, 24.69.

4.18. His-Gly-Lys-$NHNH_2$ (3)

Using the general procedure for removal of all the semi-permanent groups from742 mg (1.0 mmol) of Boc-His-(Tos)-Gly-Lys(Z)-$NHNH_2$ 287 mg (81%) of the title compound were obtained as a colorless powder. Mp 96–98 °C $[\alpha]_D^{20}$ −8.0 (*c* 1.0, H_2O). FAB-MS 355 $[M+H]^+$. IR (KBr) 3348, 3340, 3335, 3330, 3316, 2489, 1652, 1500, 1460, 1410 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.801$ (s, 1H), 8.553 (t, $J = 6.60$ Hz, 1H), 8.475 (s, 2H), 8.422 (s, 2H), 7.995 (d, $J = 6.56$ Hz, 1H), 7.943 (s, 1H), 7.494 (s, 1H), 6.446 (s, 1H), 4.776 (dt, $J = 6.76$ Hz, $J = 5.96$ Hz, 1H), 4.352 (dt, $J = 6.56$ Hz, $J = 4.76$ Hz, 1H), 4.293 (d, $J = 6.60$ Hz, 2H), 3.012 (d, $J = 4.86$ Hz, 2H), 2.795 (t, $J = 4.50$ Hz, 2H), 2.245 (m, $J = 4.76$ Hz, 2H), 1.804 (m, $J = 4.64$ Hz, 2H), 1.534 (m, $J = 4.52$ Hz, 2H), 1.267 (m, $J = 4.64$ Hz, 2H). Amino acid analysis: calcd, His/Gly/Lys = 1.0:1.0:1.0. Found, His/Gly/Lys = 0.98:1.00:0.99. Anal. Calcd for $C_{14}H_{26}N_8O_3$: C, 47.45; H, 7.39; N, 31.62. Found: C, 47.30; H, 7.17; N, 31.44.

4.19. Hydrocortisone-21-*O*-β-carbonylpropionic acid (5a)

The solution of 4.0 g (11.0 mmol) of hydrocortisone, 5.0 g (50.2 mmol) of succinic anhydride and 25 mL of pyridine was stirred at room temperature for 24 h. The reaction mixture was poured into the mixture of 100 g of ice, 30 mL of concentrated hydrochloric acid and 100 mL of water. The formed precipitates were collected and dried to provide 4.90 g (98%) of the title compound as a colorless powder. Mp 165–166 °C $[\alpha]_D^{20}$ 152.3 (*c* 1.0, $CHCl_3$). FAB-MS 463 $[M+H]^+$. IR (KBr) 3230, 3210, 3016, 1742, 1708, 1671, 1460 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 10.188$ (s, 1H), 5.628 (s, 1H), 4.804 (s, 2H), 3.954 (dt, $J = 12.88$ Hz, $J = 2.33$ Hz, 2H), 3.628 (dt, $J = 12.88$ Hz, $J = 2.33$ Hz, 2H), 3.156 (m, $J = 7.99$ Hz, 1H), 2.602 (m, $J = 8.12$ Hz, 2H), 2.454 (m, $J = 7.98$ Hz, 2H), 2.384 (m, $J = 8.60$ Hz, 2H), 2.212 (m, $J = 8.06$ Hz, 1H), 2.123 (m, $J = 7.96$ Hz, 2H), 2.112 (s, 1H), 2.101 (s, 1H), 1.943 (m, $J = 8.12$ Hz, 1H), 1.851 (m, $J = 7.95$ Hz, 2H), 1.741 (m, $J = 8.04$ Hz, 1H), 1.708 (m, $J = 7.03$ Hz, 2H), 1.236 (s, 3H), 1.152 (s, 3H), 0.966 (m, $J = 8.00$ Hz, 2H). Anal. Calcd for $C_{25}H_{34}O_8$: C, 64.92; H, 7.41; O, 27.67. Found: C, 64.79; H, 7.22.

4.20. Hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester (6a)

The solution of 500 mg (1.1 mmol) of hydrocortisone-21-*O*-carbonyl-β-propionic acid and 165 mg (1.2 mmol) of *p*-nitrophenol in 5 mL of anhydrous THF was stirred at 0 °C and 240 mg (1.2 mmol) of DCC were added. The reaction mixture was stirred at 0 °C for 2 h and then at room temperature for 24 h. The reaction mixture was filtrated and the filtrate was evaporated to dryness. The obtained residue was triturated with ether repeatedly to give 600 mg (95%) of the title compound as a colorless powder. Mp 100–102 °C $[\alpha]_D^{20}$ 108.2 (*c* 1.0, $CHCl_3$). FAB-MS 584 $[M+H]^+$. IR (KBr) 3230, 3210, 3030, 3010, 1712, 1708, 1641, 1601, 1580, 1520, 1500, 1490, 1460 1350 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 8.092$ (d, $J = 7.88$ Hz, 2H), 8.084 (d, $J = 7.88$ Hz, 2H), 5.655 (s, 1H), 4.845 (s, 2H), 4.321 (m, $J = 6.88$ Hz, 1H), 3.985 (dt, $J = 12.78$ Hz, $J = 3.62$ Hz, 2H), 3.622 (dt, $J = 12.78$ Hz, $J = 3.62$ Hz, 2H), 3.323 (s, 1H), 3.144 (m, $J = 7.00$ Hz, 1H), 2.441 (m, $J = 7.69$ Hz, 2H), 2.403 (m, $J = 7.96$ Hz, 2H), 2.224 (s, 1H), 2.145 (s, 2H), 1.863 (m, $J = 7.24$ Hz, 2H), 1.757 (m, $J = 7.09$ Hz,, 1H), 1.711 (m, $J = 7.81$ Hz, 2H), 1.246 (s, 3H), 0.978 (m, $J = 6.98$ Hz, 2H), 0.655 (s, 3H).

4.21. Prednisolone-21-*O*-β-carbonylpropionic acid (5b)

Using the same procedure as that used for preparation of hydrocortisone-21-*O*-β-carbonylpropionic acid with 4.0 g (11.0 mmol) of prednisolone instead of hydrocortisone 4.66 g (92%) of the title compound were obtained as a colorless powder. Mp 174–176 °C $[\alpha]_D^{20}$ 131.7 (*c* 1.0, $CHCl_3$). FAB-MS 461 $[M+H]^+$. IR (KBr) 3239, 3209, 3016, 1740, 1700, 1642, 1461 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 10.202$ (s, 1H), 7.244 (d, $J = 7.66$ Hz, 1H), 6.142 (d, $J = 7.66$ Hz, 1H), 6.065 (s, 1H), 4.812 (s, 2H), 4.321 (m, $J = 7.68$ Hz, 1H), 3.948 (dt, $J = 12.01$ Hz, $J = 3.56$ Hz, 2H), 3.615 (dt, $J = 12.01$ Hz, $J = 3.56$ Hz, 2H), 3.346 (s, 1H), 3.340 (s, 1H), 3.335 (m, $J = 8.20$ Hz, 2H), 2.345 (m, $J = 7.94$ Hz, 2H), 2.136 (m, $J = 7.90$ Hz, 1H), 1.910 (m, $J = 7.84$ Hz, 2H), 1.901 (m, $J = 7.88$ Hz, 1H), 1.801 (m, $J = 8.10$ Hz, 1H), 1.730 (m, $J = 8.01$ Hz, 2H), 1.312 (s, 3H), 1.105 (m, $J = 7.98$ Hz, 2H), 0.781 (s, 3H).

4.22. Prednisolone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester (6b)

Using the same procedure as that used for preparation of hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester with 500 mg (1.1 mmol) of prednisolone-21-*O*-β-carbonylpropionic acid instead of hydrocortisone-21-*O*-β-carbonylpropionic acid 575 mg (90%) of the title compound were obtained as a colorless powder. Mp 114–116 °C, $[\alpha]_D^{20}$ +95.8 (*c* 1.0, $CHCl_3$). FAB-MS 582 $[M+H]^+$. IR (KBr) 3232, 3215, 3025, 3009, 1710, 1705, 1638, 1604, 1578, 1517, 1500, 1486, 1460 1352 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 8.092$ (d, $J = 7.62$ Hz, 2H), 8.084 (d, $J = 7.62$ Hz, 2H), 7.233 (d, $J = 7.64$ Hz, 1H), 6.130 (d, $J = 7.64$ Hz, 1H), 6.040 (s, 1H), 4.845 (s, 2H), 4.275 (m, $J = 7.55$ Hz, 1H), 3.985 (dt, $J = 11.98$ Hz, $J = 3.42$ Hz, 2H), 3.622 (dt, $J = 11.98$ Hz, $J = 3.42$ Hz, 2H), 3.367 (s, 1H), 3.335 (m, $J = 8.20$ Hz, 2H), 3.320 (s, 1H), 2.263 (m, $J = 7.94$ Hz, 2H), 2.245 (m, $J = 8.10$ Hz, 1H), 1.922 (m, $J = 8.12$ Hz, 1H), 1.902 (m, $J = 7.90$ Hz, 2H), 1.782 (m, $J = 8.00$ Hz, 1H), 1.701 (m, $J = 7.00$ Hz, 2H), 1.030 (m, $J = 7.45$ Hz, 2H), 1.246 (s, 3H), 0.978 (2H), 0.695 (s, 3H). Anal. Calcd for $C_{31}H_{37}NO_{10}$: C, 63.80; H, 6.39; N, 2.40. Found: C, 63.67; H, 6.18; N, 2.31.

4.23. Hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH (7a)

(A) Under stirring the solution of 200 mg (0.63 mmol) of Glu-Asp-Gly-OH in 1 mL of water, 1 mL of THF and 0.5 mL of DMF was adjusted to pH 9 with *N*-methylmorpholine. To the solution 408 mg (0.7 mmol) of hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester were added. The reaction mixture was stirred at room temperature for 40 h and TLC ($CHCl_3$–CH_3OH–H_2O = 1:1:0.15) indicated the complete disappearance of Glu-Asp-Gly-OH. After evaporation the residue was washed with ether and ethyl acetate to provide the crude product. The crude product was then purified by silica gel chromatography ($CHCl_3$–CH_3OH–H_2O = 1:1:0.15) to provide 240 mg (50%) of the title compound. Mp 218–220 °C, $[\alpha]_D^{20}$ +88 (*c* 0.3, DMF), FAB-MS (*m*/*z*) 764 $[M+H]^+$, IR (KBr) 3342, 3320, 3315, 3006, 1740, 1702, 1669, 1655, 1460 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 10.601$ (s, 1H), 10.600 (s, 1H), 10.421 (s, 1H), 8.144 (d, $J = 6.81$ Hz, 1H), 8.084 (d, $J = 6.70$ Hz, 1H), 7.986 (t, $J = 6.50$ Hz, 1H), 5.507 (s, 1H), 4.986 (s, 2H), 4.265 (dt, $J = 6.81$ Hz, $J = 4.88$ Hz, 1H), 4.010 (dt, $J = 6.70$ Hz, $J = 5.00$ Hz, 1H), 4.109 (d, $J = 6.50$ Hz, 2H), 3.898 (dt, $J = 12.44$ Hz, $J = 3.42$ Hz, 2H), 3.564 (dt, $J = 12.44$ Hz, $J = 3.42$ Hz, 2H), 3.215 (m, $J = 7.93$ Hz, 1H), 2.654 (d, $J = 5.15$ Hz, 2H), 2.616 (m, $J = 8.03$ Hz, 2H), 2.450 (m, $J = 7.888$ Hz, 2H), 2.347 (m, $J = 8.442$ Hz, 2H), 2.312 (s, 1H), 2.208 (s, 1H), 2.204 (m, $J = 8.04$ Hz, 1H), 2.140 (m, $J = 7.94$ Hz, 2H), 2.134 (m, $J = 4.88$ Hz, 2H), 2.059 (t, $J = 4.94$ Hz, 2H), 1.932 (m, $J = 8.12$ Hz, 1H), 1.825 (m, $J = 7.87$ Hz, 2H), 1.727 (m, $J = 8.01$ Hz, 1H), 1.716 (m, $J = 7.00$ Hz, 2H), 1.256 (s, 3H), 0.955 (m, $J = 7.95$ Hz, 2H), 0.680 (s, 3H). Anal. Calcd for $C_{36}H_{49}N_3O_{15}$: C, 56.61; H, 6.47; N, 5.50; O, 31.42. Found: C, 56.45; H, 6.28; N, 5.67.

(B) Under stirring and at 0 °C to the solution of 291 mg (0.63 mmol) of hydrocortisone-21-*O*-β-carbonylpropionic acid and 81 mg (0.7 mmol) of *N*-hydroxysuccinimide in 10 mL of anhydride THF 140 mg (0.7 mmol) of DCC were added. The reaction mixture was stirred at 0 °C for 2 h and then at room temperature for 24 h. The reaction mixture was filtrated and to the filtrate 200 mg (0.63 mmol) of Glu-Asp-Gly-OH were added. The reaction mixture was stirred at room temperature for 40 h and TLC ($CHCl_3$–CH_3OH–H_2O = 1:1:0.15) indicated the complete disappearance of Glu-Asp-Gly-OH. After evaporation the residue was washed with ether and ethyl acetate to provide the crude product. The crude product was then purified by silica gel chromatography

($CHCl_3$–CH_3OH–H_2O = 1:1:0.15) to provide 380 mg (79%) of the title compound as a colorless powder. Mp 218–220 °C, $[\alpha]_D^{20}$ +88 (*c* 0.3, DMF), FAB-MS (*m*/*z*) 764 $[M+H]^+$.

4.24. Prednisolone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH (7b)

(A) Using the same procedure as (A) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH with 408 mg (0.7 mmol) of prednisolone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester instead of hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester 237 mg (48%) of the title compound were obtained as a colorless powder. Mp 178–180 °C, $[\alpha]_D^{20}$ +64 (*c* 0.3, DMF), FAB-MS (*m*/e) = 762 $[M+H]^+$. IR (KBr) 3410, 3339, 3006, 1740, 1700, 1645, 1461 cm^{-1}. ^1H NMR (DMSO-d_6): δ = 10.554 (s, 1H), 10.506 (s, 1H), 10.491 (s, 1H), 8.232 (d, J = 6.78 Hz, 1H), 8.101 (d, J = 6.70 Hz, 1H), 7.937 (t, J = 6.51 Hz, 1H), 7.232 (d, J = 7.62 Hz, 1H), 6.138 (d, J = 7.62 Hz, 1H), 6.043 (s, 1H), 4.998 (s, 2H), 4.270 (dt, J = 6.78 Hz, J = 4.69 Hz, 1H), 4.033 (dt, J = 6.70 Hz, J = 5.10 Hz, 1H), 4.008 (d, J = 6.51 Hz, 2H), 3.908 (dt, J = 12.50 Hz, J = 3.46 Hz, 2H), 3.570 (dt, J = 12.50 Hz, J = 3.46 Hz, 2H), 3.335 (s, 1H), 3.323 (m, J = 8.55 Hz, 2H), 3.315 (s, 1H), 2.670 (d, J = 5.10 Hz, 2H), 2.338 (m, J = 8.03 Hz, 1H), 2.252 (m, J = 7.90 Hz, 2H), 2.230 (t, J = 4.97 Hz, 2H), 2.131 (m, J = 4.85 Hz, 2H), 1.902 (m, J = 8.04 Hz, 1H), 1.834 (m, J = 7.85 Hz, 2H), 1.775 (m, J = 7.96 Hz, 1H), 1.688 (m, J = 7.02 Hz, 2H), 1.298 (s, 3H), 1.005 (m, J = 7.95 Hz, 2H), 0.677 (s, 3H). Anal. Calcd for $C_{36}H_{47}N_3O_{15}$: C, 56.76; H, 6.22; N, 5.52; O, 31.50. Found: C, 56.57; H, 6.35; N, 5.66.

(B) Using the same procedure as (B) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH with 291 mg (0.63 mmol) of prednisolone-21-*O*-β-carbonylpropionic acid instead of hydrocortisone-21-*O*-β-carbonylpropionic acid 370 mg (75%) of the title compound were obtained as a colorless powder. Mp 178–180 °C, $[\alpha]_D^{20}$ +64 (*c* 0.3, DMF), FAB-MS (*m*/e) = 784 $[M+H]^+$.

4.25. Hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Glu-OH (7c)

(A) Using the same procedure as (A) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH with 215 mg (0.63 mmol) of His-Gly-Glu-OH instead of Glu-Asp-Gly-OH 257 mg (52%) of the title compound were obtained as a colorless powder. Mp 157–158 °C, $[\alpha]_D^{20}$ +82 (*c* 0.3, DMF), FAB-MS (*m*/e) 786 $[M+H]^+$, IR (KBr) 3422, 3350, 3344, 3008, 1735, 1700, 1671, 1658, 1465 cm^{-1}. ^1H NMR (DMSO-d_6): δ = 12.305 (s, 1H), 10.060 (s, 1H), 10.056 (s, 1H), 8.243 (d, J = 6.76 Hz, 1H), 8.015 (d, J = 6.62 Hz, 1H), 7.988 (t, J = 6.64 Hz, 1H), 5.624 (s, 1H), 4.986 (dt, J = 6.76 Hz, J = 4.78 Hz, 1H), 4.966 (s, 2H), 4.562 (dt, J = 6.62 Hz, J = 4.98 Hz, 1H), 3.996 (d, J = 6.64 Hz, 2H), 3.928 (dt, J = 12.10 Hz, J = 3.55 Hz, 2H), 3.564 (dt, J = 12.10 Hz, J = 3.55 Hz, 2H), 3.248 (m, J = 7.93 Hz, 2H), 3.101 (d, J = 4.78 Hz, 2H), 2.586 (m, J = 8.05 Hz, 2H), 2.451 (m, J = 7.88 Hz, 2H), 2.404 (m, J = 8.44 Hz, 2H), 2.224 (t, J = 4.78 Hz, 2H), 2.200 (m, J = 8.02 Hz, 1H), 2.116 (s, 1H), 2.101 (s, 1H), 2.093 (m, J = 4.78 Hz, 2H), 2.125 (m, J = 7.88 Hz, 2H), 1.934 (m, J = 8.09 Hz, 1H), 1.861 (m, J = 7.94 Hz, 2H), 1.745 (m, J = 8.00 Hz, 1H), 1.710 (m, J = 7.02 Hz, 2H), 1.288 (s, 3H), 1.026 (m, J = 7.85 Hz, 2H), 0.685 (s, 3H). Anal. Calcd for $C_{38}H_{51}N_5O_{13}$: C, 58.08; H, 6.54; N, 8.91; O, 26.47. Found: C, 58.25; H, 6.33; N, 8.72.

(B) Using the same procedure as (B) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH with 215 mg (0.63 mmol) of His-Gly-Glu-OH instead of Glu-Asp-Gly-OH 396 mg (80%) of the title compound were obtained as a colorless powder. Mp 157–158 °C, $[\alpha]_D^{20}$ +82 (*c* 0.3, DMF), FAB-MS (*m*/e) = 786 $[M+H]^+$.

4.26. Prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Glu-OH (7d)

(A) Using the same procedure as (A) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Glu-OH with 408 mg (0.7 mmol) of prednisolone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester instead of hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester 227 mg (46%) of the title compound were obtained as a colorless powder. Mp 172–174 °C, $[\alpha]_D^{20}$ +55.4 (*c* 0.3, DMF), FAB-MS (*m*/e) 784 $[M+H]^+$, IR (KBr) 3433, 3355, 3340, 3008, 1744, 1702, 1656, 1640, 1600, 1589, 1463 cm^{-1}. ^1H NMR (DMSO-d_6): δ = 12.323 (s, 1H), 10.114 (s, 1H), 10.088 (s, 1H), 8.234 (d, J = 6.74 Hz, 1H), 8.022 (d, J = 6.69 Hz, 1H), 7.996 (t, J = 6.65 Hz, 1H), 7.430 (s, 1H), 7.321 (d, J = 7.52 Hz, 1H), 6.881 (s, 1H), 6.140 (d, J = 7.52 Hz, 1H), 6.088 (s, 1H), 4.981 (s, 2H), 4.557 (dt, J = 6.74 Hz, J = 4.75 Hz, 1H), 4.325 (m, J = 7.90 Hz, 1H), 4.314 (dt, J = 6.69 Hz, J = 4.82 Hz, 1H), 3.956 (d, J = 6.65 Hz, 2H), 3.915 (dt, J = 12.55 Hz, J = 3.51 Hz, 2H), 3.570 (dt, J = 12.55 Hz, J = 3.51 Hz, 2H), 3.508 (s, 1H), 3.392 (m, J = 8.44 Hz, 2H), 3.102 (d, J = 4.75 Hz, 2H), 3.318 (s, 1H), 2.305 (m, J = 7.95 Hz, 1H), 2.235 (t, J = 4.58 Hz, 2H), 2.230 (m, J = 7.86 Hz, 2H), 2.023 (m, J = 4.82 Hz, 2H), 1.910 (m, J = 7.78 Hz, 2H), 1.878 (m, J = 8.08 Hz, 1H), 1.741 (m, J = 7.84 Hz, 1H), 1.706 (m, J = 6.98 Hz, 2H), 1.305 (s, 3H), 1.043 (m, J = 7.69 Hz, 2H), 0.687 (s, 3H). Anal. Calcd for $C_{38}H_{49}N_5O_{13}$: C, 58.23; H, 6.30; N, 8.93. Found: C, 58.05; H, 6.16; N, 8.77.

(B) Using the same procedure as that in (B) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH with 291 mg (0.63 mmol) of prednisolone-21-*O*-β-carbonylpropionylpropionic acid instead of hydrocortisone-21-*O*-β-carbonylpropionic acid 380 mg (77%) of the title compound were obtained as a colorless powder. Mp 172–174 °C, $[\alpha]_D^{20}$ +55.4 (*c* 0.3, DMF), FAB-MS (*m*/e) 784 $[M+H]^+$.

4.27. Hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH (8a) and N^{α}-(hydrocortisone-21-*O*-β-carbonylpropionyl)-His-Gly-N^{ε}-(hydrocortisone-21-*O*-β-carbonylpropionyl)-Lys-OH (8b)

(A) Using the same procedure as (A) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH with 214 mg (0.63 mmol) of His-Gly-Lys-OH instead of Glu-Asp-Gly-OH 222 mg (45%) of **8a** and 163 mg (21%) of **8b** were obtained as a colorless powder.

Compound **8a**: Mp 125–127 °C, $[\alpha]_D^{20}$ +70 (*c* 0.2, DMF), FAB-MS (*m*/*z*) 785 $[M+H]^+$. IR (KBr) 3420, 3415, 3358, 3350, 3344, 3010, 1742, 1700, 1670, 1658, 1641, 1460 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.331$ (s, 1H), 12.020 (m, 2H), 10.513 (s, 1H), 8.204 (d, $J = 6.79$ Hz, 1H), 8.116 (d, $J = 6.66$ Hz, 1H), 8.011 (t, $J = 6.55$ Hz, 1H), 7.411 (s, 1H), 6.739 (s, 1H), 6.061 (s, 1H), 4.977 (s, 2H), 4.501 (dt, $J = 6.79$ Hz, $J = 4.82$ Hz, 1H), 4.345 (m, $J = 7.91$ Hz, 1H), 4.305 (dt, $J = 6.66$ Hz, $J = 4.69$ Hz, 1H), 3.992 (d, $J = 6.55$ Hz, 2H), 3.920 (dt, $J = 12.32$ Hz, $J = 3.61$ Hz, 2H), 3.568 (dt, $J = 12.32$ Hz, $J = 3.61$ Hz, 2H), 3.347 (m, $J = 8.41$ Hz, 2H), 3.336 (s, 1H), 3.320 (s, 1H), 3.168 (d, $J = 4.82$ Hz, 2H), 2.802 (m, $J = 4.33$ Hz, 2H), 2.622 (m, $J = 8.03$ Hz, 2H), 2.423 (m, $J = 7.76$ Hz, 2H), 2.275 (m, $J = 7.88$ Hz, 2H), 2.238 (m, $J = 8.07$ Hz, 1H), 1.936 (m, $J = 8.08$ Hz, 1H), 1.905 (m, $J = 7.85$ Hz, 2H), 1.888 (m, $J = 4.77$ Hz, 2H), 1.782 (m, $J = 8.02$ Hz, 1H), 1.703 (m, $J = 6.97$ Hz, 2H), 1.586 (m, $J = 4.66$ Hz, 2H), 1.322 (s, 3H), 1.275 (m, $J = 4.67$ Hz, 2H), 1.045 (m, $J = 7.98$ Hz, 2H), 0.674 (s, 3H). Anal. Calcd for $C_{39}H_{56}N_6O_{11}$: C, 59.68; H, 7.19; N, 10.71. Found: C, 59.52; H, 7.30; N, 10.54.

Compound **8b:** Mp 111–113 °C, FAB-MS (*m*/*z*) 1229 $[M+H]^+$. IR (KBr) 3434, 3428, 3346, 3331, 3324, 3010, 1745, 1702, 1669, 1654, 1643, 1462 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 12.331$ (s, 1H), 10.045 (s, 1H), 8.463 (d, $J = 6.70$ Hz, 1H), 8.126 (t, $J = 6.55$ Hz, 1H), 8.112 (d, $J = 6.65$ Hz, 1H), 8.005 (t, $J = 6.44$ Hz, 1H), 7.432 (s, 1H), 6.741 (s, 1H), 5.603 (s, 1H), 5.593 (s, 1H), 4.998 (s, 2H), 4.994 (s, 2H), 4.493 (dt, $J = 6.70$ Hz, $J = 4.84$ Hz, 1H), 4.127 (dt, $J = 6.65$ Hz, $J = 4.64$ Hz, 1H), 3.984 (d, $J = 6.55$ Hz, 2H), 3.915 (dt, $J = 12.40$ Hz, $J = 3.58$ Hz, 2H), 3.896 (dt, $J = 12.43$ Hz, $J = 3.62$ Hz, 2H), 3.612 (dt, $J = 12.40$ Hz, $J = 3.58$ Hz, 2H), 3.609 (dt, $J = 12.43$ Hz, $J = 3.62$ Hz, 2H), 3.604 (m, $J = 7.88$ Hz, 1H), 3.514 (m, $J = 7.78$ Hz, 1H), 3.442 (m, $J = 4.61$ Hz, 2H), 2.810 (t, $J = 4.62$ Hz, 2H), 2.607 (m, $J = 8.08$ Hz, 2H), 2.600 (m, $J = 8.02$ Hz, 2H), 2.460 (m, $J = 7.81$ Hz, 2H), 2.428 (m, $J = 7.69$ Hz, 2H), 2.407 (m, $J = 8.44$ Hz, 2H), 2.395 (m, $J = 8.54$ Hz, 2H), 2.210 (m, $J = 8.00$ Hz, 1H), 2.207 (m, $J = 8.02$ Hz, 1H), 2.133 (m, $J = 7.88$ Hz, 2H), 2.124 (m, $J = 7.87$ Hz, 2H), 2.115 (s, 1H), 2.111 (s, 1H), 2.101 (s, 1H), 2.008 (s, 1H), 1.961 (m, $J = 8.10$ Hz, 1H), 1.942 (m, $J = 8.09$ Hz, 1H), 1.861 (m, $J = 7.55$ Hz, 2H), 1.840 (m, $J = 7.58$ Hz, 2H), 1.747 (m, $J = 7.12$ Hz, 2H), 1.740 (m, $J = 7.05$ Hz, 2H), 1.762 (m, $J = 7.01$ Hz, 1H), 1.743 (m, $J = 8.01$ Hz, 1H), 1.728 (m, $J = 4.65$ Hz, 2H), 1.702 (m, $J = 4.66$ Hz, 2H), 1.315 (m, $J = 6.62$ Hz, 2H), 1.224 (s, 3H), 1.215 (s, 3H), 0.945 (m, $J = 7.98$ Hz, 2H), 0.921 (m, $J = 7.88$ Hz, 2H), 0.654 (s, 3H), 0.651 (s, 3H). Anal. Calcd for $C_{64}H_{88}N_6O_{18}$: C, 62.52; H, 7.21; N, 6.84. Found: C, 62.36; H, 7.35; N, 6.96.

(B) Using the same procedure as that in (B) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH with 214 mg (0.63 mmol) of His-Gly-Lys-OH instead of Glu-Asp-Gly-OH 247 mg (50%) of **8a** (Mp 125–127 °C, $[\alpha]_D^{20}$ +70 (*c* 0.2, DMF), FAB-MS (*m*/*z*) 785 $[M+H]^+$) and 142 mg (20%) of **8b** (Mp 111–113 °C, FAB-MS (*m*/*z*) = 1229 $[M+H]^+$) were obtained as a colorless powder.

4.28. Prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH (8c) and N^{α}-(Prednisolone-21-*O*-β-carbonylpropionyl)-His-Gly- N^{ε}-(prednisolone-21-*O*-β-carbonylpropionyl)-Lys-OH (8d)

(A) Using the same procedure as (A) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH with 408 mg (0.7 mmol) of prednisolone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester instead of hydrocortisone-21-*O*-β-carbonylpropionic acid *p*-nitrophenolic ester 206 mg (42%) of **8c** and 114 mg (23%) of **8d** were obtained as a colorless powder.

Compound **8c**: Mp 134–136 °C, $[\alpha]_D^{20}$ +49 (*c* 0.2, DMF), FAB-MS (*m*/*z*) 783 $[M+H]^+$, IR (KBr) 3434, 3421, 3360, 3346, 3340, 3010, 1745, 1700, 1665, 1652, 1640, 1600, 1580, 1464 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.385$ (s, 1H), 12.269 (s, 2H), 10.321 (s, 1H), 8.212 (d, $J = 6.85$ Hz, 1H), 8.103 (d, $J = 6.62$ Hz, 1H), 7.992 (t, $J = 6.55$ Hz, 1H), 7.415 (s, 1H), 7.149 (d, $J = 7.62$ Hz, 1H), 6.812 (s, 1H), 6.165 (d, $J = 7.62$ Hz, 1H), 6.101 (s, 1H), 4.966 (s, 2H), 4.502 (dt, $J = 6.85$ Hz, $J = 4.82$ Hz, 1H), 4.412 (dt, $J = 6.62$ Hz, $J = 4.74$ Hz, 1H), 4.356 (m, $J = 7.94$ Hz, 1H), 4.021 (d, $J = 6.55$ Hz, 2H), 3.993 (dt, $J = 12.88$ Hz, $J = 2.35$ Hz, 2H), 3.651 (dt, $J = 12.88$ Hz, $J = 2.35$ Hz, 2H), 3.340 (s, 1H), 3.319 (s, 1H), 3.196 (m, $J = 8.44$ Hz, 2H), 3.05 (d, $J = 4.82$ Hz, 2H), 2.804 (t, $J = 4.49$ Hz, 2H), 2.338 (m, $J = 7.86$ Hz, 2H), 2.285 (m, $J = 8.08$ Hz, 1H), 1.916 (m, $J = 8.10$ Hz, 1H), 1.906 (m, $J = 7.88$ Hz, 2H), 1.829 (m, $J = 7.88$ Hz, 2H), 1.756 (m, $J = 7.98$ Hz, 1H), 1.732 (m, $J = 4.74$ Hz, 2H), 1.702 (m, $J = 7.02$ Hz, 2H), 1.612 (m, $J = 4.58$ Hz, 2H), 1.305 (s, 3H), 1.114 (m, $J = 7.66$ Hz, 2H), 0.688 (s, 3H). Anal. Calcd for $C_{39}H_{54}N_6O_{11}$: C, 59.83; H, 6.95; N, 10.73; O, 22.48. Found: C, 59.66; H, 7.02; N, 10.54.

Compound **8d**: Mp 119–121 °C, FAB-MS (*m*/*z*) 1225 $[M+H]^+$. IR (KBr) 3436, 3431, 3366, 3355, 3342, 3010, 1742, 1738, 1706, 1695, 1660, 1648, 1640, 1601, 1591, 1464 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.248$ (s, 1H), 10.110 (s, 1H), 8.224 (d, $J = 6.77$ Hz, 1H), 8.137 (d, $J = 6.66$ Hz, 1H), 8.121 (t, $J = 4.40$ Hz, 1H), 7.902 (t, $J = 6.50$ Hz, 1H), 7.438 (s, 1H), 7.334 (d, $J = 7.54$ Hz, 1H), 7.330 (d, $J = 7.57$ Hz, 1H), 6.786 (s, 1H), 6.140 (d, $J = 7.54$ Hz, 1H), 6.139 (d, $J = 7.57$ Hz, 1H), 6.062 (s, 1H), 6.055 (s, 1H), 4.998 (s, 2H), 4.996 (s, 2H), 4.877 (dt, $J = 6.77$ Hz, $J = 4.85$ Hz, 1H), 4.477 (dt, $J = 6.66$ Hz, $J = 4.45$ Hz, 1H), 4.352 (m $J = 7.94$ Hz, 1H), 4.350 (m $J = 7.91$ Hz, 1H), 2 3.991 (dt, $J = 12.85$ Hz,

$J = 2.42$ Hz, 2H), 3.988 (dt, $J = 12.80$ Hz, $J = 2.55$ Hz, 2H), 3.929 (d, $J = 6.50$ Hz, 2H), 3.891 (dt, $J = 4.65$ Hz, $J = 4.54$ Hz, 2H), 3.651 (dt, $J = 12.85$ Hz, $J = 2.42$ Hz, 2H), 3.648 (dt, $J = 12.80$ Hz, $J = 2.55$ Hz, 2H), 3.401 (m, $J = 8.52$ Hz, 2H), 3.388 (m, $J = 8.33$ Hz, 2H), 3.341 (s, 1H), 3.340 (s, 1H), 3.316 (s, 1H), 3.310 (s, 1H), 3.021 (d, $J = 4.85$ Hz, 2H), 2.311 (m, $J = 8.02$ Hz, 1H), 2.320 (m, $J = 8.01$ Hz, 1H), 2.218 (m, $J = 7.88$ Hz, 2H), 2.216 (m, $J = 7.86$ Hz, 2H), 1.912 (m, $J = 8.00$ Hz, 1H), 1.908 (m, $J = 8.02$ Hz, 1H), 1.914 (m, $J = 7.81$ Hz, 2H), 1.910 (m, $J = 7.83$ Hz, 2H), 1.786 (m, $J = 4.76$ Hz, 2H), 1.755 (m, $J = 7.98$ Hz, 1H), 1.737 (m, $J = 7.96$ Hz, 1H), 1.728 (m, $J = 7.01$ Hz, 2H), 1.715 (m, $J = 7.04$ Hz, 2H), 1.706 (m, $J = 4.66$ Hz, 2H), 1.514 (m, $J = 4.55$ Hz, 2H), 1.314 (s, 3H), 1.310 (s, 3H), 1.033 (m, $J = 8.00$ Hz, 2H), 1.031 (m, $J = 8.02$ Hz, 2H), 0.755 (s, 3H), 0.749 (s, 3H). Anal. Calcd for $C_{64}H_{84}N_6O_{18}$: C, 62.73; H, 6.91; N, 6.86. Found: C, 62.55; H, 6.79; N, 6.94.

(B) Using the same procedure as (B) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH with 291 mg (0.63 mmol) of prednisolone-21-*O*-β-carbonylpropionic acid instead of hydrocortisone-21-*O*-β-carbonylpropionic acid 256 mg (52%) of **8c** (Mp 134–136 °C, $[\alpha]_D^{20}$ +49 (*c* 0.2, DMF), FAB-MS (*m*/*z*) 783 $[M+H]^+$) and 162 mg (21%) of **8d** were obtained as a colorless powder (Mp 119–121 °C, FAB-MS (*m*/*z*) 1225 $[M+H]^+$).

4.29. N^{α}-(Hydrocortisone-21-*O*-β-carbonylpropionyl)-His-Gly-N^{ε}-(prednisolone-21-*O*-β-carbonylpropionyl)-Lys-OH (9a)

Using the same procedure as (B) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH from 494 mg (0.63 mmol) of hydrocortisone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH and 291 mg (0.63 mmol) of prednisolone-21-O-β-carbonylpropionic acid 600 mg (total yield 78%) of the mixed bis-steroid substituted peptide hydrocortisone-His-Gly-Lys-(prednisolone)-OH (**9a**) were obtained as a colorless powder. Mp 122–125 °C, FAB-MS (*m*/*z*) 1227 $[M+H]^+$. IR (KBr) 3436, 3425, 3361, 3354, 3346, 3010, 1746, 1710, 1666, 1650, 1642, 1600, 1585, 1464 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.249$ (s, 1H), 10.12 (s, 1H), 8.209 (d, $J = 6.79$ Hz, 1H), 8.119 (d, $J = 6.65$ Hz, 1H), 8.121 (t, $J = 4.52$ Hz, 1H), 7.997 (t, $J = 6.59$ Hz, 1H), 7.459 (s, 1H), 7.307 (d, $J = 7.69$ Hz, 1H), 6.836 (s, 1H), 6.139 (d, $J = 7.69$ Hz, 1H), 6.071 (s, 1H), 5.606 (s, 1H), 4.989 (s, 2H), 4.893 (s, 2H), 4.887 (dt, $J = 6.79$ Hz, $J = 4.84$ Hz, 1H), 4.518 (dt, $J = 6.65$ Hz, $J = 4.74$ Hz, 1H), 4.352 (m, $J = 7.94$ Hz, 1H), 3.987 (d, $J = 6.59$ Hz, 2H), 3.979 (dt, $J = 12.72$ Hz, $J = 2.60$ Hz, 2H), 3.965 (dt, $J = 12.80$ Hz, $J = 2.65$ Hz, 2H), 3.887 (dt, $J = 6.65$ Hz, $J = 4.74$ Hz, 2H), 3.641 (dt, $J = 12.72$ Hz, $J = 2.60$ Hz, 2H), 3.637 (dt, $J = 12.80$ Hz, $J = 2.65$ Hz, 2H), 3.380 (m, $J = 8.44$ Hz, 2H), 3.339 (s, 1H), 3.215 (m, $J = 7.88$ Hz, 1H), 3.322 (s, 1H), 3.020 (d, $J = 4.84$ Hz, 2H), 2.611 (m, $J = 8.10$ Hz, 2H), 2.465 (m, $J = 7.92$ Hz, 2H), 2.343 (m, $J = 8.44$ Hz, 2H), 2.224 (m, $J = 8.04$ Hz, 1H), 2.214 (m, $J = 7.90$ Hz, 2H), 2.149 (m, $J = 8.04$ Hz, 1H), 2.128 (m, $J = 7.95$ Hz, 2H), 2.123 (s, 1H), 2.104 (s, 1H), 1.957 (m, $J = 8.00$ Hz, 1H), 1.927 (m, $J = 8.10$ Hz, 1H), 1.903 (m, $J = 7.87$ Hz, 2H), 1.859 (m, $J = 7.92$ Hz, 2H), 1.838 (m, $J = 4.84$ Hz, 2H), 1.769 (m, $J = 7.96$ Hz, 1H), 1.733 (m, $J = 8.01$ Hz, 1H), 1.731 (m, $J = 7.01$ Hz, 2H), 1.698 (m, $J = 7.01$ Hz, 2H), 1.510 (m, $J = 4.74$ Hz, 2H), 1.369 (m, $J = 4.80$ Hz, 2H), 1.309 (s, 3H), 1.220 (s, 3H), 1.031 (m, $J = 8.00$ Hz, 2H), 0.950 (m, $J = 7.98$ Hz, 2H), 0.709 (s, 3H), 0.656 (s, 3H). Anal. Calcd for $C_{64}H_{86}N_6O_{18}$: C, 62.63; H, 7.06; N, 6.85. Found: C, 62.47; H, 7.18; N, 6.72.

4.30. N^{α}-(Prednisolone-21-*O*-β-carbonylpropionyl)-His-Gly-N^{ε}-(hydrocortisone-21-*O*-β-carbonylpropionyl)-Lys-OH (9b)

Using the same procedure as (B) used for the preparation of hydrocortisone-21-*O*-β-carbonylpropionyl-Glu-Asp-Gly-OH from 494 mg (0.63 mmol) of prednisolone-21-*O*-β-carbonylpropionyl-His-Gly-Lys-OH and 291 mg (0.63 mmol) of hydrocortisone-21-*O*-β-carbonylpropionic acid 570 mg (total yield 74%) of the mixed bis-steroid substituted peptide prednisolone-His-Gly-Lys-(hydrocortisone)-OH (**9b**) were obtained as a colorless powder. Mp 130–132 °C, FAB-MS (*m*/*z*) 1227 $[M+H]^+$. IR (KBr) 3434, 3421, 3362, 3349, 3343, 3008, 1745, 1706, 1668, 1650, 1642, 1603, 1583, 1465 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 12.202$ (s, 1H), 10.101 (s, 1H), 8.471 (d, $J = 6.78$ Hz, 1H), 8.198 (d, $J = 6.70$ Hz, 1H), 8.131 (t, $J = 4.60$ Hz, 1H), 8.121 (t, $J = 6.56$ Hz, 1H), 7.413 (s, 1H), 6.751 (s, 1H), 7.365 (d, $J = 7.55$ Hz, 1H), 6.138 (d, $J = 7.55$ Hz, 1H), 6.058 (s, 1H), 5.597 (s, 1H), 4.898 (s, 2H), 4.879 (s, 2H), 4.857 (dt, $J = 6.78$ Hz, $J = 4.84$ Hz, 1H), 4.518 (dt, $J = 6.70$ Hz, $J = 4.74$ Hz, 1H), 4.322 (m, $J = 7.92$ Hz, 1H), 3.981 (dt, $J = 12.66$ Hz, $J = 2.62$ Hz, 2H), 3.939 (d, $J = 6.56$ Hz, 2H), 3.979 (dt, $J = 12.62$ Hz, $J = 2.60$ Hz, 2H), 3.887 (dt, $J = 4.65$ Hz, $J = 4.58$ Hz, 2H), 3.615 (dt, $J = 12.66$ Hz, $J = 2.62$ Hz, 2H), 3.602 (dt, $J = 12.62$ Hz, $J = 2.60$ Hz, 2H), 3.385 (m, $J = 8.52$ Hz, 2H), 3.342 (s, 1H), 3.321 (s, 1H), 3.145 (m, $J = 7.88$ Hz, 1H), 3.021 (d, $J = 4.84$ Hz, 2H), 2.611 (m, $J = 8.08$ Hz, 2H), 2.430 (m, $J = 7.92$ Hz, 2H), 2.351 (m, $J = 8.54$ Hz, 2H), 2.346 (m, $J = 7.90$ Hz, 2H), 2.212 (m, $J = 8.00$ Hz, 1H), 2.210 (m, $J = 8.02$ Hz, 1H), 2.138 (s, 1H), 2.135 (m, $J = 7.95$ Hz, 2H), 2.107 (s, 1H), 1.939 (m, $J = 8.08$ Hz, 1H), 1.912 (m, $J = 8.05$ Hz, 1H), 1.906 (m, $J = 7.88$ Hz, 2H), 1.838 (m, $J = 7.88$ Hz, 2H), 1.832 (m, $J = 4.76$ Hz, 2H), 1.777 (m, $J = 7.96$ Hz, 1H), 1.734 (m, $J = 8.00$ Hz, 1H), 1.711 (m, $J = 6.98$ Hz, 2H), 1.687 (m, $J = 7.01$ Hz, 2H), 1.512 (m, $J = 4.67$ Hz, 2H), 1.309 (s, 3H), 1.280 (m, $J = 7.84$ Hz, 2H), 1.210 (s, 3H), 0.926 (m, $J = 8.01$ Hz, 2H), 0.754 (s, 3H), 0.685 (s, 3H). Anal. Calcd for $C_{64}H_{86}N_6O_{18}$: C, 62.63; H, 7.06; N, 6.85. Found: C, 62.79; H, 7.17; N, 6.68.

4.31. 3-(His-Gly-Lys-NH-N)-Hydrocortisone (10a)

(A) The solution of 120 mg (0.35 mmol) of His-Gly-Lys-NH-NH_2, 134 mg (0.35 mmol) of hydrocortisone in 5 mL of methanol was adjusted to pH 2 with glacial acetic acid and stirred at room temperature for 48 h and TLC ($CHCl_3$–CH_3OH–H_2O = 1:1:0.15) indicated the

complete disappearance of His-Gly-Lys-NH-NH_2. After evaporation the residue was washed with ethyl acetate to provide the crude product. The crude product was crystallized in DMF–ether (1:5) to provide 180 mg (75%) of the title compound as a colorless powder. Mp 218–220 °C (dec.), $[\alpha]_D^{20}$ +173 (*c* 0.3, DMF), FAB-MS (*m/z*) 699 $[M+H]^+$. IR (KBr) 3442, 3435, 3358, 3350, 3342, 3008, 1740, 1709, 1661, 1648, 1640, 1462 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.312$ (S, 1H), 12.234 (S, 2H), 12.107 (t, $J = 4.45$ Hz, 2H), 10.451 (s, 1H), 8.002 (d, $J = 6.60$ Hz, 1H), 7.976 (t, $J = 6.65$ Hz, 1H), 7.395 (s, 1H), 6.824 (s, 1H), 5.478 (s, 1H), 4.845 (s, 2H), 4.558 (dt, $J = 6.60$ Hz, $J = 4.75$ Hz, 1H), 4.051 (t, $J = 4.86$ Hz, 1H), 3.993 (d, $J = 6.65$ Hz, 2H), 3.167 (m, $J = 7.88$ Hz, 1H), 3.054 (d, $J = 4.85$ Hz, 2H), 2.878 (m, $J = 4.61$ Hz, 2H), 2.650 (m, $J = 8.08$ Hz, 2H), 2.415 (s, 1H), 2.401 (m, $J = 7.87$ Hz, 2H), 2.325 (m, $J = 8.54$ Hz, 2H), 2.238 (m, $J = 8.00$ Hz, 1H), 2.126 (m, $J = 7.87$ Hz, 2H), 2.115 (s, 1H), 2.103 (s, 1H), 1.942 (m, $J = 8.08$ Hz, 1H), 1.786 (m, $J = 4.76$ Hz, 2H), 1.868 (m, $J = 7.89$ Hz, 2H), 1.742 (m, $J = 8.01$ Hz, 1H), 1.720 (m, $J = 4.59$ Hz, 2H), 1.708 (m, $J = 7.02$ Hz, 2H), 1.542 (m, $J = 4.61$ Hz, 2H), 1.270 (s, 3H), 0.921 (m, $J = 7.98$ Hz, 2H), 0.665 (s, 3H). Anal. Calcd for $C_{35}H_{54}N_8O_7$: C, 60.15; H, 7.79; N, 16.03. Found: C, 60.02; H, 7.65; N, 16.21.

(B) The solution of 260 mg (0.35 mmol) of Boc-His-(Tos)-Gly-Lys(Z)-$NHNH_2$, 134 mg (0.35 mmol) of hydrocortisone in 15 mL of methanol was adjusted to pH 2 with glacial acetic acid and stirred at room temperature for 48 h and TLC ($CHCl_3$–CH_3OH–H_2O = 1:1:0.15) indicated the complete disappearance of Boc-His(Tos)-Gly-Lys(Z)-$NHNH_2$. After evaporation the residue was dissolved in 50 mL of ethyl acetate. The solution was washed successively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride and the organic phase was dried over anhydrous sodium sulfate. After filtration and evaporation under reduced pressure the resulted residue was purified by silica gel chromatography ($CHCl_3$/CH_3OH, 30:1) to provide the protective intermediates, which was mixed with 6 mL of dimethyl sulfide, 6 mL of phenyl methyl ether, and 10 mL of CF_3COOH–CF_3SO_3H (4:1). The mixture was stirred at 0 °C for 2 h. After removal of CF_3COOH–CF_3SO_3H the residue was triturated with ether and the residue was purified on Sephadex G_{10} and HPLC to provide 200 mg (total yield 82%) of the title compound as a colorless powder. Mp 218–220 °C (dec.), $[\alpha]_D^{20}$ +173 (*c* 0.3, DMF), FAB-MS (*m/z*) 699 $[M+H]^+$.

(C) Using the general procedure for removal of all the semi-permanent groups from380 mg (0.35 mmol) of **11a** 195 mg (80%) of the title compound were obtained as a colorless powder. Mp 218–220 °C (dec.), $[\alpha]_D^{20}$ +173 (*c* 0.3, DMF), FAB-MS (*m/z*) 699 $[M+H]^+$.

4.32. 3-(His-Gly-Lys-NH-N)-Prednisolone (10b)

(A) Using the same procedure as that in (A) used for the preparation of 3-(His-Gly-Lys- NH-N)-hydrocortisone with 134 mg (0.35 mmol) of prednisolone instead of hydrocortisone 171 mg (70%) of the title compound as a colorless powder. Mp 233–235 °C (dec.), $[\alpha]_D^{20}$ +145.5 (*c* 0.3, DMF), FAB-MS (*m/z*) 697 $[M+H]^+$. IR (KBr) 3441, 3429, 3359, 3350, 3340, 3000, 1740, 1702, 1662, 1653, 1640, 1605, 1583, 1460 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 12.221$ (s, 1H), 12.146 (d, $J = 4.70$ Hz, 2H), 12.120 (s, 2H), 10.190 (s, 1H), 8.006 (d, $J = 6.69$ Hz, 1H), 7.983 (t, $J = 6.55$ Hz, 1H), 7.464 (s, 1H), 7.308 (d, $J = 7.58$ Hz, 1H), 6.775 (s, 1H), 6.127 (d, $J = 7.58$ Hz, 1H), 6.076 (s, 1H), 4.677 (s, 2H), 4.667 (dt, $J = 6.69$ Hz, $J = 4.58$ Hz, 1H), 4.354 (m, $J = 7.86$ Hz, 1H), 4.049 (t, $J = 4.84$ Hz, 1H), 3.979 (d, $J = 6.55$ Hz, 2H), 3.398 (m, $J = 8.44$ Hz, 2H), 3.332 (s, 1H), 3.311 (s, 1H), 3.044 (d, $J = 4.84$ Hz, 2H), 2.835 (m, $J = 4.58$ Hz, 2H), 2.454 (s, 1H), 2.235 (m, $J = 8.03$ Hz, 1H), 2.218 (m, $J = 7.95$ Hz, 2H), 1.922 (m, $J = 7.99$ Hz, 1H), 1.906 (m, $J = 7.89$ Hz, 2H), 1.779 (m, $J = 4.77$ Hz, 2H), 1.773 (m, $J = 8.01$ Hz, 1H), 1.725 (m, $J = 7.01$ Hz, 2H), 1.559 (m, $J = 4.57$ Hz, 2H), 1.451 (m, $J = 4.64$ Hz, 2H), 1.299 (s, 3H), 1.033 (m, $J = 7.87$ Hz, 2H), 0.738 (s, 3H). Anal. Calcd for $C_{35}H_{52}N_8O_7$: C, 60.33; H, 7.52; N, 16.08. Found: C, 60.42; H, 7.41; N, 16.22.

(B) Using the same procedure as that in (B) used for the preparation of 3-(His-Gly-Lys- NH-N)-hydrocortisone with 134 mg (0.35 mmol) of prednisolone instead of hydrocortisone 195 mg (80%) of the title compound as a colorless powder. Mp 233–235 °C (dec.), $[\alpha]_D^{20}$ +145.5 (*c* 0.3, DMF), FAB-MS (*m/z*) 697 $[M+H]^+$.

(C) Using the general procedure for removal of all the semi-permanent groups from 380 mg (0.35 mmol) of **11b** 200 mg (82%) of the title compound were obtained as a colorless powder. Mp 233–235 °C (dec.), $[\alpha]_D^{20}$ +145.5 (*c* 0.3, DMF), FAB-MS (*m/z*) 697 $[M+H]^+$.

4.33. 3-[Boc-His(Tos)-Gly-Lys(Z)-NH-N]-Hydrocortisone (11a)

Using the same procedure as that used for the preparation of 3-(His-Gly-Lys-NH-N)-hydrocortisone with 260 mg (0.35 mmol) of Boc-His(Tos)-Gly-Lys(Z)-$NHNH_2$ instead of His-Gly-Lys-NH-NH_2 304 mg (80%) of the title compound were obtained as a colorless powder. Mp 133–135 °C, $[\alpha]_D^{20}$ +125.6 (*c* 0.3, DMF), FAB-MS (*m/z*) 1087 $[M+H]^+$. IR (KBr) 3443, 3438, 3354, 3346, 3340, 3002, 1740, 1703, 1662, 1650, 1640, 1609, 1507, 1462, 1450, 1438, 1392, 1378, 1365, 741, 690 cm^{-1}. 3431, 3410, 3365, 3342, 3334, 3090, 3072, 3031, 1750, 1690 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 12.24$ (s, 1H), 8.661 (d, $J = 6.58$ Hz, 1H), 8.112 (d, $J = 6.58$ Hz, 1H), 8.100 (t, $J = 6.55$ Hz, 1H), 7.988 (t, $J = 6.56$ Hz, 1H), 7.805 (d, $J = 7.84$ Hz, 2H), 7.891 (s, 1H), 7.326 (d, $J = 7.78$ Hz, 2H), 7.240 (t, $J = 7.85$ Hz, 1H), 7.224 (t, $J = 7.81$ Hz, 2H), 7.105 (d, $J = 7.86$ Hz, 2H), 7.015 (s, 1H), 5.480 (s, 1H), 5.255 (s, 2H), 4.564 (s, 2H), 4.501 (dt, $J = 6.80$ Hz, $J = 6.58$ Hz, 1H), 4.306 (dt, $J = 6.58$ Hz, $J = 5.87$ Hz, 1H), 3.975 (d, $J = 6.56$ Hz, 2H), 3.395 (m, $J = 6.55$ Hz, 2H), 3.176 (m, $J = 7.94$ Hz, 1H), 3.022 (d, $J = 6.80$ Hz, 2H), 2.588 (m, $J = 8.08$ Hz, 2H), 2.401 (m, $J = 7.97$ Hz, 2H), 2.386 (s, 3H), 2.230 (s, 1H), 2.310 (m, $J = 8.55$ Hz, 2H), 2.123 (m, $J = 7.88$ Hz, 2H), 2.115 (s, 1H), 2.102 (s, 1H), 2.206 (m, $J = 7.88$ Hz,

1H), 1.945 (m, $J = 8.09$ Hz, 1H), 1.848 (m, $J = 7.91$ Hz, 2H), 1.744 (m, $J = 7.99$ Hz, 1H), 1.781 (m, $J = 5.88$ Hz, 2H), 1.704 (m, $J = 7.02$ Hz, 2H), 1.706 (m, $J = 7.01$ Hz, 2H),1.530 (m, $J = 5.40$ Hz, 2H), 1.432 (s, 9H), 1.276 (s, 3H), 0.972 (m, $J = 7.89$ Hz, 2H), 0.706 (s, 3H). Anal. Calcd for $C_{55}H_{74}N_8O_{13}S$: C, 60.76; H, 6.86; N, 10.31. Found: C, 60.88; H, 7.01; N, 10.17.

4.34. 3-[Boc-His(Tos)-Gly-Lys(Z)-NH-N]-prednisolone (11b)

Using the same procedure as that used for the preparation of 3-(His-Gly-Lys-NH-N)-prednisolone with 260 mg (0.35 mmol) of Boc-His(Tos)-Gly-Lys(Z)-$NHNH_2$ instead of His-Gly-Lys-NH-NH_2 292 mg (77%) of the title compound were obtained as a colorless powder. Mp 145–147 °C $[\alpha]_D^{20}$ +120.6 (*c* 0.3, DMF), FAB-MS (*m*/*z*) 1085 $[M+H]^+$. IR (KBr) 3440, 3432, 3356, 3349, 3335, 3002, 1744, 1702, 1660, 1650, 1641, 1601, 1580, 1462, 1395, 1380, 1365, 740, 692 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 12.561$ (s, 1H), 8.656 (d, $J = 6.78$ Hz, 1H), 8.113 (d, $J = 6.70$ Hz, 1H), 8.102 (t, $J = 4.61$ Hz, 1H), 8.006 (t, $J = 6.60$ Hz, 1H), 7.852 (s, 1H), 7.758 (d, $J = 7.80$ Hz, 2H), 7.330 (d, $J = 7.72$ Hz, 2H), 7.321 (d, $J = 7.59$ Hz, 1H), 7.242 (t, $J = 7.74$ Hz, 1H), 7.227 (d, $J = 7.70$ Hz, 2H), 7.114 (d, $J = 7.80$ Hz, 2H), 7.030 (s, 1H), 6.140 (d, $J = 7.74$ Hz, 1H), 6.059 (s, 1H), 5.152 (s, 2H), 4.856 (s, 2H), 4.508 (dt, $J = 6.78$ Hz, $J = 4.85$ Hz, 1H), 4.355 (m, $J = 7.94$ Hz, 1H), 4.295 (dt, $J = 6.70$ Hz, $J = 4.77$ Hz, 1H), 3.985 (m, $J = 4.51$ Hz, 2H), 3.982 (d, $J = 6.61$ Hz, 2H), 3.394 (m, $J = 8.55$ Hz, 2H), 3.346 (s, 1H), 3.321 (s, 1H), 3.068 (d, $J = 4.85$ Hz, 2H), 2.388 (s, 3H), 2.320 (s, 1H), 2.245 (m, $J = 7.90$ Hz, 1H), 2.233 (m, $J = 7.96$ Hz, 1H), 1.922 (m, $J = 8.11$ Hz, 1H), 1.906 (m, $J = 7.78$ Hz, 2H), 1.786 (m, $J = 4.77$ Hz, 2H), 1.772 (m, $J = 7.96$ Hz, 1H), 1.704 (m, $J = 7.01$ Hz, 2H), 1.556 (m, $J = 4.56$ Hz, 2H), 1.44 (s, 9H), 1.356 (m, $J = 4.66$ Hz, 2H), 1.315 (s, 3H), 1.032 (m, $J = 7.88$ Hz, 2H), 0.702 (s, 3H). Anal. Calcd for $C_{55}H_{72}N_8O_{13}S$: C, 60.87; H, 6.69; N, 10.32. Found: C, 60.67; H, 6.54; N, 10.50.

5. Bioassays of the peptides and steroid–urotoxins

5.1. Lymphocyte proliferation assay

The spleen of BALB/C mice euthanized by means of carbon dioxide was collected. The collected spleen was pushed gently through a stainless steel mesh screen and the cells were suspended with RPMI-1640 solution in a sterile petri dish (50 mL). The suspensions of the spleen cells were then layered on top of a Histopaque solution (3 mL) in a sterile conical tube. After centrifugation (1000*g*, 10 min) the lymphocyte band was taken and washed with RPMI-1640 via an additional centrifugation (1000*g*, 10 min). The final cell pellet was suspended in RPMI-1640, which contained penicillin (100 IU/mL), streptomycin (100 mg/mL), heat-inactivated rat serum (2.5%), and 2-mercaptoethanol (5×10^{-5} M). The cells were counted using a hemocytometer.

To each well in a 96-well microplate 100 μL of the cell suspension, 100 L of RPMI-1640 containing ConA (final concentration 5 μg/mL) or LPS (final concentration 10 μg/mL) and know concentration solution of tested compounds were added to make a final cell count of 2×10^5 cells/well. The plates were incubated at 37 °C in a humidified incubator containing air/CO_2 (95:5). After 44 h of incubation to each well 10 μL of the solution of MTT (5 mg/mL) were added and the plate was incubated for an additional 4 h. After centrifugation (1000*g*, 10 min) the precipitates were dried and the residue was dissolved with 150 μL of DMSO and the solution was gently shaken. The optical density (OD) of the solution was read at 570 nm with Biorad and their inhibitory activities were represented by the tested OD values.

5.2. Phagocytosis of mouse peritoneal macrophages

BALB/C mice were injected intraperitoneally with 1.5 mL of sodium thioglycollate solution (3%). On day 4 the peritoneal macrophages were harvested in Hank's balanced salt solution (HBSS). After washing with RPMI-1640 (supplemented with fetal calf serum and antibiotics, penicillin and streptomycin) for three times the cells were suspended in RPMI-1640 and the cell density was adjusted to 2×10^6 cells/mL. To each well in a 96-well microplate 100 μL of the cell suspension were added and then cultured in the presence of 10 μL of known concentration solution of the tested compound in NS at 37 °C with air/CO_2 (95:5). After 24 h to the residue in the cultured well 100 μL of 0.1% solution of neutral red-stained zymosan were added and incubated at 37 °C for another 30 min. After washing with HBSS for three times 100 μL of triton X-100 (10%) were added. The solution was stood over night and then gently shaken. The OD of the solution was read at 570 nm with Biorad and the inhibitory activity was represented by the tested OD value.

5.3. Cardiac tissue transplantation

BALB/C mice (male, ranged in age from 12 to 14 weeks at the time of transplantation, from the Animal Center of Beijing Medical University) were used as the host animals, which were housed six animals in a wooden cage $6 \times 12 \times 6$ inches and the normal food and water were available at all times. Donor tissue was obtained from C_{57}bl/6 mice ranging in age from near full term fetuses up to 48 h old. Utilizing one ear of a given host mouse the 'pockets' were on the dorsum of the ear was made by slitting the skin over the auricular artery at the base of the ear. The split-heart transplanted was approximately 3×3 mm in size. All tissue was grafted promptly following removal from the donor except for a period of 1–3 min in 0.9% saline solution while the host site was prepared. The donor tissue was then eased into the base of the 'pocket' near the distal edge of the ear. Usually within 3–5 days pulsatile activity of the transplant could be observed. One day after transplantation the mice were randomly divided into 11 groups. The animals of negative control were given an injection (ip)

of 0.1 mL of DMSO/H_2O (1:4, v/v), the animals of positive control were given an injection (ip) of a corresponding dosage of hydrocortisone in 0.1 mL of DMSO/H_2O (1:4, v/v), and the animals of steroid–urotoxin treating were given an injection (ip) of a corresponding dosage of hydrocortisone–urotoxin or prednisolone–urotoxin in 0.1 mL of DMSO/H_2O (1:4, v/v) per day until the electrocardiograms of the grafts disappeared. Six days after transplantation the animals were anesthetized with sodium pentobarbital at a dosage of 75 μg/g body weight and the electrocardiograms of the grafts were recorded using the physiography. The interval between the tissue transplantation and the disappearance of pulsatile activity was defined as the survival time of a graft. The data are listed in Tables 7 and 8. The statistical analysis of the data were carried out by the use of ANOVA test, $p < 0.05$ is considered significant.

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