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Synthesis and anti-tumor activity evaluation of Matijin-Su derivatives

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ABSTRACT

A series of Matijin-Su (**MTS**, *N*-(*N*-benzoyl-L-phenylalanyl)-*O*-acetyl-L-phenylalanol) derivatives was synthesized and evaluated for their anti-tumor activities in hepatocellular carcinoma cells. The IC_{50} of compounds **1**, **3**, **4**, **11**, **13** were less than 20 μM, and compound **1** and **3** showed an IC_{50} value of less than 9 μM. Expansion inhibition could be found significantly in compound **1** and **3**-treated human hepatoma cell HepG2 and PLC/PRF/5, while both compounds exhibit lower toxicity to human hepatocyte cell line L-02. Compound **1** and **3** could induce cell cycle arrest at G1/S phase. This may be attributed to increase level of intracellular reactive oxygen species (ROS). Up-regulation of p38 MAPK activity in responding the ROS stabilize p53 and activate p21 transcription, the critical regulatory in G1/S checkpoint. Observations in this study shed light on the potential of **MTS** derivatives compound **1** and **3** as novel suppressors to human liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC), amounting for 80–90% of liver cancer, has nowadays become one of the most common and prevalent human malignancies in the world [1,2]. Every year there are approximately 500,000 new cases of HCC worldwide, 80% of which happens in Asia and Africa [3]. With geographic-dependent characteristic, some major risk factors for HCC have been identified. The people of African and Asian are vulnerable to HCC caused by infectious hepatitis B virus (HBV) and hepatitis C virus, tobacco use and aflatoxin exposure [4–6]. In Europe, the United States and Japan, hepatitis virus, alcohol and tobacco use are the main factor that induced HCC in patients with liver diseases. Therapy of hepatocellular carcinoma remains far from developed.

Matijin-Su (**MTS**, *N*-(*N*-benzoyl-L-phenylalanyl)-*O*-acetyl-L-phenylalanol, Fig. 1), a dipeptide derivative, was isolated from a Chinese ethnic drug Matijin (*Dichondra repens* Forst.) which has been widely used in the treatment of chronic liver diseases in China. The anti-HBV activities of **MTS** and its derivatives were found in our previous studies [7,8]. Some dipeptide derivatives have been recognized for decades and showed diverse biological activities, including anti-inflammatory [9], antimalarial [10] and antithrombotic activities [11]. Certain peptides containing phenylalanine, serine, or alanine residues have shown anti-tumor activity by enhancing nucleic acid or protein metabolism [12]. Furthermore, Hsieh reported the synthesis and cytotoxic evaluation of a series of Fmoc-based dipeptides (Fig. 2) [13]. Since the various biological activities of dipeptide derivatives, we evaluated several **MTS** derivatives **10**–**13**, previously synthesized by our group [7], against HepG2 human liver cancer cell lines. The results showed that the IC_{50} of compound **11**, **12** and **13** were 14.21, 67.70, and 18.26 μM respectively. Encouraged by these good results, we synthesized a series of new **MTS** derivatives, and evaluated their anti-hepatocellular carcinoma activities in order to investigate the anti-tumor activity of **MTS** derivatives.

2. Results and discussion

2.1. Chemistry

The procedure for the synthesis of the target compounds **1**–**13** was illustrated in Scheme 1. The substituted L-phenylalanine methyl ester (**I**) was first reacted with substituted benzoyl acid or isonicotinic acid in the presence of dicyclohexyl carbo-diimide (DCC) and 4-dimethylaminopyridine (DMAP) to give compound **II**, which was then hydrolyzed in the NaOH (1 M) to give compound **III**, which was then reacted with substituted L-phenylalanine methyl ester hydrochloride or substituted L-phenylalanol in the presence of isobutyl chloroformate (IBCF) and *N*-methylmorpholine (NMM) to afford the compounds **1**–**13**.

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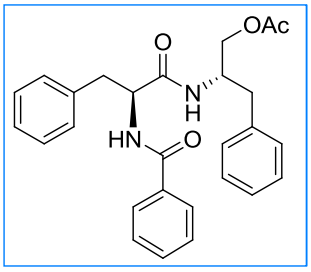


Fig. 1. The structure of **MTS**.

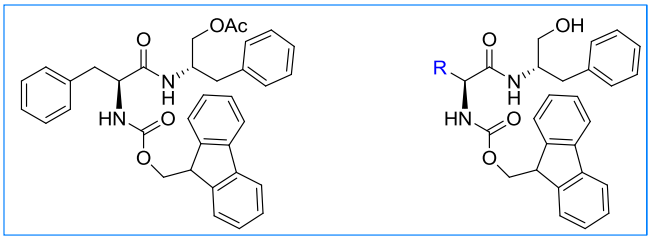
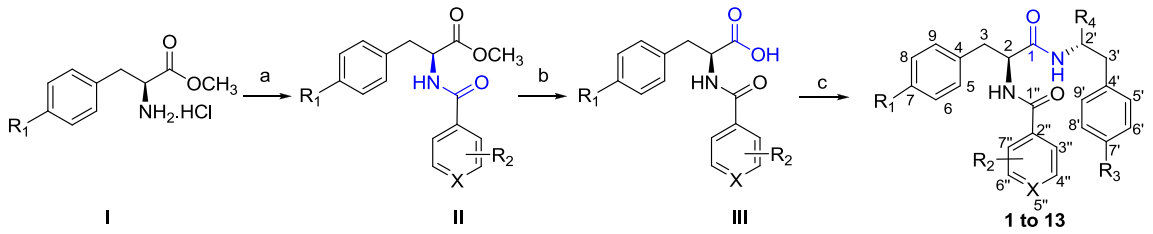


Fig. 2. Hsieh reported Fmoc-based dipeptides [13].

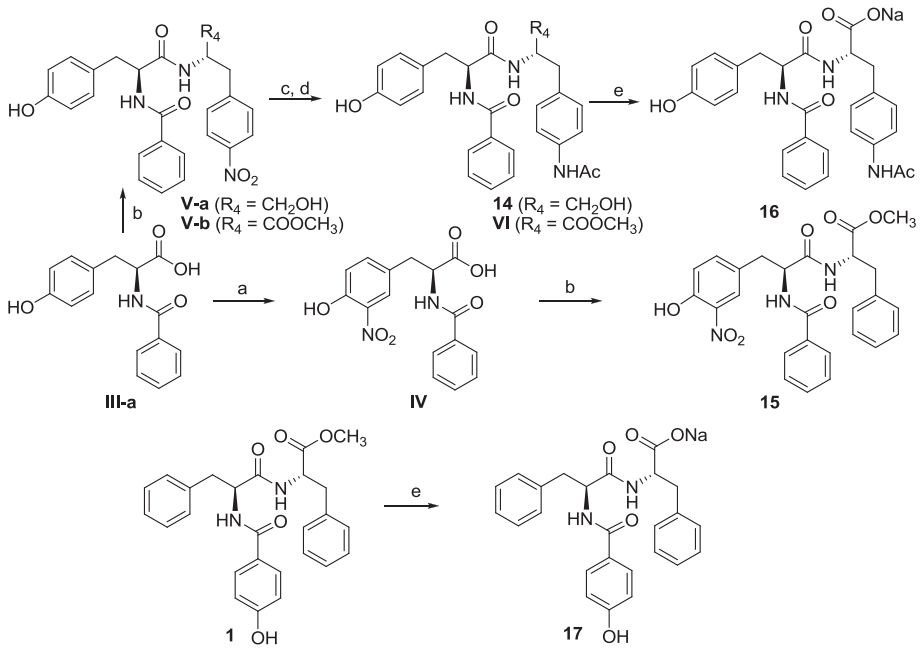
The procedure for the synthesis of the target compounds **15**–**17** was illustrated in Scheme 2. The reaction of *N*-benzoyl-L-tyrosine (**III-a**) with HNO_3 (22%) give a 6-NO_2 derivative (**IV**), which was reacted with L-phenylalanine methyl ester hydrochloride to afford **15**. The conduction of *N*-benzoyl-L-tyrosine (**III-a**) and 4-nitro-L-phenylalanol give the compound **V-a**. Then the compound **14** could be easily obtained through a hydrogenation of **V-a** followed by an acetylation of the resulted arylamine product. The compound **VI** was also synthesized using the same procedure as the synthesis of **14**. At this stage, the final compound **16** was synthesized by hydrolysis of compound **VI**. Meanwhile the synthesis of **17** was accomplished by the basic hydrolysis of compound **1**.

2.2. Evaluation of anti-tumor bioactivity of synthetic compounds

To test the anti-tumor activity of the synthetic compounds, especially in hepatocellular carcinoma, HepG2 cell and PLC/PRF/5 cell viability after drug intervention was evaluated by MTT assay using doxorubicin as positive control. Potent inhibition on the HepG2 cells survival was observed in compound **1**, **2**, **3**, **4**, **11**, **13**, in which compounds **1** and **3** exhibited the most prominent anti-tumor activity with IC_{50} lower than 10 μM (6.29 and 8.37 μM). Weak anti-tumor activity was also observed in compound **9**, **12**, **14** (118.00, 67.70, 105.30 μM). Other compounds reveal no

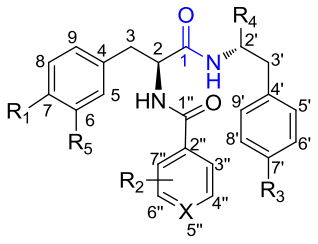


Scheme 1. Synthesis of compounds **4a**–**4n**. *Reagents and conditions*: (a) substituted benzoic acid or isonicotinic acid, DCC, DMAP, CH_2Cl_2, rt. 8 h; (b) 1.0 M NaOH, MeOH, rt.; and (c) 4-substituted L-phenylalanine methyl ester hydrochloride or 4-substituted L-phenylalanol, CH_2Cl_2, IBCF, NMM, −5 °C.



Scheme 2. Synthesis of compounds **15**–**17**. *Reagents and conditions*: (a) 22% HNO_3, 30 °C, 4 h; (b) L-phenylalanine methyl ester hydrochloride (or 4-nitro-L-phenylalanine methyl ester hydrochloride or 4-nitro-L-phenylalanol), CH_2Cl_2, IBCF, NMM, −5 °C, MeOH, H_2, Pd/C (20%), rt, 3 h; (c) Ac_2O, pyr, rt, 5 h; (d) $NaOH/H_2O$, $EtOH/CHCl_3$, rt, 3 h; and (e) the structures of the synthetic target compounds are listed in Table 1.

Table 1
The structures of the synthetic target compounds **1**–**17** and their anti-tumor bioactivity results.



Compounds	R_1	R_2	R_3	R_4	X	Dose that causes 50% cell death (μM)		
						HepG2	PLC/PRF/5	L02
1	H	5″-OH	H	$COOCH_3$	CH	6.29 ± 0.28	8.99 ± 0.12	60.05 ± 3.37
2	OH	5″-CH_3	H	CH_2OH	CH	25.64 ± 3.35	–	25.64 ± 1.94
3	OH	5″-NO_2	H	$COOCH_3$	CH	8.37 ± 0.79	15.36 ± 0.41	N/A
4	OH	5″-CH_3	H	$COOCH_3$	CH	11.74 ± 3.21	–	N/A
5	OH	H	H	$COOCH_3$	CH	N/A	–	48.87 ± 2.11
6	OH	4″-NO_2	H	CH_2OH	CH	N/A	–	N/A
7	OH	5″-NHAc	H	CH_2OH	CH	N/A	–	N/A
8	OH	H	H	CH_2OH	N	N/A	–	N/A
9	H	5″-OH	H	CH_2OH	CH	118.00 ± 4.98	–	N/A
10	NO_2	H	H	CH_2OH	CH	N/A	–	103.41 ± 5.89
11	NO_2	H	H	$COOCH_3$	CH	14.21 ± 2.01	–	N/A
l2	NO_2	H	OH	$COOCH_3$	CH	67.70 ± 2.84	–	N/A
13	NO_2	5″-CH_3	OH	$COOCH_3$	CH	18.26 ± 1.45	–	93.15 ± 2.43
14	H	H	NHAc	CH_2OH	CH	105.30 ± 5.98	–	89.93 ± 2.99
15	7-OH, 6-NO_2	H	H	$COOCH_3$	CH	38.40 ± 3.31	–	105.92 ± 6.63
16	OH	H	NHAc	COONa	CH	N/A	–	N/A
17	H	5″-OH	H	COONa	CH	N/A	–	N/A
Doxorubicin						1.00 ± 0.07	–	

Note: "N/A": not available; "–" did not be tested.

significant action on the HepG2 survival. The toxic profile of compounds **1**–**17** was investigated on hepatocellular cells L02. The inhibition of compound **1** and compound **3** on PLC/PRF/5 cell was also investigated. The results were shown in Table 1.

2.3. Compound *1* and *3* induce intracellular ROS production and cell cycle arrest in HepG2 cells

Increasing of intracellular ROS was observed in compound **1** and **3**-treated HepG2 cells, as indicated by Fig. 3(a). 10 μM treatment of compound **1** and **3** is sufficient to boost up ROS generation in HepG2 cells, and both compound **1** and **3** exhibit similar capacity in inducing ROS. In L-02 cells, compound **1** and **3** exhibits less potent in inducing ROS production. Furthermore, to determine if induction of ROS by **MTS** derivatives is correlated with the cytotoxicity, we examined ROS level in HepG2 cells treated with compound **9**, a less potent derivative. Interestingly, we found that ROS level in compound **9**-treated cells was much lower than that in compound **1** and **3**-treated cells. To further evaluate the action of compound **1** and **3**-induced ROS production on the expansion of HepG2 cells. Cell cycle analysis was conducted. It was observed that both compound **1** and **3** could retard the cell cycle transition at G1/S checkpoint, which was in line with the increased ROS production in cells with compound **1** and **3** interventions (Fig. 3b). Cell population displays higher percentage at G0/G1 phase under compound **1** and **3** interventions, indicating that a majority of cells were arrested before they could pass through G1/S checkpoint. Previous study showed that increased intracellular ROS level could retard the cell cycle transition and blocked the cells are G0/G1 phase [14]. As ROS is the major internal source of oxidative stress,

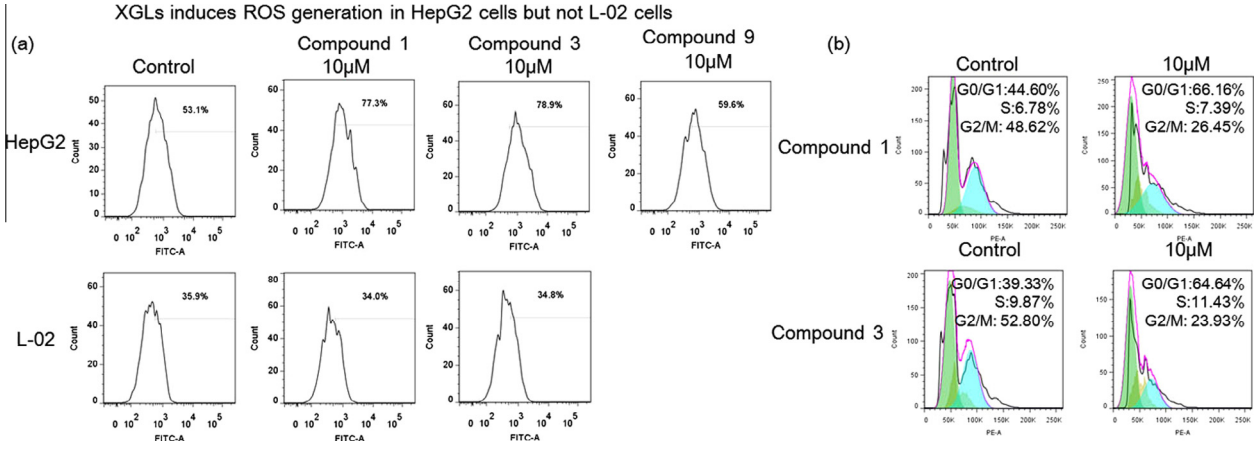


Fig. 3. Compound **1** and **3** induces ROS production (**a**) and G1/S arrest (**b**) in HepG2 cells.

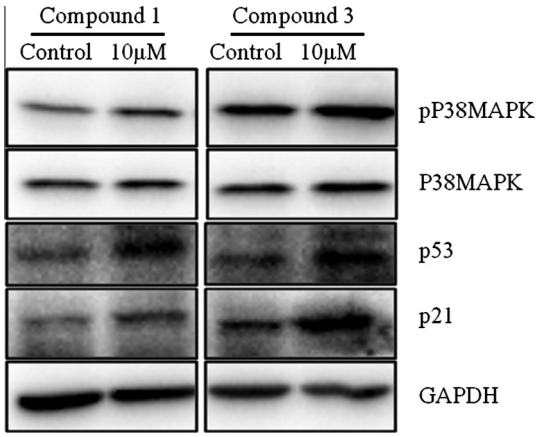


Fig. 4. Compound **1** and **3** up-regulated p38 MAPK/p53/p21 pathway in HepG2 cells.

it is predictable that up-regulation of ROS level could therefore bring oxidative damage to cell DNA, and subsequently blocks cell cycle transition and cell expansion [15]. Consistently, our observations indicated that the ROS induction by compound **1** and **3**, in line with G1/S cycle arrest in HepG2 cells, may be the responsible mechanism underlying tumor cell inhibition by compound **1** and **3**.

*2.4. Compound **1** and **3** activate p38 MAPK/p53/p21 signaling in HepG2 cells*

To further elaborate the action of compound **1** and **3** on ROS production and cycle arrest in HepG2 cells, we examined the involvement of related cellular signaling transduction after intervention. Consistent with the ROS up-regulation, the responding pathway p38 MAPK was activated (Fig. 4). Increased phosphorylation of p38 AMPK signaling was shown to be correlated with the stabilization of p53 tumor suppressor [16], which consequently restore its activity on p21 transcription [16]. Interestingly, increased endogenous levels of p53 and p21 expressions were observed in compound **1** and **3**-treated HepG2 cells (Fig. 4), which may be attributed to the ROS-induced activation of p38 MAPK. As p21 is one of the most important blockers across G1/S cell cycle transition [17], the increased expression of p21 in compound **1** and **3**-treated HepG2 cells may lead to retardness of cell cycle transition across G1/S phases. The overall regulatory scheme was shown in Fig. 5.

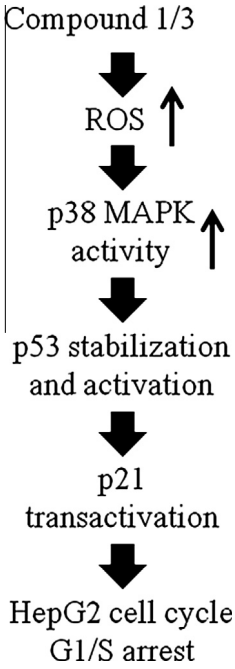


Fig. 5. Regulatory scheme of compound **1** and **3** in HepG2 cells.

3. Conclusion

A series of **MTS** derivatives was synthesized and their anti-tumor activities were evaluated in hepatocellular carcinoma cells HepG2 and PLC/PRF/5. The major findings are as follows:

(a) In general, a series of **MTS** derivatives could be potent HepG2 inhibitors (there were 5 derivatives whose IC_{50} were less than 20 μM), and two of them showed an IC_{50} value of less than 9 μM.
(b) The methyl carboxylate (MeOOC–) substitution at 2′-position might be one of the key pharmacophores for their cytotoxicity activities.
(c) The substitution with deferent groups on ring A, B and C of the derivatives of **MTS** might also greatly change the anti-HepG2 activities (or cytotoxicities): (1) the substitution (NO_2–, Me– or OH–) at position-5″ on the benzoyl ring C was an important feature in conferring relatively potent inhibitory activity; (2) the NO_2– substitution at position-7 on ring A might enhance the inhibitory activity, but the NO_2– substitution at position-8 on ring A might weaken the inhibitory activity; and (3) the OH– substitution at position-7′ on ring B might greatly reduce the inhibitory activity.
(d) Both hydroxymethyl and sodium carboxylate substitution at 2′-position might enormously reduce the inhibitory activity.
(e) Compound **1** and **3** could induce production of intracellular ROS, which activates p38 AMPK pathway to enhance expression of p53 and p21 tumor suppressors, and consequently retard HepG2 cells from G1/S transition and proliferative expansion.

4. Experimental

4.1. General

Melting points were measured with a model XT-4 apparatus and are uncorrected. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were performed on a Varian Unit INOVA-400 spectrometers in $CDCl_3$ or DMSO-*d*6. Chemical shifts (δ) are expressed in parts per million (ppm) relative to tetramethylsilane (0 ppm). Mass spectra (MS) were recorded on a Agilent MS-5973 spectrometer. High resolution mass spectra (HRMS) were recorded with a Bruker micrOTOF spectrometer in electrospray ionization (ESI) mode.

4.2. Organic synthesis

*4.2.1. General procedure for the synthesis of compounds **1**–**13***

A substituted L-phenylalanine methyl ester (**I**) (10 mmol) was added to solution of substituted benzoyl acid (or isonicotinic acid) (10 mmol), DCC (2.47 g, 12 mmol) and DMAP (200 mg, as catalyst) in dry CH_2Cl_2 (150 mL). The reaction was stirred at room temperature under N_2 for overnight. The resulting precipitate was filtered off and the solvent was removed *in vacuo*. The residue was subjected to flash chromatograph to afford the compound **II** (in 80–95% yield). The compound **II** (5 mmol) was then dissolved in a solution of NaOH (1.0 M in MeOH, 20 mL), after stirring at room temperature for 3 h, the reaction mixture was acidified to pH 2–3

with concentrated hydrochloric acid, and partitioned between ethyl acetate and water. The organic phase was separated and washed with brine, dried over Na_2SO_4 and evaporated *in vacuo* to afford the compound **III** which was directly engaged in the next step without further purification. To a solution of compound **III** (1.0 mmol), L-tyrosine methyl ester hydrochloride (or L-phenylalanine methyl ester hydrochloride) (1.1 mmol) and NMM (2.3 mmol) in CH_2Cl_2 (50 mL) was added dropwise IBCF (1.1 mmol) at 0 °C. The mixture was stirred for 30 min and the bulk of CH_2Cl_2 was removed *in vacuo*. The residue was dissolved in ethyl acetate and washed sequentially with water, 5% HCl, saturated $NaHCO_3$ solution and brine, dried with Na_2SO_4. Removal of the solvent gave a residue which was recrystallized from ethyl acetate to afford target compound **1**–**13**.

4.2.2. N-(N-5″-hydroxy-benzoyl-L-phenylalanyl)-L-phenylalanine methyl ester (**1**)

(Colorless needle, yield 61.9%) mp: 151.0–153.0 °C, ^{1}H NMR (400 MHz, DMSO): δ 9.96 (s, 1H, Ar-OH), 8.44 (d, J = 7.2 Hz, 1H, NHCO), 8.24 (d, J = 8.0 Hz, 1H, NHCO), 7.64 (d, J = 8.4 Hz, 2H, H-3″, 7″), 7.31–7.12 (m, 10H, H-5-9, 5′-9′), 6.75 (d, J = 8.8 Hz, 2H, H-4″, 6″), 4.69 (m, 1H, H-2), 3.49 (m, 1H, H-2′), 3.57 (s, 3H, OCH_3), 3.06–2.88 ppm (m, 4H, H-3, 3′); ^{13}C NMR (100 MHz, DMSO): δ 171.8 (C-1, 1′), 165.9 (C-1″), 160.2 (C-5″), 139.4 (C-4), 137.0 (C-4′), 129.4 (C-3″, 7″), 129.17 (×2), 129.13 (×2), 128.3 (C-5′, 9′), 128.0 (C-5, 9), 126.6 (C-7′), 126.2 (C-7), 124.7 (C-2″), 114.7 (C-4″, 6″), 54.4 (C-2), 53.7 (C-2′), 51.8 (OCH_3), 36.9, 36.6 ppm; ESI–HRMS: m/z [M + Na]$^+$ Calcd for $C_{25}H_{24}N_2O_5Na$: 455.1583, Found 455.1589.

4.2.3. N-(N-5″-methyl-benzoyl-L-tyrosyl)-L-phenylalanol (**2**)

(Colorless needle, yield 31.6%) mp: 193.0–195.0 °C, ^{1}H NMR (400 MHz, DMSO): δ 9.17 (s, 1H, ArNHCO), 8.36 (d, J = 8.4 Hz, 1H, NHCO), 7.88 (d, J = 8.4 Hz, 1H, NHCO), 7.72 (d, J = 8.0 Hz, 2H, H-3″, 7″), 7.26–7.00 (m, 9H, H-4″, 6″, H-5, 9, H-5′-9′), 6.61 (d, J = 8.4 Hz, 2H, H-6, 8), 4.83 (t, 1H, OH), 4.58 (m, 1H, H-2), 3.88 (m, 1H, H-2′), 3.36–3.25 (m, 2H, H-1′), 2.93–2.62 (m, 4H, H-3, 3′), 2.34 ppm (s, 3H, Ar-CH_3); ^{13}C NMR (100 MHz, DMSO): δ 171.2 (C-1), 165.9 (1″), 155.7 (C-7), 141.1 (5″), 139.1 (C-4′), 131.4 (C-2″), 130.1 (C-5, 9), 129.2 (C-6′, 8′), 128.7 (C-4″, 6″), 128.4 (C-4), 128.1 (C-5′, 9′), 127.5 (C-3″, 7″), 125.9 (C-7′), 114.8 (C-6, 8), 62.2 (C-1′), 55.2 (C-2), 52.5 (C-2′), 36.5, 36.4, 21.0 ppm (Ar-$\underline{C}H_3$); ESI–HRMS: m/z [M + Na]$^+$ Calcd for $C_{26}H_{28}N_2O_4Na$: 455.1947, Found 455.1925.

4.2.4. N-(N-5″-nitro-benzoyl-L-tyrosyl)-L-phenylalanine methyl ester (**3**)

(Colorless needle, yield 60.3%) mp: 212.0–214.0 °C, EI-MS m/z: 491 (M$^+$), 325, 285, 180, 163 (100), 147, 120, 107, 91; ^{1}H NMR (400 MHz, DMSO): δ 9.17 (s, 1H, Ar-OH), 8.89 (d, J = 8.4 Hz, 1H, NHCO), 8.60 (d, J = 7.2 Hz, 1H, NHCO), 8.29 (d, J = 8.4 Hz, 2H, H-4″, 6″), 7.99 (d, J = 8.8 Hz, 2H, H-3″, 7″), 7.26–7.18 (m, 5H, H-5′-9′), 7.11 (d, J = 8.0 Hz, 2H, H-5, 9), 6.61 (d, J = 8.4 Hz, 2H, H-6, 8), 4.68 (m, 1H, H-2), 4.49 (m, 1H, H-2′), 3.57 (s, 3H, OCH_3), 3.08–2.78 ppm (m, 4H, H-3, 3′); ^{13}C NMR (100 MHz, DMSO): δ 171.9, 171.5, 164.5 (C-1″), 155.8 (C-7), 149.1 (C-5″), 139.7 (C-2″), 137.1 (C-4′), 130.1 (C-5, 9), 129.1 (C-6′, 8′), 128.9 (C-3″, 7″), 128.3 (C-5′, 9′), 128.1 (C-4), 126.6 (C-7′), 123.5 (C-4″, 6″), 114.9 (C-6, 8), 55.1 (C-2), 53.8 (C-2′), 51.9 (OCH_3), 36.5, 36.3 ppm; ESI–HRMS: m/z [M + Na]$^+$ Calcd for $C_{26}H_{25}N_3O_7Na$: 514.1590, Found 514.1581.

4.2.5. N-(N-5″-methyl-benzoyl-L-tyrosyl)-L-phenylalanine methyl ester (**4**)

(Colorless needle, yield 65.2%) mp: 206–208 °C, EI-MS m/z: 460 (M$^+$), 325, 254, 180, 162, 147, 136, 119(100), 107, 91; ^{1}H NMR (400 MHz, DMSO): δ 9.20 (s, 1H, Ar-OH), 8.50 (d, J = 7.2 Hz, 1H, NHCO), 8.40 (d, J = 8.4 Hz, 1H, NHCO), 7.70 (d, J = 6.8 Hz, 2H, H-3″, 7″), 7.29–7.03 (m, 9H, H-4″, 6″, H-5, 9, H-5′-9′), 6.63 (d, J = 8.4 Hz, 2H, H-6, 8), 4.65 (m, 1H, H-2), 4.50 (m, 1H, H-2′), 3.58 (s, 3H, OCH_3), 3.09–2.81 (m, 4H, H-3, 3′), 2.34 ppm (s, 3H, Ar-CH_3); ^{13}C NMR (100 MHz, DMSO: δ 171.9 (C-1), 171.9 (C-1′), 166.1 (C-1″), 155.7 (C-7), 141.2 (C-5″), 137.1 (C-4′), 131.3 (C-2″), 130.2 (C-5, 9), 129.2 (C-6′, 8′), 128.7 (C-4″, 6″), 128.4 (C-4), 128.3 (C-5′, 9′), 127.5 (C-3″, 7″), 126.6 (C-7′), 114.9 (C-6, 8), 56.2 (C-2), 54.9 (C-2′), 51.9 (OCH_3), 36.6, 36.2, 21.0 ppm (Ar-$\underline{C}H_3$); ESI–HRMS: m/z [M + Na]$^+$ Calcd for $C_{27}H_{28}N_2O_5Na$: 483.1896, Found 483.1888.

4.2.6. N-(N-benzoyl-L-tyrosyl)-L-phenylalanine methyl ester (**5**)

(Colorless needle, yield 50.7%) mp: 200.0–202.0 °C, EI-MS m/z: 446 (M$^+$), 325, 240, 180, 163, 147, 120, 105 (100), 91, 77; ^{1}H NMR (400 MHz, DMSO): δ 9.19 (s, 1H, Ar-OH), 8.52–8.47 (m, 2H, NHCO × 2), 7.76 (d, J = 6.8 Hz, 2H, H-3″, 7″), 7.50 (t, 1H, H-5″), 7.43 (t, 2H, H-4″, 6″), 7.25–7.18 (m, 5H, H-5′-9′), 7.10 (d, J = 8.4 Hz, 2H, H-5, 9), 6.61 (d, J = 8.4 Hz, 2H, H-6, 8), 4.63 (m, 1H, H-2), 3.78 (m, 1H, H-2′), 3.57 (s, 3H, OCH_3), 3.07–2.78 ppm (m, 4H, H-3, 3′); ^{13}C NMR (100 MHz, DMSO): δ 171.93, 171.89, 166.2 (C-1″), 155.7 (C-7), 137.1 (C-4′), 134.1 (C-2″), 131.4 (C-5″), 130.2 (C-5, 9), 129.2 (C-6′, 8′), 128.4 (×3, C-4″, 6″, C-4), 128.3 (C-5′, 9′), 127.5 (C-3″, 7″), 126.7 (C-7′), 114.9 (C-6, 8), 54.9 (C-2), 53.8 (C-2′), 52.0 (OCH_3), 36.6, 36.2 ppm; ESI–HRMS: m/z [M + Na]$^+$ Calcd for $C_{26}H_{26}N_2O_5Na$: 469.1739, Found 469.1742.

4.2.7. N-(N-4″-nitro-benzoyl-L-tyrosyl)-L-phenylalanol (**6**)

(Colorless needle, yield 73.0%) mp: 225.5–227.0 °C, EI-MS m/z: 463 (M$^+$), 445, 297, 285, 206, 150 (100), 120, 107, 91, 60; ^{1}H NMR (400 MHz, DMSO): δ 9.15 (s, 1H, Ar-OH), 8.92 (d, J = 8.0 Hz, 1H, NHCO), 8.62 (s, 1H, H-3″), 8.37 (d, J = 8.0 Hz, 1H, H-5″), 8.22 (d, J = 7.6 Hz, 1H, H-7″), 7.94 (d, J = 8.0 Hz, 1H, NHCO), 7.76 (t, 1H, H-6″), 7.21–7.08 (m, 7H, H-5′-9′, H-5, 9), 6.61 (d, J = 8.0 Hz, 2H, H-6, 8), 4.80 (t, 1H, OH), 4.64 (m, 1H, H-2), 3.89 (m, 1H, H-2′), 3.33–3.25 (m, 2H, H-1′), 2.96–2.62 ppm (m, 4H, H-3, 3′); ^{13}C NMR (100 MHz, DMSO): δ 170.7 (C-1), 163.9 (C-1″), 155.7 (C-7), 147.6 (C-4″), 139.0 (C-4′), 135.6 (C-2″), 133.9 (C-7″), 130.1 (C-5, 9), 130.0 (C-6″), 129.1 (C-6′, 8′), 128.2 (C-4), 128.0 (C-5′, 9′), 125.8 (C-7′, C-5″), 122.2 (C-3″), 114.8 (C-6, 8), 62.2 (C-1′), 55.3 (C-2), 52.5 (C-2′), 36.5, 36.4 ppm; Anal. Calcd for $C_{25}H_{25}N_3O_6$: C 64.79, H 5.44; N 9.07, Found: C 64.55, H 5.67; N 8.91.

The compounds **7**–**9** [7] and **10**–**14** [8] have been synthesized in our Lab formally.

4.2.8. N-(N-benzoyl-6-nitro-L-tyrosyl)-L-phenylalanine methyl ester (**15**)

A solution of *N*-benzoyl-L-tyrosine (**III-a**) (5.7 g, 20 mmol) in DMF (40 mL) was added dropwise to 22% HNO_3 (40 mL) at 30 °C. After stirring at 30 °C for additional 4 h, the reaction was extracted with EtOAc (100 ml). The organic phase was washed with water, dried (Na_2SO_4) and evaporated *in vacuo* to give the compound **IV** as pale yellow waxy solid (5.63 g, 85.0%). To a solution of **IV** (0.66 g, 2.0 mmol) and L-phenylalanine methyl ester hydrochloride (0.47 g, 2.2 mmol) in CH_2Cl_2 (40 mL) and DMF (10 mL) under an atmosphere of argon was successively added *N*-methylmorpholine (NMM, 0.51 ml, 4.6 mmol), isobutylchloroform (IBCF, 0.27 ml, 2.14 mmol) at −5 °C, the reaction mixture was stirred at this temperature for 15 min and then wormed up to room temperature and stirred for 9 h. Then CH_2Cl_2 was removed and the residue was diluted with water before it was extracted with EtOAc, the combined organic phase was then successively washed with saturated $NaHCO_3$ and brine, dried with $MgSO_4$, filtrated and evaporated *in vacuo*. Purification by recrystallization from EtOAc afforded the final compound **15** (0.54 g, 55%) as a yellow crystal. Mp 201.0–202.5 °C, EI-MS m/z: 491 (M$^+$), 473, 370, 285, 208, 192, 162, 122, 105 (100), 91, 77; ^{1}H NMR (400 MHz, DMSO-*d*6): δ 8.61–8.56 (2H, m, NHCO × 2), 7.91 (s, 1H, H-5), 7.76 (d, J = 8.4 Hz, 2H, H-3″,

7″), 7.51–7.41 (m, 5H, H-9, 4″-6″, ArOH), 7.26–7.18 (m, 5H, H-5′-9′), 7.03 (d, *J* = 8.4 Hz, 1H, H-8), 4.70 (m, 1H, H-2), 4.50 (m, 1H, H-2′), 3.57 (s, 3H, OCH_3), 3.07–2.87 ppm (m, 4H, H-3, 3′); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 171.8, 171.3, 166.3 (C-1″), 150.8 (C-7), 137.0 (C-4′), 136.4 (C-9), 136.1 (C-6), 133.9 (C-2″), 131.4 (C-5″), 129.5 (C-4), 129.1 (C-6′, 8′), 128.3 (C-5′, 9′), 128.2 (C-4″, 6″), 127.4 (C-3″, 7″), 126.6 (C-7′), 125.4 (C-5), 118.8 (C-8), 54.4 (C-2), 53.8 (C-2′), 51.9 (OCH_3), 36.6, 35.6 ppm; ESI–HRMS: *m*/*z* $[M + Na]^+$ Calcd for $C_{26}H_{25}N_3O_7Na$: 514.1590, Found 514.1603.

*4.2.9. Sodium N-(N-benzoyl-L-tyrosyl)-7-acetamido-L-phenylalaninate (**16**)*

To a solution of **VI** (408 mg, 0.83 mmol) in EtOH/$CHCl_3$ (1:1, v/v, 60 mL) was added aqueous NaOH (1 M, 0.84 mL), and the mixture was stirred overnight at room temperature. The solvents were removed under reduced pressure and the residue was recrystallized from EtOAc to afford compound **16** as a white solid (243 mg, 57.3%). ^1H NMR (400 MHz, DMSO-*d*6): δ 9.95 (s, 1H, ArNHCO), 9.47 (s, 1H, ArOH), 8.86 (d, *J* = 8.4 Hz, 1H, NHCO), 7.78–7.74 (m, 3H, H-3″, 7″, NHCO), 7.48 (m, 3H, H-6′, 8′, H-5″), 7.34 (t, 2H, H-4″, 6″), 7.08 (d, *J* = 8.4 Hz, 2H, H-5, 9), 7.01 (d, *J* = 8.8 Hz, 2H, H-5′, 9′), 6.61 (d, *J* = 8.4 Hz, 2H, H-6, 8), 4.44 (m, 1H, H-2), 3.95 (m, 1H, H-2′), 3.09–2.78 (m, 4H, H-3, 3′), 1.97 ppm (S, 3H, CH_3-CO); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 172.6 (C-1′), 170.0 (C-1), 168.0 (ArNHCO), 165.3 (C-1″), 155.8 (C-7), 137.2 (C-7′), 136.0 (C-2″), 133.6 (C-4′), 132.9 (C-5″), 130.0 (C-5, 9), 129.9 (C-5′, 9′), 129.4 (C-4″, 6″), 128.7 (C-4), 128.3 (C-3″, 7″), 118.4 (6′, 8′), 115.0 (C-6, 8), 56.4 (C-2′), 55.5 (C-2), 36.5, 36.1, 23.9 ppm (CH_3CO); ESI–HRMS: *m*/*z* $[M + Na]^+$ Calcd for $C_{27}H_{26}N_3O_6Na_2$: 534.1617, Found 534.1638.

*4.2.10. Sodium N-(N-5″-hydroxy-benzoyl-L-phenylalanyl)-L-phenylalaninate (**17**)*

Compound **17** was prepared from **1** according to the same procedure as for compound **16** as white solid (203 mg, 47.4%). ^1H NMR (400 MHz, DMSO-*d*6): δ 8.45 (d, *J* = 8.4 Hz, 1H, NHCO), 7.62 (d, *J* = 8.4 Hz, 2H, H-3″, 7″), 7.44 (br, 1H, NHCO), 7.29–7.05 (m, 10H, H-5–9, 5′-9′), 6.76 (m, 3H, ArOH, H-4″, 6″), 4.50 (m, 1H, H-2), 4.02 (m, 1H, H-2′), 3.11–2.92 (m, 4H, H-3, 3′); ^{13}C NMR (100 MHz, DMSO-*d*6): δ 170.2 (C-1, 1′), 166.0 (C-1″), 160.6 (C-5″), 139.1, 139.0, 129.8 (C-3″, 7″), 129.4 (C-6, 8), 129.1 (C-6′, 8′), 128.1 (C-5′, 9′), 127.5 (C-5, 9), 126.1 (C-7), 125.5 (C-7′), 124.5 (C-2″), 114.7 (C-4″, 6″), 55.7 (C-2′), 55.4 (C-2), 37.2, 36.8 ppm; ESI–HRMS: *m*/*z* $[M + Na]^+$ Calcd for $C_{25}H_{23}N_2O_5Na_2$: 477.1402, Found 477.1391.

4.3. MTT assay

Cell viability assay to test the inhibitory activity of synthetic compounds **1**–**17** was conducted on HepG2 cells by MTT assay. HepG2 cells were seeded in 96-well tissue culture plate with high-glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA). 24 h after seeded, cells were treated with series concentrations of compounds **1**–**17** respectively and incubated for 24 h. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well with final concentration of 0.5 mg/mL and incubated for 4 h. Then medium was discarded and 200 μL of DMSO was added to each well to dissolve the blue crystal. The absorbance was read by a microplate reader (Bio-Rad, USA) and cell viability after drug intervention was normalized with vehicle treated cells. Each experiment was conducted in triplicate repeat. The similar procedures were used for the evaluation the inhibition rate of compounds **1**–**17** on L-02 cells and the inhibition rate of compounds **1** and **3** on PLC/PRF/5 cells. Human hepatic cell line L-02 was obtained from Sun Yat-Sen University (Guangzhou, China). Human hepatocellular carcinoma cell line PLC/PRF/5 was obtained from ATCC (USA). Cells were cultured in DMEM medium (high glucose) supplement with 10% FBS and 1% ampicillin/streptomycin in humid incubator with 5% CO_2 at 37 °C.

4.4. Staining of intracellular ROS

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) was used to probe the intracellular ROS. Cells with intervention was trypsinized and harvested in PBS, and stained with 20 μM DCFH-DA in dark for 30 min. The stained cells were then subject to flow cytometry analysis after wash (FACSCanto II Analyzer, BD, USA). The results were analyzed with Flow Jo 7.6 (USA).

4.5. Cell cycle analysis

HepG2 cells was synchronized by starvation in FBS-free DMEM medium overnight and then released in FBS-supplemented DMEM containing compound **1** and **3** for 24 h. Cells were harvested by scrapping and fixed in 70% ethanol overnight at 4 °C. Then cells were stained with 50 μM propidium iodide (PI) in PBS for 40 min in dark. Cells were then subject to flow cytometry analysis after wash. The results were analyzed with Flow Jo 7.6 (USA).

4.6. Immunoblotting

Cells with treatment were collected and protein was extracted. 10 μg of total protein was separated on SDS PAGE by electrophoresis. The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane and blocked with 5% Bovine serum albumin (BSA) in TBST. The membrane was then incubated with primary antibodies overnight at 4 °C with rotation followed by TBST wash. Then incubation was conducted with secondary antibodies conjugated with horseradish peroxidase (HRP). Protein expression was probed under chemiluminescence detector (Biorad, USA) with ECL select as substrate (GE, Germary).

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