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Synthesis and biological evaluation of a series of novel celastrol derivatives with amino acid chain

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The synthesis of celastrol analogues containing amino acid ester at the C-29 position and their evaluation for cytotoxic activities in vitro were reported. The MTT test showed that a set of derivatives with lower IC_{50} values than that of the positive control group cisplatin and the parent compound celastrol, which exhibited greater antiproliferative activities. The most potent title compounds **2a** *and* **2e** *exhibited cytotoxic activities in vitro against Hela and A549 cell lines with IC_{50} values of 0.371 μM and 0.237 μM, 0.235 μM and 0.109 μM, respectively. The apoptosis assay demonstrated that* **2a** *and* **2e** *can induces of A549 cell apoptosis in low concentrations. These results showed that* **2a** *and* **2e** *may be promising for further research as antitumor agents.*

Keywords: *Celastrol • Synthesis • derivatives • Amino acid • cytotoxic activities*

Introduction

The natural product celastrol[1] (Figure 1), a pentacyclic triterpene extracted from the roots and barks of Tripterygium Wilfordi (thunder god vine) plant, is a natural compound with numerous beneficial pharmacological properties,which including treatment of inflammation, edema, joint pain, rheumatoid arthritis, antimalarial and antitumor[2][3].The increasing evidence has revealed that celastrol exhibited cytotoxicity against various cancer cell lines in vittro and in vivo[4-10], such as pancreatic cancer, hepatocelluar carcinomas, glioma xenografts, prostate cancer, squamous cancer, non-small cell lung carcinoma. Further studies have showed that the antitumor activities of celastrol may cause by targeting numerous signaling pathways, such as Hsp90[11], NF-kB[9], TNF-α [12], Notch[13], p-Akt[14]. These signaling path ways may paly an important role in diverse cancers.Therefore, celastrol may be the most promising lead compound for potential antitumor agent. Moreover, the latest research suggest that celastrol is a leptin sensitizer[15] and a promising agent for the pharmacological treatment of obesity; and celastrol exhibits anti-fibrotic effect by regulating collagen production against bleomycin-induced pulmonary fibrotic[16].

Despite celastrol has diverse important biological activities, its poor solubility, weak oral bioavailability and high toxicity seriously limits the development and application of this compound in treatment of cancer. Otherwise, the raw materials of celastrol is decreasing and there is very low content of celastrol in plants. For these reasons, many researchers are interested in exploring of novel celastrol analogues, which are better pharmacological activities.

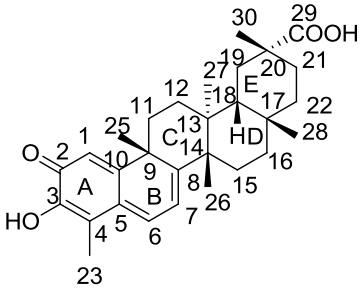


Figure.1 *The Structure of Celastrol*

The previous structure modifications of celastrol mainly concentrate on C-29-carboxylic acid group and C-3-hydroxy group. And the increasing research have been demonstrating that esterification and amidation of C-29-carboxylic acid group and C-3-hydroxy group of celastrol may increase or decline its bioactivity by bonding with different pharmacophores. Abbas designed and synthesized a series of celastrol analogues by acylating C-29-carboxylic acid group, whereas all the derivatives exhibited weaker cytotoxic activities than that of celastrol. Sun[17] and klaic[18] preparated a series of celastrol derivatives by modifying the C-29-carboxylic acid group and C-3-hydroxy group of celastrol, the bioassays demonstrated that some title analogues which containing hydrophilicity group exhibited higher antiproliferative activities than the parent compound; moreover, the further structure-activity relationship (SAR) research showed that the intact quinine methide moiety was essential for it cytotoxic activities in various cancer cell lines and neuroprotective effect, simultaneously, the studies comfirm that an amide linkage is more appropritate for identification of protein target. Tang[19] designed and synthesized a new class of C-6-indole substituted celastrol analogues and biological evaluation of some title compounds displayed better cytotoxic activities. Tang[20], Wang[21] ,Shan[22], Zhang[23] and Figueiredo[24][25] also designed and synthesized a series of celastrol derivatives by modifying the C-29-carboxylic acid group and C-3-hydroxy group, and the relation tests of anticancer activities were progressed to explore the potential anticancer agents. Unfortunately, although much effort has been made to modify the structure of celastrol, the desired analogues of celastrol are far from being completely explored.

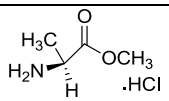
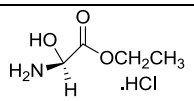
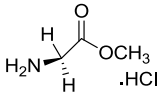
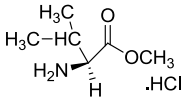
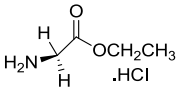
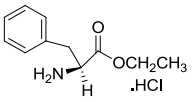
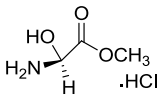
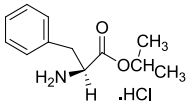
Recently, it was became a research hot-spot in medicinal chemistry that many natural products with good biological activities and drugs were modified by various amino acids, amino acids derivatives and some oligopeptides[26-30]. Firstly, many amino acids were considered as the prefect carriers of prodrugs base on they are more biological compatibility and less toxicity; secondly, some amino acids participate in regulating of gene expression, protein-synthesis and signaling pathways[31], such as arginine, glutamine, leucine, tryptophan, threonine; then the metabolic activities of the cancers cell are more vigorous, frequent and greater demand for amino acids than the normal cell lines; finally, most oral drugs were absorbed by intestinal epithelial cells, in which various membrane transporters are more distributed and higher expressed, including oligopeptide transporter protein and amino acid transporters[32-35]. For example, peptide transporter 1(PepT1), which is predominantly expressed in intestinal epithelial cells and can effectively identify and absorb the agents, which contains amino acids, amino acids derivatives and small peptides[36][37] in their structure. Interestingly, the above mentioned researchs were also showed that PepT1 is higher expressed in some tumor cell lines, such A549,HT1080,Capan-2,AsPc-1, whereas it has lower expressed in healthly tissues like lung and liver[38][39]. So many drugs, which contained the structure of amino acids, amino acids derivatives and oligopeptides, have the potential of targeting and might be identified and absorbed more easier by intestinal epithelial cells.

In this study,base on ameliorating celastrol solubility,reducing celastrol toxicity and achieving celastrol targeting effect,meanwhile to further exploring the structure-activity relationship (SAR) against various cancer cell lines,we designed and synthesized a new series of celastrol analogues, which the C-29-carboxylic acid group of celastrol was modified by multiple amino acid ester.The cytotoxic activities in vitro of these derivatives were measured.

Results and Discussion

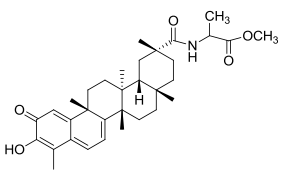
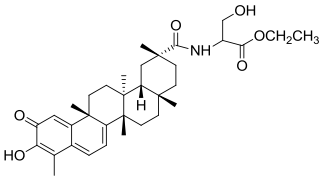
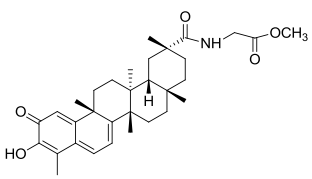
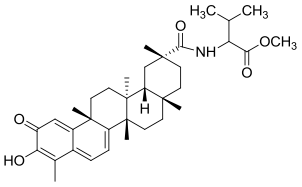
This paper presents the synthesis of the amino acid esters derivatives of celastrol by the reaction with selected amino acids such as L-Alanine, L-Glycine, L-Serine, L-Valine, L-Phenylalanine, which were outline in Scheme 1. Intermediates **1a~1h** *were prepared according to the previously reported procedure[40] by a classic method, namely, sulfinyl chloride. All the title compounds* **1a~1h** *were purified by recrystallization by using anhydrous ether and methanol or ethanol, the yield after the purification was between 80% and 90%. The structure of the intermediates and the yields are summarized in Table 1.*

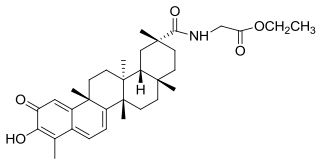
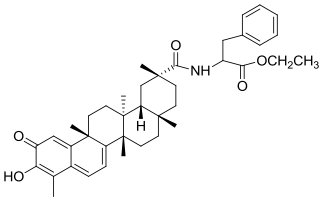
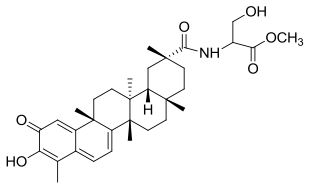
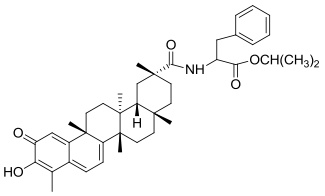
Table 1 *The structure and yields(%)obtained for* **1a~1h**

Compd	*Structure*	*Yields (%)*	*Compd*	*Structure*	*Yields (%)*
1a		*90*	**1e**		*87*
1b		*84*	**1f**		*84*
1c		*85*	**1g**		*80*
1d		*80*	**1h**		*90*

*The synthesis of the series of celastrol derivatives (***2a**~**2h***) were outlined in Scheme 1B and Table 2. Celastrol was used as lead compound and the synthesis was executed in a straightforward method, the carboxyl group at C29 of celastrol was reacted with the amino group of the intermediates in the presence of 1-hydroxybenzotriazole (HOBt) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) in CH_2Cl_2. All the derivatives (***2a**~**2h***) were prepared in a similar procedure, and their structures and yields were shown in Table 2, the eight title compounds were achieved in low yields between 30 % and 40 %. All the synthesized derivatives (***2a**~**2h***) were characterized by IR spectra, ^{1} H, ^{13}C NMR and mass spectra. The IR spectra of these title compounds revealed two absorption bands in the ranges 3300-3400 and 1590-1660 cm^{-1} indicating the -CONH- group. The compound* **2g** *was taken to illustrate structure identification as follows. In the ^{1} H NMR spectrum of* **2g**, *the signals for benzene ring protons and NH appeared at 7.09-7.25 ppm and 6.99 ppm respectively, and all other protons of* **2g** *appeared at the position were corresponding with the general studies. And the ^{13}C NMR date clearly showed the presence of the six carbon atoms of benzene ring with chemical shifts between 127-136 ppm. In addition, the presence of a molecular ion peak at m/z=626.4[M+H]+ in the mass spectra (ESI-MS) further ascertained the structure of compound* **2g**.

Table 2 *The structure and yields(%)obtained for* **2a~2h**

Compd	*Structure*	*Yields(%)*	*Compd*	*Structure*	*Yields (%)*
2a		*40*	**2e**		*40*
2b		*32*	**2f**		*35*

2c		*37*	**2g**		*37*
2d		*30*	**2h**		*33*

*The cytotoxic activities of title compounds were evaluated by MTT method using in vitro assay against Hela, A549 and HepG-2 cell lines. The results of the research on the cytotoxic activities and the cells viability of the title compounds (***2a~2h***) were summarized in Table 3, Figure 2, Figure 3 and Figure 4, respectively. All the tested compounds exhibited moderate to potent antiproliferative activities against the HepG-2 cell line. Compared with celastrol, the synthesized products showed significantly enhance cytotoxicity against Hela and A549 cell lines, except for* **2g** *and* **2h**. *Particularly, the cytotoxicity of* **2a** *(IC_{50} = 0.235 μM) and* **2e** *(IC_{50} = 0.109 μM) against A549 cell lineis nearly 5-fold and 10-fold, 25-fold and 39-fold respectively, higher than the reference compound Celastrol (IC_{50} = 1.069 μM) and the positive contral cisplatin (IC_{50} = 6.0 μM), and the further study works with* **2a** *and* **2e** *could be concentrated on Hela and A549 cell line. It is interesting that the antiproliferative activities of* **2g** *and* **2h** *were generally weaker than the activities of others title compounds and celastrol against Hela and A549 cell lines, this is probably illustrated that aromatic amino acids substitutions were not key structures for maintaining the lead compound cytotoxic activities. Based on the IC_{50} value of* **2b** *and* **2c**, **2d** *and* **2e**, **2g** *and* **2h**, *the size of eater group of amino acids has an important effect on the cytotoxicity against three cancer cell lines: the title compound with ethyl ester group express a stronger activities than that of those with methyl ester group. In general, the tested compounds displayed stronger cytotoxic activities against A549 and Hela cell line than HepG-2 cell lines, this finding is consistent with the previous reports that because A549 has expression of PepT1 while the others do not[35][36], and the agents with the structure of amino acids, amino acids derivatives might be identified by PepT1. And we hypothesize that a selecting cytotoxicity of the title compounds (***2a~2h***) to A549 cell line.*

Table 3 *Cytotoxic activities of the title derivatives toward the selected tumor cell lines*

Compounds	*Cytotoxic activities[a], IC_{50} (μM)*		
	Hela	**A549**	**HepG-2**
Celastrol[b]	*0.947±0.44*	*1.069±0.06*	*4.121±0.15*
Cisplatin[c]	*7.3±0.24*	*6.3±0.084*	*20.3±0.63*
2a	*0.371±0.16*	*0.235±0.06*	*2.077±0.1*
2b	*0.27±0.09*	*0.895±0.1*	*2.119±0.15*
2c	*0.229±0.1*	*0.545±0.1*	*1.184±0.11*
2d	*0.29±0.03*	*0.451±0.06*	*2.086±0.06*
2e	*0.237±0.09*	*0.109±0.05*	*1.598±0.09*
2f	*0.569±0.17*	*0.668±0.19*	*2.099±0.05*

2g	*2.148±0.19*	*2.314±0.60*	*2.652±0.81*
2h	*1.464±0.08*	*2.512±0.07*	*1.275±0.05*

a The values were means ± stand deviation of three determinations; b c positive control.

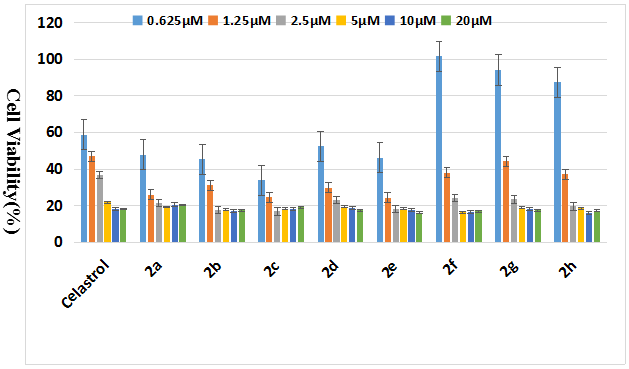


Figure 2. *The Hela cells were treated with compounds* **2a-2h***(0.625, 1.25, 2.5,5.0,10.0,20.0 μ M) and cell viability was determined by MTT assay. Columns with different background, in each graph, differ significantly (P < 0.05).*

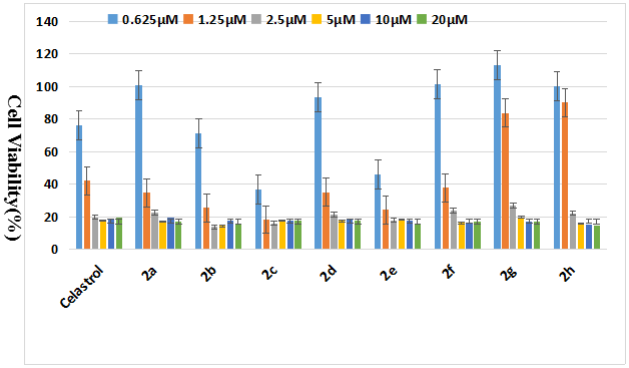


Figure 3. *The A549 cells were treated with compounds 2a-2h(0.625, 1.25, 2.5,5.0,10.0,20.0 μ M) and cell viability was determined by MTT assay.Columns with different background, in each graph, differ significantly (P < 0.05).*

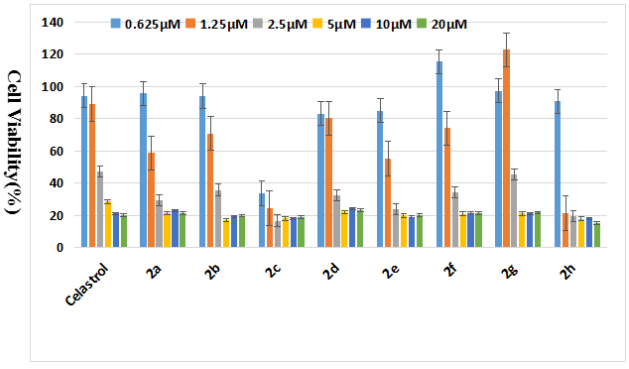


Figure 4. *The HepG-2 cells were treated with compounds 2a-2h(0.625, 1.25, 2.5,5.0, 10.0,20.0 μ M) and cell viability was determined by MTT assay.Columns with different background, in each graph, differ significantly (P < 0.05).*

AO/EB stains assay is general method to measure the result of agent induced cells apoptosis. In general, the nuclear DNA of cells with intactcell membrane could be stained by AO dye as bright green nucleus under a fluorescent microscope; and EB could only stain cells that the membrane were damaged,which appear orange-red spots. In order to observe the morphological feature changes, A549 cells were treated with the test compounds **2a** *and* **2e** *at different concentrations (0.2, 0.4, 0.6 μM) for 24 h, celastrol used as a positive control, and then were imaged under a fluorescent microscope. As seen in Figure 5, the normal cellular morphology were maintained in the control group; While the apoptosis cells were stained as bright green spots and their morphology occured deformation, including membrane blebbing, condensed and fragmented chromatin, nuclear shrinkage. In addition, compared with the concentration of 0.2 μM (*$\mathbf{2a_1}$/$\mathbf{2e_1}$/**C-1***), the morphology of apoptosis cells were more obviously changed that exposure to 0.6 μM (*$\mathbf{2a_3}$/$\mathbf{2e_3}$/**C-3***) of the tested compound. These results demonstrated that the tested compounds (***2a/2e/Celastrol***) significantly induced apoptosis of A549 cells.*

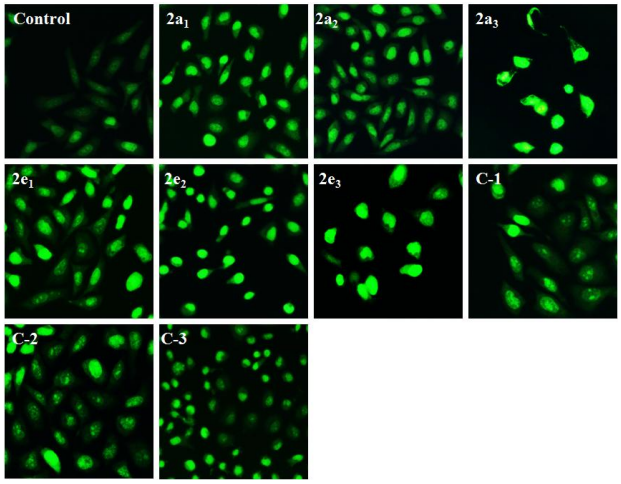


Figure 5. *Apoptosis on A549 cell line induced by test compound* **2a** , **2e** *and* **Celastrol** *(***C-1,C-2,C-3***). (control group:0.0μM;*$\mathbf{2a_1}$/$\mathbf{2e_1}$/**C-1***:0.2 μM;* $\mathbf{2a_2}$/$\mathbf{2e_2}$/**C-2***:0.4 μM;* $\mathbf{2a_3}$/$\mathbf{2e_3}$/**C-3***:0.6 μM).*

To further investigate whether these celastrol derivatives inhibited cancer cell lines growth and proliferation through induction of apoptosis, compounds **2a** *and* **2e** *were selected against A549 cell, celastrol used as a positive control. After treated with compound* **2a** ,**2e** *and celastrol for 24 h at different concentrations (0, 0.2, 0.4, 0.6 μM), the A549 cells were costained with Annexin V-FITC / PI and were measured by using flow cytometry analysis, the results were showed in Figure 6. In comparison with untreated control group, the tested compounds* **2a** *and* **2e** *caused early and late celluar apoptosis at all the setting concentrations. The percentages in the early and late apoptosis cells are 2.38 % and 5.73 % for* $\mathbf{2a_1}$*, 2.70 % and 5.72 % for* $\mathbf{2a_2}$*, 2.89 % and 6.80 % for* $\mathbf{2a_3}$*, 1.53 % and 4.41% for* $\mathbf{2e_1}$*, 2.59 % and 4.75 % for* $\mathbf{2e_2}$*, 5.56 % and 6.34 for* $\mathbf{2e_3}$*, respectively. In general, the percentages in the late apoptosis are greater than early apoptosis, and the apoptosis effect of 2e are stronger than* **2a**. *The percentages of apoptosis A549 cells in total cells after exposed with* **2a**, **2e** *and celastrol at the concentrations of 0.2, 0.4, 0.6 μM was 8.11 %, 8.42 %, 9.69 % , 5.94 %, 7.34 %, 11.90 % and 3.18 %, 3.92 % ,4.59 %, respectively, which demonstrated that compounds* **2a** *and* **2e** *significantly increased the apoptosis effect in a concentration-dependent manner, and compound* **2a** *and* **2e** *exhibited stronger effect of apoptosis than that of the positive control celastrol.*

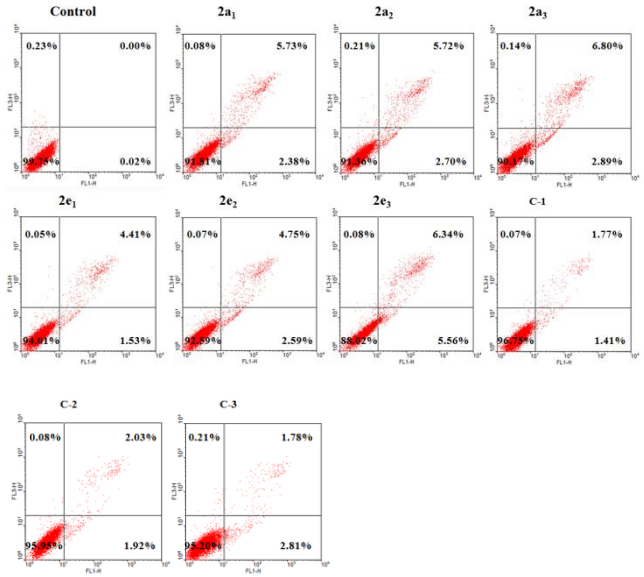


Figure 6. *(A) Active compounds* **2a** *and* **2e** *induced apoptosis of A549 cell line in flow cytometry assay compared with control group (0.0 μM) and celastrol.(B) A549 cancer cell were exposed to 0.2 μM (***$2a_1/2e_1$***/C-1),0.4μM (***$2a_2/2e_2$***/C-2) and 0.6 μM (***$2a_3/2e_3$***/C-3) for 24 h, respectively. (C) UL:necrotic cells, UR:late/secondary apoptosis viable cells, LL: normal cells, LR: early/primary apoptosis viable cells*

Conclusions

Eight novel of celastrol derivatives were synthesized and their structure were characterized in detail. Summing up, in vitro bioassays showed that celastrol derivatives displayed strongly cytotoxic activities against Hela, A549 and HepG-2 cell lines. All the new title derivatives exhibited better antiproliferative activity against Hela and A549 cell lines except **2g** *and* **2h**, *which were modified by aromatic amino acids. Particularly, the cytotoxicities of* **2a** *and* **2e** *against Hela and A549 cell lines with* IC_{50} *ranging from 0.109 to 0.371 μM, the effect of cytotoxic activities are significantly stronger than that of celastrol and cisplatin. The most potent compound* **2e**, *showed 10 and 55-fold higher antiproliferative activity against A549 cell lines than celastrol and cisplatin, respectively, which made it a promising celastrol derivative for the development of new antitumor agent. Moreover, the AO/EB and Annexin V-FITC / PI assay demonstrated that the* **2a** *and* **2e** *exhibited moderate cytotoxic activities by inducing the A549 cell early and late apoptosis in a concentration-dependent manner.*

Experimental Section

All the chemicals and reagents were purchased from commercial sources and were used without further purification. All the solvents were dried by the standard methods wherever needed. All the reactions were executed with a DF-101S collector-type thermostat heating magnetic stirrer (Yuhua Instruments Company, Gongyi). Melting points (uncorrected) were determined on a X-6 micro apparatus and the values given are uncorrected. ^{1}*H-NMR and* ^{13}*C-NMR spectra were recorded on Bruker AV-500 MHz instruments in* $CDCl_3$ *using tetramethylsilane (TMS) as the internal standard. IR spectra were determined on KBr pellets with a FTIR spectrometer in the range from 4000 to 400* cm^{-1}. *Electrospray ionization mass spectra(ESI-MS) were carried out by using Angilent technologies LC/MS. Column and thin-layer chromatography were performed on silica gel (200-300 mesh) and silica gel* GF_{254} *(Qingdao Marine Chemical Factory). And the title compounds were also purified by column chromatography on a Sephadex LH-20 column.*

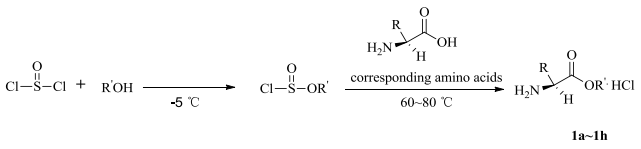
General procedure for the synthesis of ***1a~1h***

A schematic of the synthetic procedure of the hydrochloride of amino acid ester is shown in Scheme1 A. And the suitable ratio of solid to liquid are following : Amino acids： SOCl$_2$*：Alcohols =1eq：1.2~2.5eq ：19~25eq.*

*Methanol (12.8 ml, 0.3165 mol) was added under condition of ice-salt-bath and was stirred at -5 ℃. Then $SOCl_2$ (1.4 ml, 0.0195 mol) was added slowly into the methanol after 15 min and added finished completely within 30 min. The reaction mixture was stirred at ice-salt- bath (-5 ℃) for 1 h. Then L-serine (1.8 g, 0.015 mol) was added into the reaction mixture and was stirred at room temperature for 2 h. Then the reaction mixture was heated slowly to 60 ℃ and was stirred for 2 h. Subsequently, the residue was concentrated in vacuo to remove the methanol and $SOCl_2$ that unreacted. Finally, the residue was purified by crystallization from anhydrous methanol- anhydrous ether (1∶5) to give L-Serine methyl ester hydrochloride (***1d***). The synthetic procedures of* **1a~1c** *and* **1e~1h** *are similar to the* **1d** *expect the heat temperature and reaction time.*

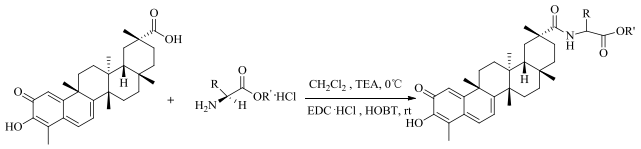
General procedure for the synthesis of ***2a~2h*** *(Scheme1 B)*

To a DCM (6 ml) solution of celastrol (45 mg, 0.10 mmol) was added HOBT (41 mg,0.30 mmol) and EDC • HCl (57 mg, 0.30 mmol) under condition of ice-salt- bath (-5 ℃). Then TEA (70 μL) was added after 20 min, and the reaction solution was stirred at -5 ℃ for 1 h. Then the corresponding amino acid esters hydrochloride (2-3eq) was added, the reaction mixture was stirred at room temperature for 12~18 h. Then the reaction mixture was washed thrice with water and the organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo to give the crude product. The purification was implemented by chromatography on a silica gel column (eluent: AcOEt/petro ether,1∶5~1∶2,v/v) and chromatography on a Sephadex LH-20 column (eluent: Methanol) to give the desired title products **2a~2h**.



1a~1h

A



Celastrol **1a~1h** **2a~2h**

a: R=CH_3 (L-Alanine) R'=CH_3

b: R=H (L-Glycine) R'=CH_3

c: R= H (L-Glycine) R'=CH_2CH_3

d: R= —OH (L-Serine) R'=CH_3

e: R= —OH (L-Serine) R'=CH_2CH_3

f: R= CH(CH3)2 (L-Valine) R'= CH_3

g: R= (L-Phenylalanine) R'=CH_2CH_3

h: R= (L-Phenylalanine) R'= CH(CH3)2

B

Scheme 1 *Synthesis Route of Compounds* **1a~1h** *and* **2a~2h**

Methyl2-((2R,4aS,6aS,12bR,14aS,14bR)-10-hydroxyl-2,4a,6a,9,12b,14a-hexamethyl-11-oxo-1,2,3,4,

4a,5,6,6a,11,12b,13,14,14a,14b-tetradecahydropicene-2carboxamido) propanoate (2a).*Red powder. Yield 40 %. M.p.131-140 ℃.IR(KBr):3376.26;2943.93;1741.72;1647.55;1581.55;1513.20;1489.88;1288.*

27;1221.95;1084.70.^{1}*H-NMR(500 MHz,*$CDCl_3$*):7.01(d,1H,J = 4.0,H-6); 6.52(s,1H,H-1); 6.39(d,1H,J =*

8.0,H-7);6.33(d, 1H, J = 8.0,NH);4.42(m,1H,CH);3.70(s,3H,OCH_3);2.20(s,3H,CH_3-23);1.43(s,3H,CH_3-25); 1.33(d,3H,J=8.0,$CHCH_3$);1.25(s,3H,CH_3-26);1.17(s,3H,CH_3-30);1.12(s,3H,CH_3-28);0.58(s,3H,CH_3-27).^{13}CNMR(500 MHz,$CDCl_3$):10.0(C-23);18.0($CHCH_3$);18.4(C-27);21.6(C-26);28.5(C-11);29.3(C-12);29

.8(C-15);30.6(C-30);30.8(C-20);31.4(C-28);33.3(C-16);33.5(C-19);34.6(C-21);36.1(C-22);38.0(C-25);39.2(C-13);40.1(C-9);42.8(C-14);44.2(C-18);44.9(C-17);47.7(NHCH);52.3(OCH_3);116.9(C-7);117

.9(C-3);119.4(C-1);126.2(C-4);127.2(C-6);133.9(C-5);145.9(C-8);164.6(C=O);170.1(C-10);173.8(C-2);177.1(C-29). ESI-MS:$C_{33}H_{45}O_5N$ calc 535,found 536 m/z$[M+H]^+$.

Methyl2-((2R,4aS,6aS,12bR,14aS,14bR)-10-hydroxy-2,4a,6a,9,12b,14a-hexamethyl-11-oxo-1,2,3,4,4a

,5,6,6a,11,12b,13,14,14a,14b-tetradecahydropicene-2-carboxamido)acetate (2b).2b *was synthesized by a similar procedure that described for* **2a**. *Red powder. Yield 42%. M.p.129-135 °C. IR (KBr): 3367.3, 2920.6, 1754.7, 1647.5, 1592.4, 1513.6, 1439.0, 1204.0, 1083.5. ^{1}H-NMR (500 MHz,$CDCl_3$): 7.00(d,1H,J = 10.0,H-6);6.51(s,1H, H-1); 6.33(d, 1H, J = 10.0, H-7); 6.30 (t, 1H, J = 5.0,NH); 3.90 (dd, 2H, J = 10.0, 5.0, $NHCH_2$); 3.71 (s, 3H, OCH_3); 2.19(s, 3H, CH_3-23); 1.42 (s, 3H, CH_3-25); 1.24 (s,3H, CH_3-26); 1.17 (s,3H,CH_3-30); 1.11(s,3H, CH_3-28); 0.56 (s,3H,CH_3-27).^{13}C-NMR(500 MHz,$CDCl_3$):10.3(C-23);18.2(C-27);21.8(C-26);28.7(C-11);29.5(C-12);29.8(C-15);30.1(C-30);30.9(C*

-20);31.2(C-28);31.7(C-16);33.6(C-19);34.9(C-21);36.4(C-22);38.3(C-25);39.4(C-13);40.4(C-9);41

.4(NHCH);43.0(C-14);44.4(C-18);45.1(C-17);52.5(OCH_3.);117.1(C-7);118.1(C-3);119.6(C-1);127.4(C

-4);134.1(C-6);146.1(C-5);164.8(C-8);170.3(C=O);170.9(C-10);178.0(C-2);178.4(C29).ESI-MS:

$C_{33}H_{43}O_5N$ calc 521,found 522 m/z$[M+H]^+$.

Ethyl2-((2R,4aS,6aS,12bR,14aS,14bR)-10-hydroxy-2,4a,6a,9,12b,14a-hexamethyl-11-oxo-1,2,3,4,4a

,5,6,6a,11,12b,13,14,14a,14b-tetradecahydropicene-2-carboxamido)acetate (2c).2c *was synthesized by a similar procedure that described for* **2a**. *Red powder. Yield, 37 %. M.p.122-128 °C. IR (KBr): 3346.7; 2925.4; 1741.9; 1644.3; 1592.4; 1513.8; 1439.5; 1202.6; 1085.2. ^{1}H-NMR (500 MHz, $CDCl_3$), 7.00 (dd, 1H, J = 10.0, 5.0, H-6); 6.51 (d,1H, J = 0.5, H-1); 6.33 (d, 1H, J = 10.0,H-7);6.29 (t, 1H, J = 5.0,NH); 4.17 (m, 2H, OCH_2); 3.90 (dd, 2H, J=10.0, 5.0, $NHCH_2$); 2.19 (s, 3H, CH_3-23); 1.43 (s, 3H, CH_3-25); 1.25 (s, 3H,CH_3-26); 1.25 (t, 3H, J = 5.0, CH_3); 1.17 (s, 3H, CH_3-30);1.12(s,3H,CH_3-28);0.56(s,3H,CH_3-27).^{13}C-NMR(500 MHz,$CDCl_3$):10.3(C-23);14.4(OCH_2CH_3);18.2(*

C-27);21.8(C-26);28.7(C-11);29.5(C-12);30.1(C-15);30.9(C-30); 31.3(C-20); 31.7(C-28); 33.6(C-16);33.7(C-19);34.9(C-21);36.4(C-22);38.3(C-25);39.4(C-13);40.4(C-9);41.6($NHCH_2$);43.1(C-14);44

.5(C-18);45.1(C-17);61.7(OCH_2CH_3);117.1(C-7);118.1(C-3);119.6(C-1);127.5(C-4);134.1(C-6);146.1

(C-5);164.8(C-8);170.3(C=O);170.4(C-10); 177.9(C-2);178.4(C-29).ESI-MS:$C_{33}H_{45}O_5N$ calc 535,found 536 m/z$[M+H]^+$.

Methyl3-hydroxy-2-((2R,4aS,6aS,12bR,14aS,14bR)-10-hydroxy-2,4a,6a,9,12b,14a-hexamethyl-11-oxo-1,2,3,4,4a,5,6,6a,11,12b,13,14,14a,14b-tetradecahydropicene-2-carboxamido)propanoate(2d).2d *was synthesiz-ed by a similar procedure that described for* **2a**. *Red powder. Yield 30 %. M.p.135-140 °C. IR (KBr): 3379.3; 2952.5; 1742.1; 1659.3; 1591.6; 1513.2; 1439.6; 1280.8; 1082.9; ^{1}H-NMR (500 MHz, $CDCl_3$): 7.02 (dd, 1H,J = 5.0, H-6);6.80 (d, 1H, J = 10.0, NH);6.51(d, 1H, J = 1.0, H-1); 6.34 (d, 1H, J = 5.0, H7); 4.49 (m, 1H, CH); 3.86 (ddd, 2H, J = 27.5, 12.5, 5.0 , CH_2OH); 3.73 (s, 3H, OCH_3); 2.19 (s, 3H, CH_3-23); 1.42 (s, 3H, CH_3-25); 1.25 (s, 3H, CH_3-26); 1.20 (s, 3H, CH_3-30); 1.12 (s, 3H, CH_3-28); 0.59 (s,3H,CH_3-27).^{13}C-NMR(500 MHz, $CDCl_3$):10.3(C-23);18.3(C-27);21.8(C-26);28.7(C-11);29.5(C-12);30.0(C-15);30.8(C-30);31.1(C-20);31.7(C-28);33.5(C-16);33*

.8(C-19);34.8(C-21);36.3(C-22);38.2(C-25);39.5(C-13);40.5(C-9);43.1(C-14);44.4(C-18);45.2(C-17

);52.8(OCH_3);54.8(NHCH);63.4(CH_2OH);117.4(C-7);118.2(C-3);119.5(C-1);127.4(C-4);134.4(C6);146.1

(C-5);165.0(C-8);170.6(C=O);171.3(C-10);177.9(C-2);178.7(C-29).ESI-MS:$C_{34}H_{49}O_5N$ calc 551,found 552 m/z[M+H]$^+$.

Ethyl3-hydroxy-2-((2R,4aS,6aS,12bR,14aS,14bR)-10-hydroxy-2,4a,6a,9,12b,14a-hexamethyl-11-oxo-1,2,3,4,4a,5,6,6a,11,12b,13,14,14a,14b-tetradecahydropicene-2-carboxamido-)Propanoate(2e).2e *was synthesized by a similar procedure that described for* **2a**. *Red powder. Yield 40 %. M.p.148-153 ℃.IR (KBr) cm^{-1}: 3365.9; 2939.4; 1737.2; 1643.4; 1591.9; 1513.2; 1440.4; 1287.7; 1223.1; 1084.9. ^{1}HNMR (500 MHz, $CDCl_3$): 7.01 (dd, 1H, J = 7.0, 1.5, H-6); 6.79 (d, 1H, J = 10.0, NH); 6.51(d, 1H, J= 1.00 , H-1); 6.34 (d, 1H, J = 10.0, H-7); 4.47 (m, 1H, CH); 4.17 (m, 2H, CH_2OH); 3.86 (m, 2H, OCH_2); 2.19 (s, 3H, CH_3-23); 1.42 (s, 3H, CH_3-25); 1.25 (s, 3H, CH_3-26); 1.24 (t, 3H, OCH_2CH_3); 1.20 (s, 3H, CH_3-30); 1.12(s, 3H, CH_3-28); 0.60 (s, 3H, CH_3-27). ^{13}C-NMR (500 MHz,$CDCl_3$): 10.37(C-23);14.17(CH_3);18.39(C-27);21.86(C-26);28.80(C-11);29.55(C-12);30.06(C-15);*

30.09(C-30);31.11(C-20);31.72(C-28);33.60(C-16);33.88(C-19);34.84(C-21);36.41(C-22);38.29(C-25)

;39.51(C-13);40.53(C-9);43.15(C-14);44.46(C-18);45.19(C-17);54.96(NHCH);62.09(OCH_2);63.64(CH_2OH)

;117.36(C-7);118.21(C-3);119.62(C-1);127.50(C-4);134.34(C-6);146.17(C-5);164.95(C-8);170.54(C=

O);170.84(C-10);178.46(C-2);178.76(C-29).ESI-MS:$C_{34}H_{47}O_6N$ calc 565,found 566 m/z[M+H]$^+$.

Methyl2-((2R,4aS,6aS,12bR,14aS,14bR)-10-hydroxy-2,4a,6a,9,12b,14a-hexamethyl-11-oxo-1,2,3,4,4a

,5,6,6a,11,12b,13,14,14a,14b-tetradecahydropicene-2-carboxamido)-3-methylbutano-ate(2f).2f *was synthesiz-ed by a similar procedure that described for* **2a**.*Red powder. Yield 35 %. M.p.205-208 ℃.IR (KBr): 3368.5; 2988.4; 1728.1; 1665.5; 1607.9; 1517.2; 1439.8; 1285.5; 1202.8; 1082.2. ^{1}H-NMR (500 MHz,$CDCl_3$): 7.03 (d, 1H, J = 10.0, H-6); 6.53 (s, 1H, H-1); 6.34 (d, 1H, J=5.0,H-7); 6.32 (d, 1H, J = 10.0, NH); 4.42 (dd, 1H, J=10.0, 5.0, NHCH); 3.67 (s, 3H, OCH_3); 2.52 (m, 1H, $CHCH_3$); 2.21 (s, 3H, CH_3-23); 1.42 (s, 3H, CH_3-25); 1.25 (s, 3H, CH_3-26); 1.18 (s, 3H, CH_3-30); 1.13 (s, 3H, CH_3-28); 0.88 (d, 3H, J=5.00, $CHCH_3$); 0.87 (d, 3H, J=5.00, $CHCH_3$); 0.53(s,3H,CH_3-27).^{13}C-NMR(500 MHz, $CDCl_3$): 10.41(C-23);17.96(CH_3);18.20(CH_3);18.98(C-27);21.97(C*

-26);28.81(C-11);29.48(C-12);30.25(C-15);30.80($CHCH_3$);30.96(C-30);31.68(C-20);31.76(C-28);33.4

9(C-16);34.12(C-19);34.91(C-21);36.42(C-22);38.24(C-25);39.45(C-13);40.82(C-9);43.21(C-14);44.

40(C-18);45.23(C-17);52.32(OCH_3);56.85(NHCH);117.25(C-7);118.18(C-3);119.60(C-1);127.51(C-4);

134.31(C-6);146.16(C-5);164.96(C-8);170.54(C=O);173.03(C-10);177.95(C-2);178.43(C-29).ESI-MS:

$C_{35}H_{49}O_5N$ calc 563,found 564 m/z[M+H]$^+$.

Ethyl2-((2R,4aS,6aS,12bR,14aS,14bR)-10-hydroxy-2,4a,6a,9,12b,14a-hexamethyl-11-oxo-1,2,3,4,4a

,5,6,6a,11,12b,13,14,14a,14b-tetradecahydropicene-2-carboxamido)-3-Phenyl propanote (2g).2g *was synthesized by a similar procedure that described for 2a.Red powder. Yield 37 %. M.p.97-103 ℃.IR (KBr): 3389.5; 2924.3; 1736.7; 1662.9; 1593.8; 1514.0; 1439.4; 1374.9; 1287.3; 1202.3; 1080.2. 1H-NMR (500 MHz,$CDCl_3$): 7.07~7.25 (m, 5H, 5 Ph); 7.06 (d, 1H, J = 6.0, H-6); 6.99 (dd, 1H, J = 7.0, 1.0, NH); 6.52 (d, 1H, J = 1.0, H-1); 6.31 (d, 1H, J = 7.5, H-7); 4.68 (dd, 1H, J = 12.5, 5.5, CH); 4.10 (m, 2H, OCH_2); 3.07 (ddd, 2H, J = 19.5, 13.5, 5.5, CH_2); 2.19 (s, 3H, CH_3-23); 1.42 (s, 3H, CH_3-25);1.23 (s, 3H, CH_3-6); 1.20 (t, 3H, J = 7.5, CH_3); 1.10 (s, 3H,CH_3-30);1.09(s,3H,CH_3-28);0.52(s,3H,CH_3-27).^{13}CNMR(500 MHz,$CDCl_3$):10.38(C-23);14.17(CH_3);18.2*

6(C-27);21.93(C-26);27.06(CH_2);28.79(C-11);29.47(C-12);30.12(C-15);30.84(C-30);30.94(C-20);31.

73(C-28);33.62(C-16);34.85(C-19);36.41(C-21);37.98(C-22);38.29(C-25);39.41(C-13);40.58(C-9);43.

15(C-14);44.36(C-18);45.16(C-17);52.95(CH);61.68(OCH_2);117.08(C-7);118.12(C-3);119.71(C-1);127.

17(para-Ph-C);127.50(C-4);128.56(ortho-Ph-2C);129.51(meta-Ph-2C);134.07(C-6);136.16(Ph-C);146.

14(C-5);164.88(C-8);170.42(C=O);172.04(C-10);177.24(C-2);178.46(C-29). ESI-MS:$C_{40}H_{51}O_5N$ calc 625,found 626 m/z[M+H]$^+$.

Isopropyl2-((2R,4aS,6aS,12bR,14aS,14bR)-10-hydroxy-2,4a,6a,9,12b,14a-hexamethyl-11-oxo-1,2,3,4,

4a,5,6,6a,11,12b,13,14,14a,14b-tetradecahydropicene-2-carboxamido)-3-phenyl propa-noate (2h).2h *was synthesized by a similar procedure that described for* **2a**.*Red powder. Yield 33 %. M.p. 92-96 ℃.IR (KBr): 3371.7; 2938.3; 1731.3; 1651.1; 1594.1; 1514.0; 1439.3; 1375.1; 1287.0; 1105.8.* ^{1}*H-NMR (500 MHz, $CDCl_3$): 7.09~7.25 (m, 5H, 5 Ph); 7.08 (d, 1H, J = 6.5, H6); 6.98 (dd,1H, J=6.0, 1.0, NH); 6.51 (d, 1H, J = 1.0, H-1); 6.30 (d, 1H, J = 6.0, H-7); 4.91 (m, 1H, OCH); 4.65 (dd, 1H, J = 13.0, 1.0, CH); 3.05 (ddd, 2H, J = 20.0, 14.0 ,6.0 ,CH_2); 2.19 (s,3H, CH_3-23);1.41(s, 3H,CH_3-25);1.22 (s, 3H,CH_3-26); 1.14 (d,3H,J = 6.0,CH_3); 1.11 (d,3H,J = 6.5,CH_3);1.10 (s,3H,CH_3-30);1.09(s,3H,CH_3-28);0.51(s,3H,CH_3-27).*^{13}*C-NMR(500 MHz,$CDCl_3$):10.37(C-23);18.28(C-27);21.77(2×*

CH_3);21.89(C-26);28.78(C-11);29.46(C-12);30.09(C-15);30.87(C-30);30.91(C-20);31.71(C-28);33.61

(C-16);33.61(CH_2);34.83(C-19);36.40(C-21);38.03(C-22);38.28(C-25);39.40(C-13);40.55(C-9);43.13

(C-14);44.34(C-18);45.13(C-17);53.01(CH);69.57(OCH);117.08(C-7);118.08(C-3);119.69(C-1);127.11

(para-,Ph-C);127.46(C-4);128.48(ortho-,Ph-2C);129.55(meta-,Ph-2C);134.04(C-6);136.17(Ph-C);146.

10(C-5);164.86(C-8);170.43(C=O);171.51(C-10);177.29(C-2);178.45(C-29).ESI-MS:$C_{41}H_{53}O_5N$ calc 639

,found 662 m/z[M+Na]$^+$.

Cell lines and cell culturing

Three cell lines used in this research were purchased from the American Type Culture Collection. The cell lines were all maintained in RPMI1640 medium and were incubated at 37 ℃in a 5% CO_2 incubator.

MTT Assay

To evaluate the cytotoxic activities of synthesized analogues, the antiproliferative activities of **2a**~**2h** *were screened in a MTT assay[23-25] in three human cancer cell lines: human non-small cell lung cancer (A549), Hela Cells and human hepatoma cells (HepG-2). In general, the cells were seeded in 96-well culture plates and incubated overnight at 37 ℃ in a 5 % CO_2 incubator. All the title tested compounds were then added to the wells to achieve final concentrations as (0.625, 1.25, 2.5 5.0 10.0, 20.0 μM), which were gained by dissolving in DMSO and diluting with PBS (DMSO final concentration < 0.02 %). Cisplatin and celastrol were also used as the positive control. After 48 h incubation, 20 μL of the MTT solution (5 mg/mL in PBS) were added to each well and the plates were incubated for 4 h. Then the medium was removed from each well and DMSO (100 μL) was added to solubilize the MTT formazan. The optical density was then measured at 490 nm on a microplate spectrophotometer. The cytotoxicities of the title compounds were expressed as IC_{50} value that was defined as the concentration at 50 % of cell survival relative to the control. All experiment was repeated in triplicate.*

Cell apoptosis assay by AO/EB staining methods

A549 cell line were routinely placed on chamber slides in six-well plates (2×10^5 cells per well) and incubated for 24 h. Then RPMI 1640 with 10 % of PBS was added to each well and incubated at 37 ℃ in a 5 % CO_2. The medium was discarded and replaced with medium (DMSO final concentration < 0.02 %) containing the title compounds **2a** *and* **2e** *(0, 0.2, 0.4, 0.6 μM) for 24 h. Subsequently, the medium was removed and the cell were washed with PBS three times, and fixed with 4 % formalin. Then cells were stained with AO/EB dye mix (100 μg/ml AO, 100 μg/ml EB) for 10 min. Finally, the results of induction of apoptosis were visualized by a fluorescence microscope (Nikon, Yokohama, Japan).*

Cell apoptosis assay by flow cytometry

A549 cell were seeded in six-well tissue culture plates (2× 105 cells per well) at 37 ℃ in a 5 % CO_2. Then different concentrations of **2a** *and* **2e** *(0, 0.2, 0.4 , 0.6 μM) were added into each well and incubated for 48 h at 37 ℃ in a 5 % CO_2. Cells were trypsinized, washed with PBS and fixed with 70 % ethanol after incubating, and maintained at 4 ℃ for overnight. Then cells were costained with Annexin V-FITC and PI for 15 min in the dark. The results of apoptosis of cells were detected by a FACSCalibur flow cytometry (Beckman Dickinson &Co., Franklin Lakes, NJ)and the date was processed by WinMDI software. The minimum number of cells analyzed for per sample was 10000.*

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Author Contribution Statement

Chaohai Pang and Dingyong Wang performed all the synthesis and wrote the acticle.Chunhua Liu and Xuejin Wu checked the manuscript.Dingyong Wang and Jinhui Luo are correspondance author.

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