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The synthesis, distribution, and anti-hepatic cancer activity of YSL

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Abstract—YSL was prepared stepwise from C terminal to N terminal with the side chain un-protective amino acids, Boc-Leu-OMe, Boc-Ser-OH, and Boc-Tyr-OH, as the starting materials in 39.5% total yield (31.2 g/per batch). With the side chain un-protective Boc-(3,5-dibromo)-Tyr-OH and HCl·Ser-Leu-OMe as the starting materials (3,5-^{3}H-Tyr)-Ser-Leu-OH was obtained in 29% yield. The determination of radioactive quantity in the urine and feces indicated that even after the administration for 130 h only 8.4% (5.35% in urine and 3.05% in feces) of total radioactive quantity from the metabolite of [3,5-^{3}H-Tyr]-Ser-Leu-OH were monitored. The distribution study revealed the relative accumulation level of the individual tissue was arranged in the sequence of spleen > liver > kidney > lung > heart > muscle > brain. Selecting hepatic cancer as the target YSL significantly increased the survival time of H22 tumor cells implanted mice.

1. Introduction

It is well known that animal tissues are the important source of bioactive peptides.[1–5] In the pepsin promotion degradation of porcine spleen a random peptide library was obtained, among which one was assigned to Tyr-Ser-Leu-OH (YSL), which was also obtained 20 years ago from mammalian leucine aminopeptidase promotion degradation of α-BCP (Ala-Pro-Arg-Leu-Arg-Phe-Tyr-Ser-Leu), a neurotransmitter released by the bag cells in abdominal ganglion of the marine snail *Aplysia*[6] and no bioactivity was mentioned. In the preliminary screening YSL exhibited immuno-modulating effects, such as enhancing concanavalin (ConA) induced proliferation of mouse spleen lymphocytes, phagocytosis of mouse peritoneal macrophages, and the activity of NK cells. In our opinion to develop the preliminary screening results a practical synthetic route has to be established and the therapeutic target has to be defined. As a practical synthetic route of YSL the starting materials should be minimal protective and the preparation scale should be large. In order to define the therapeutic target of YSL the corresponding in vivo distribution should be identified. In the in vivo distribution research of peptides or proteins both ELISA methods[7–13] and isotope labeling[14,15] may be employed to strengthen the monitor sensitivity. Since the molecular weight of YSL is too low to obtain the corresponding antibody, the isotope labeling is obviously the best choice. Though the phenyl group in Tyr residue of YSL is a convenient function group to be labeled with isotope ^{125}I, the introduction of ^{125}I at 3 and 5 positions of the phenyl will un-proportionally increase the molecular weight of YSL from 381 to 581. In this case the other means such as ^{3}H and ^{14}C labeling have to be considered. Based on the economical reason the oriental ^{3}H-labeling YSL is considered for the in vivo pharmacokinetics process. In the present paper the normal protective strategy and minimal protective strategy of the synthesis of YSL were described, the in vivo distribution was examined by the use of [3,5-^{3}H-Tyr]-Ser-Leu-OH as the probe, and the effects of YSL on a tumor model were described.

Keywords: YSL; [3,5-^{3}H-Tyr]-Ser-Leu-OH; Distribution; Anti-hepatic cancer.
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2. Results and discussion

2.1. The minimal protective strategy is suitable for preparation of YSL in large scale

In general YSL may be prepared either by normal protective strategy or by minimal protective strategy. According to Scheme 1 YSL was prepared stepwise from C terminal to N terminal with the protective amino acids, Boc-L-Leu-OBzl, Boc-Ser(Bzl)-OH, and Boc-Tyr(Dcb)-OH, as the starting materials. The total yield of YSL was 350 mg (44.5%). According to Scheme 2 YSL was prepared stepwise from C terminal to N terminal with the side chain un-protective amino acids, Boc-L-Leu-OMe, Boc-Ser-OH, and Boc-Tyr-OH, as the starting materials. The total yield of YSL was 31.2 g (39.5%). As a common strategy for peptide synthesis when the sequence permits some side chain of amino acid such as the hydroxyl group of Thr and Ser may not be protected and its relatively weak nucleophilicity may be used to realize the selective acylation of amino group and thus to realize the minimal protective synthesis. The results of the present paper suggested that with the minimal protective strategy though the yield of YSL was decreased by 5.4% the scale was enlarged by about 1000 times. In the preparation of (3,5-^{3}H-Tyr)-Ser-Leu-OH the side chain un-protective Boc-Ser-OH, Boc-L-Leu-Ome, and Boc-(3,5-dibromo)-Tyr-OH were also used as the starting materials. The total yield of (3,5-^{3}H-Tyr)-Ser-Leu-OH was 29% (Scheme 2).

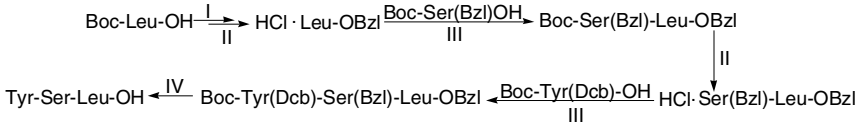
Removing Boc group the protective dipeptide or tripeptide was mixed with the solution of hydrogen chloride in ethyl acetate, the product was immediately separated as precipitates, and the reaction took less than 1 h. Perhaps the quick precipitation made the product to be out off the reaction system and thus the acylation of its side chain was avoided. Preventing racemization the sponification of the tripeptidyl ester was carried out at −4 °C strictly. The specific optical rotation of YSL from solution method and that of YSL from solid method (Fmoc chemistry) were compared and no difference was found between them.

2.2. In vivo [3,5-^{3}H-Tyr]-Ser-Leu-OH was degraded into Tyr and Ser-Leu-OH

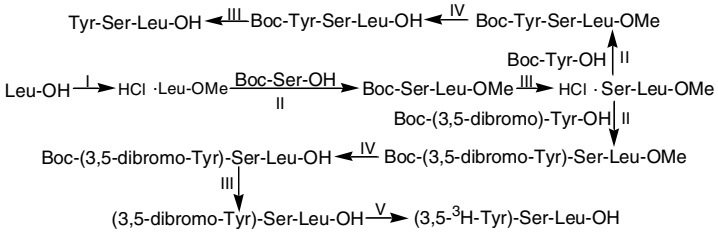
With [3,5-^{3}H-Tyr]-Ser-Leu-OH as tracer the in vivo disposition of YSL was investigated. After administration via intravenous bolus in rat and Rhesus macaque their plasma radioactivity was determined. Two minutes later there was no more YSL in the tested plasma. The result demonstrated that in vivo [3,5-^{3}H-Tyr]-Ser-Leu-OH was degraded within 2 min. The urine and feces of YSL received mice were collected. No YSL was found from the mouse urine and feces collected within 1–6 h. The data supported that in vivo YSL was degraded quickly. The urine and feces collected after the administration for 130 h still gave 8.4% (5.35% in urine and 3.05% in feces) radioactivity of the total metabolite of [3,5-^{3}H-Tyr]-Ser-Leu-OH. The data suggested that the excretion of the metabolites of YSL is rather slow. At 37 °C [3,5-^{3}H-Tyr]-Ser-Leu-OH was incubated in rat plasma for 2 min two metabolites were monitored by HPLC, one gave the same retention time as that of Tyr and another gave the same retention time as that of Ser-Leu-OH. Accordingly it could be deduced that in vivo YSL was degraded into Tyr and Ser-Leu-OH.

2.3. [3,5-^{3}H-Tyr]-Ser-Leu-OH distributed to the tissues after metabolism

After intravenous administration of [3,5-^{3}H-Tyr]-Ser-Leu-OH solution (9.0×10^8 dpm/mL, 0.35 mg/mL) via caudal vein the heart, liver, spleen, lung, kidney, brain, muscle, and genitals of the mice were separated. The tissue dpm value was determined by a scintillation counter and it was noticed that [3,5-^{3}H-Tyr]-Ser-Leu-OH widely distributed in the mouse body. The relative accumulation level of the individual tissue was arranged in the sequence of spleen > liver > kidney > lung > heart > muscle > brain (corresponding to 3.48 ± 1.13, 1.89 ± 0.12,



Scheme 1. Synthetic route of YSL with normal protective strategy. Reagents: (I) $CsCO_3$, $C_6H_5CH_2Br$; (II) $HCl/CH_3CO_2C_2H_5$ (6 mol/L); (III) DCC/HOBt/NMM with corresponding carboxyl component; (IV) HF.



Scheme 2. Synthetic route of YSL with minimal protective strategy. Reagents: (I) HCl/CH_3OH; (II) DCC/HOBt/NMM with corresponding carboxyl component; (III) $HCl/CH_3CO_2C_2H_5$ (6 mol/L); (IV) $NaOH/H_2O/CH_3OH$; (V) $^3H_2$ and 10% Pd/C.

1.60 ± 0.26, 1.41 ± 0.24, 1.01 ± 0.21, 0.64 ± 0.19, and 0.62 ± 0.18 dpm, respectively).

Since in plasma YSL was quickly degraded into Tyr and Ser-Leu-OH it is critical to define the contribution of [3,5-^{3}H-Tyr]-Ser-Leu-OH and 3,5-^{3}H-Tyr-OH to the monitored tissue radioactivity. Accordingly the distribution of 3,5-^{3}H-Tyr-OH was also observed. After intravenous administration of 3,5-^{3}H-Tyr-OH solution (9.0×10^8 dpm/mL, 0.35 mg/mL) via caudal vein the heart, liver, spleen, lung, kidney, brain, muscle, and genitals of the mice were separated. The tissue dpm value was determined by scintillation count and it was noticed that 3,5-^{3}H-Tyr-OH also distributed widespread in the mouse body. The relative accumulation level of the individual tissue was arranged in another sequence of lung > kidney > spleen > liver > heart > muscle > brain (corresponding to 1.49 ± 0.16, 1.33 ± 0.06, 1.24 ± 0.02, 0.77 ± 0.15, 0.68 ± 0.03, 0.60 ± 0.01, 0.51 ± 0.04). The obviously different distribution of 3,5-^{3}H-Tyr-OH and [3,5-^{3}H-Tyr]-Ser-Leu-OH suggested that after the administration of [3,5-^{3}H-Tyr]-Ser-Leu-OH the monitored tissue radioactivity reflected rather the level of itself than that of its metabolite 3,5-^{3}H-Tyr-OH. Thus the metabolism of YSL possibly follows its distribution. Based on the high affinity of [3,5-^{3}H-Tyr]-Ser-Leu-OH with the mouse liver in the present study hepatic cancer was selected as its target.

2.4. YSL significantly prolong the survival time of H22 tumor cells implanted mice

The suspension of 4×10^7 H22 viable tumor cells and 0.2 mL sterile saline were implanted intraperitoneally into female Kun-Ming mice (18–22 g, 6 weeks old). Each animal was given an injection (ip) with YSL per day for therapeutic group, with sterile saline for control group, and with cyclophosphamide for positive control. The survival time was recorded in days following tumor implantation. The antitumor activity of YSL was represented by the mean survival time.[16,17] The data are listed in Table 1 and the statistical analysis of the data were carried out by the use of ANOVA test, $p<0.05$ is considered significant. The experiments showed that 10 μg/kg/d of YSL significantly prolonged the survival time of murine H22 tumor cells implantation mice.

Table 1. The influence of YSL on H22 tumor in mice

Compound	Dosage (μg/kg)	Survival time (day)
NS	0.5 mL	18.53 ± 1.37
Cyclophosphamide	20	21.94 ± 4.54[b]
YSL	10	25.53 ± 14.14[a]
	20	25.82 ± 14.29[a]
	40	30.47 ± 17.89[b]
	80	35.06 ± 20.90[b,c]

$n=17$.
[a] Compare to NS, $p<0.05$.
[b] Compare to NS, $p<0.01$.
[c] Compare to cyclophosphamide, $p<0.05$.

Table 2. The influence of YSL on BEL-7402 human hepatic cancer in nude mice

Compound	Dosage (μg/kg)	Tumor weight (g)
NS	0.5 mL	2.0258 ± 0.9147
Cyclophosphamide	20	0.2611 ± 0.1484[a]
YSL	5	0.8345 ± 0.4957
	80	0.6327 ± 0.4970[a]
	160	0.5627 ± 0.3230[a]

$n=10$.
[a] Compare to NS, $p<0.05$.

2.5. YSL significantly decreased the weight of BEL-7402 human hepatic tumor in nude mice

To the small tear at the subcutaneous part of the abdominal cavity of the nude mice a 2–4 mm^3 BEL-7402 tumor mass was implanted. The animals were given an injection (ip) with YSL per day for therapeutic group, with sterile saline for control group, and with cyclophosphamide for positive control. The experiments showed that 80 μg/kg/d of YSL significantly decreased the weight of BEL-7402 human hepatic tumor in nude mice[18,19] (Table 2). Because of its low dosage (80 μg/kg) and low metabolism stability though a lot of efforts were made no YSL can be detected from the BEL-7402 human hepatic tumor cells in nude mice.

2.6. Experimental

All chemicals were commercially available. The palladium on activated carbon (10%) was prepared by ourselves. Ultraviolet spectra were recorded on the UV-210 spectrometer using water as solvent. Radiochemical purity was determined in a thin layer radioscanner Model RTLS-A. Tritium was counted using a Packard Liquid Scintillation counter, Model FG-353G. Elemental analysis was determined with Elementary Analyzer Model 1106. MS spectra were recorded on HP-5988 GC–MS spectrometer.

2.7. 3,5-Dibromotyrosine

The solution of 3.0 mL of bromine (3.12 g, 0.056 mol) in 15 mL of acetic acid was dropped into the stirred suspension of 5.0 g (0.028 mol) of L-tyrosine and a clear red solution was obtained. The reaction mixture was stirred at 50–60 °C for 2 h and then allowed to cool. The formed precipitates of 3,5-dibromotyrosine hydrobromide were collected by filtration and washed with ether to give 7.0 g of the title compound. The filtrate was evaporated and the residue was treated with ether to provide another 3.8 g of the title compound. The total yield of crude title compound was 92%. The crude product was dissolved in 30 mL of water and the solution was adjusted to pH 6 with ammoniasol union (25%). The formed crystals were collected by filtration and washed with ether to provide 6.5 g (69.4%) of pure title compound. IR (KBr): 3359, 3352–3221, 3011, 1760, 1686, 1600, 1595, 1492, 1460, 1380, 760, 702 cm^{-1}. ^{1}H NMR (DMSO-d_6): $\delta=11.25$ (s, 1H), 11.02 (s, 1H), 9.50 (d, $J=6.42$ Hz, 2H), 7.08 (s, 2H), 4.02 (m, $J=5.68$ Hz,

1H), 3.12 (d, $J=5.75$ Hz, 2H). FAB-MS (*m/e*), 340 $[M+H]^+$, $[\alpha]_D^{20}$ +1.3 (*c* 5, 4% HCl). Anal. Calcd for $C_9H_9O_3NBr_2$: C, 31.89; H, 2.68; N, 4.13. Found: C, 31.81; H, 2.72; N, 4.06.

2.8. Boc-3,5-dibromotyrosine

The solution of 2.1 g (0.00478 mol) of L-3,5-dibromotyrosine in 20 mL of $NaOH/H_2O$ solution (1 mol/L) was stirred at 0 °C for 0.5 h, to which the solution of 1.78 g (0.00817 mol) of Boc_2O in 2 mL of THF were added. The reaction mixture was stirred at room temperature for another 24 h. After evaporation in vacuum the residue was extracted with ether to remove the *tert*-butyl carboxylic acid and the aqueous phase was acidified with a dilute solution of $KHSO_4$ to pH 2–3. The separated aqueous phase was extracted with ethyl acetate for three times. The organic layer was combined, washed with water twice, dried over anhydrous sodium sulfate, filtered, and evaporated in vacuum to give 2.4 g (85.2%) of the title compound as a colorless powder. Mp 105–108 °C. ^{1}H NMR (DMSO-d_6): $\delta=11.23$ (s, 1H), 11.05 (s, 1H), 8.25 (d, $J=6.45$ Hz, 1H), 7.09 (s, 2H), 4.02 (m, $J=5.64$ Hz, 1H), 3.12 (d, $J=5.66$ Hz, 2H), 1.45 (s, 9H). FAB-MS (*m/e*): 450 $[M+H]^+$, $[\alpha]_D^{20}$ +13 (*c* 2, MeOH). Anal. Calcd for $C_{14}H_{17}Br_2NO_5$: C, 38.29; H, 3.90; N, 3.19. Found: C, 38.40; H, 3.79; N, 3.30.

2.9. Boc-Ser-Leu-OMe

The solution of 20.02 g (0.121 mmol) of L-leucine methyl ester hydrochloride, 17.2 g (0.127 mmol) of HOBt, 24.87 g (0.121 mol) of Boc-Ser-OH, and 15.6 mL (0.121 mmol) of *N*-methylmorpholine were dissolved in 100 mL of anhydride tetrahydrofurane. At 0 °C to the stirring solution 26.24 g (0.127 mol) of DCC were added. The reaction mixture was adjusted to pH 8–9 with *N*-methylmorpholine. The reaction mixture was stirred at 0 °C for 3 h and then at room temperature for 12 h. The precipitated *N*,*N*′-dicyclohexylurea was removed by filtration and the filtrate was evaporated in vacuum. The residue was dissolved in ethyl acetate and the solution was washed successively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride and the organic phase was separated and dried over anhydrous sodium sulfate. After filtration and evaporation under reduced pressure 39.3 g (99%) of the title compound were obtained as a syrup. IR (KBr): 3434, 3350, 1730, 1635, 1396, 1385 cm^{-1}. ^{1}H NMR (DMSO-d_6): $\delta=8.21$ (d, $J=6.85$ Hz, 1H), 8.15 (d, $J=6.79$ Hz, 1H), 4.90 (m, $J=5.88$ Hz, 1H), 4.66 (m, $J=5.86$ Hz, 1H), 3.60 (s, 3H), 3.08 (d, $J=5.45$ Hz, 2H), 2.02 (s, 1H), 1.78 (t, $J=5.63$ Hz, 2H), 1.82 (m, 1H), 1.42 (s, 9H), 1.03 (d, $J=5.55$ Hz, 6H). FAB-MS (*m/e*): 333 $[M+H]^+$, $[\alpha]_D^{20}$ −36 (*c* 2, MeOH). Anal. Calcd for $C_{15}H_{28}N_2O_6$: C, 54.20; H, 8.49; N, 8.43. Found: C, 54.33; H, 8.29; N, 8.61.

2.10. HCl·Ser-Leu-OMe

The solution of 41.2 g (0.124 mol) of Boc-Ser-Leu-OMe and 4 mL of hydrogen chloride in ethyl acetate (4 mol/L) was stirred at 0 °C for 2 h. The colorless precipitates were collected by filtration and washed with ethyl acetate and ethyl ether repeatedly to give 28.5 g (99%) of the title compound, as a colorless powder. Mp 128–129 °C. IR (KBr): 3434, 3423, 3357, 1735, 1630, 1394, 1386 cm^{-1}. ^{1}H NMR (DMSO-d_6): $\delta=9.42$ (d, $J=6.87$ Hz, 2H), 8.01 (d, $J=6.75$ Hz, 1H), 4.45 (m, $J=5.75$ Hz, 1H), 4.05 (m, $J=5.75$ Hz, 1H), 3.60 (s, 3H), 3.06 (d, $J=5.47$ Hz, 2H), 2.18 (s, 1H), 1.85 (t, $J=5.64$ Hz, 2H), 1.81 (m, 1H), 1.04 (d, $J=5.55$ Hz, 6H). FAB-MS (*m/e*): 233 $[M+H]^+$, $[\alpha]_D^{20}$ −18 (*c* 2, MeOH). Anal. Calcd for $C_{10}H_{21}ClN_2O_4$: C, 44.69; H, 7.88; N, 10.42. Found: C, 44.54; H, 7.64; N, 10.21.

2.11. Boc-Tyr-Ser-Leu-OMe

At 0 °C to the stirring solution of 27.45 g (0.097 mol) of Boc-L-Tyr-OH, 13.85 g (0.102 mol) of HOBt, 26.23 g (0.097 mol) of HCl·Ser-Leu-OMe, 15.6 mL (0.121 mmol) of *N*-methylmorpholine, and 125 mL of anhydride THF 21.13 g (0.102 mol) of DCC were added. The reaction mixture was adjusted to pH 8–9 with *N*-methylmorpholine. After stirring at 0 °C for 3 h the reaction mixture was stirred at room temperature for 12 h. The precipitated *N*,*N*′-dicyclohexylurea was removed by filtration and the filtrate was evaporated in vacuum. The residue was dissolved in ethyl acetate and the solution was washed successively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride and the organic phase was separated and dried over anhydrous sodium sulfate. After filtration and evaporation under reduced pressure 47.5 g (99%) of the title compound were obtained as a colorless powder. IR (KBr): 3434, 3350, 3346, 3012, 1737, 1627, 1600, 1591, 1489, 1452, 1396, 1385, 710 cm^{-1}. Mp 147–148 °C. ^{1}H NMR (DMSO-d_6): $\delta=11.00$ (s, 1H), 8.18 (d, $J=6.83$ Hz, 1H), 8.16 (d, $J=6.75$ Hz, 1H), 8.10 (d, $J=6.85$ Hz, 1H), 7.31 (d, $J=7.26$ Hz, 2H), 7.18 (d, $J=7.26$ Hz, 2H), 4.90 (d, $J=5.69$ Hz, 1H), 4.84 (m, $J=5.61$ Hz, 1H), 4.65 (m, $J=5.84$ Hz, 1H), 4.04 (d, $J=5.66$ Hz, 2H), 3.62 (s, 3H), 3.13 (d, $J=5.47$ Hz, 2H), 2.02 (s, 1H), 1.85 (t, $J=5.65$ Hz, 2H), 1.81 (m, 1H), 1.42 (s, 9H), 1.05 (d, $J=5.57$ Hz, 6H). $[\alpha]_D^{20}$ −22 (*c* 2, MeOH), FAB-MS (*m/e*): 496 $[M+H]^+$. Anal. Calcd for $C_{24}H_{37}N_3O_8$: C, 58.17; H, 7.53; N, 8.48. Found: C, 58.11; H, 7.38; N, 8.31.

2.12. Boc-Tyr-Ser-Leu-OH

The solution of 49.5 g (0.10 mol) of Boc-Tyr-Ser-Leu-OMe, 500 mL of methanol, and 300 mL of aqueous NaOH (2 mol/L) was stirred at 0 °C for 5 h. The reaction mixture was adjusted to pH 6 with $KHSO_4$ (2 mol/L) and evaporated in vacuum. The residue was again adjusted to pH 1 with $KHSO_4$ (2 mol/L). The formed precipitates were collected by filtration to offer 38.0 g (96%) of the title compound as a colorless powder. Mp 103–105 °C. IR (KBr): 3432, 3353, 3344–2109, 1735, 1622, 1605, 1591, 1492, 1450, 1394, 1387, 710 cm^{-1}. ^{1}H NMR (DMSO-d_6): $\delta=11.04$ (s, 1H), 11.00 (s, 1H), 8.12 (d, $J=6.85$ Hz, 1H), 8.05 (d, $J=6.76$ Hz, 1H), 7.89 (d, $J=6.84$ Hz, 1H), 7.32 (d, $J=7.28$ Hz, 2H), 7.16 (d, $J=7.28$ Hz, 2H), 4.92 (d, $J=5.67$ Hz, 1H), 4.64 (m, $J=5.63$ Hz, 1H), 4.49 (m, $J=5.82$ Hz, 1H), 4.02 (d,

$J = 5.68$ Hz, 2H), 3.03 (d, $J = 5.52$ Hz, 2H), 2.04 (s, 1H), 1.81 (t, $J = 5.66$ Hz, 2H), 1.79 (m, 1H), 1.44 (s, 9H), 1.02 (d, $J = 5.52$ Hz, 6H). FAB-MS (*m/e*): 481 $[M+H]^+$, $[\alpha]_D^{20}$ −7 (*c* 2, MeOH). Anal. Calcd for $C_{23}H_{35}N_3O_8$: C, 57.37; H, 7.33; N, 8.73. Found: C, 57.44; H, 7.55; N, 8.58.

2.13. Tyr-Ser-Leu-OH

The solution of 38.0 g (0.08 mol) of Boc-Tyr-Ser-Leu-OH and 400 mL of the solution of hydrogen chloride in ethyl acetate (6 mol/L) was stirred at room temperature for 3 h. The reaction mixture was evaporated in vacuum and the residue was dissolved in 40 mL of water. The solution was adjusted to pH 6 with ammonia spirit, the formed precipitates were collected by filtration and recrystallized in ethanol to provide 31.2 g (85%) of the title compound as a colorless powder. The purity was higher than 98%. IR (KBr): 3405, 3362, 3359, 3352–3221, 3012, 1762, 1684, 1602, 1591, 1496, 1462, 1381, 762, 706 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 11.02$ (s, 1H), 10.41 (s, 1H), 9.66 (d, $J = 6.83$ Hz, 2H), 8.21 (d, $J = 6.74$ Hz, 1H), 8.01 (d, $J = 6.82$ Hz, 1H), 7.30 (d, $J = 7.31$ Hz, 2H), 7.14 (d, $J = 7.31$ Hz, 2H), 4.65 (d, $J = 5.66$ Hz, 1H), 4.56 (m, $J = 5.65$ Hz, 1H), 4.05 (m, $J = 5.78$ Hz, 1H), 4.10 (d, $J = 5.66$ Hz, 2H), 3.17 (d, $J = 5.56$ Hz, 2H), 2.06 (s, 1H), 1.83 (t, $J = 5.64$ Hz, 2H), 1.76 (m, 1H), 1.05 (d, $J = 5.52$ Hz, 6H). FAB-MS (*m/e*): 382 $[M+H]^+$, $[\alpha]_D^{20}$ −6 (*c* 2, H_2O). Anal. Calcd for $C_{18}H_{27}N_3O_6 \cdot 1/2H_2O$: C, 55.37; H, 7.14; N, 10.76. Found: C, 55.68; H, 7.28; N, 10.76.

2.14. Boc-(3,5-dibromo-Tyr)-Ser-Leu-OMe

At 0 °C to the stirring solution of 817 mg (3.043 mmol) of HCl·Ser-Leu-OMe, 484 mg (3.585 mmol) of HOBt, 1600 mg (3.64 mmol) of Boc-3,5-dibromotyrosine, 0.39 mL (3.045 mol) of *N*-methylmorpholine and 40 mL of anhydrous tetrahydrofurane, 739 mg (3.59 mmol) of DCC was added. The reaction mixture was adjusted to pH 8–9 with *N*-methylmorpholine. The reaction mixture was stirred at 0 °C for 3 h and then at room temperature for 12 h. The precipitated *N*,*N*′-dicyclohexylurea was removed by filtration and the filtrate was evaporated in vacuum. The residue was dissolved in 100 mL of ethyl acetate and washed with saturated solution of $NaHCO_3$, saturated solution of NaCl, and the solution was washed successively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride. The organic phase was separated and dried over anhydrous sodium sulfate, filtered, and evaporated in vacuum to offer 2.1 g (87%) of the title compound as a colorless powder. Mp 91–93 °C. IR (KBr): 3438, 3354, 3349, 3010, 1730, 1625, 1604, 1592, 1484, 1456, 1394, 1386, 1092, 710 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 11.30$ (s, 1H), 8.17 (d, $J = 6.85$ Hz, 1H), 8.14 (d, $J = 6.76$ Hz, 1H), 8.11 (d, $J = 6.84$ Hz, 1H), 7.36 (s, 2H), 4.92 (d, $J = 5.67$ Hz, 1H), 4.82 (m, $J = 5.63$ Hz, 1H), 4.67 (m, $J = 5.86$ Hz, 1H), 4.02 (d, $J = 5.67$ Hz, 2H), 3.63 (s, 3H), 3.12 (d, $J = 5.46$ Hz, 2H), 2.01 (s, 1H), 1.86 (t, $J = 5.67$ Hz, 2H), 1.82 (m, 1H), 1.43 (s, 9H), 1.03 (d, $J = 5.56$ Hz, 6H). FAB- MS (*m/e*): 655 $[M+H]^+$, $[\alpha]_D^{20}$ −8 (*c* 2, MeOH). Anal. Calcd for $C_{24}H_{35}Br_2N_3O_8$: C, 44.12; H, 5.40; N, 6.43. Found: C, 44.30; H, 5.38; N 6.29.

2.15. Boc-(3,5-dibromo-Tyr)-Ser-Leu-OH

The solution of 2.1 g (3.22 mmol) of Boc-(3,5-dibromo-Tyr)-Ser-Leu-OMe, 50 mL of methanol, and 20 mL of aqueous NaOH (2.0 mol/L) was stirred at 0 °C for 5 h. The reaction mixture was adjusted to pH 6 with $KHSO_4$ (2 mol/L) and evaporated in vacuum. The residue was again adjusted to pH 1 with $KHSO_4$ (2 mol/L). The separated aqueous phase is extracted with ethyl acetate for three times. The extractions were pooled, washed with water twice, dried over anhydrous sodium sulfate, filtered, and evaporated in vacuum to give 1.9 g (98.5%) of the title compound as a colorless powder. Mp 102–105 °C. IR (KBr): 3441, 3352, 3347–2245, 3011, 1740, 1628, 1605, 1590, 1486, 1455, 1392, 1384, 1097, 710 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 11.02$ (s, 1H), 11.00 (s, 1H), 8.13 (d, $J = 6.84$ Hz, 1H), 8.08 (d, $J = 6.74$ Hz, 1H), 7.94 (d, $J = 6.83$ Hz, 1H), 7.33 (s, 2H), 4.91 (d, $J = 5.65$ Hz, 1H), 4.66 (m, $J = 5.65$ Hz, 1H), 4.47 (m, $J = 5.84$ Hz, 1H), 4.05 (d, $J = 5.66$ Hz, 2H), 3.05 (d, $J = 5.54$ Hz, 2H), 2.02 (s, 1H), 1.83 (t, $J = 5.65$ Hz, 2H), 1.76 (m, 1H), 1.42 (s, 9H), 1.04 (d, $J = 5.54$ Hz, 6H). FAB-MS (*m/e*): 641 $[M+H]^+$, $[\alpha]_D^{20}$ −1 (*c* 2, MeOH). Anal. Calcd for $C_{23}H_{33}Br_2N_3O_8$: C, 43.21; H, 5.20; N, 6.57. Found: C, 43.40; H, 5.32; N, 6.41.

2.16. (3,5-Dibromo-Tyr)-Ser-Leu-OH

The solution of 1.9 g (2.97 mmol) of Boc-(3,5-dibromo-Tyr)-Ser-Leu-OH and 20 mL of the solution of hydrogen chloride in ethyl acetate (4 mol/L) was stirred at 0 °C for 3 h. The reaction mixture was filtrated to collect the precipitates. The precipitates were washed with ethyl acetate and ethyl ether repeatedly to provide 1.5 g (88%) of the title compound as a colorless powder. IR (KBr): 3438, 3354, 3352, 3342–2245, 3010, 1730, 1629, 1608, 1590, 1487, 1459, 1380, 1098, 712 cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 11.04$ (s, 1H), 10.40 (s, 1H), 9.64 (d, $J = 6.85$ Hz, 2H), 8.23 (d, $J = 6.76$ Hz, 1H), 8.05 (d, $J = 6.84$ Hz, 1H), 7.32 (s, 2H), 4.63 (d, $J = 5.67$ Hz, 1H), 4.54 (m, $J = 5.67$ Hz, 1H), 4.07 (m, $J = 5.76$ Hz, 1H), 4.11 (d, $J = 5.67$ Hz, 2H), 3.16 (d, $J = 5.58$ Hz, 2H), 2.04 (s, 1H), 1.85 (t, $J = 5.66$ Hz, 2H), 1.74 (m, 1H), 1.07 (d, $J = 5.54$ Hz, 6H). FAB-MS 540 $[M+H]^+$, $[\alpha]_D^{20}$ +11 (*c* 2, H_2O). Anal. Calcd for $C_{18}H_{25}Br_2N_3O_6$: C, 40.09; H, 4.67; N, 7.79. Found: C, 40.00; H, 4.81; N, 7.62.

2.17. [3,5-^{3}H-Tyr]-Ser-Leu-OH

In a 20 mL reaction flask 5 mg (0.01 mmol) of H-(3,5-dibromoTyr)-Ser-Leu-OH, 12 mg of calcium carbonate, 12 mg of 10% Pd/C, and 2 mL of DMF–water (1:1) were mixed. The reaction flask was put into liquid nitrogen to cool and then evacuated to 1×10^{-3} mmHg. To the reaction flask tritium was introduced and 650 mmHg of vacuum was maintained untill the sample thawed. The reaction mixture was stirred at room temperature for 1 h and then cooled with liquid nitrogen. The excess tritium was recovered and backed into the tritium storage tank. The catalyst Pd/C was removed. To the residue ethanol was added repeatedly followed by evaporation to remove the labile tritium and give the crude product,

which was purified on silica paper (ethyl acetate–pyridine–acetic acid–water = 30:20:6:11 v/v, $R_f = 0.56$) and then washed with water. The chemical quantity was determined by UV spectrometer and the radioactivity was counted with liquid scintillation counter. The radiochemical purity was determined by paper chromatography followed by scanning with thin layer radioscanner, and the radiochemical purity and specific activity were >98% and 36 Ci/mmol, respectively. HPLC analysis (chromatograph column: waters symmetry C18, 5 μm, 3.9 mm×150 mm; mobile phase: A, 0.1%TFA/H_2O; B, 0.1%TFA/CH_3CN; gradient elution: 0–20 min from 95%B to 65%B, 20–30 min from 65%B to 95%B; flow rate: 1.5 mL/min; detector: waters 2487 dual λ absorbance detector; detection wavelength: 215 nm) confirmed that both YSL and its labeled derivative had same retention time (13 min).

2.18. Distribution study in mice

Fifty mice were divided into five groups (10/group) in random. The work solution (9.0×10^8 dpm/mL, 0.35 mg/mL) was prepared and administrated (151.6 μg/kg, 2.5×10^9 dpm/kg) via caudal vein of the mice. The mice in each group were put to death at 20 min, 1, 1.5, 4, and 12 h after administration, respectively. The heart, liver, spleen, lung, kidney, brain, muscle, and genitals of the mice were separated and washed with water. From each tissue 100 mg of sample were taken and its dpm value was determined by scintillation. The relatively accumulation level was calculated according to the following equation.

$$\text{relative accumulation level} = \frac{\text{dpm in tissue/weight of tissue}}{\text{total dpm given/weight of mouse}} \times 100\%$$

2.19. Anti murine H22 tumor assay

Kun-Ming mice (18–22 g, 6 weeks old) were divided into six groups (20/group) in random. The suspension of 4×10^7 H22 viable tumor cells suspended in 0.2 mL sterile saline was implanted intraperitoneally into the mice. One day later the animal of cyclophosphamide treating group was injected (ip) 20 μg/kg of cyclophosphamide per day for at most 60 days. After 5 days the animal of NS treating group was injected (ip) 0.5 mL of NS per day for 60 days. After 5 days the animal of YSL treating group was injected (ip) 10, 20, 40, or 80 μg/kg of YSL per day for at most 60 days. The survival time of the mice in the individual group was recorded in days following tumor implantation.

2.20. Inhibition effect of YSL on BEL-7402 human hepatic tumor in nude mice

Health BALB/C(nu/nu) nude mice (18–22 g, 4–5 week old) were divided into five groups (10/group) in random. On the abdominal cavity of the nude mice a small tear was made into which a 2–4 mm^3 BEL-7402 tumor mass was implanted. On the next day of the tumor implantation the animal of cyclophosphamide treating group was injected (ip) 20 μg/kg of YSL per day for 60 days, the animal of NS treating group was injected (ip) 0.5 mL of NS per day for 60 days, the animal of YSL treating group was injected (ip) 10, 5, 80, or 160 μg/kg of YSL per day for 60 days. Then the nude mice were executed and the tumors were taken out and weighed.

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