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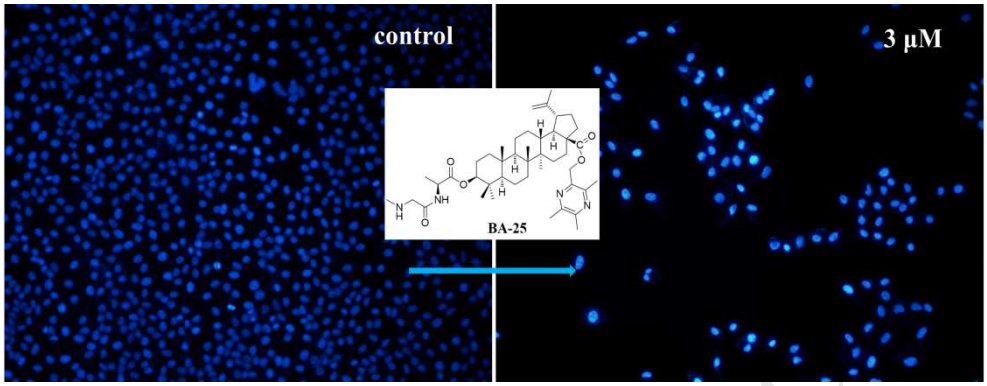
Graphical Abstract

New series of TBA amino acid/dipeptide derivatives were synthesized and evaluated for their antitumor activity. **BA-25** was found to be the most active one.



Original article

Combination of Amino Acid/Dipeptide with Ligustrazine-Betulinic Acid as Antitumor Agents

Bing Xu[a], Wen-Qiang Yan[a], Xin Xu[a], Gao-Rong Wu[a], Chen-Ze Zhang[a], Yao-Tian Han[a], Fu-Hao Chu, Rui Zhao[a], Peng-Long Wang[a,*], Hai-Min Lei[a,*]

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Abstract: The lead compound **TBA**, 3β-Hydroxy-lup-20(29)-ene-28-oic acid-3, 5, 6-trimethylpyrazin-2-methyl ester, which exhibited promising antitumor activity and induced tumor cell apoptosis in various cancer cell lines, had previously been reported. Moreover, reports have revealed that the introduction of amino acid to betulinic acid could improve selective cytotoxicity as well as water solubility. Thus, a series of novel **TBA** amino acid and dipeptide derivatives were designed, synthesized and screened for selective cytotoxic activity against five cancer cell lines (HepG2, HT-29, Hela, BCG-823 and A549) and the not malignant cell line MDCK by standard MTT assay. Most of the tested **TBA**-amino acid and dipeptide analogues showed stronger anti-proliferative activity against all tested tumor cell lines than **TBA**. Among them, **BA-25** exhibited the greatest cytotoxic activity on tumor cell lines (mean IC_{50} = 2.31±0.78 μM), that was twofold than the positive drug cisplatin (**DDP**), while it showed lower cytotoxicity on MDCK cell line than DDP. Further cell apoptosis analyses indicated **BA-25**-induced apoptosis was associated with loss of mitochondrial membrane potential and increase of intracellular free Ca^{2+} concentration.

Keywords: Betulinic acid, Amino acids derivatives, Dipeptide derivatives, Selective cytotoxicity, Structure-activity relationships, Apoptosis;

1. Introduction

Cancer is one of the major diseases which threats human life and health seriously [1-2]. The cytotoxic agents are still one of the main clinical treatments for the malignant tumor. However, these drugs usually have some severe side effects with poor patients compliance, therefore the discovery of the targeted anti-tumor drugs is of great significance [3-6]. Because of their strong selective cytotoxicity and potent apoptosis induction activity, pentacyclic triterpenoids and their derivatives had become the focus of scientific interest [7-10].

Ligustrazine (2,3,5,6-tetramethylpyrazine, TMP), a major effective component of traditional Chinese medicine Rhizoma Chuanxiong (*Ligusticum, chuanxiong Hort*), has been used for the treatment of cardiovascular and cerebrovascular diseases in the clinic in China for many years [11-13]. Recently, ligustrazine was found to possess anticancer activity *in vivo* and *in vitro*, it could induce cancer cell apoptosis and reverse multidrug resistance in tumors [14-16]. Meanwhile, recent researches revealed that the introduction of ligustrazine to the anti-tumor components could increase their cytotoxicity and selectivity [17, 18]. This has stimulated interest in using ligustrazine as the scaffold to synthesize new anticancer agents by combination it with other anti-tumor ingredients [16, 19-21]. In our previous study, we successfully synthesized a series of novel ligustrazine-triterpenes derivatives and observed that these derivatives possessed potent selective cytotoxicity, of which, 3β-Hydroxy-lup-20(29)-ene-28-oic acid-3, 5, 6-trimethylpyrazin-2-methyl ester (**TBA**) displayed promising selective cytotoxicity (IC_{50} < 5.23 μM) [17-19, 21, 22]. In addition, reports have shown that the introduction of amino acid or dipeptide to triterpenes could improve selective cytotoxicity as well as water solubility [8, 21, 23-25]. Based on the above, we attempted the synthesis of several **TBA** amino acids and dipeptide derivatives **BA-X** by introducing various amino acids or dipeptides to the C3 of **TBA**, in order to improve its antitumor activities and tumor targeting. All newly synthesized compounds were fully characterized by ^{1}H-NMR, ^{13}C-NMR, HRMS and tested for cytotoxic activity against a panel of tumor cell lines and normal cell line, including HepG2, HT-29, Hela, BGC823, A549 and MDCK. Meanwhile, the

preliminary anti-tumor mechanisms of the most potent compound were also investigated by fluorescence staining observation and flow cytometric analysis in present study. In addition, the structure-activity relationships of these derivatives were briefly discussed.

2. Results and discussion

2.1. Chemistry

The designed derivatives were prepared following the procedures in Scheme 1-4. The compound **TBA** (**BA-01**) was prepared according to our previous study with some modifications [19]. According to the literature procedure, the intermediate (3,5,6-trimethylpyrazin-2-yl)methanol (**4**) was successively obtained [26]. Then the intermediate **4** was further reacted with tosyl chloride (TsCl) in tetrahydrofuran (THF) in the presence of triethylamine (TEA) and 4-dimethylaminopyridine (DMAP) to yield the important intermediate 2-(chloromethyl)-3,5,6-trimethylpyrazine (**5**) (Scheme 1). Subsequently, it underwent alkylation reaction in N, N-dimethylformamide (DMF) with betulinic acid (BA) to afford the compound **TBA** (**BA-01**) (Scheme 2).

(Insert Scheme 1)

(Insert Scheme 2)

The **TBA** amino acid derivatives **BA-02---BA-15** (Scheme 3, Table 1) were obtained by 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) mediated esterification from the corresponding protected (N-Boc, N-Cbz) amino acids and **TBA**. Deprotection was performed with trifluoroacetic acid (TFA) in dry dichloromethane (DCM) or by treating the compounds with Pd/C (10%) in methanol (MeOH). It was worth noting that, to avoid the formation of undesirable by-products, the hydroxyl groups of Cbz-L-serine and Cbz-L-threonine should be protected with tert-Butyldimethylsilyl chloride (TBDMSCl) before esterification with **TBA** in the synthesis of **BA-14** and **BA-15**. Removal of the TBDMS groups was achieved using

1M tetrabutylammonium fluoride (TBAF) solution in THF (Scheme 3).

(Insert Scheme 3)

(Insert Table 1)

(Insert Table 1)

In similar fashions, the TBA dipeptide derivatives **BA-16---BA-27** were prepared according to the procedures in Scheme 4. In brief, the TBA amino acids derivatives **BA-02**, **BA-04**, **BA-06** and **BA-12** underwent peptide coupling reactions with the corresponding N-Boc protected amino acids (L-Gly, L-Sar, L-Pro, L-Ala) in the presence of EDCI, 1-hydroxybenzotriazole (HOBt) and N, N-Diisopropylethylamine (DIPEA) in dry DCM to afford the corresponding **TBA** dipeptide intermediates, which were further treated with TFA in dry DCM to give the final compounds **BA-16---BA-27** (Table 2). The structures of all target derivatives were confirmed by spectral (^{1}H-NMR, ^{13}C-NMR and HRMS) analysis.

(Insert Scheme 4)

(Insert Table 2)

2.2. Biology

2.2.1 Cytotoxicity Assay

The *in vitro* antitumor activity of **TBA** amino acids and dipeptide derivatives was evaluated on five tumor cell lines (HepG2, HT-29, Hela, BCG-823, A549) using the standard MTT assay, and their toxicity was tested using MDCK cells. The IC_{50} values were summarized in Table 3. As shown in Table 3, after combination with amino acid or dipeptide, most of the targeted compounds showed significantly improved cytotoxicity on all tested tumor cell lines compared to the starting material **TBA**. The cytotoxicity detection also revealed that most of **TBA** amino acids derivatives (such as **BA-02**, **BA-09**, **BA-10**, **BA-12** et al) and nearly all **TBA** dipeptide derivatives exhibited better antiproliferative activities than the positive drug DDP, while they showed lower cytotoxicity than DDP on MDCK cell line. Among the candidates,

BA-25 was the most active one, which exhibited perfect antiproliferative activities (mean IC_{50} = 2.31±0.78 μM) on all tested cancer cell lines. For example, the IC_{50} values of **BA-25** for HT-29, Hela and BGC-823 (1.70±0.34 μM, 1.74±0.99 μM, 1.79±0.28 μM) is much lower than those of the positive drug cisplatin (DDP) (4.10±1.17 μM, 5.60±0.78 μM, 4.25±0.32 μM). Meanwhile **BA-25** exhibited higher cytotoxic selective towards MDCK cells (IC_{50} = 10.84±0.27 μM) than DDP. It is worth noting that, although the compound **BA-10, BA-23** and **BA-24** exhibited slightly lower cytotoxicity than **BA-25** on the tested cancer cells, their pronounced cytotoxic selective (therapeutic index (TI)>10) towards MDCK cells distinguished them from this series. Their IC_{50} value for MDCK cells was more than 40 uM (see Table 3).

From the obtained results, it was observed that **TBA** aliphatic amino acids derivatives exhibited better anti-proliferative activities than those aromatic amino acids and heterocyclic amino acids derivatives, as exemplified by **BA-02**, **BH-04**, **BA-10**, **BA-12** > **BA-03**, **BA-11**; Structure-activity relationship analysis among the **TBA** aliphatic amino acids derivatives also revealed that the compounds with small molecule aliphatic amino acids seemed to be more active than those with high molecular weight aliphatic amino acids, such as **BA-02**, **BA-04** > **BA-07**, **BA-08**. In addition, it was observed the **TBA** basic amino acids derivatives seemed to be more active than those acidic amino acids derivatives, such as **BA-10** > **BA-05**. The result also revealed that the **TBA** dipeptide derivatives were more active than the amino acids derivatives. Potency is an important criterion for assessing leads, but it is not the only consideration when selecting a lead compound for further optimization into a drug [27, 28]. The selectivity, solubility and hydrophobicity were also essential to the perfect drug candidates, because these properties were closely associated with absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of the compounds [29-31]. Thus the compound **BA-10**, **BA-23**, **BA-24** and **BA-25** was selected for further pharmacodynamics and pharmacokinetic evaluation, including the *in vivo* antitumor activity and plasma stability, *in vivo* pharmacokinetics, in the hope of producing drug candidates for drug development. And the results will be reported in due course. Meanwhile the most active compound **BA-25** (mean IC_{50} = 2.31±0.78 μM) was selected for further analysis to study its mechanism of growth inhibition on HepG2 cell line in this study.

(Insert Table 3.)

2.2.2. Analyses of apoptosis

2.2.2.1. Morphological detection of apoptosis using Giemsa staining

To investigate the mechanism of HepG2 cell death induced by **BA-25**, the morphologic changes of cells were observed by Giemsa staining. As shown in Fig. 1, the morphology of HepG2 cells in the negative control was normal; When the cells treated with **BA-25** (1.5, 3, 6 μM) for 72 h, typical signs for apoptosis were found. Even at low concentration of **BA-25** (1.5 μM), cells had showed typical apoptosis morphological features, such as cell shrinkage, chromatin margination and nuclear fragmentation. With the increase of the concentration of **BA-25**, the characteristic of apoptosis was more and more obvious. When the concentration of **BA-25** was raised up to 6 μM, almost all cells showed typical apoptotic morphological changes such as nuclear condense, nuclear fragmentation. These results indicated that **BA-25** significantly induced HepG2 cells apoptosis.

(Insert Fig. 1)

2.2.2.2. Morphological detection of apoptosis using DAPI staining

To further characterize the effects of apoptosis induction of **BA-25** on HepG2, the nucleus morphological changes in **BA-25**-treated HepG2 were observed by DAPI staining. After **BA-25** treatment for 72 h, HepG2 cells showed nuclear morphological changes typical of apoptosis in a dose-dependent manner, such as nuclear condensation, nuclear fragmentation and the formation of apoptotic bodies (Fig. 2).

(Insert Fig. 2)

2.2.2.3. Detection of apoptosis using Annexin V-FITC/PI staining

To further perform a comprehensive view on the apoptosis induced by **BA-25**, apoptotic rates were analyzed by flow cytometry using an Annexin V-FITC/PI staining. As shown in Fig. 3, the HepG2 cells demonstrated a remarkable response to the apoptotic effect of **BA-25** in a dose-dependent fashion. The apoptotic effect was determined by counting the apoptosis ratios (including the early and late apoptosis ratios). Following different concentrations (2, 3, 4 μM) of **BA-25** treatment, the apoptosis ratios increased from 6.3% of the control to 10.9%, 17.8%, 36.8%,

176 respectively. These results indicated that **BA-25** had the potential to induce HepG2
177 cell apoptosis.

178 (Insert Fig. 3)

179 2.2.2.4. Measurement of mitochondrial membrane potential

180 Disruption of mitochondrial membrane potential ($\Delta\Psi m$) is one of the earliest
181 intracellular events that occur following the onset of apoptosis [32, 33]. To study
182 whether the mitochondrial events were involved in the apoptosis induced by **BA-25**,
183 $\Delta\Psi m$ was measured quantitatively using flow cytometry with cell-permeable cationic
184 dye Rhodamine 123 (Rh123). Rh123 accumulates in normal mitochondria due to its
185 high negative charge, and the reduction of $\Delta\Psi m$ will lead to the release of Rh123 and
186 reduction of its fluorescence intensity [34]. As shown in Fig. 4, the fluorescent
187 intensity decreased in a dose-dependent manner. HepG2 cells treated with **BA-25** (2
188 μM, 3 μM, 4 μM) for 72 h exhibited a lower fluorescent intensity (MFI: 6416, 5411,
189 2147) compared with untreated HepG2 cells (MFI: 26410) (Fig. 4).

190 (Insert Fig. 4)

191 2.2.2.5. Assessment of intracellular Free Ca^{2+}

192 A sustained increase in intracellular Ca^{2+} concentrations is recognized as a factor for
193 cell death or injury [35]. The membrane-permeable Ca^{2+}-sensitive fluorescent dye
194 Fluo-3AM was used to evaluate the level change of intracellular free Ca^{2+} in HepG2
195 cell line after co-culture with **BA-25**. The Fluo-3 AM can across the cell membrane
196 and be cut into Fluo-3 with the cells. The Fluo-3 can specifically combine with the
197 Ca^{2+} and has a strong fluorescence with an excitation wavelength of 488 nm [36, 37].
198 As shown in Fig. 5, with the increase of **BA-25** concentration, intracellular free Ca^{2+}
199 fluorescence increased dramatically (MFI 2743, 3760, 6353) compared with the control
200 group (MFI 1992). The results indicated that the increase of intracellular Ca^{2+} was
201 related with **BA-25**-induced HepG2 cell apoptosis (Insert Fig. 5).

202 **3. Conclusions**

203 In this study, we successfully synthesized 27 novel **TBA** amino acid and dipeptide
204 derivatives by attaching different amino acids or dipeptides to the C3 of **TBA**. Their
205 cytotoxicity was determined by the standard MTT assay. The result indicated that

most of the synthesized **TBA**-amino acid and dipeptide analogues showed significantly improved selective cytotoxicity against all tested tumor cell lines compared to **TBA**. From the obtained result, we also observed that the compounds with small molecule aliphatic amino acids seemed to be more active than those with high molecular weight aliphatic amino acids, such as **BA-02, BA-04** > **BA-07, BA-08**; The **TBA** dipeptide derivatives were more active than the amino acids derivatives. Among all the new derivatives, compound **BA-25** showed the greatest cytotoxicity on tumor cell lines (IC_{50} = 2.31±0.78 μM), that was twofold than the positive drug DDP, while it showed lower cytotoxicity on MDCK cell line than DDP. Further anti-tumor mechanistic investigation showed that **BA-25**-induced apoptosis in HepG2 cells was involved loss of the mitochondria membrane potential and increase of intracellular free Ca^{2+} concentration. In addition, although the compound **BA-10**, **BA-23** and **BA-24** exhibited slightly lower cytotoxicity than **BA-25** on the tested cancer cells, their pronounced cytotoxic selective towards MDCK cells distinguished them from this series. And the compound **BA-10**, **BA-23**, **BA-24** and **BA-25** was selected for further pharmacodynamics and pharmacokinetic evaluation, including the *in vivo* antitumor activity and plasma stability, *in vivo* pharmacokinetics, in the hope of producing drug candidates for drug development. These results suggested that the attempt to discover high selectivity anti-tumor lead compounds by introduction of amino acid or oligopeptides to cytotoxic agents was viable.

4. Experimental Section

4.1. Chemistry

Reagents were bought from commercial suppliers without any further purification. Melting points were measured at a rate of 5 °C/min using an X-5 micro melting point apparatus (Beijing, China) and were not corrected. Reactions were monitored by TLC using silica gel coated aluminum sheets (Qingdao Haiyang Chemical Co., Qingdao, China). The specific rotation was measured using an Autopol I (Rudolph Instruments) automatic polarimeter at 25 °C in MeOH. NMR spectra were recorded on a BRUKER AVANCE 500 NMR spectrometer (Fällanden, Switzerland) with tetramethylsilane (TMS) as an internal standard; chemical shifts δ were given in ppm and coupling

constants *J* in Hz. HR-MS were acquired using a Thermo Sientific TM LTQ Orbitrap XL hybrid FTMS instrument (Thermo Technologies, New York, NY, USA). Cellular morphologies were observed using an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan).

*(3,5,6-trimethylpyrazin-2-yl)methanol (**4**)*. Compound **4** was prepared according to the method described by Deng et al. [26].

*2-(chloromethyl)-3,5,6-trimethylpyrazine (**5**)*. To a solution of the important intermediate (3,5,6-trimethylpyrazin-2-yl)methanol (**4**) (10mmol) in dry tetrahydrofuran (20 mL), TsCl (13mmol), TEA (20mmol) and DMAP (2mmol) were added. The mixture was allowed to stir at room temperature for 12 h. Then the solution was evaporated under vacuum and washed with brine. After drying the organic layer over anhydrous Na_2SO_4 and evaporating the solvent under vacuum, the crude product was purified by flash chromatography (silica gel, petroleum ether: acetone = 10:1).

*3β-Hydroxy-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**TBA**)*. Compound **5** (5.0 mmoL) and betulinic acid (5.0 mmoL) were dissolved in 25 mL dry DMF, then K_2CO_3 (5.0 mmoL) was added and the mixture was at 25 °C for 12 h. Then the reaction mixture was poured into ice-water and the crude product was extracted with ethyl acetate. After drying the organic layer over anhydrous Na_2SO_4 and evaporating the solvent under vacuum, the crude product was purified by flash chromatography (silica gel, petroleum ether: acetone = 10:1). White powder, yield 54.1%, mp: 184.6–185.4 °C, $[\alpha]_D$ = +16 (c 0.50, MeOH); ^{1}H-NMR ($CDCl_3$) (ppm): 0.78, 0.80, 0.82, 0.96, 0.98, 1.67 (s, 3H, 30-CH_3 of BA), 2.51 (s, 3H, -CH_3), 2.53 (s, 3H, -CH_3), 2.57 (s, 3H, -CH_3), 3.02 (m, 1H), 3.19(m, 1H), 4.61, 4.74 (each, brs, 1H, =CH_2), 5.20, 5.23 (each, d, *J* = 12.5 Hz, 1H, -CH_2); ^{13}C-NMR ($CDCl_3$) (ppm): 14.7, 15.4, 15.9, 16.1, 18.3, 19.4, 20.4 (-CH_3), 20.9, 21.4 (-CH_3), 21.6 (-CH_3), 25.5, 27.4, 28.0, 29.7, 30.6, 32.1, 34.4, 36.9, 37.2, 38.1, 38.7, 38.9, 40.7, 42.4, 46.9, 49.5, 50.6, 55.4, 56.7, 64.3 (-CH_2), 79.0, 109.6, 145.4, 148.7, 148.9, 150.5, 150.9, 175.5 (-COO-); HRMS (ESI) m/z: 591.45212 $[M + H]^+$, calcd. for $C_{38}H_{58}N_2O_3$ 590.44474.

*4.1.1. General Procedure for the Preparation of TBA amino acids derivatives **BA-02---BA-13***

4.1.1.1. General procedure for esterification at carbon C3 (method A)

The compound **TBA** (1 equiv.) was dissolved in dry DCM (25 mL), DMAP (0.1 equiv.) and the protected amino acid (1.3 equiv.) were added. After addition of EDCI (1.5 equiv.), the mixture was stirred at 25 °C for 12 h. The reaction mixture was diluted with 50 ml CH_2Cl_2, then successively washed with water and brine (10 mL each), dried over sodium sulphate and filtered, and the solvent was evaporated. Then the crude product was purified by flash chromatography (silica gel, dichloromethane: methanol = 40:1).

4.1.1.2. General procedure for deprotection (method B)

To a solution of the Boc-protected compound in dry DCM (10 mL), TFA (1 mL/10 mL DCM) was added. The mixture was allowed to stir in ice bath for 2 h. After completion of the reaction (as monitored by TLC), the solution was evaporated and washed with a saturated sodium carbonate solution (20 mL). The aqueous layer was extracted with DCM (3 × 25 mL), the combined organic extracts were washed with brine (20 mL), dried over sodium sulfate, filtrated and evaporated. Purification was performed by flash chromatography (silica gel, dichloromethane: methanol = 40:1).

4.1.1.3. General procedure for the deprotection (method C)

Pd/C (10%; 80 mg) was added to a solution of the Cbz-protected compound in 30 mL MeOH. The mixture was stirred at room temperature for 12 h. After completion of the reaction (as monitored by TLC), the solvent was filtered to remove Pd/C. The filtrate was concentrated in vacuum and the residue was purified by flash chromatography (silica gel, dichloromethane: methanol = 40:1).

3β-(Glycyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-02).*** Obtained from **TBA** by method A and method B as a colorless powder; Yield: 75.3%; mp: 104.7–105.4 °C, $[\alpha]_D$ = +22 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77, 0.83, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (s, each, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -CCHCH_2-), 3.44-3.47 (m, 2H, -CH_2NH_2), 4.50-4.53 (m, 1H, -OCOCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.6, 18.3, 19.5, 20.6 (-CH_3), 21.0, 21.5 (-CH_3), 21.8 (-CH_3), 23.9, 25.6, 28.1,

30.7, 32.2, 34.4, 37.1, 37.2, 38.0, 38.2, 38.5, 40.8, 42.5 (-CH_2NH_2), 47.0, 49.6, 50.6, 55.6, 56.8, 64.5 (-CH_2), 81.9 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 173.9 (-$CONH_2$), 175.7 (-COO-); HRMS (ESI) m/z: 648.47040 $[M+H]^+$, calcd. for $C_{40}H_{61}N_3O_4$ 647.46621.

*3β-(L-Phenylalanyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-03**).* Obtained from **TBA** by method A and method C as a colorless powder; Yield: 65.3%; mp: 82.0–82.7 °C; $[\alpha]_D$ = +186 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.76, 0.77, 0.80, 0.82, 0.94 (s, each, 3H, 5×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.82-2.86 (m, 1H, -CH_2CH-), 2.96-3.02 (m, 1H, -CCHCH_2-), 3.15-3.18 (m, 1H, -CH_2CH-), 3.72-3.76 (m, 1H, -$CHNH_2$), 4.49-4.52 (m, 1H, -OCOCH-), 4.59, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-), 7.19-7.33 (m, 5H, -C_6H_5); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.6, 15.9, 16.2, 16.6, 18.1, 19.3, 20.5 (-CH_3), 20.9, 21.4 (-CH_3), 21.7 (-CH3), 23.7, 25.5, 28.0, 29.6, 30.5, 32.0, 34.2, 36.9, 37.1, 37.9, 38.1, 38.3, 40.7, 41.1, 42.4, 46.9, 49.5, 50.4, 55.4, 56.1, 56.7, 64.4 (-CH_2), 81.9, 109.7 (-CH=C-), 126.8, 128.6, 129.3, 137.3, 145.3, 148.8, 150.5, 156.0 (-CH=C-), 174.4, 175.5 (-COO-); HRMS (ESI) m/z: 738.51666 $[M+H]^+$, calcd. for $C_{47}H_{67}N_3O_4$ 737.51316.

*3β-(L-Alanyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-04**).* Obtained from **TBA** by method A and method B as a colorless powder; Yield: 73.0%; mp: 126.3–127.2 °C, $[\alpha]_D$ = +20 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.84, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (brs, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -CCHCH_2-), 3.45-3.55 (m, 1H, -$CHNH_2$), 4.48-4.51 (m, 1H, -OCOCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 19.5, 20.6 (-CH_3), 20.8, 21.0, 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.1, 29.8, 30.7, 31.1, 32.2, 34.4, 37.0, 37.2, 38.1, 38.2, 38.5, 40.8, 42.5, 47.0, 50.5 (-$CHNH_2$), 50.6, 55.5, 56.8, 64.5 (-CH_2), 81.6 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0,

150.6, 151.1 (-CH=C-), 175.7 (-COCH-), 176.3 (-COO-); HRMS (ESI) m/z: 662.48627 $[M+H]^+$, calcd. for $C_{41}H_{63}N_3O_4$ 661.48186.

3β-(L-Aspartyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-05).*** Obtained from **TBA** by method A and method C as a colorless powder. Yield: 45.6%; mp: > 210 °C, $[\alpha]_D$ = +36 (c 0.50, MeOH); HRMS (ESI) m/z: 706.47534 $[M+H]^+$, calcd. for $C_{42}H_{63}N_3O_6$ 705.47169.

3β-(L-Prolyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-06).*** Obtained from **TBA** by method A and method B as a colorless powder; Yield: 72.7%; mp: 147.4–148.3 °C, $[\alpha]_D$ = +13 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77, 0.83, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (brs, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -CCHCH_2-), 3.22-3.25 (m, 1H, -CH_2CH_2-), 4.10-4.13 (m, 1H, -NHCHCH_2-), 4.47-4.56 (m, 1H, -OCOCH-), 4.59, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.2, 19.5, 20.6 (-CH_3), 21.0, 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 24.6, 25.5, 28.2, 29.7, 29.8, 30.6, 32.1, 34.3, 37.0, 37.2, 38.2, 38.4, 40.8, 42.5, 46.4, 47.0, 49.6, 50.6, 55.5, 56.8, 59.7 (-NHCH-), 64.5 (-CH_2), 83.5 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 171.5 (-COCH-), 175.7 (-COO-); HRMS (ESI) m/z: 688.50122 $[M+H]^+$, calcd. for $C_{43}H_{65}N_3O_4$ 687.49751.

3β-(L-Leucyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-07).*** Obtained from **TBA** by method A and method B as a colorless powder; Yield: 58.4%; colorless powder; mp: 81.8–82.5 °C, $[\alpha]_D$ = +24 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.83 (s, each, 3H, 2×-CH_3, methyl of BA), 0.84 (brs, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -CCHCH_2-), 3.48 (brs, 1H, -CHNH_2), 4.48-4.51 (m, 1H, -OCOCH-), 4.59, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0,

16.3, 16.8, 18.3, 19.5, 20.6 (-CH_3), 21.0, 21.5 (-CH_3), 21.8 (-CH_3), 22.0, 23.1, 23.8, 25.0, 25.6, 28.2, 29.8, 30.7, 32.2, 34.4, 37.1, 37.2, 38.1, 38.2, 38.5, 40.8, 42.5, 47.0, 49.6, 50.6, 55.6, 56.8 (-$CHNH_2$), 64.5 (-CH_2), 69.1, 81.9 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 175.7 (-COO-); HRMS (ESI) m/z: 704.53546 $[M+H]^+$, calcd. for $C_{44}H_{69}N_3O_4$ 703.52881.

3β-(L-Isoleucyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-08).*** Obtained from **TBA** by method A and method B as a colorless powder; Yield: 60.5%; mp: 88.6–89.4 °C, $[\alpha]_D$ = +27 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.82 (s, each, 3H, 2×-CH_3, methyl of BA), 0.85 (brs, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -CCHCH_2-), 3.48 (brs, 1H, -$CHNH_2$), 4.50-4.53 (m, 1H, -OCOCH-), 4.59, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, J = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 11.9, 12.0, 14.8, 15.9, 16.0, 16.3, 16.9, 18.3, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.9, 24.8, 25.6, 26.2, 28.2, 29.8, 30.7, 32.2, 34.4, 37.1, 37.2, 37.9, 38.2, 38.5, 40.8, 42.5, 47.0, 49.6, 55.6, 56.8 (-CH-NH_2), 59.3, 64.5 (-CH_2), 82.5 (-OCOCH-), 109.8 (-CH=C-), 136.9, 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 175.7 (-COO-); HRMS (ESI) m/z: 704.52814 $[M+H]^+$, calcd. for $C_{44}H_{69}N_3O_4$ 703.52881.

3β-(L-Pyroglutamyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-09).*** Obtained from **TBA** by method A as a colorless powder; Yield: 52.8%; mp: 118.2–119.1 °C, $[\alpha]_D$ = +24 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.82, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.83 (brs, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -CCHCH_2-), 4.22-4.24 (m, 1H, -NHCH-), 4.52-4.55 (m, 1H, -OCOCH-), 4.59, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, J = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 18.5, 20.5, 21.0 (-CH_3), 21.5 (-CH_3), 21.7 (-CH_3), 23.8, 25.2, 25.6, 28.2, 29.4, 29.7, 29.8, 30.7, 32.1, 34.3, 37.0, 37.2, 38.1, 38.2, 38.4, 40.8, 42.5, 47.0 (-CH-NH-), 49.6, 50.6, 55.5, 55.8, 56.8, 64.4 (-CH_2), 82.7 (-OCOCH-), 109.8 (-CH=C-), 145.6,

148.8, 149.1, 150.6, 151.0 (-CH=C-), 171.7 (-COCH-), 175.7 (-COO-), 177.7 (-CONH-); HRMS (ESI) m/z: 702.48419. $[M+H]^+$, calcd. for $C_{43}H_{63}N_3O_5$ 701. 47677.

3β-(L-Lysyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-10).*** Obtained from **TBA** by method A and method C as a colorless powder; Yield: 56.8%; mp: >220 °C, $[\alpha]_D$ = +30 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 0.67, 0.88, 0.89, 0.91, 1.00 (s, each, 3H, 5×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.52, 2.53, 2.60 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.93-3.01 (m, 2H, -CH_2NH_2), 3.01-3.04 (m, 1H, -CCHCH_2-), 3.57-3.60 (m, 1H, -CHNH_2), 4.52-4.55 (m, 1H, -OCOCH-), 4.61, 4.71 (brs, each, 1H, =CH_2), 5.23, 5.27 (d, each, 1H, *J* = 20Hz, -OCH_2-); ^{13}C-NMR (125 MHz, CD_3Cl+ CD_3OD): δ (ppm) 14.0, 15.1, 15.5, 15.9, 17.6, 18.5, 19.3 (-CH_3), 20.3 (-CH_3), 20.4 (-CH_3), 21.7, 23.1, 25.0, 26.4, 27.4, 29.1, 30.0, 31.5, 31.6, 33.7, 36.3, 36.6, 37.4, 37.7, 37.9, 38.7, 40.2, 41.9, 46.6, 48.9, 50.0, 52.9, 55.0, 56.3 (-CH-NH_2), 63.5 (-CH_2), 82.5 (-OCOCH-), 111.5 (-CH=C-), 145.2, 148.6, 149.3, 150.0, 150.8 (-CH=C-), 168.7 (-COCH-), 175.4 (-COO-); HRMS (ESI) m/z: 719.54608. $[M+H]^+$, calcd. for $C_{44}H_{70}N_4O_4$ 718.53971.

3β-(L-Tryptophanyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-11).*** Obtained from **TBA** by method A and method C as a colorless powder; Yield: 61.2%; mp: 119.8–120.6 °C, $[\alpha]_D$ = +30 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78 (s, 6H, 2×-CH_3, methyl of BA), 0.83 (s, 6H, 2×-CH_3, methyl of BA), 0.94 (s, 3H, -CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.49, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.95-3.02 (m, 2H, -CH_2NH_2), 3.35-3.37 (m, 1H, -CCHCH_2-), 3.70-3.81 (m, 1H, -CHNH_2), 4.45-4.53 (m, 1H, -OCOCH-), 4.68, 4.72 (brs, each, 1H, =CH_2), 5.18, 5.21 (d, each, 1H, *J* = 15Hz, -OCH_2-), 7.05-7.20, 7.29-7.37, 7.60-7.63 (m, 4H, -C_6H_5); ^{13}C-NMR (125 MHz, $CDCl_3$) : δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.1, 29.8, 30.1, 30.7, 32.2, 34.4, 37.1, 37.2, 38.1, 38.2, 38.5, 40.8, 42.5, 47.0, 49.6, 50.6, 54.9, 55.5, 56.8 (-CH-NH_2), 64.5 (-CH_2), 82.5 (-OCOCH-), 109.8 (-CH=C-), 110.3, 111.6, 111.4, 118.8, 119.6, 122.3, 123.8 (-NHCH-), 127.3, 136.5, 145.5, 148.9, 148.9, 150.6, 151.1 (-CH=C-), 175.7 (-CO-); HRMS (ESI) m/z: $[M+H]^+$ 777.53125,

calcd. for $C_{49}H_{68}N_4O_4$ 776.52406.

3β-(L-Sarkosyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-12).*** Obtained from **TBA** by method A and method B as a colorless powder; Yield: 76.9%; mp: 172.6–173.4 °C, $[\alpha]_D$ = +42 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$) : δ (ppm) 0.77, 0.84, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (brs, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.40, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -CCHCH_2-), 3.52 (brs, 2H, -CH_2NH-), 4.48-4.51 (m, 1H, -OCOCH-), 4.56, 4.59 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.6, 18.3, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.9, 25.6, 28.2, 29.8, 30.7, 32.2, 34.4, 34.6, 37.0, 37.2, 38.0, 38.2, 38.5, 40.8, 42.5, 47.0, 49.6, 50.6, 50.7, 55.6, 56.8 (-CH-NH_2), 64.5 (-CH_2), 83.2 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 168.6 (-COCH-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 662.48975, calcd. for $C_{41}H_{63}N_3O_4$ 661.48186.

3β-(L-Valyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-13).*** Obtained from **TBA** by method A and method C as a colorless powder; Yield: 52.3%; mp: > 220 °C, $[\alpha]_D$ = +20 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.82, 0.94 (s, each, 3H, 3×-CH_3, methyl of BA), 0.85 (brs, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -CCHCH_2-), 3.33-3.34 (m, 1H, -CHNH_2), 4.49-4.52 (m, 1H, -OCOCH-), 4.59, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.8, 18.3, 17.1, 18.3, 19.5, 19.6, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.9, 25.6, 28.2, 29.8, 30.7, 31.6, 32.2, 34.4, 37.1, 37.2, 38.0, 38.2, 38.5, 40.8, 42.5, 47.0, 49.6, 50.6, 55.6, 56.8 (-CH-NH_2), 60.2, 64.5 (-CH_2), 82.2 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 690.52112, calcd. for $C_{43}H_{67}N_3O_4$ 689.51316.

4.1.2. General Procedure for the Preparation of TBA amino acids derivatives ***BA-14---BA-15***

It was worth noting that, to avoid the formation of by-products, the hydroxyl groups of Cbz-L-serine Cbz-L-threonine should be protected with TBDMSCl before esterification with **TBA** in the synthesis of **BA-14** and **BA-15**. To a magnetically stirred solution of the corresponding N-Cbz-protected amino acid (N-Cbz-L-serine N-Cbz-L-threonine) (1.0 equiv.) and imidazole (2.0 equiv.) in dry N, N-dimethyl formamide (10 mL) at 0°C, the TBDMSCl (1.5 equiv.) was added. Then the reaction mixture was allowed to warm to room temperature and stirred for 24h. After completion of the reaction (as monitored by TLC), the reaction mixture was poured into ice-water, and the sediment was filtered and washed with water. Then the residue was dissolved with DCM, dried over sodium sulfate and evaporated under vacuum, and the crude product (N-Cbz-TBDMS-L-serine, N-Cbz-TBDMS-L-threonine) was obtained.

The starting material **TBA** (1 equiv.) was dissolved in dry DCM (20 mL), DMAP (0.1 equiv.) and the protected amino acid (1.3 equiv.) were added. After addition of EDCI (1.5 equiv.), the mixture was stirred at 25 °C for 12 h. Then the solution was washed with brine. After drying the organic layer over anhydrous Na_2SO_4 and evaporating the solvent under vacuum, the crude product was purified by flash chromatography (silica gel, dichloromethane: methanol = 40:1).

The Cbz-protected compound was dissolved in 20 mL MeOH, Pd/C (10%; 80 mg) was added. The mixture was allowed to stir at room temperature for 12 h and filtered to remove Pd/C. The filtrate was concentrated in vacuum and the residue was further reacted with 1.5 equivalents of 1M TBAF in THF for 1.5 h at 25 °C. Then the solvent was evaporated under vacuum. Purification was performed by flash chromatography (silica gel, dichloromethane: methanol = 40:1).

3β-(L-Threonyl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-14).*** Yield: 46.7%; colorless powder; mp: 103.1-103.9 °C, $[\alpha]_D$ = -4 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.85, 0.86 (s, each, 3H, 3×-CH_3,

methyl of BA), 0.82 (brs, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.97-3.00 (m, 1H, -CCHCH_2-), 3.39-3.58 (m, 1H, -CHNH_2), 3.85-3.98 (m, 1H, -CHOH), 4.54-4.55 (m, 1H, -OCOCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.16, 5.19 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.8, 18.3, 19.5 (CH_3CH-), 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.9, 25.5, 27.7, 28.2, 29.0, 29.7, 30.6, 32.1, 34.3, 37.0, 37.2, 38.0, 38.2, 38.5, 40.8, 42.5, 47.0, 49.6, 50.5, 55.6 (-CH-NH_2), 56.8, 64.5 (-CH_2), 67.2 (-CHOH), 82.6 (-OCOCH-), 95.8, 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 175.75 (-CO-); HRMS (ESI) m/z: $[M+H]^+$ 692.49957, calcd. for $C_{42}H_{65}N_3O_5$ 691.49242.

3β-(L-Seryl)-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester ***(BA-15).*** Yield: 47.5%; colorless powder; mp: 133.2-134.1 °C, $[\alpha]_D$ = +16 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77, 0.82, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.83 (brs, 6H, 2×-CH_3, methyl of BA), 1.66 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.97-3.00 (m, 1H, -CCHCH_2-), 3.32-3.35 (s, 1H, -CHNH_2), 3.63 (brs, 1H, -OH), 3.72-3.75 (m, 1H, -CH_2OH), 3.86-3.94 (m, 1H, -CH_2OH), 4.52-4.56 (m, 1H, -OCOCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.16, 5.19 (d, each, 1H, *J* = 15Hz,-OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.9, 24.3, 25.5, 28.1, 29.7, 30.6, 32.1, 34.3, 37.0, 37.2, 38.1, 38.2, 38.4, 40.8, 42.5, 47.0, 49.6, 50.5, 55.5 (-CH-NH_2), 56.8, 62.5 (-CH_2OH), 64.5 (-CH_2), 82.4 (-OCOCH-), 82.7, 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 678.48480, calcd. for $C_{41}H_{63}N_3O_5$ 677.47677.

4.1.3. General Procedure for the Preparation of TBA dipeptide derivatives ***BA-16---BA-27***

The corresponding TBA amino acid derivatives (**BA-02**, **BA-04**, **BA-06**, **BA-12**) (1.0 equiv.), DMAP (0.1 equiv.) and N-Boc-protected amino acids (Boc-L-glycine, Boc-L-alanine, Boc-L-sarcosine, Boc-L-proline) were dissolved in 25 mL dry DCM,

EDCI (0.1 equiv.) was added. The mixture was stirred at room temperature overnight. Then the solution was washed with brine, dried over sodium sulfate, filtered and the solvent was evaporated. Purification was performed by flash chromatography (silica gel, dichloromethane: methanol = 40:1). Then the Boc-protected compounds were further reacted with TFA (1mL per 10mL DCM) in dry DCM in ice bath for 2 h. After completion of the reaction (as monitored by TLC), the solution was evaporated and washed with a saturated sodium carbonate solution (20 mL). The aqueous layer was extracted with DCM (3 × 25 mL), the combined organic extracts were washed with brine (20 mL), dried over sodium sulfate, filtrated and evaporated. Purification was performed by flash chromatography (silica gel, dichloromethane: methanol = 30:1).

*3β-[(L-Glycyl)-L-Glycyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-16**).* Yield: 52.8%; colorless powder; mp: 130.8-131.7 °C, $[\alpha]_D$ = +15 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 0.67, 0.98 (s, each, 3H, 2×-CH_3, methyl of BA), 0.88 (brs, 9H, 3×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.52, 2.53, 2.60 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.98-3.04 (m, 1H, -CCHCH_2-), 3.35 (s, 2H, -CH_2NH_2), 3.98 (s, 2H, -CH_2NH-), 4.50-4.53 (m, 1H, -OCOCH-), 4.61, 4.71 (brs, each, 1H, =CH_2), 5.23, 5.26 (d, each, 1H, *J* = 15Hz, -OCH_2-), 5.51 (s, 1H, -NH-). ^{13}C-NMR (125 MHz, CD_3OD): δ (ppm) 15.1, 16.4, 16.7, 16.9, 19.2, 19.5, 20.4, 21.2 (-CH_3), 21.4 (-CH_3), 22.0 (-CH_3), 24.6, 26.7, 28.4, 30.7, 31.6, 33.1, 35.4, 37.8, 38.2, 39.0, 39.6, 41.9 (-CH_2-NH-), 42.1, 43.5 (-CH_2-NH_2), 45.6, 50.6, 51.7, 54.8, 56.8, 57.9, 65.0 (-CH_2), 83.4 (-OCOCH-), 110.4 (-CH=C-), 147.1, 150.3, 150.7, 151.7, 152.5 (-CH=C-), 160.0, 171.3 (-CONH-), 175.7 (-COCH_2-), 177.0 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 705.49500, calcd. for $C_{42}H_{64}N_4O_5$ 704.48767.

*3β-[(L-Sarkosyl)-L-Sarkosyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester **(BA-17)***. Yield: 62.3%; colorless powder; mp: 118.4-119.2 °C, $[\alpha]_D$ = +12 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.76, 0.81, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 1.66 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 3.04-3.17 (m, 2H, -CH_2NH-), 2.98 (s, 3H, CH_3-N-), 2.98-3.04 (m, 1H, -CCHCH_2-), 3.96 (s, 3H,

CH_3-NH-), 3.96-4.12 (m, 2H, -CH_2N-), 4.50-4.51 (m, 1H, -OCOCH-), 4.58, 4.70 (brs, each, 1H, =CH_2), 5.16, 5.19 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.4, 19.5, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.1, 29.7, 30.7, 32.2, 33.4, 34.4, 35.8 (CH_3N-), 37.0, 37.3, 38.0, 38.2, 38.8, 39.0, 40.8, 42.5, 47.0, 49.6, 50.6, 51.8, 55.4 (-CH-NH_2), 56.8, 64.5 (-CH_2), 79.1 (CH_3NH-), 82.9 (-OCOCH-), 109.7 (-CH=C-), 145.5, 148.9, 149.0, 150.7, 151.1 (-CH=C-), 163.3, 168.4 (-CH_2COO-), 175.7 (-CO-); HRMS (ESI) m/z: $[M+H]^+$ 733.52527, calcd. for $C_{44}H_{65}N_3O_5$ 732.51897.

*3β-[(L-Prolyl)-L-Sarkosyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-18**)*. Yield: 60.7%; colorless powder; mp: 158.5-159.3 °C, $[\alpha]_D$ = +52 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.81, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (s, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.50, 2.53, 2.56 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.95-2.99 (m, 1H, -CCHCH_2-), 3.11 (s, 3H, CH_3N-), 3.42, 3.46 (brs, each, 1H, -CH_2-NH-), 3.68-3.71 (m, 1H, -CHNH-), 4.51-4.53 (m, 1H, -OCOCH-), 4.59, 4.71 (brs, each, 1H, =CH_2), 5.18, 5.21 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 19.5, 20.4, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.9, 25.6, 28.2, 29.4, 29.8, 30.7, 32.1, 34.3, 36.4, 37.0, 37.2, 38.0, 38.2, 38.5, 40.8, 42.5, 47.0, 49.6, 50.4, 50.6, 55.5, 56.8, 58.3, 62.4, 64.4 (-CH_2), 83.1 (-OCOCH-), 109.8 (-CH=C-), 145.8, 148.6, 149.3, 150.6, 151.1 (-CH=C-), 163.3, 168.0 (-CH_2COO-), 169.3 (-CON-), 175.6 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 759.54059, calcd. for $C_{46}H_{70}N_4O_5$ 758.53462.

*3β-[(L-Alanyl)-L-Glycyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester **(BA-19)***. Yield: 58.9%; colorless powder; mp: 117.8-118.7°C, $[\alpha]_D$ = +17 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77, 0.83, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (s, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.87-2.95 (s, each, 2H, -NH_2-), 2.96-3.01 (m, 1H, -CCHCH_2-), 3.61-3.63 (m, 1H, -CH-), 4.01 (d, 2H, -NHCH_2-), 4.50-4.53 (m, 1H, -CHCH_3), 4.58, 4.70 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H,

J = 15HZ, -OCH_2-), 7.80 (s, 1H, -NH-CH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.6, 18.3, 19.5, 21.0, 20.6 (-CH_3), 21.4, 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.1, 29.7, 30.7, 32.2, 34.4, 37.0, 37.2, 38.0, 38.2, 38.5, 39.0, 40.8, 41.4 (-NH-CH_2-), 42.5, 47.0, 49.6, 50.6 (-$CHNH_2$), 50.7, 55.5, 56.8, 64.5 (-CH_2), 82.6, 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 169.9 (-CH_2-COO-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 719.51080, calcd. for $C_{43}H_{66}N_4O_5$ 718.50332.

*3β-[(L-Prolyl)-L-Glycyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-20**).* Yield: 59.3%; colorless powder; mp: 122.6-123.5 °C, $[\alpha]_D$ = +4 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77, 0.83, 0. 93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (s, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.99-3.03 (m, 2H, -CH_2NH-), 3.07-3.12 (m, 1H, -CCHCH_2-), 3.94-3.97 (m, 1H, -NHCH-), 4.00-4.02 (m, 2H, -CH_2CH_2-), 4.49-4.52 (m, 1H, -OCOCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz,-OCH_2-), 8.19 (s, 1H, -NH-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.6, 18.3, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 26.1, 28.2, 29.8, 30.7, 30.8, 32.2, 34.4, 37.1, 37.2, 38.0, 38.2, 38.5, 40.8 (-NHCH-), 41.4, 42.5, 47.0, 47.3, 49.6, 50.6, 55.5, 56.8, 60.5, 62.4 (-CHNH-), 64.5 (-CH_2), 82.6 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 150.6, 151.1 (-CH=C-), 169.9 (-CONH-), 175.7 (-COOCH-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 745.52527, calcd. for $C_{45}H_{68}N_4O_5$ 744.51897.

*3β-[(L-Sarkosyl)-L-Glycyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-21**).* Yield: 57.6%; colorless powder; mp: 117.8-118.6 °C, $[\alpha]_D$ = +16 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77 (s, each, 3H, -CH_3, methyl of BA), 0.82 (brs, 9H, 3×-CH_3, methyl of BA), 0.93 (s, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.97-3.01 (m, 1H, -CCHCH_2-), 3.32 (s, 2H, -NHCH_2-), 3.78-4.03 (m, 2H, -CH_2CO-), 4.50-4.53 (m, 1H, -OCOCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.16, 5.19 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125

MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.6, 18.3, 19.4, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 21.9, 22.0, 23.7, 25.6, 28.0, 29.7, 30.7, 32.2, 34.4, 34.6, 37.0, 37.2, 38.0, 38.2, 38.5, 40.8, 42.5, 47.0, 49.6, 50.6, 55.5 (-$NHCH_2$-), 56.8, 64.5 (-CH_2), 82.6 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.1, 151.1 (-CH=C-), 168.6 (-CONH-), 170.8 (-COOCH-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 719.51044, calcd. for $C_{43}H_{66}N_4O_5$ 718.50332.

*3β-[(L-Alanyl)-L-Alanyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-22**).* Yield: 53.1%; colorless powder; mp: 118.3-119.2 °C, $[\alpha]_D$ = -2 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.94 (s, each, 3H, 2×-CH_3, methyl of BA), 0.82-0.83 (m, 9H, 3×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.96-3.01 (m, 1H, -$CCHCH_2$-), 3.56-3.60 (m, 1H, NH_2CH-), 4.49-4.52 (m, 1H, -OCOCH-), 4.52-4.57 (m, 1H, -NHCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-), 7.75-7.76 (d, 1H, -NH-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 18.7, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.1, 29.8, 30.7, 32.2, 34.4, 37.1, 37.2, 38.1, 38.2, 38.4, 40.8, 41.6, 42.5, 47.0, 48.4 49.6 (NH_2CH-), 50.6, 55.5, 56.8 (-NHCH-), 64.5 (-CH_2), 82.4 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.1, 151.1 (-CH=C-), 172.8 (-COOCH-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 733.52606, calcd. for $C_{44}H_{68}N_4O_5$ 732.51897.

***3**β-[(L-Glycyl)-L-Alanyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester **(BA-23)**.* Yield: 55.3%; colorless powder; mp: 172.4-173.3 °C, $[\alpha]_D$ = -2 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.83, 0.94 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (s, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.54 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.97-3.01 (m, 1H, -$CCHCH_2$-), 3.56 (brs, 2H, -CH_2NH_2), 4.49-4.52 (m, 1H, -OCOCH-), 4.58 (m, 1H, -NHCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-), 7.87 (s, 1H, -NH-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 18.8, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.1, 29.8, 30.7, 32.2, 34.4, 37.1, 37.2, 38.1, 38.2, 38.4, 40.8, 42.5 (NH_2CH_2-), 47.0, 48.2, 49.6, 50.6, 50.7, 55.5

(-NHCH-), 56.8, 64.5 (-CH_2), 82.3 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 172.9 (-COCH-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 719.51044, calcd. for $C_{43}H_{66}N_4O_5$ 718.50332.

*3β-[(L-Prolyl)-L-Alanyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-24**).* Yield: 56.4%; colorless powder; mp: 113.7-114.5 °C, $[\alpha]_D$ = -6 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77, 0.79, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.81-0.86 (m, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.49, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.65-2.70 (m, 1H, -NHCH-), 2.91-3.03 (m, 2H, -CH_2NH-), 3.04-3.08 (m, 1H, -CCHCH_2-), 3.82-3.83 (m, 1H, -CHNH-), 4.47-4.56 (m, 1H, -OCOCH-), 4.70, 4.73 (brs, each, 1H, =CH_2), 5.16, 5.19 (d, each, 1H, *J* = 15Hz, -OCH_2-), 8.07-8.09 (d, 1H, -NH-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.1, 29.7, 30.7, 31.0, 32.2, 34.3, 37.0, 37.2, 38.0, 38.2, 38.5, 40.8, 42.5, 47.0, 47.3, 49.6, 50.0, 50.6 (-NHCH-), 55.5, 56.8, 60.5, 62.8, 64.5 (-CH_2), 78.9, 82.2 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 170.8 (-CONH-), 174.7 (-COOCH-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 759.54053, calcd. for $C_{46}H_{70}N_4O_5$ 758.53462.

*3β-[(L-Sarkosyl)-L-Alanyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-25**).* Yield: 58.7%; colorless powder; mp: 136.5-137.3 °C, $[\alpha]_D$ = -2 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77, 0.83, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.82 (s, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.63-2.73 (m, 1H, -NHCH-) 2.94-3.01 (m, 1H, -CCHCH_2-), 3.35-3.44 (m, 2H, -NHCH_2-), 4.48-4.51 (m, 1H, -OCOCH-), 4.58, 4.71 (brs, each, 1H, =CH_2), 5.17, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-), 7.80-7.82 (d, 1H, -NH-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 18.7, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.1, 29.8, 30.7, 32.2, 34.4, 36.2, 37.1, 37.2, 38.1, 38.2, 38.4, 40.8, 42.5, 47.0, 48.2, 49.6, 50.6, 53.7, 55.5, 56.8, 64.5 (-CH_2), 82.4 (-OCOCH-), 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 172.7

(-CONH-), 175.7 (-COO-); HRMS (ESI) m/z: $[M+H]^+$ 733.52521, calcd. for $C_{44}H_{68}N_4O_5$ 732.51897.

*3β-[(L-Glycyl)-L-Sarkosyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-26**).* Yield: 78.5%; colorless powder; mp: 117.2-118.1 °C, $[\alpha]_D$ = +14 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.77, 0.79, 0.93 (s, each, 3H, 3×-CH_3, methyl of BA), 0.81 (s, 6H, 2×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.50, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.97-3.01 (m, 1H, -CCHCH_2-), 3.00-3.06 (m, 2H, -CH_2NH_2), 3.95-4.13 (m, 3H, CH_3NH-), 4.48-4.56 (m, 2H, -CH_2-), 4.58, 4.70 (brs, each, 1H, =CH_2), 5.16, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 15.5, 16.0, 16.3, 16.7, 18.3, 19.5, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 25.6, 28.1, 28.2, 29.8, 30.7, 31.1, 32.2, 34.4, 37.1, 37.2, 37.3, 38.0, 38.2, 38.5, 40.8, 42.5 (-CH_2NH_2), 47.0, 49.6, 50.6, 55.5, 56.8 (-NCH_2-), 64.5 (-CH_2), 79.1, 109.8 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 175.7 (-COOCH_2-); HRMS (ESI) m/z: $[M+H]^+$ 719.51044, calcd. for $C_{43}H_{66}N_4O_5$ 718.50332.

*3β-[(L-Alanyl)-L-Sarkosyl]-lup-20(29)-ene-28-oic acid-3,5,6-trimethylpyrazin-2-methyl ester (**BA-27**).* Yield: 79.8%; colorless powder; mp: 136.2-137.1 °C, $[\alpha]_D$ = +34 (c 0.50, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 0.78, 0.93 (s, each, 3H, 2×-CH_3, methyl of BA), 0.81 (s, 9H, 3×-CH_3, methyl of BA), 1.67 (s, 3H, 30-CH_3 of BA), 2.48, 2.49, 2.53 (s, each, 3H, 3×-CH_3, methyl of TMP), 2.87, 2.95 (s, each, 2H, -CH_2-), 4.01 (brs, 2H, -CNH_2), 4.06 (brs, 3H, -NCH_3), 4.58, 4.70 (brs, each, 1H, =CH_2), 5.16, 5.20 (d, each, 1H, *J* = 15Hz, -OCH_2-); ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 14.8, 16.0, 16.3, 16.7, 18.3, 19.5, 19.6, 20.6, 21.0 (-CH_3), 21.5 (-CH_3), 21.8 (-CH_3), 23.8, 25.6, 28.2, 29.8, 30.7, 31.6, 32.2, 34.4, 36.6 (-NCH_3), 37.0, 37.2, 38.0, 38.2, 38.5, 40.8, 41.6, 42.5, 47.0, 49.6, 50.2, 50.6, 55.5, 56.8 (-NCH_2-), 64.5 (-CH_2), 82.7, 109.81 (-CH=C-), 145.5, 148.9, 149.0, 150.6, 151.1 (-CH=C-), 162.7 (-CH_2-COO-), 175.6 (-COOCH_2-); HRMS (ESI) m/z: $[M+H]^+$ 733.52545, calcd. for $C_{44}H_{68}N_4O_5$ 732.51897.

4.2. Bio-Evaluation Methods

4.2.1. Cell Culture

The human hepatocellular carcinoma cell line (HepG2), human colon carcinoma cell line (HT-29), human cervical cancer cell line (Hela), human lung cancer cell line (A549), human gastric cancer cell line (BGC-823) and Madin-Darby canine kidney cell line (MDCK) were obtained from the Chinese Academy of Medical Sciences & Peking Union Medical College. The cultures of the cells were maintained as monolayer in RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) enicillin/streptomycin (Thermo Technologies, New York, NY, USA) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The **TBA** derivatives under study were dissolved in DMSO (Sigma, St. Louis, MO, USA) and added at required concentrations to the cell culture.

4.2.2. Cytotoxicity Assay

The cytotoxicity of the compounds was evaluated *in vitro* via the MTT method against HepG2, HT-29, Hela, BGC-823, A-549 cell lines with cisplatin as the positive control. Tumor cells growing in the logarithmic phase were seeded in 96-well plates at a density 3×10^3 cells/well and incubated overnight. The following day, cells were then treated with serial dilutions of the tested compounds for 72h. At the end of this incubation, 20μL of 5 mg/mL methylthiazol tetrazolium (MTT) was added to each well and incubation proceeded at 37°C for another 4 h. After the supernatant medium was thrown away, 150 μL dimethylsulphoxide (DMSO) were added to each well and absorbance was measured at 490 nm using a plate reader (BIORAD 550 spectrophotometer, Bio-rad Life Science Development Ltd., Beijing, China). Experiments were performed in triplicates and the values were the average of three (n = 3) independent experiments. The concentration of the compound which gives 50% growth inhibition corresponds to the IC_{50}. Tumor cell growth inhibitory rate was calculated in the following Equation (1):

$$\% \text{ inhibition} = (1 - \text{Sample group OD/Control group OD}) \times 100\% \quad (1)$$

4.2.3. Morphological detection of apoptosis using Giemsa staining

To observe the changes in cell morphology after treatment with **BA-25**, Giemsa staining was performed as previously described [19]. Briefly, exponentially growing

HepG2 cells (4×10^3 cells/well) were cultured in 6-well plates overnight and then treated with various concentrations of **BA-25** (1.5, 3, 6 μM) for an additional 72 h. Following twice washes with PBS and fixing with cold methanol, cells were stained with 6% Giemsa (Giemsa, Molecular Probes/Invitrogen Life Technologies, Carlsbad, CA, USA) solution for 5 min, washed with water and dried. The cell morphological changes were observed under a fluorescent microscope [38].

4.2.4. Morphological detection of apoptosis using DAPI staining

Morphological observation of nuclear changes was performed by DAPI staining in this assay. HepG2 cells were seeded in the six well plates at a density of 4×10^3 cells/well and were allowed to grow for 12 h. Then the cells were treated with different concentration ranges of **BA-25** (1.5, 3, 6 μM) for 72 hours. After the treatment period, cells were washed with PBS and fixed with 4% paraformaldehyde. Then the liquid was removed and the cells were stained with 1 mg/mL DAPI (DAPI, Molecular Probes/Invitrogen Life Technologies, Carlsbad, CA, USA) for 1 min in dark. After staining, cells were visualised under a fluorescence microscope [39].

4.2.5. Detection of apoptosis using Annexin V-FITC/PI staining

To determine early apoptosis and secondary necrosis, HepG2 cells were stained with annexin-V FITC apoptosis detection kit (Beijing BioDee Biotech. Co., Ltd., Beijing, China) as per manufacturer instruction. After exposure to **BA-25** (2, 3, 4 μM) for 72 h, HepG2 cells were collected, washed twice with cold PBS and centrifuged at 1000 rpm for 5 min. The resulting pellet was mixed with 200 μL binding buffer of the Annexin V-FITC kit, then 10 μL FITC labeled annexin V was added and mixed gently. After incubation at 4°C for 15min in the dark, the cells were washed twice and resuspended in 300 μL binding buffer containing 10 μL PI. Then the cells were immediately analyzed with a flow cytometry [40].

4.2.6. Measurement of mitochondrial membrane potential

Evaluation of mitochondrial membrane potential in HepG2 cells was performed by flow cytometry using the rhodamine 123 (Rh123) staining. HepG2 cells (1×10^5 cells/ well) in logarithmic growth phase were incubated in 6-well culture plate for 24 h. Then the cells were exposed to 2, 3 and 4 μM **BA-25** for 72h and harvested by

trypsinnization. After twice washes with cold PBS, the cells were incubated at 37°C with 10 μg/mL Rh123 (Rh123, Beijing BioDee Biotech. Co., Ltd., Beijing, China) for 30 min. Following twice washes with PBS, fluorescent intensities were determined by flow cytometry with excitation and emission wavelength set at 488 nm and 530 nm, respectively [33, 36].

4.2.7. Assessment of intracellular Free Ca^{2+}

For the measurement of intracellular free Ca^{2+}, HepG2 cells were seeded in 6-well plate at 1×10^5 cells/well and were allowed to grow for 24h. After **BA-25** (2, 3, 4 μM) treatment for 72h, the cells were harvested and washed twice with cold PBS, then resuspended in HBSS buffer with 10 μM Fluo-3AM (Fluo-3AM, Shanghai Beyotime Biotech. Co., Ltd., Shanghai, China), and incubated for 30 min at 37 °C in the dark. The cells were then subjected to flow cytometric analysis at 488 nm excitation wavelength [36, 37].

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Author Contributions

Ideas and experiment design: Bing Xu, Peng-Long Wang and Hai-Min Lei; Chemistry and Biology: Bing Xu, Wen-Qiang Yan, Xin Xu, Yao-Tian Han; Analysis and interpretation of data: Rui Zhao, Fu-Hao Chu, Gao-Rong Wu; Writing and review of the manuscript: All the authors; Study supervision: Haimin Lei, Penglong Wang.

Conflicts of Interest

The authors declare no conflict of interest.

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Captions to the Tables, Fig.s and Schemes.

Table1 The structures of **TBA** amino acids derivatives **BA-02---BA-15**.

Table 2 The structures of **TBA** dipeptides derivatives **BA-16---BA-27**.

Table 3 The IC_{50} values of **TBA** amine acids or dipeptide derivatives **BA-X** for different tumor cells and MDCK cells.

Fig. 1. Morphological detection of apoptosis using Giemsa staining (200×): (**a**) Control group; (**b**) 1.5 μM; (**c**) 3 μM; and (**d**) 6 μM. The cell morphology was observed under the light microscope after Giemsa staining. The most representative fields are shown. Arrows indicate the typical apoptotic cell.

Fig. 2. Morphological detection of apoptosis using DAPI staining (200×): (**a**) control group; (**b**) 1.5 μM; (**c**) 3 μM; and (**d**) 6 μM. The cell morphology was observed under the fluorescence microscope after DAPI staining. The most representative fields are shown. Arrows indicate the typical apoptotic cell.

Fig. 3. Detection of apoptosis using Annexin V-FITC/PI staining: (**a**) control group; (**b**) 2 μM; (**c**) 3 μM; and (**d**) 4 μM.

Fig. 4. Effect of **BA-25** on mitochondrial membrane potential: (**a**) control group; (**b**) 2 μM; (**c**) 3 μM; and (**d**) 4 μM. Cells were determined by flow-cytometric analysis stained with Rhodamine 123 for 30 min. Results are expressed as mean fluorescent intensity (MFI).

Fig. 5. Effect of **BA-25** on intracellular free Ca^{2+} in HepG2 cells: (**a**) control group; (**b**) 2 μM; (**c**) 3 μM; and (**d**) 4 μM. After being treated with 2.0, 3.0 and 4.0 μM **BA-25** for 72 h, cells were determined by flow-cytometric analysis stained with Fluo-3AM for 30 min. Results are expressed as mean fluorescent intensity (MFI).

Scheme 1. Synthesis of the intermediate 2-(chloromethyl)-3,5,6-trimethylpyrazine (**5**). *Reagents and Conditions:* (a) aceticacid (AcOH), 30% H_2O_2, reflux, 90°C, 6h; (b)

928 acetic anhydride (Ac_2O), reflux, 105°C, 2h; (c) THF: MeOH: H_2O=3:1:1, NaOH, 1h;
929 (d) THF, TsCl, TEA, DMAP, 12h.

930 **Scheme 2.** Synthesis of the derivative **TBA (BA-01)**. *Reagents and Conditions:* (a)
931 dry DMF, dry K_2CO_3, 25 °C, 12h.

932 **Scheme 3.** Synthesis of the **TBA** amino acids derivatives **BA-02---BA-15**. *Reagents*
933 *and Conditions:* Boc-amino acids or Cbz-amino acids, DCM, DMAP, EDCI, 25°C,
934 12h; (b) TFA in dry DCM, 0°C, 2h or Pd/C (10%), MeOH, 25°C, 12h; (c)TBDMSCl,
935 Imidazole, DMF, 25°C, 12h; (d) TBAF, THF, 25 °C, 1.5h.

936 **Scheme 4.** Synthesis of the **TBA** dipeptide derivatives **BA-16---BA-27**. *Reagents and*
937 *Conditions:* (a) Boc-amino acids, HOBt, EDCI, DIPEA, 25°C, 12h; (b) TFA in dry
938 DCM, 0°C, 2h.

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950 **Table1** The structures of **TBA** amino acids derivatives **BA-02---BA-15**

Compound	R	Compound	R
BA-02	L-Gly	BA-09	L-Pyr
BA-03	L-Phe	BA-10	L-Lys
BA-04	L-Ala	BA-11	L-Trp
BA-05	L-Asp	BA-12	L-Sar
BA-06	L-Pro	BA-13	L-Val
BA-07	L-Leu	BA-14	L-Thr
BA-08	L-IIe	BA-15	L-Ser

951 **Table 2** The structures of **TBA** dipeptides derivatives **BA-16---BA-27**

compound	R_1	R_2
BA-16	L-Gly	L-Gly
BA-17	L-Sar	L-Sar
BA-18	L-Sar	L-Pro
BA-19	L-Gly	L-Ala
BA-20	L-Gly	L-Pro
BA-21	L-Gly	L-Sar
BA-22	L-Ala	L-Ala
BA-23	L-Ala	L-Gly
BA-24	L-Ala	L-Pro
BA-25	L-Ala	L-Sar
BA-26	L-Sar	L-Gly
BA-27	L-Sar	L-Ala

952 **Table 3** The IC_{50} values of **TBA** amine acids or dipeptide derivatives **BA-X** for
953 different tumor cells and MDCK cells

Compd	IC_{50} (μM)					
	HepG2	HT-29	Hela	BGC-823	A549	MDCK
BA-01	5.70±1.77	7.41±2.34	8.00±1.89	6.87±0.21	3.56±0.31	18.20±0.18

BA-02	6.77±0.67	3.97±0.59	4.24±0.70	3.87±0.22	3.62±0.31	5.82±0.50
BA-03	>40	>20	>40	>20	>40	>40
BA-04	>20	7.74±0.14	6.62±1.41	6.16±0.58	6.33±0.17	>40
BA-05	>20	>20	>20	>20	>20	>40
BA-06	4.54±0.46	2.73±0.35	6.33±0.02	3.47±0.2	3.36±0.48	10.15±4.05
BA-07	>40	>20	>20	>20	>20	>40
BA-08	12.73±0.8	15.89±2.9	>20	>20	14.52±5.3	>40
BA-09	5.86±0.45	3.78±0.89	6.59±0.34	4.70±0.42	4.11±0.56	9.24±0.21
BA-10	2.39±0.70	2.64±0.5	2.62±0.36	2.16±1.10	1.26±0.83	>40
BA-11	>40	7.41±2.34	>20	>20	>20	>40
BA-12	4.5±0.65	1.10±0.67	3.96±1.08	3.97±2.15	3.05±0.34	9.08±0.22
BA-13	5.91±0.05	>20	8.63±0.75	7.14±1.70	6.90±0.37	>40
BA-14	3.64±0.49	6.66±0.92	8.39±0.58	7.05±0.53	4.18±0.81	>40
BA-15	4.03±0.22	4.35±1.31	2.74±0.97	6.21±0.29	4.39±2.76	8.56±1.19
BA-16	4.0±0.87	3.19±0.4	2.40±0.04	2.88+0.42	1.95±0.58	7.45±1.15
BA-17	3.94±0.02	8.32±1.07	5.08±1.61	4.10±0.13	4.83±0.01	10.91±1.4
BA-18	3.75±0.36	5.98±0.29	3.02±0.12	1.83±1.07	3.91±1.13	11.28±0.21
BA-19	2.27±0.67	3.67±0.42	3.19±0.97	4.99±0.33	3.25±0.15	16.23±5.90
BA-20	3.48±1.13	4.11±1.05	2.20±0.43	0.84±0.39	2.98±1.61	6.63±0.41
BA-21	5.27±1.29	3.37±2.59	7.38±0.38	2.07±0.99	3.02±1.08	20.64±5.89
BA-22	1.93±0.49	4.18±1.20	4.49±0.82	2.51±1.68	2.54±0.44	10.21±1.14
BA-23	2.17±0.26	4.38±2.39	5.57±0.55	1.49±0.63	2.45±0.80	>40
BA-24	3.39±1.01	3.56±0.07	2.33±0.46	2.52±0.16	4.08±0.45	>40
BA-25	3.09±1.49	1.70±0.34	1.74±0.99	1.79±0.28	3.25±1.10	10.84±0.27
BA-26	4.94±0.04	3.31±0.28	2.62±1.13	3.43±0.42	2.96±0.76	8.08±0.13
BA-27	4.27±0.25	3.86±1.33	4.55±1.49	4.38±0.57	5.18±1.33	14.56±1.88
DPP	3.42±0.68	4.1±1.17	5.60±0.78	4.25±0.32	3.85±0.63	12.38±1.23

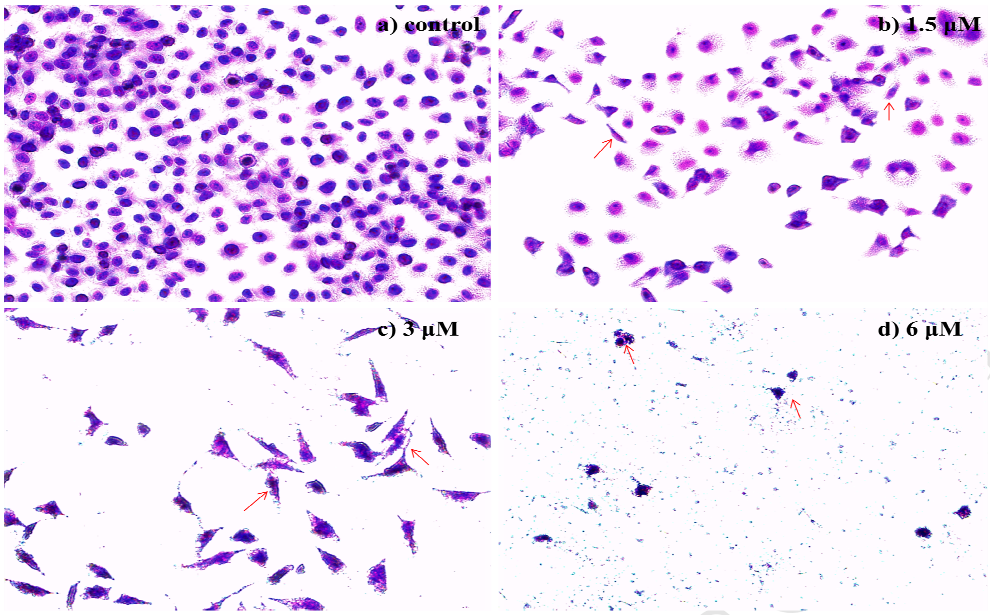


Fig. 1. Morphological detection of apoptosis using Giemsa staining (200×).

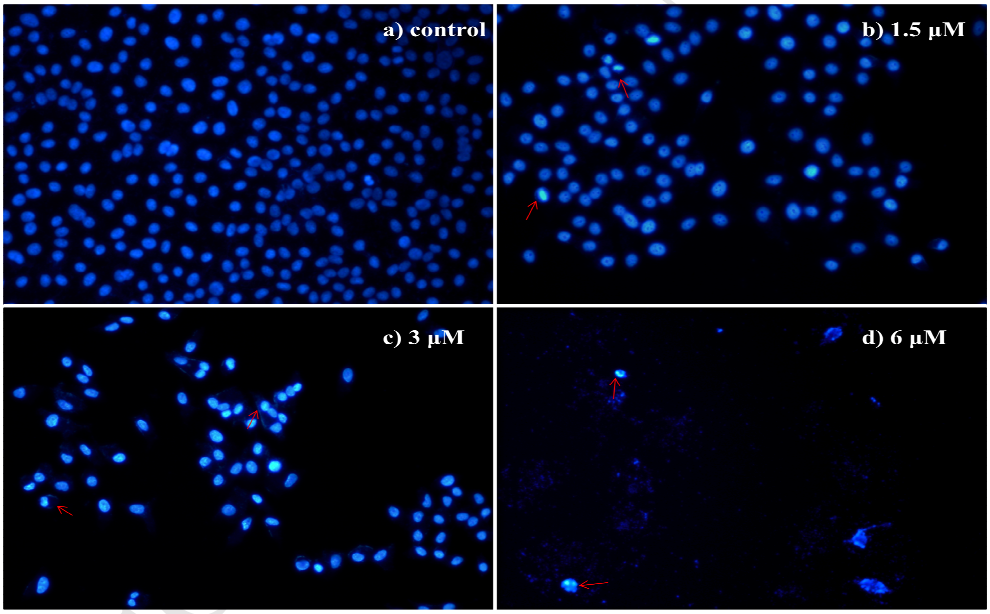
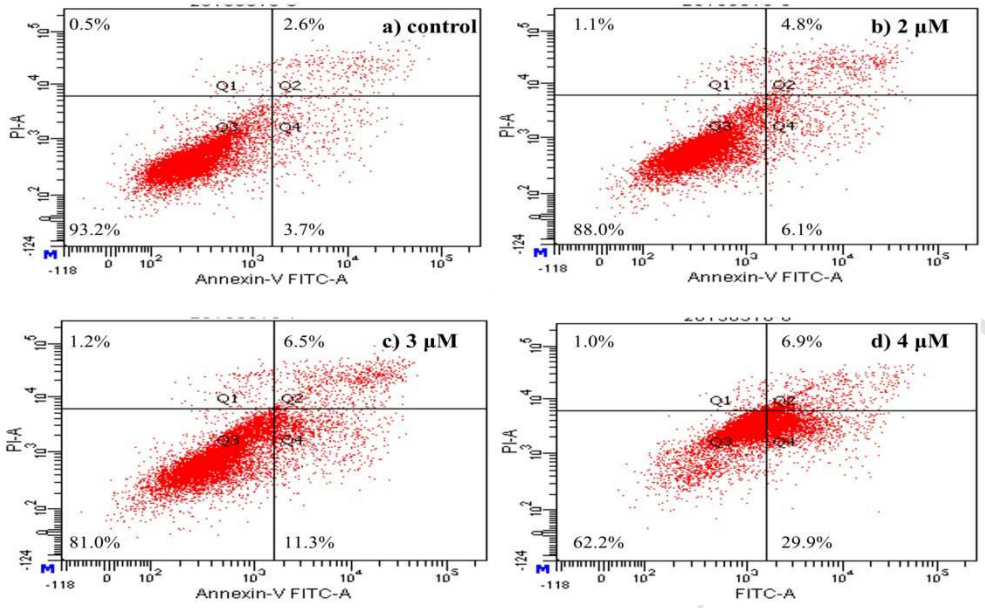


Fig. 2. Morphological detection of apoptosis using DAPI staining (200×).



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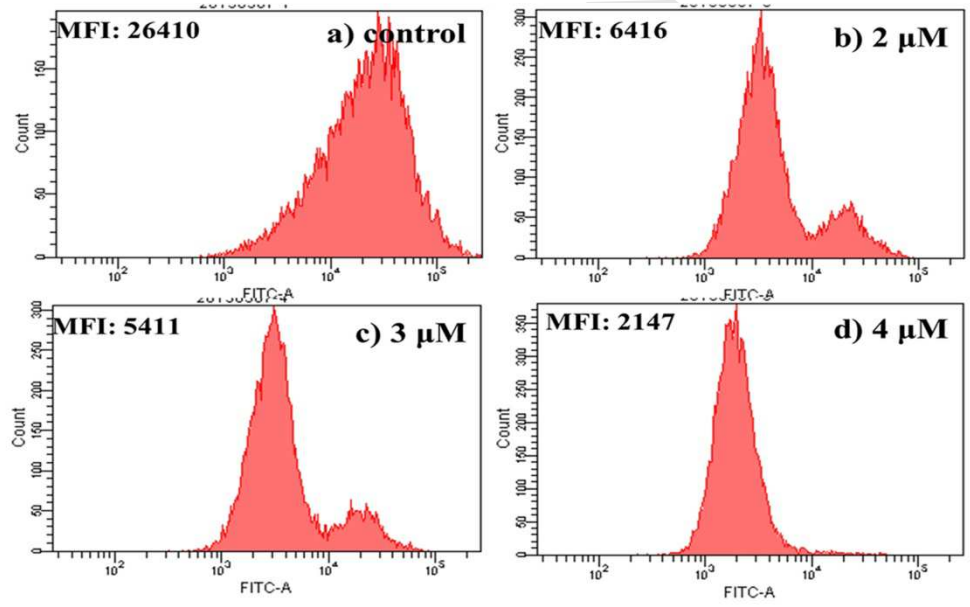
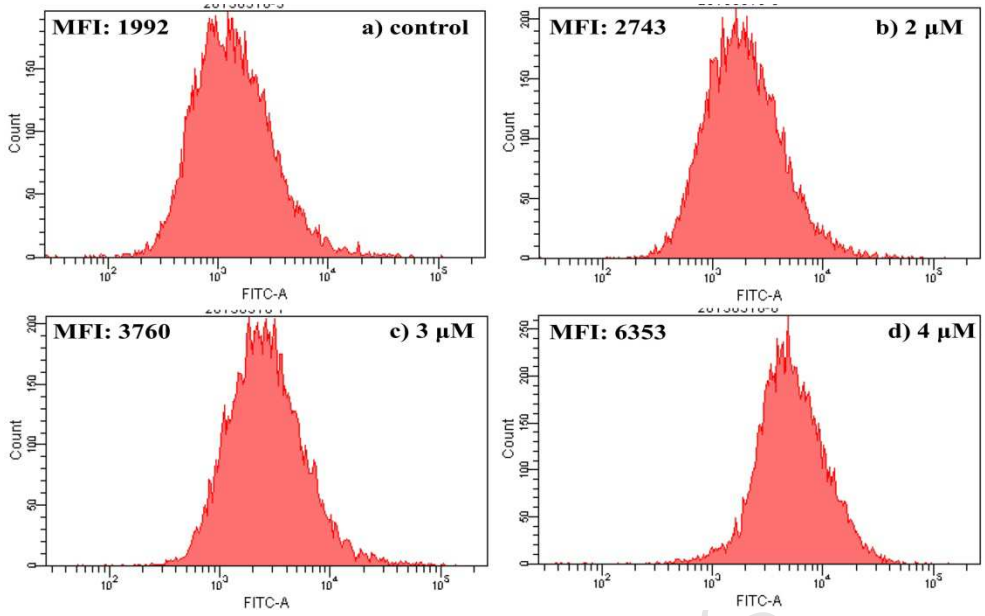
972 **Fig. 3.** Detection of apoptosis using Annexin V-FITC/PI staining.



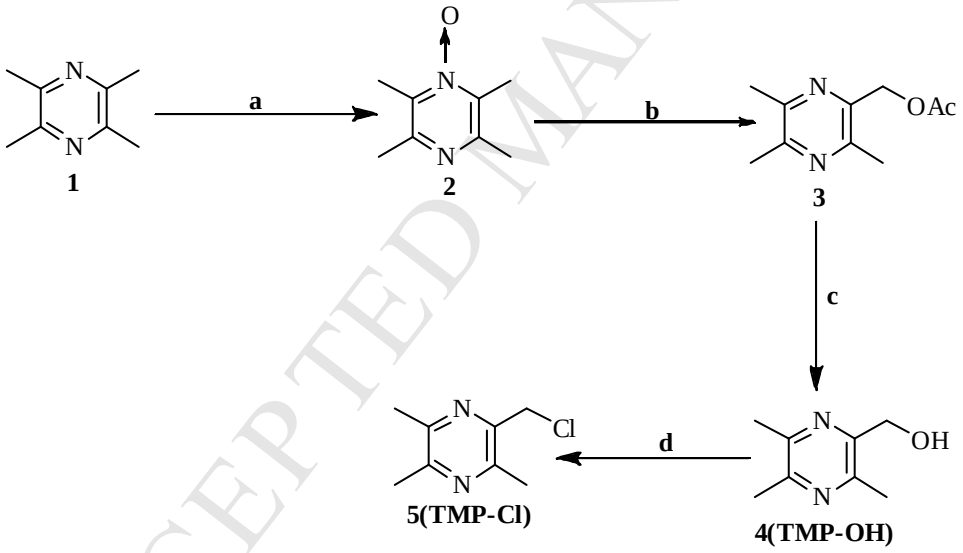
973

974 **Fig. 4.** Effect of **BA-25** on mitochondrial membrane potential.



975

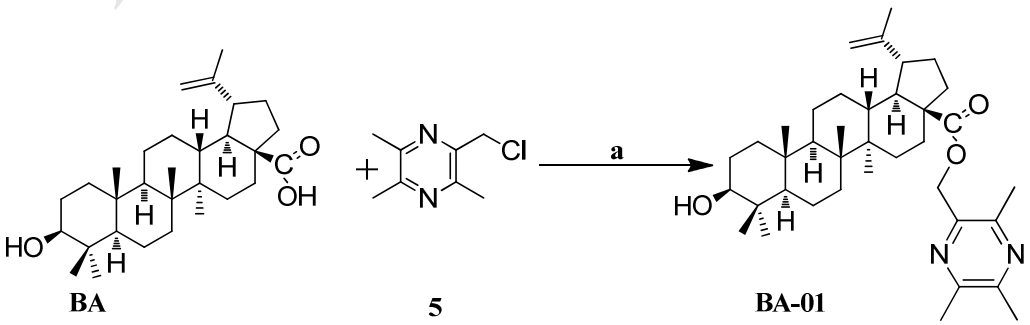
976 **Fig. 5.** Effect of **BA-25** on intracellular free Ca^{2+} in HepG2 cells.



977

978 **Scheme 1.** Synthesis of the intermediate 2-(chloromethyl)-3,5,6-trimethylpyrazine

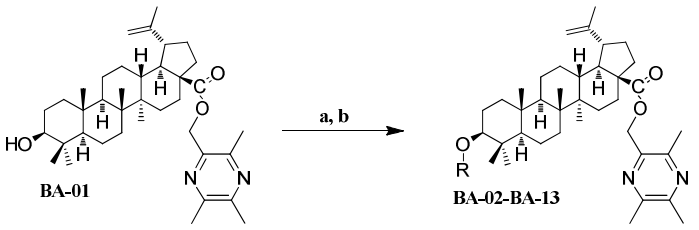
979 (**5**).



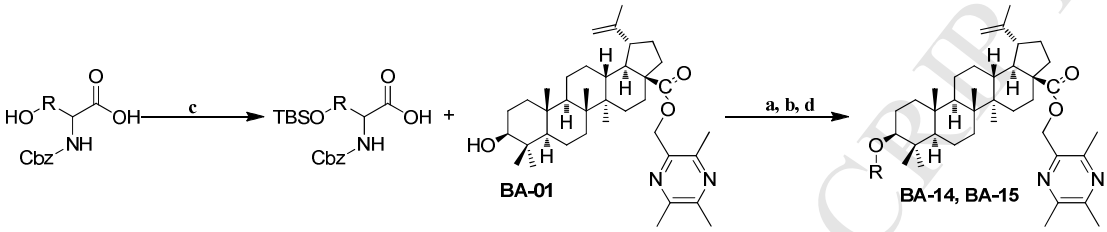
980

981 **Scheme 2.** Synthesis of the derivative **TBA (BA-01**).

982

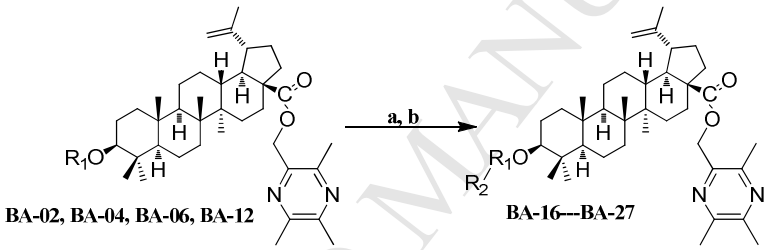


983



984 **Scheme 3.** Synthesis of the **TBA** amino acids derivatives **BA-02---BA-15**.

985



986 **Scheme 4.** Synthesis of the **TBA** dipeptide derivatives **BA-16---BA-27**.

Highlights

a. Twenty-six **TBA** amino acid/dipeptide derivatives were synthesized.

b. **TBA** amino acid/dipeptide derivatives showed potent cytotoxicity on tumor cell, and low toxicity on normal cell.

c. **BA-25**-induced apoptosis was associated with loss of mitochondrial membrane potential.

d. **BA-25**-induced apoptosis was associated with increase of intracellular free Ca^{2+} concentration.